医療福祉機器技術分野
E-041

平成７年度
新エネルギー・産業技術総合開発機構委託業務
（提案公募型・最先端分野研究開発事業）
研究成果報告書

人工臓器用生体機能賦活化型素材の開発技術

平成８年１２月

横浜市立大学

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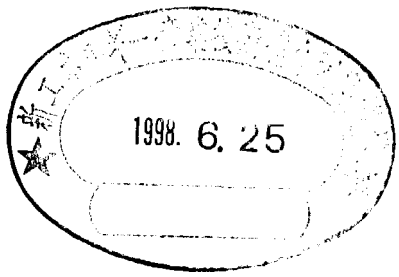

平成８年１２月

横浜市立大学

平成７年度　新エネルギー・産業技術総合開発機構委託業務　研究成果報告書概要

作製年月日	平成８年１２月３１日
分野／ プロジェクト番号	分野：医療福祉機器技術分野　　番号：E-041
研究機関名	横浜市立大学
代表者部署・役職	医学部外科学第一講座・講師
代表者氏名	野一色泰晴
プロジェクト名	人工臓器用生体機能賦活化型素材の開発技術
研究期間	平成８年１月１６日　～　８年１２月３１日
研究の目的	生体機能を賦活化させる素材を創出することにより、高機能人工臓器製造のための次世代医療産業用基盤技術を作る。具体的な目的として、1.高分子材料のマイクロ加工、マイクロ化加工、2.生体材料の生体親和性処理、3.生分解性材料の導入、4.生理活性物質および細胞の固定による徐放、5.自家組織片の素材としての活用、等である。これらの組み合わせにより積極的に生体機能賦活化を計り、トータルシステムの機能要素技術を示す。
成果の要旨	合成高分子材料のうち、ポリウレタン材料については無数のマイクロ孔を開けることによって血液成分のうちフィブリンやアルブミン等を付着させて、細胞親和性を得た。そしてその成果をもとに内径1.5mm,長さ100mmの人工血管をラットに植え込み開存させ続けた。生体高分子材料のうち、Collagenの微細繊維を布製人工血管に絡ませ、さらにそれをサクシニール化することによってコラーゲンに微少分子間吸着水をつけさせて低血栓性のTubeを作らせて、これを人工血管とした。その結果、低血栓性及び細胞親和性を得ることとなり新生内膜を急速に得ることに成功した。生体材料の一つである自家組織の有効利用として、我々は骨髄組織を多孔性のポリテトラフロロエチレン管に絡ませて人工血管を作製した。その結果、移植された骨髄組織より血管新生を促し、内皮細胞誘導因子を産生させる成長因子を発現させて急速な新生内膜形成を得た。
成果発表・特許等の状況	1996年5月28日より開催される第5回国際バイオマテリアル会議および1996年6月26日より開催される第24回日本血管外科学会で報告、さらに1997年6月30日より開催される国際人工臓器学会にて報告予定。
今後の予定	前述の研究成果を一つの人工血管などに応用し、長期動物実験を積み重ねて設計通りの成果が得られるか否かの検討を行う予定。

（様式－３）

Summary of New Energy and Industrial Technology Development Organization Entrusted R&D Report for FY 1996

Date of preparation	December 31, 1996
Field / Project number	Field: Medical and walfare Support Technology No. E-041
Research organization	Yokohama City University
Post of the research coordinator	Assistant Professor Yokohama City University School of Medicine
Name of the research coordinator	Yasuharu Noishiki (signature) Yasuharu Noishiki
Title of the project	A new technology of artificial organ materials which can induce host biocompatibility
Duration of the project	January 16, 1996 ～ December 31 , 1996
Purpose of the project	The aim of this project is to produce a highly biocompatible materials for next generation's artificial organs using the following methods: 1, Micromodification of polymr materials. 2, Biocompatible treatment for biological materials. 3, Application of bioabsorbable materials. 4, Bioactive substance immobilization. and 5, Use of autologous tissue as artificial organ materials.
Summary of the results	As a synthetic polymer material, microporous polyurethane was used for a small diameter vascular prosthesis. The graft with this technology was successfully implanted in rat abdomical aortic position. The graft of 1.5 mm in internal diameter and 10 cm in length showed excellent patency with nice endothelialization. As a biological material, microfibers of collagen was used for a sealing substance of vascular prothesis. The microfibers absorbed a large amount of water, which could prevent blood leakage from the graft wall. The graft showed non-thrombogenic property and excellent host cell affinity, resulted in rapid neointima formation. As to autologous tissue, bone marrow was used, since marrow cells can differentiate into any mesenchimal cells with synthesis of growth factors. Marrow cell transplanted vascular prothesis showed rapid capillary ingrowth. These results indicated that the newly designed materials had suitable properties for materials of next generation's artificial organs.
Publication, patents, etc.	We represented these results in the 5th World Biomaterial Congress and The Annual Meeting of the Americal Society for Artificial Internal Organs. Now, we are considering the patent application for some of these findings.
Future plans	We are planning to apply these technology to develop new artificial organs, especially in the field of vascular prostheses.

まえがき

人工臓器には拡がりがあり夢がある。そして技術革新によりそれが実現可能である。昔人類は鳥の翼をまねて飛行努力をした。結局飛行の理論をつかみ、技術革新と努力の積み重ねた結果、鳥よりも高く、遠く、早く飛べるようになった。もし、人類が移植にこだわっていれば、そして鳥の翼の移植に成功したとしたら、天使のような翼を得たかもしれないが、快適な空の遠距離旅行も無理であるし、月にも行けない。

人工臓器の歴史はわずか40年であり、医学全体の4000年に比べるときわめて浅い。しかし、その歴史の新しさにかかわらず、人工臓器は従来困難視されていた多くの疾患の治療に大きな貢献をしてきた。そして、さらに高機能の人工臓器の要望が高まっている。人工臓器は今日まで生体の臓器の代用物を作ることを使命としてきた。しかし、今日では免疫複合体を除去してリウマチを治療する臓器（装置）を開発したり、脂肪を吸着除去して動脈硬化症を治療するような血液浄化臓器も登場し、「神の作り忘れた臓器」の創造への道を歩んでいる。また0.1ミクロンの人工赤血球（生体の赤血球は約8ミクロン）のようにマイクロ化に成功して極度に狭窄した血管をも通過して酸素を送るように、天然臓器を越える可能性がでてきた。

このような今日にあって、飛ぶための飛行の理論を得たように、我々は人工臓器のための生体の順応性の理論を正しく理解することに成功し、新しい素材の設計指針を得た。この成果をもとにこの度は新しい設計理論にもとずく高機能人工臓器造成のための素材開発基盤技術の資料を得ることを研究の目的とした。

産業技術としての直接的波及効果としては、人工血管、人工腱、人工弁、人工心臓血液接触面および生体組織との接合部、人工肝臓や人工脾臓などのハイブリッド型人工臓器用基材、人工皮膚、人工気管など、次世代用人工臓器のあらゆる面で技術革新となりうることから、この関連の我が国の医療産業が21世紀において指導的立場に出る可能性が出てきた。

今日の低成長時代においても国民医療費は着実に増え続けており、一部の医療産業も成長している。しかし日本製CTが全国の病院で活躍しても患者一人一人がその特許料を外国に支払っていることを考えると、医療産業における日本発の独自技術が余りにも少ないことに残念さを感じる。もし、日本でその基礎研究が推進され、工業所有権を得て、医療産業が育成できれば国民医療費も低下させうるし、相当な外貨もかせぎ得る。このような事から、将来の医療の大きな柱の一つと予測される人工臓器に対し、その基礎研究を育て、基盤技術を確立することがいかに大切かが理解できる。米国をはじめ諸外国では高騰する医療裁判費用の影響から診断用医療機器の開発は進められても治療用機材の開発には余り力が注がれなくなってきた。特にPL法施行後は生体内植え込み用人工臓器開発には後ろ向きとなっている。しかし我が国においては、工業用材料をはじめ、多くの特色ある材料の研究が過去20年間続けられ、その成果が顕著となってきた。本研究の提案者は材料研究プロジェクトに人工臓器分野で20年間研究を継続してきた。その結果、生体順応性の理論を見い出し、これを生体機能賦活化に応用する糸口を得た。これを活用することは医療産業の一面を大きく発展させ得るものと思われる。

この度の研究成果をまとめるにあたり、国内の主な繊維メーカーからは最近のトラッキング問題、PL問題の関係より研究協力をいたただくことができなかった。しかしながら、これに替わる技術として我々独自の方法で、ある程度の成果を得ることができた。短い研究期間の成果であるがここにそれを報告する。

研究チーム名簿

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馬拉提　　（招聘研究員）

補助研究員　　4名

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40) 成長できる人工血管の開発の基礎研究

第1章

合成高分子材料のマイクロ加工、マイクロ加工化技術

はじめに人工臓器、特に植え込み型人工臓器用素材としては工業的製品を転用しているものが多く、近年になり人工臓器専用も作られるようになったが、その設計思想としては、生体に無害なもの、いわゆるステルス性が最重要視されているのが現状である。すなわち、今日の人工臓器は「静かなる異物」の組み合わせで成り立っている。しかし、今日、人工臓器にはさらなる高機能が要求されており、21世紀には極めて高い技術が人工臓器製造に要求され医療を支えてゆく必要に迫られている。しかし今日の技術の延長線上ではこの要求に応じられない。我々の設計思想は次世代人工臓器用素材として、材料が生体内に入ることにより、かえって生体組織を活性化し、治癒促進をさせる「積極的賦活化物」を利用するもので、結果的に人工臓器に高機能を発揮させることを目的としている。

この度の研究ではこの目的のため高分子材料を加工することにより生体組織を賦活化する工夫をおこなったので紹介する。

1. ポリエステル繊維のマイクロ加工、マイクロ加工化

植え込み用人工臓器素材の中で最も使用されているのがポリエステル繊維である。これは衣服用に作られたものを転用しており、その繊維断面は円形で約20ミクロンの直径をもつ。しかし、カーボンファイバーやグラスファイバーのように細くすればするほど繊維は柔軟性を増すとともに強度も増加する。実際ポリエステル繊維は最新の技術により直径0.1ミクロン程度まで細くすることが可能である。この超極細繊維を用いると生体内に存在するコラーゲン繊維により近くなるとともに、前述した通り細胞が積極的に寄り添うように付着しはじめる。これは細胞のもつ本能的性質の一つであり形態追従効果（Contact guidance）として知られている。この本能的性質を引き出すことで細胞の材料による誘導が可能となり人工臓器の高機能化が実現できるとともに、高齢者でも細胞の活性化が生じて安全な手術が期待される。

このような背景のもとで我々は過去に行った超極細ポリエステル繊維を用いて作成した人工血管の植え込み結果の再検討を行った。本人工血管は設計当時は超極細繊維を用い、これを強力なウォータージェット操作によって絡ませ、その絡まりによって人工血管切断端のほつれを防止すること、および極細繊維を用いることによって、人工血管を緻密に織っても硬くならないことなどを目的としていた。しかしこの度の研究の再検討の目的は、形態追従効果によって細胞が活発に誘導されて、内膜治癒が加速されるか否かという点に解析の主眼を置いた。

材料と方法

1) 超極細繊維製人工血管の作成

一般に広く使用されているポリエステル繊維（0.2デニール）布の内表面を超極細ポリエステル繊維[2)]（0.12デニール, 繊維断面直径3.0ミクロン）で覆った特殊な布で内径7 mm, 長さ5.7 cmの管を作成し、布製人工血管（有孔性：200 cc／cm^2, 120mmHg, H^2O）を作成した。対照として、市販のダクロン製人工血管（2.0デニール, 繊維断面直径20ミクロン, 有孔性：400）を採用した。

2) 動物実験

体重8～12 kgの成犬12頭を全身麻酔下に右側開胸し、胸部下行大動脈を約5 cm切除し、ここに作成した人工血管6本を植え込んだ。手術中に感染防止のため合成ペニシリンを使用したが、その他、抗血栓性薬剤はもとより、一切の薬剤は術前後を通じて使用しなかった。実験動物は一般状態をはじめ、大腿動脈拍動等の観察を行い、植え込み2日目より99日に至るまでの間に間隔をおいて屠殺し、試料を採取した。得られた各試料は肉眼的、光顕的、走査電顕的に観察した。

3) 光顕的、電顕的観察

光顕的観察用試料としては、採取後、各試料を1%グルタールアルデヒド（0.2 M, リン酸バッファー, PH 7.4）液中で固定したのち、流水中で洗滌した。次にアルコール系列で脱水し、親水性樹脂（JB-4, ポリサイエンス社）に包埋し、ガラスナイフでで切片を作成した。各切片はヘマトキシリン、エオジン染色、レゾルシンフクシン弾性線維染色で染色後観察した。

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3) 光顕、電顕的観察

光顕、電顕的観察では、植え込み2日目の実験群の初期血栓層は一様に薄く、起毛した超極細繊維がかろうじてフイブリン層に埋もれている程度であり、10～100 μ の厚さであった（図3）。植え込み15日目では吻合部付近および部分的ではあるが中央部でも内皮細胞の出現がみられた。新生内膜は薄く、超極細繊維の起毛の高さと一致していた。起毛繊維間隙には多数の線維芽細胞の侵入を認めた（図4）。植え込み21日目の例でも、域間隙のフイブリン域に形成質細胞と多数の線維芽細胞の侵入を認めた。この線維芽細胞の侵入は起毛繊維の間隙に含まれたフイブリン層内に限られており、起毛繊維のない部分のフイブリン層内には侵入していなかった。15日目に比べて、21日目では治癒はさらに進み、中央部でも広く内皮細胞の被覆がみられた（図4）。61日目の例では、新生内膜は全面にわたって完成しており、内皮細胞が完全に内面を覆っていた（図5）。85日目の例でも治癒は完了していた。なお、硝子様変性、脱落、潰瘍化、石灰化等は認められなかった。これに対し、対照群では。吻合部の治癒は実験群と同様に進行したものの、中央部では遅延しており、99日目の例でも中央部は内皮細胞に覆われておらず、表面に新鮮な赤色血栓の付着を認めた。壁内部でのポリエステル繊維の間隙への細胞侵入は悪く、線維芽細胞の侵入はごく僅かであった（図6）。

考察

超極細繊維は東レで開発された鹿革感触の人工皮革、エクセーヌ用原糸であり、これで作った布はたとえ緻密に編んでも硬くならないという特徴をもっている。作成した人工血管は低有孔性であるにもかかわらず、しなやかで軟らかく、人工血管に鹿革感触を導入したものである。この布は起毛してあり、ビロード様の感触があるが、ビロード布のような厚みはなく均一に薄い。この柔軟性は、従来の低有孔性人工血管を用いた手術時の、人工血管の堅さに由来する不都合、不便さ、扱いにくさ等[3]をことごとく解消してしており、有孔性200であっても、従来の1200ないし2000程度の軟らかさをもっている。この柔軟な人工血管を用いれば、石灰化を来した病的血管に縫縮しつつ吻合してゆく血管手術で、手術中の苦労を大幅に軽減するのみならず、吻合部からの出血も減少させられよう。

また一方、試作人工血管の大きな特徴として、形成される新生内膜の急速な治癒、安定性が挙げられる。一般に高有孔性においては治癒が早い。初期血栓層内の赤血球は急速に流れ出し、2日目にはフイブリン網となる。低有孔性では赤血球が流れだし難く、治癒が遅れる[3]。しかし、試作人工血管においては、高有孔性に似て急速である。

初期血栓層[1]、フイブリン層の薄いのも特徴の一つである。繊細な繊維からなる柔らかな起毛がフイブリン層を薄く保持していると思われる。起毛の高さにフイブリン層はとどまっており、それ以上肥厚していない。これは起毛繊維の軟らかさが何らかの作用を果たしていると考えられている。従来の硬い起毛ではこのような現象はみられていない。また一方、内皮細胞の被覆にも特徴がある。一般に低有孔性人工血管では内皮細胞の被覆はほとんど吻合部からの連続的伸展に限られており[4]、外膜側から細血管が人工血管繊維間隙を貫通して内膜面へ開口し、そして集落形成という内皮細胞被覆形態は望みが

たい。それにもかかわらず、試作人工血管の中央部でも内皮細胞被覆がみられたのは、フイブリン層が内皮細胞の生着に有利な状況をもっていたと思われる。吻合部では若い内皮細胞が分裂、増殖をしており、これが血流に流されてフイブリン層上に付着することもあるので[4]、この生着に有利であれば、内皮細胞は集落も作りやすいと思われる。15日目、21日目の例にみられるように、起毛された超極細繊維間隙には多数の線維芽細胞侵入があり、起毛繊維のない部分のフイブリン層ではそれが認められないというのは、超極細繊維の存在が細胞侵入、増殖に良好な環境を作っていると思われる。細胞は超極細繊維から何らかの情報をとらえ、急速に繊維間隙に侵入し、増殖してゆく、これを養うべく細血管も侵入するであろうし、この細血管が人工血管内面に開口すれば、細血管由来の内皮細胞集落を作ることも当然考えられる。このように新生内膜内部での急速治癒が内皮細胞の内表面へ生着増殖するのに好影響を与えると考えられる現象は、従来の太い繊維を用いた場合には見られないものである。従来の人工血管では、新生内膜内部での治癒よりも内皮細胞が内表面を被覆する過程が先に完成するし、また治癒も遅延するものと思われていた。本研究での人工血管では繊維の太さを細胞と同じ、もしくはそれより細くすることによって、細胞との親和性を大幅に改善させた結果、治癒を著しく促進させることとなった。繊維の太さと細胞との親和性の研究は、繊維の形態をも含めて、どの太さで、どの形態が細胞にとって最適かという研究を今後進めてゆくことで、人工血管に限らず、人工臓器開発研究に貢献してゆけるものと思われる。

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2. ポリウレタンのマイクロ加工、マイクロ多孔質処理による生体適合性獲得

1) 緒言

我々はこれまでの一連の研究を通して、内面構造と漏水生が小口径人工血管の開存性と内皮化に及ぼす影響を検討してきた[1~6]。そして、これまでの動物実験では、異なった漏水率（0, 2, 7, 11, 39 ml / min / cm^2）のミクロ多孔質の管壁をもつ4種類のポリウレタン製人工血管（内径 1.5 mm, 長さ 1.5 - 2 cm）をラットの腹部大動脈に直線状に端々吻合で植え込み、術後3ヶ月間まで評価した。その結果、漏水率が0または2.7 ml / min / cm^2と小さな人工血管は内面構造が平滑膜かミクロ多孔質であるかにかかわらず、早期に血栓性閉塞をきたした。また漏水率が11ml / min / cm^2で内面構造が散在性小孔を伴った平滑筋である人工血管は開存性は良好であったが、内皮化はほとんど認められなかった。一方、39 ml / min / cm^2という試験された人工血管のなかでは最も大きな漏水率をもち、かつ内面構造がミクロ多孔質である人工血管は植え込み3ヶ月後に良好な開存性とほぼ完全な内皮化が得られた[1)]。すなわち、満足すべき開存性を得るためには、漏水率が約 11 ml / min / cm^2以上であること。そして、良好な開存性かつ高度な内皮化を達成するためには（1）漏水率が39 ml / min / cm^2 ないしはそれ以上であること、あるいは（2）漏水率が39 ml / min / cm^2 ないしはそれ以上であり、かつ内面構造がミクロ多孔質であることが望ましいことが示唆された（表1）。

そこで今回の研究の目的は、（1）内面構造がミクロ多孔質であるポリウレタン製小口径人工血管は3ヶ月間の植え込み実験では良好な結果が得られているので、やはり内面構造がミクロ多孔質であるポリウレタン製小口径人工血管を作成し、1年以上の長期間植え込み実験により、開存性、安全性、内皮化等を評価すること、（2）過去の実験では長さ約2 cmのグラフトを直線状に植え込むモデルを主として用いたが、本研究では全てのグラフトを長さ10 cmのループ状グラフトとして植え込むという厳しいモデルで評価すること、（3）グラフトの漏水率を39 ml / min / cm^2 より大きくした方が、開存性と内皮化のさらなる向上が認められるか否かを評価すること、である。

表1
ミクロ多孔質ポリウレタン製小口径人工血管の植え込み後3ヶ月までの開存率と内皮化

人工血管	内腔面	漏水率	開存性	内皮化
PUG-S-0	平滑膜	0	不良	---
PUG-S-11	平滑膜＋散在性小孔	11	良好	不良
PUG-2.7	ミクロ多孔質	2.7	不良	---
PUG-39	ミクロ多孔質	39	良好	良好

（内径 1.5 mm, 長さ 1.5 - 2 cm の直線状グラフトの植え込みモデル）漏水率単位：（ml / min / cm2）

2) 材料と方法

I. 人工血管作製法

まず、再結晶塩化ナトリウムを以下の方法で作成した。100 g の塩化ナトリウムを280 ml の脱イオン水に溶解し、80 - 100 °C で 30 分間撹拌する。この溶液を濾過して遺残塩化ナトリウムを除去する。この溶液に 200 ml のエタノールを添加し、塩化ナトリウムの顆粒を生じさせ、濾過し、溶媒で洗滌し、その後 24 時間乾燥させて再結晶塩化ナトリウムを得た。

次に、再結晶塩化ナトリウムとポリウレタンをビーカーに入れ、テトラハイドロフランとジメチルホルママイドを加えてポリウレタンと再結晶塩化ナトリウムとの縣濁液を作成した。外径 1.5 mm のガラス棒をこの溶液に浸して付着させた後、ガラス棒を取り出して、直ちに脱イオン水の中に入れた。そして 30 分後に取り出して、個体化したポリウレタンからガラス棒を注意深く抜き去ることにより、ミクロ多孔質ポリウレタン製小口径人工血管（内径 1.5 mm, 長さ 10 cm, 漏水率 146 ml / min / cm^2 ）作製した。今回の実験では、漏水率を 39 ml / min / cm^2 より大きくした方が、開存性と内皮化のさらなる向上が認められるか否かを評価する目的で漏水率が 146 ml / min / cm^2 の人工血管を作製した（図 1）。人工血管は 24 時間、流水で洗滌した。

作製された人工血管の微細構造は走査型電子顕微鏡を用いて精査した。

漏水率（hydraulicpermeability）は 120 mmHg の水圧下に人工血管から最初の 1 分間に漏出した脱イオン水の量を測定して求めた。

作製された人工血管は以下の方法で消毒した。まず、（1）滅菌脱イオン水に 10 分間浸漬した後、（2）0.1 モル塩酸に 30 分間浸漬した。そして、再び（3）滅菌脱イオン水に10 分間浸漬した後、新しく調整された滅菌脱イオン水中に保存した。

II. 人工血管の植え込み

270 - 300 g のラットにペントバルビタールナトリウムを腹腔内注射することにより、全身麻酔をかけた。腹部正中切開で開腹し、大動脈ー総腸骨動脈分岐部の中枢部の腹部大動脈を約 1 cm 剥離した。同部を切離して、長さ 10 cm のグラフトをループ状にして端々吻合で植え込んだ（n = 3）。各吻合には 10 - 0 ナイロン糸による 8 - 10 針の結節縫合を行った。抗血栓剤は術前、術後とも投与しなかった。そして 1 年以上の長期にわたり開存性、安全性および内皮化の状態を評価することにした。

3) 結果

植え込み前の人工血管の走査型電子顕微鏡観察では人工血管壁内部、内面および外面はミクロ多孔質構造を呈していた（図 1）。

現時点で 205 日目（n = 1）と 207 日目（n = 2）の生存ラットを得ている。このうち 1 匹のラットを 76 日目と 133 日目に人工血管の植え込み時と同様に、全身麻酔下に清潔手術走査開腹した。そしてグラフトおよびグラフト吻合部の大動脈の拍動を触知することによりグラフトの開存を確認した（図 2）。その後、再び開腹し、より長期の開存性、安全性および内皮化の進展過程を引き続き観察する予定とした。

すなわち我々が提唱する小口径人工血管の長期開存を得るための構想は次ぎの通りである。まず、（1）人工血管の内面をミクロ多孔質とする。さらに（2）ある一定以上の漏水性を賦与する。これは換言すれば、穿通性の小孔が人工血管壁に存在することを意味する。この構造によって、人工血管は植え込み後、早期の血栓性閉塞をおこさずに開存することができる。そして、中期から慢性期にかけて、すみやかに高度の内皮化が達成される。これは人工血管壁に存在する多数の穿通性小孔の中に宿主細胞が進入、増殖して、人工血管内面における新生内膜形成のための生物学的基盤を提供することが大きな要因と考えられる。

上述した人工血管の植え込み後の経過は、これまでの動物実験で確認されていることは既に記した。本研究では高度に達成された内皮化が慢性期において、なかでも一年以上長期間経過した後にもそのまま維持されていくのか否か評価することが大きな目的のひとつである。今回の報告ではミクロ多孔質ポリウレタン製人工血管（内径 1.5 mm, 長さ 10 cm, 漏水率 146 ml / min / cm^2 ）約 4 ヶ月半にわたり開存していることを確認した。またこれまでの実験では長さ約 2 cm の人工血管で評価したが、本研究では長さ 10 cm の人工血管という厳しい条件で評価することとした。

さらに至適漏水率についても実験的に追求してゆく方針である。これまでの実験では至適漏水率は約 11 ml / min / cm^2 以上であることは推測されるが、どれ位が上限なのかが不明である。そして満足すべき結果が得られる漏水率の価には一定の幅があるのか。もし、あるとした場合、その範囲内であれば、ほぼ同一の結果が得られるのか。あるいはその範囲にあってもかなりの差があるのかについても探求することが今後の課題である。一方、漏水性がある一定以上を越えると植え込み時に漏血がが多くなり、実際の手術に使用するにふさわしくないレベルがあると考えられる。この観点からも実用的、実際的な意味での至適漏水率を追求してゆくべきであろう。

一方、人工血管と同様に血液接触面をもつ左心補助心臓の開発においても従来は血液接触面を平滑面としてきたが[9]、最近では本研究と同様の方式が導入されはじめている。すなわち、左心補助心臓である Heart Mate ™ において、血液接触面を平滑面ではなく粗面にして血栓症の著明な減少と、内腔面の部分的内皮化が観察されたとの報告がある[10、11]。

従来、人工臓器における血液接触面の研究においては、その素材自体あるいはその化学的表面修飾に力点がおかれていた。しかし、本研究や Heart Mate ™ の例で解るように物理的表面構造さらに材料内部あるいは外面の物理的構造にも着目する必要があると考えられる。またこれらの化学的および物理的要素の組み合わせによってはさらに優れた血液接触面をもつ人工臓器が開発される可能性がある。

5) 結論

ミクロ多幸質ポリウレタン製人工血管（内径 1.5 mm, 長さ 10 cm, 漏水率 146 ml / min / cm^2 ）を作製した。このグラフトをループ状にしてラットの腎動脈下、腹部大動脈に端々吻合で植え込んだ。現時点で約 7 ヶ月目の生存ラット（n = 3）を得ている。このうち 1 匹のラットを 76 日目と 133 日目に開腹してグラフトの開存を確認した。引き続き一年

以上の長期植え込みを行い、開存性、安全性、そして内皮化の状態を評価する予定とした。

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A COLLAGEN-COATED FABRIC VASCULAR PROSTHESIS AS A PUNCTUREABLE A-V SHUNT

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Running Head: Puncutureable collagen coated vascular graft

Key Words: Coated vascular prosthesis, Blood access, Fabric vascular prosthesis, Thermal Crosslinking, Hydrous surface, Punctureable graft, Non-throimbogenic property

Abstract

A fabric vascular prosthesis sealed with succinylated collagen (SC graft) was developed as an A-V shunt graft for hemodialysis. The SC graft was soft, pliable, flexible, and puncturable with quick hemostasis. A needle puncture made a smaller hole in the SC graft wall than in a control e-PTFE graft such as is usually used for an A-V shunt. The SC graft was extremely hydrous since it is sealed with water which is absorbed into the intermolecular spaces of negatively charged collagen. Succinylated collagen suspension was injected with pressure into a knitted fabric vascular prosthesis wall (water permeability: 1200ml) so as to become entangled in the Dacron network. Then the graft was lyophilized and thermally crosslinked. Water leakage from 6 holes created by a 18 G needle puncture in vitro under water pressure of 120 mmHg was 34.5 ± 29.9 ml/min. in the SC-graft and 169.9 ± 38.5 ml/min. in the control e-PTFE graft. Hemostatic time at six 18G needle puncture sites on grafts implanted in the abdomen of 12 dogs was 4.5 ± 2.5 min. in the SC graft and 34.2 ± 11.5 min. in the controls. After implantation, the luminal surface of the SC grafts had a thinner thrombus layer than the control grafts. After one week, a thin thrombus layer covered the luminal surface of the SC grafts, and puncture sites were recognized as small dots under it, but in the controls, the thrombus layer was thick and the puncture sites could not be seen. These results suggest that the SC graft is less thrombogenic and has less blood leakage from the puncture sites than the control.

Introduction

Various kinds of vascular grafts such as autologous vein grafts, autologous subcutaneous connective tisue tubes, glutaraldehyde crosslinked bovine grafts, glutaraldehyde crosslinked umblical cord vein grafts, e-PTFE grafts, fabric grafts, polyurethane coated grafts, and spun Corethane (a polyarbonate-urethane formulated for enhanced biodegradation resistance over conventional polyurethanes) fibers-Dacron composite prostheses have been tried and used as an A-V shunt blood access for hemodialysis[1-6]. Today, e-PTFE grafts are the most frequently used in this field because of their high patency rate and easy puncturability[1,7,8,9]. Fabric grafts are, however, the most frequently used in reconstructive surgery for occlusive peripheral

arterial diseases such as occlusion of femoral arteries due to arterioscrelosis, since e-PTFE grafts more often develop intimal hyperplasia at distal anastomotic sites than fabric grafts. In the field of blood access, the intimal hyperplasia problem with e-PTFE grafts has also been discussed for some time[1]. Other disadvantages of e-PTFE grafts are excessive bleeding after needle puncture, perigraft hematoma formation, seroma formation, and aneurysmal dilatation after repeated punctures by a hemodialysis needle[10-12]. On the other hand, knitted fabric vascular prostheses are not used due to their low patency rate caused by the thrombogenic properties of Dacron fabric, even though the fabric grafts have other properties that make them superior to e-PTFE grafts, such as elasticity, softness, easy suturing, flexibility, easy punctureability, and quick hemostasis[13].

The most frequent problems with the e-PTFE graft for blood access are prolonged bleeding after puncture and the intimal hyperplasia at the distal anastomotic sites after long-term implantation[1]. In this communication, we report a new fabric vascular prosthesis coated with negatively charged collagen which can absorb a large amount of water. The graft is extremely hydrous compared with the hydrophobic properties of the control e-PTFE graft, but is expected to be non-thrombogenic and to have quick hemostasis at needle puncture sites when used for blood access. Because the observation period of the current experiment was short, we could not assess intimal hyperplasia, but we present an evaluation of puncture tests and hemostasis.

Materials and methods

1. graft preparation

Aseptically purified and succinyated atelocollagen[14,15] (Koken Co. Ltd., Tokyo, Japan) was used as a sealant, dispersed in distilled water at 40 °C, pH 7.0, to make a collagen suspension.

A porous Dacron fabric vascular prosthesis (MICRON, InterVascular S.A., Clearwater, Florida, U.S.A., 6 mm ID, water permeability: 1200 ml / min. at 120 mmHg) was used as a framework of the graft. It was enveloped in a vinyl chloride bag and connected with a syringe containing the succinylated collagen suspension, by a three-way stopcock and a connecting tube

to make a closed circulation system[16]. Collagen suspension was sieved through the prosthetic wall by pressurized injection from the syringe. The suspension that passed through the wall was injected again with the syringe. After repeating the procedure several times, the interstices of the Dacron fibers of the prosthesis were impregnated with collagen. Then the prosthesis was lyophilized and thermally crosslinked[17,18] at 130 °C for 40 hours, simultaneously sterilizing the graft. With this procedure, a prosthesis impregnated with succinylated collagen (SC graft) was prepared.

An e-PTFE graft (Gore-Tex graft, W.L.Gore & Associates, Inc. Flagstaff, Arizona, U.S.A.) was used as a control.

2. Analysis of the SC graft

Water leakage from holes created by an 18 G needle puncture in vitro under water pressure of 120 mmHg was measured at 6 puncture sites according to the water permeability test for vascular prostheses[19]. Under internal water pressure of 120 mmHg, the graft was punctured with an18 G needle at a 30 ° angle to the graft external surface. One minute after the puncture, the needle was withdrawn. Amount of water leakage through the needle hole was measured, and the average leakage of water through the 6 puncture holes was calculated. The same procedure was performed with the control e-PTFE graft.

3. Implantation of the prostheses

Adult mongrel dogs of both sexes, weighing 8 to 18 kg, were anesthetized with an initial dose of 40 to 75mg of ketamine hydrochloride (Sankyo Pharmacy Co. Ltd., Osaka, Japan) intramuscularly and 8 to 15 ml of pentobarbital (2.5 % solution) intravenously. Supplemental doses were given when required. The dogs were intubated and supported with a respirator at 20 cycles per minute and a tidal volume of 150 to 250 ml of 20 % oxygen. The peritoneal cavity was entered via a midline incision at the center of the abdomen. The abdominal aorta was exposed and mobilized by severing two pairs of lumber arteries and the inferior mesenteric artery. Five cm of the aorta was resected and replaced by the SC-graft (internal diameter, 6 mm; length, 5.5 to 6 cm). Continuous suturing with a 5-0 polyester multifilament suture (Nihon Shoji Co. Ltd., Osaka, Japan) was used. During the operation, an antibiotic (1 gm

Cephalosporin, Fujisawa Pharmacy, Osaka, Japan) was administered into the peritoneal cavity, but no anticoagulants were used at any time. The control grafts were also implanted in the same manner.

The time required to establish hemostasis at the puncture sites was measured at 6 sites on 6 SC grafts and 6 control grafts. The implanted graft was punctured with an 18 G needle at a 30 ° angle to the graft external surface. After 1 minute, the needle was withdrawn. Digital pressure was applied to the puncture hole, and every half minute the finger was slightly lifted to observe the coagulation at the puncture hole, and the time required to establish hemostasis at each puncture hole was recorded. The mean time at the 6 puncture sites was calculated.

After the hemostasis test, both the SC grafts and the controls were left in the abdominal aortic position in order to observe the healing process at the puncture site and the whole luminal surface.

All animal care was in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 80-23, revised 1985).

4. Explantation of the prostheses

Six specimens were removed at 1 hour, and 6 at 1 week after implantation from both the SC graft groups and the control groups. Before harvesting, heparin sodium (100IU/kg) was administered intravenously to prevent clotting. All the retrieved specimens were rinsed with saline solution to remove excess intraluminal blood and examined macroscopically.

5. Histological examination

For light and scanning electron microscopic observations, 4 mm sections of the prostheses were cut longitudinally from the proximal to the distal anastomoses. Each sample was cut into seven pieces, and identifying marks were placed from the proximal to the distal area to observe the entire length of the graft in sequence. The specimens were fixed with 10 % formaldehyde in phosphate buffer 0.2 mol/L, pH 7.4. Tissue for light microscopic examination was embedded in hydrophilic resin[20]. Sections were stained with hematoxylin and eosin, with the PAP

method for the staining of factor VIII of endothelial cells, and with Von Kossa staining for detection of calcification.

Sections for scanning electron microscopic observation were stained with a 1 % osmium tetroxide solution, dehydrated in a graded series of ethanol and amyl acetate, and then critical-point dried with carbon dioxide and sputter-coated with gold palladium. Examination was performed with a S-800 scanning electron microscopy (Hitachi, Tokyo, Japan; accelerating voltage; 15kV).

Results

1. Preparation of the SC grafts

Impregnation of the prosthetic wall with collagen was easy. After several repeated injections of the collagen suspension through the prosthetic wall, the collagen was trapped in the interstices of Dacron fibers of the prosthesis. After lyophylization, the graft become completely white. Thermal treatment made the coated collagen insoluble from the graft wall.

2. Prepared SC graft

The SC grafts were slightly rigid, white, and porous-looking when dry. However, once a graft dipped into saline solution, it absorbed water quickly and became soft and pliable (Fig. 1a, 1b).

3. Puncture test in vitro

Figure 1 showed an 18 G needle puncture on an SC graft and on an control. Fig. 1b shows both grafts after the needle removal. The hole on the SC graft was approximately 0.3 mm in diameter, but the hole on the control was approximately 1.2 mm. There was no remarkable resistance to the needle puncture on either graft. Water leakage through the holes was measured under pressure of 120 mm Hg at 6 puncture sites, and was 34.5 ± 29.9 ml/min. in the SC graft and 169.9 ± 38.5 ml/min. in the control e-PTFE graft.

4. Scanning electron microscopy

Holes created by the 18 G needle on the SC graft and on the control are shown in Figures 2a and 2b. Both photographs were taken at the same magnification. The control graft has a

large, round hole approximately 1.2 mm in diameter in it. The thickness of the graft wall was approximately 0.4 mm. The entire cut edge of the graft wall with a smooth surface is seen through the hole. In the SC graft, there was a small tear of 0.2 to 0.6 mm in width with a rough edge of cut and frayed Dacron fibers with broken collagen. The luminal surface of the control was composed of the ordinary expanded PTFE structure with numerous nodules and micro-fibrils. The luminal surface of the SC-graft was Dacron fibers coated with collagen with many holes of various sizes, i.e. the lumen was porous and rough (Fig. 2b).

5. Graft implantation and puncture test in vivo

Implantation of both the SC graft and the control graft was easy without any problems. The SC graft wall was soft and pliable, and easy to suture. There was no bleeding through the graft wall or the suture holes in the SC-graft. The control graft also showed no bleeding through the graft wall, But, there was a little bleeding through the suture holes just after implantation. This was controlled within two minutes without any treatment.

Average hemostatic time at 6 puncture sites on each SC-graft was 4.5 ± 2.5. In the control grafts, the average time was 34.2 ± 11.5 min.

6. Graft retrieval

After the puncture test, there was no bleeding problem at the puncture sites or the suture lines on either SC grafts or the control grafts. A small thrombus adhered to each puncture site on the graft outer surface (Fig. 3a). The luminal surface of the SC grafts at one hour was completely covered with a thin red thrombus layer. The puncture sites observed from the luminal surface were seen as small irregular spots, but there was almost no difference from the surrounding areas (Fig. 4a). The control grafts, a thick red thrombus layer covered the whole luminal surface, and the puncture sites could not be seen. (Fig. 4b).

One week after implantation and puncture, the luminal surface of the SC grafts was covered with a thin fibrin layer. A small thrombus at each puncture site was seen from the luminal surface through a thin thrombus layer, but not all the puncture sites could be seen (Fig. 4c). In the control grafts, the luminal surface was covered with a thick thrombus layer, and the puncture sites underneath the thrombus layer could not be seen (Fig. 4d).

7. Microscopical observation

One hour after puncture, the luminal surface of the SC grafts was covered with a thrombus layer approximately 0.1 mm thick. Needle holes at the puncture sites were completely occupied by fresh thrombus composed of fibrin, platelets, erythrocytes, and leukocytes (Fig. 5a). The coated collagen was present on the luminal surface, on the outer surface, and in the interstices of the Dacron fibers, i.e., inside the graft wall. But there were many vacant spaces in the collagen layer, i.e., it had numerous pores. In the control grafts, the luminal surface at one hour was covered with a thick thrombus layer approximately 1.2 mm thick. The puncture hole was also completely occupied fresh thrombus (Fig. 5b).

After one week, the SC grafts were enveloped by loose connective tissue from the outer surface. There was no perigraft hematoma, granuloma, scar, seroma, induration, or other unusual reaction around the grafts. The luminal surface of the SC grafts was covered with a thin fibrin layer 0.2 mm thick which contained a small number of erythrocytes. Small numbers of fibroblasts had already migrated into the interstices of the Dacron fibers. The luminal surface was composed of a fibrin layer without endothelial cells. There was no capillary ingrowth inside the graft wall. The puncture site had no foreign body reaction. At the anastomotic site, endothelial cell lining from the host aortic wall could be seen continuously beyond the suture line. Some fibroblasts had migrated underneath the endothelial cell layer. The coated collagen was thin and had started to be absorbed at one week. In the control groups, three out of 6 grafts had perigraft hematoma. There was no adhesion between the graft and the surrounding connective tissue. The luminal surface was still covered with a fresh thrombus layer 0.5 mm thick. There was no fibroblast migration inside the graft wall. At the puncture site, the needle holes were occupied by a fresh thrombus which contained a small number of fibroblasts, but no capillaries.

Discussion

Compared with the controls, it was evident from the results that the greatest advantage of the SC graft was the small tear-like hole created by the needle puncture, followed by quick

hemostasis at the site. Another characteristic property of the SC graft was that its luminal surface was less thrombogenic.

The time required to achieve hemostasis after the needle puncture on the SC grafts was approximately one-eighth of that on the controls. This was due to the difference in the shape, size, morphological structure, and materials of the hole and its wall. It has already been pointed out that a needle puncture makes a large hole on an e-PTFE graft[7,21]. The raw material of the e-PTFE graft is easily deformed. Therefore, the hole retained its original size after the punccture. On the other hand, in the SC graft, the fabric structure was soft and pliable because the collagen sealant retained a large amount of water[22]. The collagen network as shown in the SEM photographs would accelerate hemostasis, since collagen fibrils can easily accumulate platelets. The three-dimensional structure of native collagen is thrombogenic due to aggregation of platelets[23-25]. The hydrous collagen network produced by negatively charged collagen made the coating substrate soft and pliable. Therefore, the needle enters the collagen network by expanding it, resulting in a small hole after the needle removal due to re-swelling of the collagen network and flexibility of the Dacron fibers. The hole created by the needle puncture become a small tear after the needle puncture and had irregular edges composed of polyester fibers and collagen fibers. The small tear with the irregular edge would be effective to shorten the time of hemostasis.

The luminal surfaces of both the SC graft and the controls at one hour and one week after implantation were completely different. The SC grafts were less thrombogenic than the controls. In general, collagen induces platelet aggregation because platelets are negatively charged. However, the modified collagen is less thrombogenic[26]. Collagen fibers in the SC grafts were negatively charged and modified by succinylation. The collagen layer on the SC grafts had less opportunity to be exposed to the blood stream since the luminal surface was hydrous because of the water trapped around the collagen fibers. Water does not enhance platelet aggregation nor fibrin deposition. Consequently, the hydrous surface of the prosthesis can prevent platelet aggregation and fibrin deposition. The hydrous surface on the SC grafts was non-thrombogenic, effectively preventing excessive thrombus adhesion after implantation.

Fabric vascular prostheses without collagen coating have not been used as A-V shunt grafts because of their thrombogenicity. In the present experiments, the hydrophilic collagen coating showed the possibility of overcoming the problems with fabric vascular prostheses for A-V shunt grafts.

The e-PTFE grafts used as controls have been reported to have less thrombus[9,27] due to the non-adhesiveness[22,29] of the original raw material. Thrombus forming on an e-PTFE graft surface is expected to detach itself from the surface, maintainaning the graft patency. However, the surface has numerous cracks, nodules, and microfibrils to give the graft flexibility by expanding. These provide anchoring sites for the thrombus on the luminal surface. The thick thrombus layer on the luminal surface at one hour in the present study supports this hypothesis. On the other hands, the walls of the puncture hole were relatively smooth, as shown in the SEM photographs, due to the plasticity of the PTFE. The thrombus inside the hole needs anchoring sites for hemostasis. But there were almost no anchoring sites on the walls of the hole, as shown in Fig. 2b. The thrombus inside the hole is not stable.

During clinical hemodialysis, heparin is usually used systemically to prevent thrombus formation in the hemodialyser. A 14 or 16 G needle is used for the puncture. A thrombus layer does not form on the graft luminal surface or inside the needle hole. A certain period of time after implantation, however, new adventitial tissue composed of fibroblasts and collagen fibers with microcapilaries is created around the graft. This tissue forms thrombus at puncture sites, resulting in hemostasis. Therefore, in general, ePTFE grafts are not allowed to be punctured for 2 weeks after implantation, during the term of adventitial formation, i.e., the term for encapsulation of the graft by the host tissue. This waiting time for e-PTFE grafts is also a problem for patients, especially in emergency cases. Even if new adventitial tissue is formed after a time, the excessive bleeding may cause a perigraft hematoma.

In conclusion, the newly developed fabric prosthesis with a negatively charged collagen coating has been shown to be an improved A-V shunt for blood access. The intimal hyperplasia at the distal anastomotic sites has not been studied in the present workdue to the short

observation period. We are presently evaluate long-term patency and internal hyperplasia at the distal anastomotic sites of the SC graft.

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Figure legend

Fig. 1. Puncture of SC graft (top) and ePTFE graft (bottom) with an 18 G needle(left), and after the puncture (right).

Fig. 2. Scanning electron microscopical observations of the puncture sites in SC graft (left) and ePTFE graft (right).

Fig. 3. Outer surfaces of SC graft (left) and ePTFE graft (right) 1 hour after puncture.

Fig. 4. Luminal aspects of SC grafts (left top and left bottom) and ePTFE grafts (right top and right bottom) 1 hour (left top and right top) and 1 week (left bottom and right bottom) after puncture.

Fig. 5. Photomicrographies of cross section of SC graft (left) and ePTFE graft (right) one hour after puncture. Thin thrombus covered the luminal surface on SC graft (left). In the puncture hole, small numbers of the Dacron fibers trapps the thrombus. Thick thrombus forms on the luminal surface and inside the puncture hole on ePTFE graft (right)

ま と め

鋼材でも丸棒よりH型綱やコの字鋼材が強度面より使用され、全体的構造ではハニカム構造が利用されているように、細くした繊維にさらに表面のマイクロ加工を加えることにより強度を増し、これの立体構築によって細胞との親和性をも制御可能となってきた。

また一方次世代の医療技術として低侵襲治療が考えられ、人工血管植え込みでもカテーテルを用いた人工血管の血管内留置が考えられている。しかし、従来の繊維の太さの人工血管ではカテーテル内に挿入することに限りがある。しかるに超極細繊維を用いると強度を維持しつつも少量化が可能となるため、人工血管を用いる低侵襲治療としてはこの技術の他に考えられない。これは低侵襲治療型次世代人工臓器創成の大きな武器となりうる。

この度の合成高分子材料のマイクロ加工、マイクロ加工化の研究成果より、当初予期した細胞適合性の発揮、そしてそれによる生体機能賦活化材料の創出の糸口をつかみ、その成果の一部も明らかとなった。そして、今後はさらに低侵襲治療型次世代人工臓器への道を歩みだしたと我々は考えている。

第2章

生体高分子材料の生体親和性処理

はじめに動物や人体から得た臓器には今日の科学技術を駆使しても作り得ない微細な構造的機能的特徴があるが、従来はグルタールアルデヒドの如き毒性の強い化学物質で処置する以外、その構造維持、免疫反応抑制方法は無かった。しかし、その処理により細胞親和性はなくなり、その特徴を生かすことができなくなっていた。一方我々は毒性が少なく、親水性をも高めることができる化学処理薬を医用に導入するための糸口をつかむことができた。これより、動物や人体から得られる臓器をはじめ生体高分子材料すべてが人工臓器用素材として転用できる可能性が出てきた。

このような背景のもとに、我々は動物の血管をこの処理方法を用いて処理し、人工血管に応用し、その組織反応を観察したので報告する。

生体高分子材料を人工臓器用素材として使用する場合、それらを自家組織移植として用いる場合を除いて、一般に何らかの処理を行う。同種移植の場合で細胞成分を余り含まないような組織においては、その処理は単なる低温処理、すなわち −80〜−150℃程度に冷却することにのみで、臨床的に使用されているものもあるが、これらは例外的であって、他のすべての生体由来材料は、その抗原性を低下させるため、また強度を増すため、および植え込み後の生体内劣化を防ぐ目的で化学薬品を用いて架橋処理を行う。臨床で使用されている生体由来材料、例えばブタの心臓弁とか心脳膜などはグルタールアルデヒドを用いるのが一般的であり、そのほかの処理方法としては、フォルムアルデヒド処理、ヘキサメチレンジイソミアネイト処理などがある。これらは抗原性を低下させること、強度をもたせ生体内での劣化を防ぐ目的では優れた成績を挙げてきた。しかしながら、その欠点として、それらの薬品の毒性が挙げられる。またさらにこれらの化学薬品で処理すると、材料が硬化し、生体由来材料特有の柔軟性を失う。またさらにこれらの処理によって材料は疎水性となって、細胞との親和性を低下させる、もしくは失ってしまう。このうち細胞毒性については米国アリゾナ大学のクバピル教授による詳細な研究があって、架橋処理に用いたグルタールアルデヒドが植え込み後数年経過しても、まだ材料から除放出され続けており、これによって宿主細胞が材料に近づけない、または材料内に侵入できない状態、つまり細胞非親和性状態が続くといわれている。事実、グルタールアルデヒド処理に生体由来材料への細胞の付着、侵入はきわめて悪い。

しかし、心臓弁のように弁上に細胞や組織が付着しない方が好ましいような場合に使用される時にはこの不便さは感じないが、たの部位に使用されれば、例えば人工血管や人工心外膜、人工胸腹部壁などのような組織の付着が要求される場合においては好ましくない。

このような背景にあって我々はエポキシ基をもつ親水性の架橋剤を開発した。これをもとにこの度は細胞との親和性を検討する目的でその架橋剤の特性を検討した。

1. エポキシ化合物による生物系材料の架橋法とその特性

生物系材料は、最近、医用材料としての応用けんきゅうが盛んに行われている。特に、コラーゲンは哺乳類の結合組織に普遍的に分布しており、細胞の基質として多彩な役割を担っているので、医用材料として期待されている。しかし、コラーゲンを医用材料として応用する場合、成型加工性が合成高分子に比べ劣っている。生体内での安定性向上の観点から、生体内での安定性向上の観点から、コラーゲンを主体した材料には、架橋処理が行われることが多い。そして架橋剤としてのグルタルアルデヒドが最も広く使われているが、材料の柔軟性の維持・強度の向上という面からはあまり好ましくない。グルタルアルデヒドの欠点を補う架橋剤として、ポリエポキシ化合物が優れた特徴を持つことを見出した。すなわち、ポリエポキシ化合物で架橋を導入した材料は、柔軟性を失わずに強度が向上し、生体内で比較的長期間安定に存在するが、いずれ吸収されてしまうことがわかった。

1) 目的

生物系医用材料、特にコラーゲンを主体にした医用材料を人工臓器の材料として応用する場合、その形状の維持、強度及び柔軟性の調節などが技術的に第一に問題になる点である。これらの力学的な側面からの要請により、コラーゲンに架橋を導入することが、しばしば行われる。また、生体内での分解吸収を遅らせたり、コラーゲンの側鎖のマスクなどのためにも架橋処理が行われる。架橋処理の方法としては、紫外線・γ線などを使用する方法や架橋剤を使用する方法などが目的に応じて使われている。これらのうち、グルタルアルデヒドで架橋する方法が一番多く使われてきた。グルタルアルデヒドは、医用材料の処理に広く用いられており、架橋剤として優れているが、グルタルアルデヒドでコラーゲン線維を処理すると、材料の柔軟性が失われてしまうという欠点がある。目的によってはその方が良いかもしれないが、柔軟性が要求される人工臓器には好ましくない。特に人工血管などの様に、柔らかさが重要な要素になるようなものには適さない。一方、ポリエポキシ化合物は、繊維産業などで良く使われており、種々の化合物が市販されている。これらの中には、材料に柔軟性や親水性を付与する性質のものがある。また、ポリエポキシ化合物はエポキシ基を持つので、グルタルアルデヒドよりも広い反応性をもっている。これらのことから、ポリエポキシ化合物をコラーゲンの架橋剤として使用した場合の特性を検討した。

2) 方法

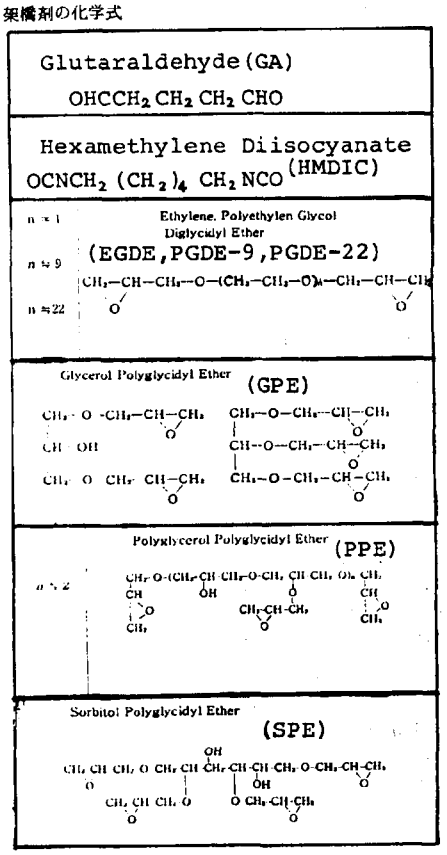
I. 架橋剤

ポリエポキシ化合物としてのエチレングリコールジグリシンジルエーテル（EGDE）、及びポリエチレングリコールジグリシンエーテル（PGDE-9,22）を取りあげた。これらは、材料に親水性を付与するといわれている[1)]。この他のポリエポキシ化合物として、グリセロールポリグリシジルエーテル（GPE）、ポリグリセロールポリグシジルエーテル（PPE）、ソルビトールポリグリシジルエーテル（SPE）を用いた。以上の試薬は、ナガセ化学工業（株）のものを使用した。

また、比較対照する架橋剤として、グルタルアルデヒド（GA）、及びヘキサメチレンジイソシアネート（HMDIC）を取りあげた。GA及びHMDICは和光純薬製のものを

使用した。架橋剤の化学式を表1に示した。

表1. 架橋剤の化学式



II. 試料

試料として、牛の心膜、アテロコラーゲンのフィルム及びスポンジを使用した。牛心膜は、脂肪を取り除いた後生理食塩水で良く洗い、0.01％フィシン（PH7.0）で処理し、エタノール、エーテルで脱脂して凍結乾燥した。また、凍結乾燥処理をしない試料も用意した。アテロコラーゲンフィルムは、牛真皮からペプシンで抽出したアテロコラーゲンを1％濃度で溶かし（pH3）、テトロンメッシュの上に1 cmの厚さで流し込み、風乾することにより作製した。アテロコラーゲンスポンジは、1％アテロコラーゲン酸性溶液を中和、ホモジナイズしてテトロンメッシュの上に5mmの厚さで流し込み、凍結乾燥することにより作製した。

III. 反応

GA及びポリエポキシ化合物は、10％NaCl水溶液又は50％メタノール水溶液に溶解して使用した。架橋剤の濃度は、反応性に違いがあるので0.01～20％の範囲で変化させた。溶液のpHは6～13、温度は、コラーゲンが変性しないように20～30℃で反応させた。反応率は未反応のε-NH^2基をTNBS法[2)]で測定することにより求めた。まず、試料5～10 mgを試験管にとり、4％$NaHCO_3$を1ml、1％TNBS（ピクリルスルホン酸ナトリウム）を1ml加え、40℃で2時間反応させる。次に濃塩酸3mlを加え110℃で1時間分解する。冷却後、ジエチルエーテルで余分なTNBSを抽出してから345 nmの波長で吸光度を測定する。架橋処理していない試料（コントロール）の吸光度をA、それぞれの試料の吸光度をBとしたとき、反応率は｜（A-B）／A｜×100％で表した。

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IV. 物性

心膜について、力学的強度と柔軟性を検討するため、引張強度及び伸び率を測定した。架橋剤で処理した心膜は、リン酸緩衝生理食塩水（PBS, pH7.4）に浸漬し、濡れた状態で測定を行った。引張強度は、TBS 式抗張力試験機で測定した。伸び率は加重をかけないときの試料の長さを　、加重をかけたときの試料の長さを　としたとき、

｜（　－　）÷　｜×100（%）で表した。また、試料の物性の一つとして、熱収縮温度を測定した。試料は、心膜を用い、PBS に浸漬した状態で 1℃／10min の割合で温度を上げ、熱収縮温度を求めた。試料の元の長さを L_1、完全に収縮したときの長さを L_2 としたとき、（$L_1 + L_2$）／2 の長さになったときの温度を収縮温度とした。

V. 組織反応

架橋剤の組織適合性を検討するため、架橋剤で処理した試料を犬に植え込んだ。試料は、アテロコラーゲンフィルム及びスポンジを、それぞれ 1cm × 3 cm に切ったものを用いた。試料の犬の背部皮下に植え込み、1, 3, 4, 6 週後に取り出して、10 %ホルマリンで固定後パラフィンに包埋した。薄片をヘマトキシリンーエオジンで染色し、光学顕微鏡で観察した。

3) 結果

図 1 に、EGDE と PGDE - 22 の反応率の濃度・時間変化を示した。分子量が大きい方が反応性が低いことがわかる。次に、PGDE - 22 とGA の反応率の pH 変化を図 2 に示した。PGDE - 22 は、ph12 以上で良く反応し、GA は pH があまり高いと GA 同志で重合するために反応率が下がっている。

PGDE - 22 及び GA で処理した心膜の引張強度、収縮温度を表 2 に示した。PGDE - 22 の引張強度は、反応率が低いときにコントロールよりも大きく、反応率が高くなると低下した。GA の引張強度は、反応率に関係なくほぼコントロールと同じ値を示している。収縮温度は、架橋処理によってコントロールよりも高くなった。GA とPGDE - 22 の収縮温度を比較すると GA のほうが全般的に高くなっている。表 2 の実験に使用した心膜は、凍結乾燥した試料を架橋処理したものである。次に、ポリエポキシ化合物、GA、HMDIC で架橋した試料について引張強度、伸び率を測定した結果を表 3 に示した。この実験に使用した心膜は、凍結乾燥などの乾燥処理やエタノールなどでの急激な脱水は、全く行われなかった。引張強度は、架橋を導入することによって向上した。また、ポリエポキシ化合物は、GA や HMDIC に比べて高い強度を与えることがわかる。伸び率は、GA とHMDIC がコントロールよりも低下しているのに対し、ポリエポキシ化合物で処理した試料はコントロールと同じかそれよりも大きな伸び率を示した。特に、PGDE - 22 及び GPE, PPE が大きな伸びを示している。表 4 、図 3 ～ 6 に皮下挿入試験の結果を示した。異物反応率は、（－）～ (卅) の 6 段階で示した[3]。（－）は、細胞が植え込み材料をとくに異物として認識しない状態。（±）は、（－）と（＋）の中間の状態。（＋）は、細胞が異物として認識し、周囲に異物性巨細胞や類上皮細胞が集まっているが、異物を取り囲む細胞の層が 10 層以下で異物反応が軽微な状態。(卄) は、（＋）と (卅) の中間の状態。(卅) は、顕著な異物反応を示し、細胞が幾重にも集ま

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り塊を形成している状態、(卌) は、(卅) より強く、細胞毒として作用し、周囲の細胞が壊死に陥る状態を示す。また、＊を付したものは吸収像が観察されたもの、＊＊は完全に吸収されてしまったものを示す。コントロールのフィルム及びスポンジは、1週間程度で吸収されてしまった。EGDE、PGDE-22、GA で処理した心膜及びフィルムは、あまり異物反応は強くなくコントロールとほぼ同じ程度であった。また、これらの架橋剤で処理したスポンジは、植え込み後初期の強い異物反応を示した。EGDE で処理した試料は、PGDE-22 や GA で処理した試料より吸収が速い傾向がある。表4及び図3～6に示した実験に使用した心膜は、凍結乾燥処理をしたものである。

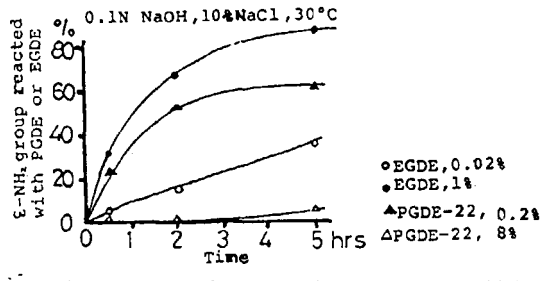

図1. (ポリ)エチレングリコールジグリシジルエーテルの反応率の濃度及び時間変化(試料は心膜)

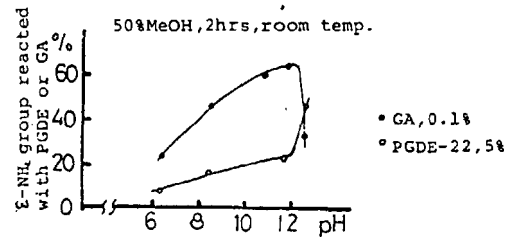

図2. ポリエチレングリコールジグリシジルエーテルとグルタルアルデヒドの反応率のpH変化(試料は心膜)

	ε-NH_2 group reacted (%)	tensile strength (kg/10mm²)	shrinkage temperature (°C)
control	0	10.5±0.4	58.0
PGDE-22	7	17.9±7.4	58.2
	38	13.0±0.4	58.6
	62	9.4±1.9	64.1
	95	2.0±0.7	85<
GA	11	12.5±5.0	71.4
	41	10.0±3.0	72.0
	56	11.7±2.7	75.0
	95	10.4±7.1	85<

表2. ポリエチレングリコールジグリシジルエーテル及びグルタルアルデヒドで処理した牛心膜の引張強度・収縮温度

	ε-NH_2 group reacted (%)	tensile strength (kg/10mm²)	elongation (%)
CONTROL	0	3.5	60
GA	48	5.2	53
HMDIC	50	4.5	49
EGDE	22	8.7	62
PGDE-9	53	13.3	60
PGDE-22	17	9.9	72
GPE	11	15.3	74
PPE	14	13.6	100
SPE	21	8.6	68

表3. ポリエポキシ化合物、グルタルアルデヒド、ヘキサメチレンジイソシアネートで処理した牛心膜の引張強度・伸び率

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4) 考察

ポリエポキシ化合物で処理した心膜は、グルタルアルデヒドやヘキサメチレンジイソシアネートに比べて引張強度が大きい。引張強度は、凍結乾燥した試料と乾燥していない試料で違っている。これは、凍結乾燥によって心膜の組織が変形してしまったためと思われる。ポリエポキシ化合物は、反応率が高いと逆に強度が低下するので、ε-アミノ基の反応率が60％以下のところで使用した方が良いことがわかった。また、グルタルアルデヒドやヘキサメチレンジイソシアネートで処理した試料は、伸び率が低下したが、ポリエポキシ化合物で処理した試料はコントロールと同じかそれ以上の伸び率を示した。これは、ポリエポキシ化合物で架橋を導入しても線維の状態が天然の状態を維持しているためと思われる。しかし、高い反応率のところで低下するのは、ポリエポキシ化合物により親水的になりすぎて線維が水を含み膨張したためと思われる。また、収縮温度は、ポリエポキシ化合物の方がグルタルアルデヒドよりも低くなっている。これもポリエポキシ化合物で処理したものの方が、より天然の状態に近いことを示している。従って、ポリエポキシ化合物で架橋を導入すると、材料の性質を損なわずに強度を向上させることができる。特に柔軟性に優れている。

ポリエポキシ化合物の架橋剤としての組織反応性は、6週間までの間でほとんど問題はなかった。スポンジの試料では、初めに顕著な異物反応があったが、これは組織に接するスポンジの表面積が非常に大きかったためと思われる。架橋処理によって、材料の吸収性が低下し、生体内で長期間安定に存在することがわかった。

5) 結論

ポリエポキシ化合物は、コラーゲンを主体とした生物系材料の架橋剤として、グルタルアルデヒドなどに比べ次の点で優れている。

1. 材料の強度を大きく向上させることができる。
2. 材料のもつ柔軟性を失うことがない。
3. 天然の組織の性質を維持したままで架橋することができ、異物反応も少ない。

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2. 架橋処理された生物材料を枠組みとした宿主細胞による器官再構築

架橋処理された生物材料が、生体内に植え込まれたあと材料内部で器官再構築の生じることを示した。一般に、生物材料が植え込み用人工臓器の素材として用いられる場合、材料はグルタールアルデヒド（ＧＡ）処理されていた。しかしＧＡ処理をうけた材料は硬くなり、疎水性となって、宿主細胞の材料内への侵入は容易でなかった。我々は、ＧＡに代って、エポキン基をもつ親水性架橋剤を生物材料処理に導入し、動物から取った頚動脈を素材として、細口径人工血管を作成した。その長期開存例をみると、人工血管壁内部に線維芽細胞や平滑筋様細胞が侵入し、新しく血管壁を形成していた。しかも、天然の血管壁構造と酷似した構造を再構築していた。一方、ＧＡ処理し、同様の実験を行った対照群の血管には、このような現象は認められなかった。このことから、器官の再構築は、用いた材料のマトリックスとその物性、及びそれの置かれた環境等が合わさって良好な状況を生み出したときに生じるものと思われる。

1）はじめに

人工臓器用素材として、合成高分子材料、金属材料、無機材料、生体高分子材料（生物材料）等が広く用いられており、これらの材料と生体間の界面現象解明も進められている。これらのうち、生物材料にあっては、人工心臓弁のような例を除き、多くは、宿主細胞が材料内部へも侵入し、材料と生体とが一体化し材料の素構築に基づいて材料の置かれた環境に順応した器官を再構築することで、宿主内で安定化し、人工臓器としての機能を発揮することが期待されている。しかし実際には材料内部への細胞の侵入、そしてそれに引き続く器官の再構築が認められるのはまれである。その原因の１つに、材料のグルタールアルデヒド（ＧＡ）処理が挙げられている。ＧＡ処理は、材料の強度を増し、生体内での分解、吸収性を低下させ、結果として、抗原性も低下させることで広く用いられている。しかし、我々はＧＡ処理による利点と欠点を理解した上で、その欠点を補うため、エポキシ基をもつ親水性架橋剤をＧＡに代って新しく導入し[1]、細口径人工血管を作成した[2]。この処理方法によると、材料はＧＡ処理をうけた時のような硬さにはならず、しかも親水性を保持することができる。この度は、この方法で作成した人工血管の長期開存例を検討した結果、人工血管壁内部に宿主細胞が侵入し、血管壁の再構築が生じていることを発見した。この現象は従来のＧＡ処理血管では決してみられなかった現象であるので、ここに報告する。

2）材料と方法

Ⅰ．人工血管の作成、および動物実験

成犬から得た新鮮な頚動脈を蒸留水中に２時間浸透し、血管壁内の諸細胞を浸透圧で膨潤させたあと、音波処理（40 KHz , 1060 W, 20 秒間）にて細胞成分を破壊し、コラーゲンと弾性板からなる天然由来の管（脱細胞管）を得た。次に2 %硫酸プロタミン水溶液を内腔に注入し、80～100 mmHg の空気圧で膨らませつつ、親水性エポキシ架橋剤；Denacol EX 512, PGPGE（ナガセ化成工業）、（5 % PGPGE の 0.1 M Na_2Co_3、100 %エ

4）考察

I. 材料による細胞との反応の相違

PGPGE管とＧＡ管の相違は、その架橋処理方法にある。両者は、ともに脱細胞管を架橋したものであるが、架橋方法の差によって両者の物性に大きな差が生じた。表1で明らかなように、ＧＡ管は硬く、PGPGE管は柔軟性がある。また、ＧＡ管が疎水性的性質を帯びているに対して、PGPGE管は親水性である。外見的にも、前者の色調が淡茶であるに対し、後者は白である。両者ともヘパリン化されている。両者の大きな差は、柔軟性と親水性の差にある。これは、ＧＡとPGPGEの分子上での差であって、同一物質を架橋しても、架橋剤の差でまったく性質の異なる材料が出来上がる。さて、その両者に対する細胞の反応であるが、材料内に侵入する数と種類、そして、侵入後の細胞配列に差がみられる。図7と図9を対比して明らかなように、PGPGE管には、多数の平滑筋様細胞と線維芽細胞が侵入していたがＧＡ管は線維芽細胞が主であり、その数もすくない。このような差は、材料の違いによる細胞反応の現れ方によるものと思われるが、さらにまた、材料の物性、例えば柔軟性の差が、侵入する細胞の種類やその配列をも支配することが考えられる。一般の布製人工血管においては、新生内膜を形成する内皮細胞と平滑筋様細胞に配列の規則性がある。すなわち、内皮細胞は、人工血管内を流れる血流方向に平行にその長軸を並べ新生してくるのに対して、平滑筋細胞は、新生血管壁全体にかかる張力、ひずみの方向に平行に配列することが判明している[5]、断裂したアキレス腱の再生時や、皮膚の傷の再生時にでも、再生する細胞は張力の影響をうけ、張力のかからない場では細胞配列に規則性が生じない[6]。このようなことから、PGPGE管ではＧＡ管に比べ、植え込まれていた間に、拍動ごとにかかる張力が人工血管の歪みを生じ、これが、平滑筋様細胞の侵入および、その配列に適した状態が作られていたと思われる。このように、同じ素材から作った材料でも処理方法によって、その物性が変化し、それに応じて細胞の反応も変化することは興味深い。

ところでPGPGE管における平滑筋様細胞の配列が一般の血管壁にみられる内側輪状、外側縦走状となったことは、単に、張力理論のみでは説明のつかない現象である。このような細胞による器官の再構築には、材料のおかれた場、環境、材料内部構造、その他すべての要因が作用し合って、その結果として行われるものと予測される。

III. 材料と環境と器官再構築

天然組織と同等の組織構造をもつ細胞組み込み型ハイブリットオーガンとして、Bellの人工皮膚[7]や人工血管[8]がよく知られている。この場合、皮膚真皮や血管壁の組織構造は、コラーゲンゲル中で培養する線維芽細胞や平滑筋細胞の働きにより形成される。すなわち、in-vitroにおける細胞培養による組織構築の例である。一方、修復しようとする組織の枠組み、すなわち、tenplateを欠損部

にインプラントして、templateないへの細胞や血管の誘導、配列を促し、生体のもつ修復力を有効に利用して組織再構築をはかることもできる。ここに発表した我々の例は、まさにこの例であるし、またYamas[9]のコラーゲンスポンジからなるskin templateもこの例にあてはまるものである。この場合、templateの構造や性質は、組織再構築に不可欠である。template内への細胞の誘導、配列に大きな影響を与える。本発表で示したよ

うに、天然組織に可及的近似の枠組み構造及び性質をもつようにtemplateをデザインすることが、理想的な組織再構築に最も重要である。

III. ヘパリン残存と血管壁再構築

植え込み後のヘパリン徐放連度を計測したこれまでの研究によると、PGPGE管もGA管においても同様に、約1ヶ月で90％以上のヘパリンが放出され、その後も引き続き減少することが明らかになっている。[2,10]。布製人工血管や結合組織管等で、無ヘパリン状態では植え込み後2週間で内皮細胞が吻合部付近に出現し、その後徐々に中央に伸展してくる[11,12]が、ヘパリン化されると内皮細胞による被覆は遅れる[13]。しかし、ヘパリンが放出され終わると、細胞の動きは活発化し、急速に血管壁構築が進行する[13]。本研究において、PGPGE管とGA管とは植え込み時のヘパリン含量に差があるものの、1ヶ月後には90％以上のヘパリンは放出され、45日目頃には諸細胞の侵入がみられることから、この時点でヘパリンは消失していると考えられる。従って、長期例でのPGPGE管とGA管との細胞活動の差はヘパリン残量によるものではなく、マトリックスの性質の差に基因するものと思われる。

5）おわりに

架橋処理されて、もはや無生物化した材料へ、これを足場として細胞が侵入し、器官の再構築を行う現象を人工血管において認めた。このように、細胞のもつ性質を引き出し本来の活動をさせて、目的とする人工臓器の機能を発揮させる方式は、新しい型の人工臓器の1つの道を示すものである。

稿を終えるにあたり、国立循環器病センター研究所、林　三郎部長に、血管壁の粘男性測定において、御指導をうけたことを記し、ここに深謝いたします。

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まとめ

以上の二つの研究結果より、本方法が従来のグルタールアルデヒド処理方法に比べて著しく優れた効果をもつことが明らかとなった。

本方法は現在臨床で用いられている生体由来材料製人工臓器をほとんどすべて変えてしまうほどの利点がある。生体由来材料の特性を生かし、細胞親和性を維持させておくことで生体内での長期安定性を獲得できる。これは、ハイブリット型人工臓器を設計する上においても重要な点であり、さらに、その上に最近話題のサイトカイン、成長因子などの効果を発現させるには、グルタールアルデヒドのような毒性の強い処理法方では、その効果が発揮できないことから、この度開発した方法の広い範囲での活用が期待できる。

第３章

生体内分解性プラスチックの医用への応用

我々は成分解性プラスティックのある種のものは生体に極めて近い弾性、柔軟性をもっていること、および生体内で徐々に分解されること、そしてさらに、その分解時に何ら異物反応を来さないことを発見した。これは今日臨床で用いられている生体内分解性材料の硬くてもろく吸収時に異物反応を示すのに比べ大きな利点である。このような特性をもつ成分解性プラスティックを用いることで新たな人工臓器の設計が可能となる。しかも、このポリマーを用いて前述のマイクロ加工を組み合わすことにより、生体機能を賦活化させうるし、目的を達成すると消耗したあとは生体の自然治癒にゆだねるという一時的な人工臓器も創造しうる。これは人工臓器の基盤技術としては前述のマイクロ加工と並ぶ技術革新と思われる。現在の公害対策用プラスティック用である生分解性プラスティックはバクテリアから産生させることもあって微量でも高価であり、まだ実用化途上であるが人工臓器用素材は少量で高価であることから、この関係の産業は将来が明るいと期待される。

この度は生体内分解性プラスチックとして、ポリエステルの一つであるP（3HB - co - 4HB）（3 - ヒドロキシ酪酸（3HB）と4 - ヒドロキシ酪酸（4HB）の共重合体を用いて、血液接触医療用材料としての応用および腹部欠損部に用いる抗癒着性膜としての応用の二つを取りあげて検討を行った。

なお、本材料の合成は日本ゼオン株式会社の化成品研究所、小松崎　茂博士との共同研究によるものであり、その合成は日本ゼオンにおいて、その動物を用いた評価は我々の大学で行ったものである。

1. バイオポリエステルの
血液接触医療用材料としての応用検討

1．緒言

1．1　目的

微生物が生産するポリエステルの一つであるP（3HB－co－4HB）（3－ヒドロキシ酪酸（3HB）と4－ヒドロキシ酪酸（4HB）の共重合体，図1に構造式を示す）は，弾性・柔軟性および生体適合性に優れた生体内分解性材料として，医療用途への応用の可能性が期待される．[1)]

非血液接触系の軟組織用医療材料としての応用検討を行った結果，組織を呼び込み器官を再建させる用途に適していると判断され，本実験ではそのような用途において臨床的価値の大きい血液接触医療用材料としての応用について検討する．

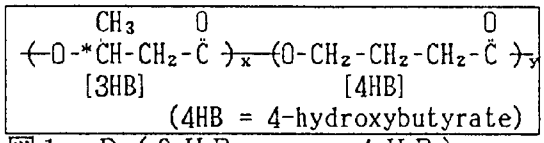

図1　P（3HB－co－4HB）

1．2　主な検討課題

血液接触系の器官再建としては主として人工血管，心臓・血管系パッチがあげられる．現在，人工血管，心臓・血管系パッチ等の血管修復材としては主にポリエステル繊維編織物や多孔性ポリテトラフルオロエチレンが使用されている．多孔性ポリテトラフルオロエチレンは屈曲性はあるが伸縮性，柔軟性に欠けるほか，人工血管内面に形成される新生内膜あるいは偽内膜（フィブリン等の血液凝固物のうすい層）の人工血管壁へのアンカリングが十分でない場合がある．また，ポリエステル繊維編織物は新生内膜あるいは偽内膜のアンカリングにすぐれており，編成方法によってかなりの伸縮性を付与することも可能であるが，漏血を防止するために有孔性を小さくする必要があり，伸縮性，柔軟性を有するものは実質的には使用されていない．

本研究ではポリエステル繊維編織物のアンカリング特性，伸縮性・柔軟性を生かし，かつ，実用的な非漏血性を有するものとするために，比較的有孔性の大きいポリエステル編物とバイオポリエステルを複合することを主な検討課題とした．これによりバイオポリエステルの弾性・柔軟性，生体適合性，生分解性，易加工性等の性質を役立てることができると考えられる．一般に有孔性の大きい人工血管ほど組織の侵入による器質化が良好であることが知られており，組織の侵入に応じてバイオポリエステルが分解していくことにより，有孔性の大きいポリエステル編物を枠組として良好な組織の再建が達成されることが期待される．

研究にあたってはバイオポリエステルの評価に関して継続して共同研究しており，また，この分野での世界的権威者である横浜市大・野一色泰晴講師に指導をお願いすることとした．

1．3　過去の研究例

ポリエステル人工血管とコラーゲン・ゼラチン等の生分解性物質を複合する試みは古くから行われ，最近市販された製品についての研究結果では良好であるとの報告もあるが，ゼラチンが剥離しやすいこと，製造工程上の問題（wet な状態ですべて無菌環境で製造されるが，工程中で菌が増殖する場合があり，最終工程で滅菌してもｴﾝﾄﾄｷｼﾝの問題が残る），架橋剤（ﾎﾙﾏﾘﾝ 等）の細胞毒性の問題等を指摘している報告もある．[2,3)] 心臓・血管系パッチとしては超極細ポリエステル繊維とコラーゲンを複合した実験例がある．[4)] これらのコラーゲン・ゼラチン系のものでは伸縮性を付与することはできない．また，やはり伸縮性はないがポリグリコール酸・ポリ乳酸共重合体等の脂肪族ポリエステルを利用する試みも行われている．[5)]

1．4　研究の進め方

野一色先生の意向を尊重して，まずこの分野での最難関課題である細径人工血管（内径6㎜以下）の検討を行い，その研究過程で得た成果をもとにしてより実用的な応用用途を探っていく方針とした．これには実験動物として主に犬を用いるため実験モデルを設定しやすいという側面も勘案されている．

ただし，実際問題としては細径人工血管はその技術的難度が非常に高い．より実現性のある用途としては，太径人工血管（すでに実績のある人工血管に漏血防止のためのシール材としてバイオポリエステルを使用し，ﾌﾟﾚｸﾛｯﾃｨﾝｸﾞ不要とする），人工腎臓透析患者の内シャント用人工血管（定常的に医師の監視下にあり，閉塞しても致命的ではなく交換が可能）等があげられる．本研究ではそれらの用途も念頭におきながら検討を進めていくこととした．

2．実験

2．1　材料

バイオポリエステル P(3HB-co-80%4HB)は当社培養生産品を使用した．ヘパリンナトリウム・塩化ベンザルコニウム錯体（ヘパリン錯体と略記）は文献[6]記載の方法にしたがって作成した．水溶性のヘパリンナトリウム をヘパリン錯体とすることによりクロロホルムに溶解することができ，クロロホルム溶液中でバイオポリエステル と均一に混合することが可能となった．卵黄レシチンは和光純薬工業製生化学用を使用した．

2．2　in vitro 血液適合性評価

内径５mm・長さ７cmのシリコーンチューブ （富士システムズ，ファイコンチューブ SH No.5）内面にバイオポリエステル クロロホルム溶液 （3 wt/vol%， ヘパリン錯体・卵黄レシチンを適当量添加する.）をコーティングし，充分に乾燥後，一端をコック（ピンチコック ホフマン式）にて封じて試料チューブとした．開放端を上方に向けて試料チューブを実験台上にセット し，チューブ内に生理食塩水を注入し，30分後に生理食塩水を除去した．ウサギ （日本白色種，♂，体重約2.5 kg）ネンブタール麻酔下で心臓穿刺法にて血液を採取し，直ちに各試料チューブ内に深さ３cm程度に注入した．所定時間（１～５分）放置後，チューブ内を生理食塩水にて洗浄し，２％グルタルアルデヒド 水溶液にて固定した．血液接触面より５mm角程度の小片を切り取り，イオン 交換水にて洗浄後，減圧乾燥した．血液接触面を試料台に固定し白金蒸着後走査型電子顕微鏡（ＳＥＭ）にて血小板・フィブリン の付着・変形を中心に観察した．

2．3　犬動脈内１時間挿入試験

各材料を内径３mm・長さ３cmのテフロンチューブ内面にコーティングし，麻酔下で犬頸動脈あるいは大腿動脈に挿入し，縫合糸にて外側から縛って固定した．１時間後取り出し，生理食塩水にて洗浄後，２％グルタルアルデヒド 水溶液にて固定し，血液接触面を前項と同様にしてＳＥＭにて観察した．

2．4　埋植用人工血管試料

内径3.5 mm，外径4.5 mmのポリエステル繊維製管（野一色先生より提供されたもの，有孔性大きい）をバイオポリエステル で処理して試料とした．バイオポリエステル 処理は次のようにして行った：①外径3.0-3.5 mmのテフロンチューブにバイオポリエステル クロロホルム溶液 （3 wt/vol% ， ヘパリン 錯体・卵黄レシチンを適当量添加する.）で１～２回コーティングする，②コーティングしたテフロンチューブの上に上述のポリエステル繊維製管をかぶせる，③一端をコック で封じた内径５mmのシリコーンチューブ 内にバイオポリエステル ジオキサン 溶液 （0.5 wt/vol%）を注入しておく，④③項のチューブ内に②項のテフロンチューブを挿入し泡抜きを手早く行った後ドライアイス/エタノール中で凍結させる，⑤一端を封じていたコック を外し（必要に応じてテフロンチューブも引き抜いて）凍結乾燥機内に入れ凍結乾燥する，⑥乾燥後シリコーンチューブ（および テフロンチューブ）を除去し所定の長さ（２～４cm）に切断して試料とする．

2．5　犬動脈内人工血管埋植試験

各試料を麻酔下で犬頸動脈あるいは大腿動脈に吻合した．動脈→静脈シャントの場合には動脈側は直線状となるように吻合し，静脈側はＴ字状となるように吻合した．所定期間埋植後（５～１９日），全身ヘパリン化し麻酔下にて脱血死させ試料を回収した．試料は生理食塩水にて軽く洗浄し切り開いて写真撮影後，10%ホルマリン 水溶液にて固定し，脱イオン 水洗浄にて脱ホルマリン 後，ヒドロキシエチルメタクリレート 樹脂包埋し (Reichert- Jung製 HISTORESIN 使用)，電動回転式ミクロトーム (Reichert-Jung 製 2055 AUTOCUT)を使用して厚さ5 μmの切片とし，ヘマトキシリン・エオジン染色あるいはトルイジンブルー染色して光学顕微鏡観察した．

2．6　埋植用パッチ試料

(1)ポリエステル伸縮布＋バイオポリエステル フィルム（貼り合わせ試料）

弾性伸度が約100 %のポリエステル製編物を裁断し，バイオポリエステル クロロホルム溶液(2 wt/vol %)中に浸漬したのち直径15cmのガラスシャーレ 上で溶媒を揮散させて，バイオポリエステル でコーティング伸縮布を作成した．バイオポリエステル クロロホルム 溶液(2 wt/vol %)40mlを直径15cmのガラスシャーレ 上に流延し徐々に溶媒を揮散させて厚さ約40μmのフィルムを作成した．このフィルムの上にバイオポリエステル でコーティング伸縮布をのせ上から軽く押さえた状態で90℃オーブン中で30分間処理した後，3×5 cmに切断して試料を得た．

(2)ポリエステル伸縮布の片面をバイオポリエステル多孔質とし片面をバイオポリエステル平滑面とした試料，

(1)で得た試料を直径5 cmのアルミカップの形状に合わせて切り取り，バイオポリエステル フィルム面を下にしてアルミカップ上に置き，90℃オーブン中で30分間処理し放冷してアルミカップに密着させた．その上にバイオポリエステル ジオキサン 溶液 (1 wt/vol %) 7 mlを注ぎ，直ちに-20 ℃の凍結乾燥機内の棚に入れ凍結させたのち，凍結乾燥して試料を得た．バイオポリエステル ジオキサン 溶液 (1 wt/vol %) のかわりに，卵黄レシチンをバイオポリエステル に対して5 wt% 含むバイオポリエステル ジオキサン 溶液 (1 wt/vol %) を使用して同様に処理してレシチン入り試料を得た．

(3)ポリエステル伸縮布の両面をバイオポリエステル多孔質とした試料

厚さ1 mmのアルミ L字材を使用して7×9 cmの長方形の型枠を作成し，バイオポリエステル コーティング伸縮布と15×13cmステンレス 製トレー 底面との間に1 mmの間隙ができるようにバイオポリエステル コーティング伸縮布を型枠にセット してステンレス 製トレー 上に置いた．バイオポリエステル ジオキサン 溶液 (2 wt/vol %) 38mlを注ぎ，直ちに-20 ℃の凍結乾燥機内の棚に入れ凍結させ，凍結乾燥した後，3×5 cmに切断して試料を得た．

2．7　犬右心パッチ実験

麻酔下において開胸し，犬の右心室の一部を切開し各パッチ試料を適宜トリミングして(1)試料ではバイオポリエステル フィルム側を，(2)試料では多孔質面側を，(3)試料では孔径100 μm程度の多孔質面側（凍結乾燥時に上側の面）をそれぞれ血液面側にして縫合し，閉胸した．所定期間（14～28日間）後に麻酔下に脱血死させ，開胸して試料を回収し，人工血管埋植試験と同様に処理して評価した．

3．結果

3．1　in vitro評価（Ⅰ）

新鮮血によるin vitro評価の結果，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ P(3HB-co-80%4HB)が積極的な血栓性の表面ではないことを確認した．また，ﾍﾊﾟﾘﾝ錯体を全体の2.5-40 wt %含ませることにより抗凝血性をある程度付与することが可能なこと，ﾍﾊﾟﾘﾝ錯体を10 wt %以上添加するとｺｰﾃｨﾝｸﾞ膜上でﾍﾊﾟﾘﾝ錯体が顕著に相分離をおこし血液との接触により容易にﾍﾊﾟﾘﾝ錯体が脱離して孔が残ること等が確認された．

3．2　犬動脈内1時間挿入試験〔ＤＡ－１，２〕

ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ のみの 試料，ﾍﾊﾟﾘﾝ錯体を全体の2.5 wt% 含む試料，ﾍﾊﾟﾘﾝ錯体を全体の10 wt%含む試料，市販のﾎﾟﾘｳﾚﾀﾝ(TECOFLEX EG80A)試料の４種を用いて犬２匹により実験した．(図２)ＳＥＭ観察（図３）の結果，①ﾎﾟﾘｳﾚﾀﾝ(TECOFLEX EG80A)と比べてほぼ同様の抗血栓性であること，②ﾍﾊﾟﾘﾝ錯体の複合により抗凝血性をある程度付与できることが確認された．ただし，この方法はﾁｭｰﾌﾞ端部の段差による乱流の影響が大きいことも明らかとなった．

3．3　犬動脈内人工血管埋植試験（Ⅰ）〔ＤＢ－１，２；　８日間埋植〕

埋植用人工血管の例を図４に示す．人工血管内面には厚さ約20μｍのﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑ層がありその外側にはﾎﾟﾘｴｽﾃﾙ繊維製管と一体化したﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質層がある．埋植前の試料を光顕にて観察した結果を図５に示す．ﾍﾊﾟﾘﾝ錯体を含有する試料ではﾍﾊﾟﾘﾝがﾄﾙｲｼﾞﾝﾌﾞﾙｰにより染色されること，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑ層は凍結乾燥操作によりかなり多孔化しておりﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 層の部分にﾍﾊﾟﾘﾝ錯体が分布していることが明らかとなった．

ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ のみの 試料，ﾍﾊﾟﾘﾝ錯体を全体の5 wt% 含む試料の２種（いずれも長さ４cm）を用いて犬２匹により８日間の埋植試験を行った．結果を図６および表１ DB-1,DB-2 に示す．試料はいずれも閉塞していた．

3．4　in vitro評価（Ⅱ）

前項までの結果から，①３㎜径程度の細径管への適用はﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ のみの 試料では難しいこと，②ﾍﾊﾟﾘﾝ錯体のﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑ中での安定性が悪く容易に脱離して有効な抗凝血性が得られないこと等が推察されたため，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ とﾍﾊﾟﾘﾝ錯体の複合法につき検討し，卵黄ﾚｼﾁﾝを共存させることによりある程度の安定化が可能なことを見いだした．そこで，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ-ﾍﾊﾟﾘﾝ錯体-卵黄ﾚｼﾁﾝ系につき検討することとし，その第一段階として in vitro 血液適合性評価を行った．その結果この系であればﾍﾊﾟﾘﾝ錯体を全体の20 wt%程度含有させてもかなり安定であり，有効な抗凝血性が期待されることが明らかとなった．

3．5　犬動脈内人工血管埋植試験（Ⅱ）〔ＤＣ－１，２；　５日間埋植〕

①ﾎﾟﾘｴｽﾃﾙ編管のみ，②ﾎﾟﾘｴｽﾃﾙ編管＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ，③ﾎﾟﾘｴｽﾃﾙ編管＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ＋ﾚｼﾁﾝ20%，④ﾎﾟﾘｴｽﾃﾙ編管＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ＋ﾚｼﾁﾝ20% ＋ﾍﾊﾟﾘﾝ錯体20% の４種の試料（いずれも長さ２cm）を用いて犬２匹により５日間の埋植試験を行った．結果を図７および表１ DC-1,DC-2 に示す．犬DC-2は埋植手術時に出血が多かったためか５日目にはすでに死亡していたが，各試料は①，②，③はいずれも血栓により閉塞していたのに対し，④の試料は２匹とも開存しており吻合部付近からのﾊﾝｽ形成が多少みられるものの血栓の付着は少なかった．光顕による解析から人工血管内面にﾍﾊﾟﾘﾝが残存していることが確認された．

3．6　犬動脈内人工血管埋植試験（Ⅲ）〔ＤＤ－１，２，３；　１７～１９日間埋植〕

前項の結果からﾍﾊﾟﾘﾝ錯体-ﾚｼﾁﾝ 添加系に可能性があると考えられたため，より長期間の実験を行うこととした．また，ﾍﾊﾟﾘﾝ錯体非添加系に関してはより血流が多いと考えられる頸動脈→頸静脈シャントとして埋植し再度評価することとした．

ﾎﾟﾘｴｽﾃﾙ編管＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ＋ﾚｼﾁﾝ20% ＋ﾍﾊﾟﾘﾝ錯体20% 試料（長さ２cm，計６本）を犬３匹大腿動脈に吻合し，19日間（内１匹は17日間）埋植した．また，ﾍﾊﾟﾘﾝ錯体を含まない人工血管（ﾎﾟﾘｴｽﾃﾙ編管＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ および ﾎﾟﾘｴｽﾃﾙ編管＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ＋ﾚｼﾁﾝ20% ）を同時に頸動脈→頸静脈シャントとして埋植した．結果を図８および表１ DD-1 ～DD-3 に示す．その結果，ﾍﾊﾟﾘﾝ錯体-ﾚｼﾁﾝ 添加系の１本のみが開存しており，他の５本は血栓により閉塞していた．開存していた１本も両吻合部よりのﾊﾟﾝﾇｽ形成が顕著であり，光顕による解析からﾊﾟﾝﾇｽ形成部の人工血管内面にﾍﾊﾟﾘﾝが残存していないことが確認された．

3．7　犬右心パッチ実験（Ⅰ）〔ＤＥ；　２８日間埋植〕

前項までの結果により細径人工血管へのﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ｺｰﾃｨﾝｸﾞの適用は難しいと判断されたことから，血栓による閉塞の可能性のより少ない用途として心臓・血管系パッチに関する実験を試みることとした．

まず，ﾎﾟﾘｴｽﾃﾙ伸縮布＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑ（貼り合わせ試料）を犬の右心のパッチとして使用する４週間の埋植試験を行った．結果を図９に示す．パッチは柔軟で右心の形状によく適応し，縫合性も良好であった．剖検の結果機能的には良好と考えられたが，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑ平滑面（血液側）と組織との接着は十分ではなく，血液凝固物とそこへ侵入した細胞とからなるﾊﾟﾝﾇｽが内側に形成し，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑとﾊﾟﾝﾇｽの間に生成した血栓が溶解してできたと推定される空間も存在していた．以前実施した腹腔内での埋植結果ではﾌｨﾙﾑ状の試料でも比較的良好な細胞の接着が得られていたが，血管系ではより接着しやすい環境（多孔構造等）が必要と推定され，次に多孔構造の付与を検討することとした．

3．8　犬右心パッチ実験（Ⅱ）〔ＤＦ－１，２；　１４日間埋植〕

ﾎﾟﾘｴｽﾃﾙ伸縮布の片面をﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ多孔質とし片面をﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ平滑面とした試料により犬２匹を使用し右心パッチとして２週間埋植した．結果を図１０に示す．パッチは機能的に良好であり，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質（血液側，孔径 100μｍ程度）と組織との接着も良好であった．ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質の血液接触面には血液凝固物とそこへ侵入した細胞とからなる厚さ50μｍ程度の層が均一に形成していた．ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質とﾎﾟﾘｴｽﾃﾙ伸縮布が一体となった部分には血液凝固物と侵入した細胞により埋められており，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ に起因する異物反応も小さかったが，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 平滑面側の最外層には薄膜が張っている形となっており，外側からﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 平滑面内への細胞の侵入はみられなかった．

試料は親水性を付与するために卵黄ﾚｼﾁﾝを５％添加したものとしないものとの２種を用い，ﾚｼﾁﾝ添加系は非常に水濡れ性がよく縫合時の取扱性に優れていたが，２週間後の評価では特に差はみられなかった．

3．9　犬右心パッチ実験（Ⅲ）〔ＤＧ－１，２；　１４日間埋植〕

ﾎﾟﾘｴｽﾃﾙ伸縮布の両面をﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質とした試料により犬２匹に右心パッチとして２週間埋植した．結果を図１１に示す．また，パッチ試料のＳＥＭ写真を図１２に示す．ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質（血液側，孔径 100μｍ程度；外側，孔径10μｍ程度）と組織との接着はほぼ良好であった．血液接触面においては，一部に埋植部位での血液の乱流にもとづくと推定される軽度のﾊﾟﾝﾇｽ（まず血液凝固物が生成しそれが器質化したと考えられる）がみられたが（犬DG-1），犬DG-2では治癒経過が特に良好であり内皮化（治癒）している部分も観察された．

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4．考察

4．1　細径人工血管への応用に関して

実験結果をもとに推論を加えてまとめを行うと，次のようになる．

①ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ P(3HB-co-80%4HB)の初期の抗血栓性はﾎﾟﾘｳﾚﾀﾝと同程度である．

②ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 表面への組織の接着性は良好である．

③ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 中にﾍﾊﾟﾘﾝを混合する場合，吻合部のﾊﾟﾝﾇｽの含ﾍﾊﾟﾘﾝﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 表面への接着性が低く，ﾊﾟﾝﾇｽが大きくなって血栓による閉塞に結びつきやすい．

これに対し，研究のねらいは，

(a)ﾍﾊﾟﾘﾝ等を併用することにより初期の抗血栓性を確保し，

(b)吻合部の生体血管側から伸びてくるﾊﾟﾝﾇｽをﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ｺｰﾃｨﾝｸﾞに接着させ過形成を防ぎ，

(c)人工血管外側から毛細血管が十分に侵入したのち，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ｺｰﾃｨﾝｸﾞが分解吸収され，人工血管内面全体の内皮化を達成する，

ことにある．

上記の推論から，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 中にﾍﾊﾟﾘﾝを混合するｱﾌﾟﾛｰﾁ では，(a)と(b)を両立させることは難しい考えられる．他のｱﾌﾟﾛｰﾁ としてはﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ の上にﾍﾊﾟﾘﾝを含むｾﾗﾁﾝ層を形成させることがあげられる．これに類似した試みとしては京都府立医大・丹生先生が野一色先生らの協力により行っている研究があり良好な結果が得られており，[7] また，ｾﾗﾁﾝ層ｺｰﾃｨﾝｸﾞが吻合部ﾊﾟﾝﾇｽ過形成防止に有効なことは東京女子医大・太田先生らの一連の透析用ｼｬﾝﾄﾁｭｰﾌﾞの検討でも明らかにされている．[8]　しかし，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ｺｰﾃｨﾝｸﾞの上にｾﾗﾁﾝ層を安定に導入するためには多孔化・ｾﾗﾁﾝ層架橋等のかなり煩雑な製造工程が必要となること，熱可塑性で加工しやすく架橋剤の細胞毒性の心配もないというﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ の物性上の特徴を生かしているとは考えられないこと等のことから，これ以上細径人工血管系への応用検討は行わないこととした．

4．2　心臓・血管系パッチへの応用に関して

犬右心パッチとしての実験結果をまとめると次のようになる．

①ﾎﾟﾘｴｽﾃﾙ伸縮布＋ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑ（貼り合わせ試料）

機能的には：良好

組織学的には：ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 平滑面（血液側）と組織との接着は十分ではない

血管系ではより接着しやすい環境（多孔構造等）が必要と推定された．

②ﾎﾟﾘｴｽﾃﾙ伸縮布の片面をﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ多孔質とし片面をﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ平滑面とした試料，

機能的には：良好

組織学的には：ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質（血液側，孔径 100μm程度）と組織の接着は良好

平滑面側は外側から平滑面内への細胞の侵入はみられなかった．

③ﾎﾟﾘｴｽﾃﾙ伸縮布の両面をﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ多孔質とした試料

機能的には：良好

組織学的には：ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 多孔質（血液側，孔径 100μm程度；外側，孔径10μm程度）と組織の接着は良好．血液接触面では一部に内皮化も観察された．

これらの結果から，③の試料は機能性・治癒経過とも優れていると考えられた．

5．結論

Ｐ（３ＨＢ－ｃｏ－８０％４ＨＢ）の細径人工血管および心臓・血管系パッチの２用途への応用について犬による埋植試験を行い，以下の結果を得た．

(1)血液適合性は市販の医療用ポリウレタンと同程度で積極的な抗血栓性はなかった．

(2)内径３㎜のポリエステル繊維製人工血管へ適用する場合，抗凝血剤なしでは１週間以内に閉塞した．ヘパリン－塩化ベンザルコニウム錯体およびレシチンを複合することにより，１週間以内の開存は確保できたが，それ以上の期間の埋植においては吻合部のパンヌス過形成にもとづく血栓による閉塞の問題を解決することができず，細径人工血管への応用は不適と判断された．

(3)右心パッチとしての評価の結果，ポリエステル伸縮布の両面をバイオポリエステル多孔質とした試料が，縫合時の操作性，パッチとしての機能性，パッチへの組織の侵入とその後の治癒性にそれぞれ優れていることが明らかとなり，心臓・血管系パッチはバイオポリエステルの最も有力な医療器材応用分野と判断された．

6．文献

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表　　犬動脈内人工血管埋植試験による評価結果[a)]

犬 No.	埋植期間（日）	部位	試料	剖検所見	病理所見
DB-1	8	右大腿動脈	内径約2.5 mm，BP	閉塞	b)
		左大腿動脈	内径約2.5 mm，BP ヘパリン錯体５％含有	閉塞	b)
DB-2	8	右大腿動脈	内径約2.5 mm，BP	閉塞	b)
		左大腿動脈	内径約2.5 mm，BP ヘパリン錯体５％含有	閉塞	b)
DC-1	5	右頸動脈	内径約3.0 mm，BP	閉塞	b)
		左頸動脈	内径約3.5 mm，ポリエステル編管のみ （埋植時にブレクロッティング 処理）	閉塞	b)
		右大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有	○開存	図７を参照．
		左大腿動脈	内径約3.0 mm，BP， 卵黄レシチン20％含有	閉塞	b)
DC-2	<5[c)]	右頸動脈	内径約3.5 mm，ポリエステル編管のみ （埋植時にブレクロッティング 処理）	閉塞	b)
		左頸動脈	内径約3.0 mm，BP	閉塞	b)
		右大腿動脈	内径約3.0 mm，BP， 卵黄レシチン20％含有	閉塞	b)
		左大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有	○開存（吻合部より，わずかにパンヌス成長あり）	b)
DD-1	19	右大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有　No.1	○開存（吻合部より，パンヌス成長あり）	図８を参照．
		左大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有　No.2	閉塞	b)
		右頸動脈→右頸静脈 シャント	内径約3.0 mm，BP	閉塞	b)
DD-2	19	右大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有　No.3	閉塞（感染あり，中枢側生体血管部で閉塞した）	b)
		左大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有　No.4	閉塞	b)
		右頸動脈→右頸静脈 シャント	内径約3.0 mm，BP， 卵黄レシチン20％含有	閉塞	b)
DD-3	17	右大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有　No.5	閉塞	b)
		左大腿動脈	内径約3.0 mm，BP，ヘパリン錯体20％・ 卵黄レシチン20％含有　No.6	閉塞	b)
		右頸動脈→右頸静脈 シャント	内径約3.0 mm，BP， 卵黄レシチン20％含有	閉塞	b)

a）略号：BP= バイオポリエステル コーティング，ヘパリン錯体= ヘパリンナトリウム・塩化ベンザルコニウム錯体．
b）閉塞した部位の標本，あるいは，死亡後時間を経過してから固定した標本であり，組織の履歴を正しく把握できないため詳しい解析は行わなかった．
c）５日目にはすでに死亡していた．

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2. バイオポリエステルの腹壁漿膜欠損モデルによる生体適合性試験

1．緒言

1．1　目的

微生物が生産するポリエステルの一つであるP（3HB－co－4HB）（3－ヒドロキシ酪酸（3HB）と4－ヒドロキシ酪酸（4HB）の共重合体，図1に構造式を示す）は，弾性・柔軟性および生体適合性に優れた生体内分解性材料として，医療用途への応用の可能性が期待される．[1)]

本報告書ではP（3HB－co－4HB）の実際的応用をめざしウサギの腹壁漿膜欠損モデルによる生体適合性試験を行い，癒着防止材等の非血液接触系の軟組織用医療材料としての応用の可能性について明らかにすることを目的とした．

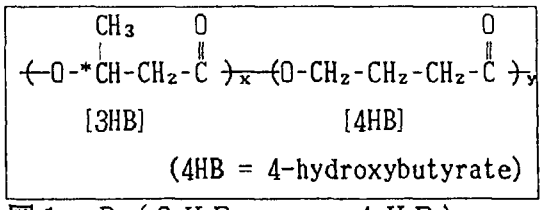

図1　P（3HB－co－4HB）

1．2　癒着防止材と腹壁漿膜欠損モデルについて

1．2．1　癒着と癒着防止材

線維性組織の形成によって生体器官が剥離不可となる状態を呼び，原因として

①外科手術の創部の治癒過程で周辺の正常組織との癒着が併発．

②外科手術時における生体組織に対する機械的あるいは化学的刺激．

③外科手術後の細菌感染，炎症，合併症．

等があげられる．

例えば開腹手術の後，腸管が他臓器・腹壁等に癒着することにより腸閉塞を起こすことがあり，再手術を余儀なくされる場合も多い．癒着防止が重要な部位としては手術件数の多い腹部のほか，脳，肺，心，手の腱等があり，それぞれ有効な癒着防止材の開発が期待されている．

従来コンドロイチン硫酸，デキストラン等の水溶液が癒着防止の目的で臨床的に使用されることがあったが大きな効果はなく，また，最近米国で開発された酸化セルロース（Johnson & Johnson,"Interceed"）についても容易に局部にのみ適用できる使いやすさは認められるもののやはり大きな効果があるとはいえない．[2)]

1．2．2　本実験の目的

今回当社で新しい生体内分解性材料であるP（3HB－co－4HB）の医療用途への応用を検討しているが，その弾性・柔軟性および生体適合性に優れている性質から癒着防止材は有力なターゲットの一つと考えられた．そこで，癒着防止材としての使用を具体的に意識した動物実験評価を実施することとした．共同研究先としては，人工血管の権威者であり，癒着防止材に関しても優れた研究を発表している横浜市立大学医学部・野一色泰晴講師にお願いすることとした．

1．2．3　癒着防止の考え方と腹壁漿膜欠損モデル実験

癒着防止の考え方としては，

①ヘパリン（抗血液凝固剤）等でフィブリン析出を阻止する

②抗炎症剤により線維芽細胞の過度の増殖を抑える

③創部にフィブリンと一体化した保護層を形成させ漿膜再生までの間癒着を軽減する

④物理的に隔壁を入れ癒着を阻止する

等があるが，P（3HB－co－4HB）の弾性・柔軟性を生かした使い方として，主として物理的な隔壁効果による癒着防止をねらうこととした．

癒着防止の実験モデルとしても種々の例があるが，

①ラットは癒着が起こりにくい傾向があり使うべきでないこと

②飼育上ウサギが使いやすいこと

③スクリーニングとして多種の試料の比較を考えていること

等から，評価部位として大きな面積を確保でき，手技の巧拙の影響が少ないと考えられるウサギの腹壁漿膜欠損モデルによることとした．

1．3　本研究における検討課題

過去にウサギを使用して健常な皮下および腹腔内でのP（3HB－co－4HB）の埋植試験を実施し，生体内分解性と生体適合性を確認しているが，今回は腹腔内癒着防止材としての使用環境，すなわち，腹腔内で傷と材料とが接した状態で

①材料と細胞との相互作用

②材料の生分解性

③癒着防止効果

④癒着防止材としての使いやすさ

等について評価することを課題とした．

評価試料としてはP（3HB－co－4HB）フィルム，とくに弾性・柔軟性・生体内分解性の点でもっとも特徴のある4HB組成80%品を中心として使用し，縫合性，生分解性，強度等を考慮して，ポリグリコール酸メッシュ等の既存生分解性材料との複合，酵素による表面処理，熱分解による分子量低減等を併用することとした．実験1では以前実施した埋植試験において実績のある膜厚約200μmの試料による基礎的評価を，実験2では膜厚約10μmの試料によるより実際的な評価をそれぞれ実施した．

2．実験

2．1　評価試料（主な試料を図2に示す）

ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ P(3HB-co-4HB) は当社培養生産品を使用した．それらのうち4HB 組成80% および90% のものについてはさらに窒素中において 200℃で20分間処理して熱分解し，分子量をMn＝約５万，Mw＝約10万程度に調整して使用した．ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ はAldrich 製を用いた．これらをそれぞれｸﾛﾛﾎﾙﾑ溶液とし９cmシャーレ１枚にポリマー各1.5 g あるいは0.075g使用して，厚さ約 200μmあるいは約10μmのﾌｨﾙﾑを作成した．これを，必要に応じてさらに一部加工したのち，４×４cmに切断して以下の試料を作成した．なお，編物との貼り合わせ２層構造のものは編物側を漿膜欠損部へあてて使用することとした．

〔実験１〕

①P(3HB-co-80%4HB)ﾌｨﾙﾑ（厚さ約 200μm）
P(3HB-co-80%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約 200μm）を90℃オーブン中で30分間溶融処理した．

②P(3HB-co-80%4HB)ﾌｨﾙﾑ（厚さ約 200μm）／ﾀﾞｸﾛﾝﾌｧﾌﾞﾘｯｸ〔貼り合わせ２層構造〕
P(3HB-co-80%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約 200μm）の上に“STRETCH DACRON FABRIC”(Meadox Medicals Inc.製）をのせ上から軽く押さえた状態で90℃オーブン中で30分間処理して，ﾌｨﾙﾑとﾀﾞｸﾛﾝﾌｧﾌﾞﾘｯｸを貼り合わせた．

③P(3HB-co-80%4HB)ﾌｨﾙﾑ（厚さ約 200μm）／ﾎﾟﾘｸﾞﾘｺｰﾙ 酸編物〔貼り合わせ２層構造〕
P(3HB-co-80%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約 200μm）の上に“ﾒﾃﾞｨﾌｨｯﾄ C (2-0　ｸﾞﾘｰﾝﾌﾞﾚｲﾄﾞ)”（日本ﾒﾃﾞｨｶﾙｻﾌﾟﾗｲ 製）の編物をのせ上から軽く押さえた状態で90℃オーブン中で30分間処理して，ﾌｨﾙﾑと編物を貼り合わせた．

④P(3HB-co-50%4HB)ﾌｨﾙﾑ（厚さ約 200μm）
P(3HB-co-50%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約 200μm）を90℃オーブン中で30分間溶融処理した．

⑤ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ(PCL)ﾌｨﾙﾑ（厚さ約 200μm）
ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ(PCL)ｷｬｽﾄﾌｨﾙﾑ（厚さ約 200μm）をそのまま使用した．

⑥ｼﾘｺｰﾝ ﾌｨﾙﾑ：市販の“ｻｲﾗｽﾃｨｯｸ医療用ｼｰﾄ (500-5)”(Dow Corning Corporation製)

〔実験２〕

⑦ﾃｷｿﾝﾒｯｼｭ：市販のﾎﾟﾘｸﾞﾘｺｰﾙ 酸編物(“ﾃｷｿﾝﾒｯｼｭ”，型番号#2，Davis+Geck，Inc.製)

⑧P(3HB-co-80%4HB)ﾌｨﾙﾑ（厚さ約20μm）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕
P(3HB-co-80%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約10μm）の上にﾃｷｿﾝﾒｯｼｭをのせ上から軽く押さえた状態で90℃オーブン中で30分間処理してﾌｨﾙﾑと編物を貼り合わせたのち，ﾌｨﾙﾑ面にもう１枚ﾌｨﾙﾑをのせて同様に処理した．

⑨P(3HB-co-80%4HB)ﾌｨﾙﾑ（厚さ約20μm，酵素処理）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕
P(3HB-co-80%4HB)ﾌｨﾙﾑ（厚さ約20μm）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕を0.1Mリン酸緩衝液(pH7.0) 中でLipase from Rhizopus arrhizus (ﾍﾞｰﾘﾝｶﾞｰ ﾏﾝﾊｲﾑ)3 μg／mℓにより30℃で１時間処理し，水洗後乾燥した．

⑩P(3HB-co-90%4HB)ﾌｨﾙﾑ（厚さ約20μm）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕
P(3HB-co-90%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約10μm）を使用して，⑧試料と同様に処理した．

⑪P(3HB-co-90%4HB)ﾌｨﾙﾑ（厚さ約20μm，酵素処理）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕
P(3HB-co-90%4HB)ﾌｨﾙﾑ（厚さ約20μm）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕を0.1Mリン酸緩衝液(pH7.0) 中でLipase from Rhizopus arrhizus (ﾍﾞｰﾘﾝｶﾞｰ ﾏﾝﾊｲﾑ)3 μg／mℓにより30℃で３時間処理し，水洗後乾燥した．

⑫P(3HB-co-80%4HB)ﾌｨﾙﾑ（厚さ約10μm）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕
P(3HB-co-80%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約10μm）の上にﾃｷｿﾝﾒｯｼｭをのせ上から軽く押さえた状態で90℃オーブン中で30分間処理してﾌｨﾙﾑと編物を貼り合わせた．

⑬P(3HB-co-90%4HB)ﾌｨﾙﾑ（厚さ約10μm）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕
P(3HB-co-90%4HB)ｷｬｽﾄﾌｨﾙﾑ（厚さ約10μm）を使用して，⑫試料と同様に処理した．

⑭ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝﾌｨﾙﾑ(厚さ約10μm）／ﾃｷｿﾝﾒｯｼｭ〔貼り合わせ２層構造〕
ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ(PCL)ｷｬｽﾄﾌｨﾙﾑ（厚さ約10μm）を使用して，⑫試料と同様に処理した．

2．2　腹壁漿膜欠損モデル実験方法

2．2．1　動物

ウサギ（日本白色種），♂，約2.5 kg.(手術前日夕よりエサをカットして用いた.)

2．2．2　手技

(1)（準備のための）麻酔〔I.V.Cannula,ゴム栓，シリンジ，注射針使用〕
①耳静脈に注入口設置　②ネンブタール（生食3 倍希釈）約2 mℓ注入

(2) 腹部を全面的に毛刈り〔電動クリッパー，掃除機使用〕

(3) ウサギ固定，消毒〔包帯，カット綿，膿盆，アリス鉗子使用〕
①イソジン　②消毒用アルコール　③術者は手術用手袋を着用

(4) オイフ　①ウサギ用　②器具置場用

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(5) 器具等準備
①器具をヒビテン液より器具置場へ　②サンプルを滅菌シャーレへ　③サンプルに抗生物質（アミペニックス）振りかけ（ｱﾐﾍﾟﾆｯｸｽは１ｇ力価のものを生食12mℓに溶かし使用）
(6) オイフカット　①腹部のオイフをハサミでカット　②オイフを１号針付糸で固定.
(7) 麻酔
ｹﾀﾗｰﾙ 10（三共）(生食10倍希釈)，ﾈﾝﾌﾞﾀｰﾙ(生食3 倍希釈）各0.5-1 mℓを適時注入.
(少な目に使用し，必要に応じて追加する.)
(8) 腹部の切開
①メスで切れ目を入れる.
②電気メスにより真ん中の線（白線Linea alba）にそって深くカット.
(正中切開すると出血が少ない.)
③中央部をピンセットで持ち上げ，電気メスで穴を開ける.
④穴にハサミを入れ，頸部方向へ腹壁を切開する.
⑤ハサミで臀部方向へ腹壁を切開する.(スキマに手を入れ，内臓をハサミで傷つけないよう注意する.)
(9) 腹壁漿膜剥離（図３(1)～(3)）
①腹壁切開部の片側をオイフごと鉗子でつまんで折り返す.
②折り返した腹壁内側の頸部側に切開部から１～２cm以上間をあけて３×３cmの正方形部を定規で計り，その各頂点に当たる部分を有鉤ピンセットでつまみその下をハサミでカットする.
③漿膜をピンセットでつまみながら，頂点部の切れ目から他の頂点部の方向へ漿膜の下にハサミを入れ漿膜を剥離し，頂点と頂点を結ぶ線分に当たる漿膜をカットする.
④３×３cmの正方形部の漿膜をピンセットでつまみ,(ハサミで剥離し）除去する.
⑤腹壁内側の臀部側に同様にして正方形部の漿膜を剥離する.
⑥腹壁切開部の反対側の片面に同様にして漿膜剥離をおこなう.
(10)サンプル縫合（貼り合わせ２層構造のものは編物側を漿膜欠損部へあてて縫合）
〔実験１〕（図３(4)）
①サンプルを漿膜剥離部に当て，ﾌﾟﾛﾘｰﾝ4-0 針付糸で中央部を縫合する.(ﾌﾟﾛﾘｰﾝ 糸の縫合は６重くらいにおこなう.)
②腹壁切開部側の頂点から順に頂点と頂点間の中点計８箇所を同様に縫合する.
③アミペニックスを振りかける.
〔実験２〕（実験１で端部および縫合結び目での癒着がみられたため改善した)(図３(5))
①サンプルを漿膜剥離部に当て，エチボンド5-0 針付糸でサンプル周囲に沿って連続的に縫合する.
②アミペニックスを振りかける.
(11)切開創の縫合
①エチボンド2-0 針付糸で腹壁を縫合.(腸管を縫わないように注意．漿膜同士がつくように注意して結ぶ)
②アミペニックスを振りかける.
③エチボンド2-0 針付糸で皮膚を縫合する.(傷口同士がつくように注意して結ぶ)
(12)術後の処置　①電気毛布の上にしばらく寝かせる．　②ケージに入れる.

２．２．３　各試料の縫合位置と実験期間
各ウサギの右腹部頸部側，右腹部尾部側，左腹部頸部側，左腹部尾部側の順にⅠ，Ⅱ，Ⅲ，Ⅳと番号を付し，各試料を以下のように配置した．また，実験期間は(1)２週間（漿膜の再生をみる），(2)２ヶ月（材料の分解吸収過程をみる）の２期間を基本とした.
〔実験１〕
ｳｻｷ G-1, G-3　Ⅰ：ｺﾝﾄﾛｰﾙ（漿膜剥離後無処置），Ⅱ：試料①，Ⅲ：試料⑥，Ⅳ：試料⑤
ｳｻｷ G-2, G-4　Ⅰ：ｺﾝﾄﾛｰﾙ，Ⅱ：試料②，Ⅲ：試料③，Ⅳ：試料④
〔実験２〕
ｳｻｷ H-1, H-2　Ⅰ：ｺﾝﾄﾛｰﾙ，Ⅱ：試料⑦，Ⅲ：試料⑧，Ⅳ：試料⑨
ｳｻｷ H-3, H-4　Ⅰ：ｺﾝﾄﾛｰﾙ，Ⅱ：試料⑦，Ⅲ：試料⑩，Ⅳ：試料⑪
ｳｻｷ H-5, H-6　Ⅰ：ｺﾝﾄﾛｰﾙ，Ⅱ：試料⑫，Ⅲ：試料⑬，Ⅳ：試料⑭

２．３　評価方法
所定期間埋植後，麻酔下で心臓穿刺により脱血死させた後，腹部を両脇部から切開して癒着の状況を観察し，癒着している部分の臓器はつけたままで腹壁部を切離し，写真撮影する.(図４，６）　試料10%ﾎﾙﾏﾘﾝで固定し，脱ｲｵﾝ 水洗浄にて脱ﾎﾙﾏﾘﾝ 後，ﾋﾄﾞﾛｷｼｴﾁﾙﾒﾀｸﾘﾚｰﾄ 樹脂包埋し（Reichert- Jung製 HISTORESIN 使用），電動回転式ﾐｸﾛﾄｰﾑ（Reichert-Jung製 2055 AUTOCUT ）を使用して厚さ5 μmの切片とし，ＨＥ染色して光顕観察した.

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3．結果

3．1　実験1・膜厚約２００μmの試料による結果

結果を表1に，主な剖検写真を図4に，主な光顕写真を図5に示す．

3．1．1　手術および術後の経過

試料はやや固い感じであった．6日目に一羽が死亡したが，餌の取りすぎが一つの要因と思われ，餌量の調節をおこなった結果他のウサギは順調な経過をとった．

3．1．2　2週間埋植の目視評価結果

6日目(ｳｻｷﾞ G-4) および14日目(ｳｻｷﾞ G-3) の結果とも試料縫合部（ﾓﾉﾌｨﾗﾒﾝﾄの硬めの糸を使用し，結紮を確実にするため6重に結んでいるため，大きな結び目ができている）の縫合糸結び目に軽い癒着が起こる傾向が観察された．

3．1．3　2週間埋植の組織学的評価

(1)ｺﾝﾄﾛｰﾙ（漿膜剝離後無処置部）は漿膜（中皮細胞）がかなり再生しているようであった．

(2)P(3HB-co-80%4HB)（溶融処理したﾌｨﾙﾑ）は線維芽細胞の付着がみられた．

(3)ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ （ｸﾛﾛﾎﾙﾑよりのｷｬｽﾄﾌｨﾙﾑ）は中皮細胞による被覆がかなり観察された．

(4)ｼﾘｺｰﾝ には細胞は付着していないが，縫合部よりの癒着の程度は大きかった．

3．1．4　2ヶ月埋植の目視評価結果

P(3HB-co-80%4HB)，P(3HB-co-50%4HB)，ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ の表面は薄く組織に覆われていた．2週間目との違いは毛細血管が顕著に見えることであった．材料表面そのものの周辺臓器との癒着は見られなかったが，試料縫合部の縫合糸結び目あるいは試料と漿膜欠損部との隙間と周辺臓器との間に軽い線維性の癒着がみられた．ｼﾘｺｰﾝ ﾌｨﾙﾑ表面にはなにも組織が付着していなかったが，ｼﾘｺｰﾝ と漿膜欠損部との隙間と周辺臓器との間には強い癒着が観察された．ｺﾝﾄﾛｰﾙは1羽では癒着はなく治癒していたが，もう1羽では腸管との間に強い癒着が観察された．

3．1．5　2ヶ月埋植の組織学的評価

(1)P(3HB-co-80%4HB)，P(3HB-co-50%4HB)，ﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ の表面は線維芽細胞を主とする薄い被包で覆われその腹腔側には漿膜（中皮細胞）が再生していた．材料と接触する面にはﾏｸﾛﾌｧｰｼﾞ がみられた．各材料自身には表面より生体成分が入りこんでおり部分的に分解していることが示唆された．材料周辺に異常な反応はみられなかった．

(2)ｼﾘｺｰﾝ ﾌｨﾙﾑ表面にはなにも組織が付着していなかったが，ｼﾘｺｰﾝ と漿膜欠損部との隙間付近では肉芽が大きく盛り上がっていた．

(3)ｺﾝﾄﾛｰﾙ（2羽それぞれ1箇所ずつ）は非癒着部では漿膜に覆われ治癒していたが，腸管との癒着部では線維芽細胞の増殖を伴い腸管の筋層と強固に癒着していた．

(4)P(3HB-co-80%4HB)とﾎﾟﾘｸﾞﾘｺｰﾙ酸編物を熱融着により貼り合わせた試料の場合ﾎﾟﾘｸﾞﾘｺｰﾙ酸繊維はまだ残存し周囲に異物巨細胞が観察された．

(5)P(3HB-co-80%4HB)とﾀﾞｸﾛﾝ織布を熱融着により貼り合わせた試料の場合にはﾀﾞｸﾛﾝ織布のまわりに肉芽が形成されP(3HB-co-80%4HB)ﾌｨﾙﾑとの間に入り込んでいた．

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3．2　実験２・膜厚約１０μｍの試料による結果

結果を表２に，主な剖検写真を図６に，主な光顕写真を図７に示す．

3．2．1　手術および術後の経過

試料は柔軟で扱いやすく，縫合性も良好であった．より柔軟なﾏﾙﾁﾌｨﾗﾒﾝﾄ 糸を使い試料の周囲に連続縫合した．術後はすべて順調な経過をとった．

3．2．2　２週間埋植の目視評価結果

目視評価では、前回の実験で不備であった端部および縫合糸結び目での癒着がほとんど解消したこと、ｺﾝﾄﾛｰﾙ（発膜剥離後無処置部）に全く癒着がないのに対しﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 部が他組織と癒着している箇所が一部にみられたこと等が観察された．

3．2．3　２週間埋植の組織学的評価

とくに4HB 80%品，90%品の場合に試料自体が崩壊しはじめるﾚﾍﾞﾙ にまで分解が進行していた．分解にともなう組織反応が起こっており，異物巨細胞も観察されたが，異物反応の程度はﾎﾟﾘｸﾞﾘｺｰﾙ酸ﾒｯｼｭとくらべて小さいかった．

3．2．4　２ヶ月埋植の目視評価結果

ｺﾝﾄﾛｰﾙには全く癒着がなかった．他試料は癒着のあるものとないものとがあり結果は一定していなかった．ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ を酵素処理して表面を粗面としたものは酵素処理しないものにくらべてやや癒着傾向が強いかという印象を受けた．

3．2．5　２ヶ月埋植の組織学的評価

厚さ約10μｍのﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ 膜（４ＨＢ組成80%，90%）はほとんど原形をとどめないほど分解しており，ﾎﾟﾘｸﾞﾘｺｰﾙ酸とほぼ同程度の分解性であった．比較に使用したﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ膜は同条件で分解の程度は低かった．ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ膜の異物反応性はﾎﾟﾘｸﾞﾘｺｰﾙ酸よりもやや小さかった．

4．考察

4．1　実験1の総合評価

実験の結果，

(1)P(3HB-co-80%4HB)ﾌｨﾙﾑの生体適合性は良好である，

(2)ﾌｨﾙﾑは薄い被包で覆われその腹腔側には漿膜が再生し他臓器との癒着を防いでいる，

(3)ﾌｨﾙﾑが表面より一部分解している，

(4)ｺﾝﾄﾛｰﾙ1箇所に強度の癒着があり，ﾌｨﾙﾑに一応の癒着防止効果があったと判断される，

(5)腹壁漿膜欠損モデルは生体内で多数の材料を同条件で評価するよいモデルと考えられる．

等が明らかとなり，ﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ ﾌｨﾙﾑは癒着防止材としても可能性があると考えられた．

実験1の試料で癒着防止材として不十分な点としては(a)2箇月間で表面より一部分解している様子がみられるものの大部分は残存していること，(b)ﾌｨﾙﾑが約200μmと厚かったためか少しゴワゴワした感じであったこと，(c)縫合部の縫合糸結び目あるいは漿膜欠損部と試料の隙間において軽い癒着がみられたこと等である．材料の分解が表面から進行するのは酵素分解に特徴的なことであり，例えば加水分解性材料であるﾎﾟﾘｸﾞﾘｺｰﾙ酸では内部でも早期に分解することが知られている．また，P(3HB-co-80%4HB)のひとつの特徴はうすい柔軟なﾌｨﾙﾑとしての物性にすぐれていることであり，これはﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ･ﾎﾟﾘｸﾞﾘｺｰﾙ酸等と異なる点である．これらのことからP(3HB-co-80%4HB)を薄いﾌｨﾙﾑとして使用して早期の分解性および材料の柔軟性を生かし，取扱い易さの点をﾎﾟﾘｸﾞﾘｺｰﾙ酸ﾒｯｼｭと複合して補う試みを中心にして，実験2においてもう一度腹壁漿膜欠損モデル実験を実施することとした．

4．2　実験2の総合評価

実験1では試料の厚さが約200μmであったため，試料は比較的固く分解は試料の表面のみであった．実験2では厚さ約10μmに薄膜化したﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ(強度保持のためﾎﾟﾘｸﾞﾘｺｰﾙ酸ﾒｯｼｭと貼り合わせてある）を使用しているため，とくに4HB 80%，90%品の場合に試料は柔軟で取扱性もよく，2週間ですでに試料自体が崩壊しはじめるﾚﾍﾞﾙ にまで分解が進行しており，2ヶ月後にはほとんど原形をとどめないほど分解が進んでいた．分解にともなう異物反応の程度はあまり大きくなく，生体内分解性材料として有用であることが確認された．これに対してﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ では約10μmの薄膜としても顕著な分解は観察されず，また，柔軟性においても劣っていた．これらの様子を示す光顕写真を図8にまとめて示す．約10μmに薄膜化して柔軟にし，かつ，縫合方法を試料周囲の連続縫合としたことにより，漿膜欠損部と試料の隙間の問題は解消した．また，試料縫合にﾏﾙﾁﾌｨﾗﾒﾝﾄ のより柔軟な糸を使った結果，縫合糸結び目の問題も解消した．

癒着防止効果という点ではｺﾝﾄﾛｰﾙ（漿膜剥離後無処置）が全く癒着せず，ｺﾝﾄﾛｰﾙと比べてよいという結果は得られなかった．この点については，より癒着しやすい実験モデル（例えば“Contaminated Peritoneal Model”[3]）により実験することも重要であり，また，抗凝固剤等の何らかのｱｸﾃｨﾌﾞ な作用を複合することも考えられる．しかし，今回の実験を通じてﾊﾞｲｵﾎﾟﾘｴｽﾃﾙ の組織との親和性の良さが印象づけられ，癒着防止よりもむしろ組織を呼び込むような役割（人工血管，人工気管等）に使用する方が適しているのではないかと考えられた．

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5．結論

ウサギによる２ヶ月間の腹壁漿膜欠損モデル試験を２回行い，以下の結果を得た．

(1)腹腔内で傷と材料とが接した状態においてもP(3HB-co-4HB)ﾌｨﾙﾑ の生体適合性は良好であり，ﾌｨﾙﾑは薄い被包で覆われその腹腔側には漿膜が再生し他臓器との癒着を防ぐ作用が観察された．

(2)厚さ約２００μｍのﾌｨﾙﾑの場合には比較的固く分解も試料の表面のみであったが，約１０μｍのﾌｨﾙﾑ（とくに4HB 80%，90%品；強度保持のためﾎﾟﾘｸﾞﾘｺｰﾙ酸ﾒｯｼｭと貼り合わせている）の場合には試料は柔軟で取扱性もよく，２ヶ月間で実質的に分解することが確認された．これに対してﾎﾟﾘｶﾌﾟﾛﾗｸﾄﾝ では約１０μｍの薄膜としても顕著な分解は観察されず，また，柔軟性においても劣っていた．

(3)癒着防止効果という点ではｺﾝﾄﾛｰﾙ（漿膜剥離後無処置）がほとんど癒着せず，ｺﾝﾄﾛｰﾙと比べてよいという結果は得られなかった．癒着防止よりもむしろ組織を呼び込むような役割（人工血管，人工気管等）に使用する方が適しているのではないかと考えられた．

6．文献

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表　　実験１の評価結果

ｳｻｷ No.	埋植期間（日）	試料	剖検所見	病理所見
G-3	14	ｺﾝﾄﾛｰﾙ	癒着なし.	漿膜（中皮細胞）がかなり再生している.
		①4HB80%(200μm)	縫合糸結び目にごく軽い癒着.	ﾌｨﾙﾑのまわりに線維芽細胞の付着がみられた.
		⑥ｼﾘｺｰﾝ	漿膜欠損部とﾌｨﾙﾑの隙間から癒着	ﾌｨﾙﾑと生体組織との付着はなかった.
		⑤PCL(200 μm)	縫合糸結び目にごく軽い癒着.	ﾌｨﾙﾑの腹腔側に中皮細胞による被覆がかなり観察された.
G-4	6[a)]	ｺﾝﾄﾛｰﾙ	癒着なし	b)
		②4HB80%(200μm)/ ﾀﾞｸﾛﾝ ﾌｧﾌﾞﾘｯｸ	縫合糸結び目を中心にやや強い癒着.	b)
		③4HB80%(200μm)/ ﾎﾟﾘｸﾞﾘｺｰﾙ酸編物	縫合糸結び目を中心にやや強い癒着.	b)
		④4HB50%(200μm)	縫合糸結び目にごく軽い癒着.	b)
G-1	61	ｺﾝﾄﾛｰﾙ	癒着なし.	漿膜に覆われ治癒していた.
		①4HB80%(200μm)	縫合糸結び目および 端部を中心に軽い癒着.	線維芽細胞主体の薄い被包で覆われ腹腔側には漿膜が再生していた
		⑥ｼﾘｺｰﾝ	漿膜欠損部とﾌｨﾙﾑの隙間から強い癒着	ﾌｨﾙﾑと漿膜欠損部との隙間で肉芽が大きく増生していた.
		⑤PCL(200 μm)	縫合糸結び目および 端部を中心に軽い癒着.	線維芽細胞主体の薄い被包で覆われ腹腔側には漿膜が再生していた
G-2	61	ｺﾝﾄﾛｰﾙ	腸管と強い癒着	線維芽細胞の増殖を伴い腸管筋層と強固に癒着していた.
		②4HB80%(200μm)/ ﾀﾞｸﾛﾝ ﾌｧﾌﾞﾘｯｸ	縫合糸結び目および 端部を中心に癒着.	ﾀﾞｸﾛﾝ繊維周辺には異物巨細胞が顕著に観察された.
		③4HB80%(200μm)/ ﾎﾟﾘｸﾞﾘｺｰﾙ酸編物	縫合糸結び目および 端部を中心に軽い癒着.	ﾎﾟﾘｸﾞﾘｺｰﾙ 酸繊維周辺には異物巨細胞が観察された.
		④4HB50%(200μm)	縫合糸結び目および 端部を中心に軽い癒着.	線維芽細胞主体の薄い被包で覆われ腹腔側には漿膜が再生していた

a) 14日間埋植の予定であったが，６日目に死亡した．死亡前日より下痢気味で剖検すると胃内に餌がぱんぱんにつまっている状態であり，餌の取りすぎが一つの要因と思われた．
b) 死亡後約半日経過してから剖検および標本の固定を行っており，組織の状態も多少変化していると考えられ，詳しい解析は行わなかった．

表　　実験２の評価結果

ウサギ No.	埋植期間（日）	試料	剖検所見	病理所見
H-2	14	コントロール	癒着なし．	漿膜（中皮細胞）がかなり再生している．
		⑦デキソンメッシュ	癒着なし．	全体を主として線維芽細胞が覆いポリグリコール酸繊維周辺に異物巨細胞
		⑧4HB80%(20 μm)/デキソンメッシュ	軽い癒着あり．	主として線維芽細胞が覆う．フィルムの分解始まっている．
		⑨4HB80%(20 μm)/デキソン(酵素)	癒着なし．	主として線維芽細胞が覆う．フィルムの分解始まっている．
H-4	14	コントロール	癒着なし．	漿膜（中皮細胞）がかなり再生している．
		⑦デキソンメッシュ	癒着なし．	全体を主として線維芽細胞が覆いポリグリコール酸繊維周辺に異物巨細胞
		⑩4HB90%(20 μm)/デキソンメッシュ	癒着あり．	主として線維芽細胞が覆う．フィルムの分解始まっている．
		⑪4HB90%(20 μm)/デキソン(酵素)	部分的に癒着あり．	主として線維芽細胞が覆う．フィルムの分解始まっている．
H-6	14	コントロール	癒着なし．	漿膜（中皮細胞）がかなり再生している．
		⑫4HB80%(10 μm)/デキソンメッシュ	部分的に癒着あり．	主として線維芽細胞が覆う．フィルムはかなり分断されている．
		⑬4HB90%(10 μm)/デキソンメッシュ	癒着なし．	主として線維芽細胞が覆う．フィルムはかなり分断されている．
		⑭PCL(10μm)/デキソンメッシュ	癒着なし．	主として線維芽細胞が覆う．フィルムの分解の程度は小さい．
H-1	70	コントロール	癒着なし．	漿膜に覆われ治癒していた．
		⑦デキソンメッシュ	全面的に癒着．	ポリグリコール酸繊維はまだ残存している．異物巨細胞あり．
		⑧4HB80%(20 μm)/デキソンメッシュ	端部に部分的に軽い癒着あり．	フィルムはコナゴナに分解されている．腹腔側には漿膜が再生している．
		⑨4HB80%(20 μm)/デキソン(酵素)	端部に部分的に軽い癒着あり．	フィルムはコナゴナに分解されている．腹腔側には漿膜が再生している．
H-3	70	コントロール	癒着なし．	漿膜に覆われ治癒していた．
		⑦デキソンメッシュ	部分的に軽い癒着あり	ポリグリコール酸繊維はまだ残存．線維芽細胞を主とする組織が覆う．
		⑩4HB90%(20 μm)/デキソンメッシュ	癒着なし．	フィルムはコナゴナに分解されている．腹腔側には漿膜が再生している．
		⑪4HB90%(20 μm)/デキソン(酵素)	全面的に癒着．	フィルムはコナゴナに分解されている．腸管の筋層と癒着している．
H-5	70	コントロール	癒着なし．	漿膜に覆われ治癒していた．
		⑫4HB80%(10 μm)/デキソンメッシュ	端部に部分的に軽い癒着あり．	フィルムはコナゴナに分解されている．腹腔側には漿膜が再生している．
		⑬4HB90%(10 μm)/デキソンメッシュ	癒着なし．	フィルムはコナゴナに分解されている．腹腔側には漿膜が再生している．
		⑭PCL(10μm)/デキソンメッシュ	癒着なし．	フィルムの分解はあまり進んでいない．異物反応せ小さい．

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まとめ

以上のm結果より生体内分解性プラスチックは異物反応をひきおこすことなく、生体内に受け入れられ、しかも400～500％の伸展性をもつという特性があり、調整によって生体組織に極めて酷似した特性をもたせることが明らかとなった。このことは、従来臨床に用いられいる生体内分解性プラスチック、例えばポリラクテックアシッド、ポリグリコーリックアシッドなどは全く伸展性が無く、またその吸収の時異物反応が見られる等の欠点があることから、我々の研究に用いたプラスチックはすべての面でこれを満足させていることから、この度の研究以外に多くの生体内植え込み用人工臓器用素材として有用であることが判明した。今後は、その可能性を求めてさらに研究を続けてゆきたい。

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第４章

サイトカイン、成長因子の固定

各種サイトカイン、成長因子は近年注目の的となっており、その基礎研究は著しい。しかし、その臨床応用はほとんど手が付けられていない。それはサイトカイン、成長因子等が微量で効果が強いがその寿命が極めて短いため、それらを固定化し、徐放出するシステムがなかったからである。我々は最近話題の bFGF（線維芽細胞成長因子）や VEGF（血管内皮細胞成長因子）などをそのサイトカインの特性を利用して吸着固定する方法を開発し、徐放出に成功した。この技術と前述の 1.2.3 の技術も組み合わせが可能なことから、次世代の機能的人工臓器素材の選択肢は大きく広がってきている。

また一方、遺伝子工学技術導入材料については、人工臓器、移植、遺伝子工学は 21 世紀医療を支える大きな柱として位置付けられており、一般に競合的関係と思われている。しかし、我々はこれらを相補的関係として相互利用することを考えている。例えば gene を導入するためのリポソーム材料は単純であるがゆえに小さな工夫が大きな効果をもたらす。また gene を導入した細胞の特性を理解すると、それに応じた材料の分子設計が可能となり、そのような材料によってマトリックスが作られるようになる。このように次世代を担う遺伝子工学技術も適切な材料との組み合わせでその真の力を発揮することができる。

この研究に関しては、この度最も力を入れて研究している分野である。しかしながら、この度の研究プロジェクトでは研究期間が余りにも短いため、まだ成果はでていない。しかしながら、ごく近い将来、明るい結果が出ると思われる。この分野の研究は世界中でしのぎををけずって競争しているため、日進月歩の研究成果が見られることが毎月の多くの関係 Journal の論文が明らかである。

我々も現在それを行っているが、現在まだ研究は完成していない。従って、近い将来その報告をする予定である。

Natural Cytokine Synthesis for Vascular Prostheses Induced from Autologously Transplanted Bone Marrow

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Running Head

Natural cytokine synthesis for vascular grafts

Abstract

A bone marrow transplanted vascular graft functioned as an autocrine artificial organ which produced growth factors and was self-regulated. In a canine study, autologous bone marrow was harvested, enmeshed into a long-fibril-length e-PTFE graft wall and implanted in the abdominal aorta. Active endothelialization started simultaneously throughout the graft, with numerous capillary ingrowth and was completed throughout the length within 3 weeks. Transplanted marrow cells survived and continued exogenous hemopoiesis with synthesis of bFGF which activated angiogenesis in the graft wall. A complete endothelial cell lining and hemopoiesis were maintained without adverse effects for up to 6 months.

Introduction

Vascular grafts in human do not endothelialize, and the graft surface is covered with fresh thrombus for a long period of time after implantation like a protracted ulcer in the blood vessel wall[1]. Great efforts to endothelialize the graft surface by cell seeding methods have been made in the last 15 years. However, none of them have been proven successful. Meanwhile, transplantation of autologous tissue fragments have been used effectively to accelerate the healing process of protracted skin ulcers and prolonged bone fractures[2,3]. Tissue fragments contain various kinds of cells. In the skin, fibroblasts act as feeder cells to epidermal cells[4]. In equivalent experiments, smooth muscle cells and fibroblasts were shown to enhance endothelial cell growth[5,6]. We have applied a similar technique onto vascular prostheses, since endothelial cell proliferation is greatly improved with an underlying base of feeder cells. Satisfactory results in both animal experiments[7,8] and in the clinic[9] were obtained with venous and adipose tissue fragmented-grafts. Capillaries for endothelialization originated from the transplanted fragments and complete endothelialization was observed in a canine study[7]. From this evidence, the procurement of autologous tissue containing a satisfactory quantity and quality of proliferative feeder cells was desired to accelerate endothelialization. In order to regulate the healing process, we designed a vascular prosthesis to have growth factors during the endothelialization. An autologous bone marrow tissue

containing various proliferative and differentiative cells with feeder cells and cytokine secretion[10] was chosen for the source of fragments and was transplanted into the vascular graft wall[11]. Under the stimulation of newly produced cytokines, a regulated, rapid creation of a new blood vessel wall by the transplanted cells was expected.

As the basic matrix, an e-PTFE graft (diameter:6 mm, length:6-8 cm) with an average fibril length of 90 μm (Baxter Vascular Systems Division, Irvine CA) was used. Approximately 0.5 ml of bone marrow was obtained from the sternum of experimental animals by a needle puncture and tirred into 20 ml of Hepes-buffered cell culture RPMI medium (Gibco Laboratories, Grand Island, NY) to create a suspension. The suspension was injected into the graft lumen several times with pressure to enmesh the marrow cells into pores of its wall. Then the bone marrow transplanted graft (BM-graft) was implanted into the same animal from which the bone marrow was obtained. As a control, the long-fibril-length e-PTFE graft without bone marrow treatment was used.

Thirty-six adult mongrel dogs of both sexes, weighing 7 to 15 kg, were used. Twenty-two of them were implanted with the BM-grafts, and fourteenwith controls. Animals were anesthetized with ketamine hydrochloride (5% solution, Sankyo Co. Ltd., Tokyo Japan) intramuscularly and pentobarbital (2.5% solution) intravenously. A 5 cm segment of the aorta was resected and replaced with the prepared graft. All animal care was in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 80-23, revised 1985).

For light microscopic observations of removed grafts, 3 mm wide sections were cut along the longitudinal direction from the proximal to the distal anastomosis. The specimens were fixed with 3% formaldehyde in phosphate buffer, and embedded in a hydrophilic resin. Thin sections were stained with Hematoxylin and Eosin, Masson and May Giemsa stain, Peroxidase Antiperoxidase (PAP) method, and Von Kossa's stain. The distribution of bFGF

was investigated by immunohistochemical staining according to a previous report[12]. Immunostaining was confirmed in sections by omitting the immunohistochemical control procedures. The number of capillaries per 1 mm^2 (2 mm by 0.5 mm) in five fields randomly chosen from the middle of each graft wall was counted on 3 week, 3 month and 6 month explants. The average number of capillaries was calculated for each implant dulation.

No animal died during the observation period (**Table 1**). Twenty out of 22 BM-grafts were patent at retrieval, and 2 were found to be occluded at 91 days. Eleven out of 14 control grafts were patent, and 3 were occluded at 124, 182 and 183 days.

The BM-grafts retrieved within 7 days were red with fresh thrombus. At 14 days, graft surface was dark red and smooth. At 18, 20 and 21 days, their luminal surfaces were smooth, glistening, and dark red without thrombus. The grafts were surrounded with red granulomatous connective tissue. At 2 and 3 months, the graft lumen was glistening and light red, and the outer surface was red. At 6 months, the grafts were soft without scar formation or abnormal reaction, and the lumen was glistening and light pink .

In the controls, within 7 days, the graft lumen was red with a thin thrombus and was smoother at 14 and 15 days. Pannus extended over the anastomotic suture lines at 27 days and it was glistening white with a length of about 1.5 cm at 83 and 150 days, while a thin red thrombus was in the center. The long-term grafts (6 months) were white on both the inner and outer surfaces.

Microscopically, after enmeshing the suspension, the pores of the BM-graft wall contained clumps of marrow cells including marrow stromal cells, polychromatophilic erythroblasts and myelocytes. No stem cells were identified. At the end of 3 days after implantation, the graft wall was occupied with many erythrocytes and marrow cells. At 4 days, the lumen was covered with a thin fibrin layer without erythrocytes. Marrow cells in the graft were immunoreactively bFGF positive (**Figure 1**). At 7 days, numerous colonies of endothelial-like cell were noticed along the entire graft lumen. These cells were stained by PAP method and Factor VIII positive, suggesting that they were endothelial cells. At 14 days,

the total area of these endothelial cell colonies occupied more than half of the graft lumen, and other areas were still covered with fibrin. Capillaries and fibroblasts had infiltrated into the adventitia and then into the graft wall. Colonies of erythroblasts, i.e., erythroblastic islands, were noticed inside the graft wall, and were always associated with capillaries. Heterophilic myelocyte in mitoses were frequently observed. At 18 days, the graft lumen was completely lined with a single layer of endothelial cells (**Figure 2A**). Subendothelial smooth muscle cells were present near the anastomoses but were not observed elsewhere. Substantial capillary ingrowth was seen in the perigraft adventitia with many capillaries infiltrating the graft wall. At 2 months, multi-layers of smooth muscle cells without hyperplasia were noticed underneath the endothelial cells. The number of erythroblastic islands decreased. At 3 months, heterophilic myelocyte mitosis was still commonly observed. Many myelocytes, megakaryocytes, and lymphocytes were present in the graft together with capillaries. There were immunohistochemically bFGF reactive. Some erythroblastic islands were still present inside the graft wall (**Figure 2B**). Tremendous capillary migration was noticed both in the graft wall and in the adventitia. At 6 months, a few erythroblastic islands were still noticed. Circumferentially arranged inner and longitudinally arranged outer multilayers of smooth muscle cells were observed underneath the endothelial cells (**Figure 2C**). Spongy bone with bone marrow tissue associated with capillaries were observed sporadically in the graft wall (**Figure 2D**). Numerous capillaries were still present (**Figure 3**), but the size of them decreased.

In the controls, no hemopoiesis phenomenon was observed in any of the grafts. The graft wall was occupied by fresh coagula at 1 - 3 days. At 7 days, the lumen was covered with a thin thrombus layer. At 14 days, the graft lumen within 2 mm from the anastomotic lines was covered with endothelial cells, while the remaining area still retained a thin thrombus layer. Fibroblasts infiltrated the adventitia along with a few capillaries. At 25 and 27 days, the endothelialized pannus extended 3 - 5 mm from the suture lines, while the other area was covered by an irregular thrombus with a thickness of less than 50 μm. Smooth muscle cells

were noticed underneath the pannus. Capillary ingrowth was rarely observed. At 3 months, the pannus extended about 15 mm from the suture lines, and was covered with endothelial cells. Multilayers of smooth muscle cells were underneath them. The other area was covered with a thin thrombus. Fibroblast migration was observed in the graft wall along with a few capillaries. At 6 months, the center area of the graft was covered with a thin fibrin layer without endothelial cells. Other areas were completely endothelialized with multilayers of smooth muscle cells. Many capillaries were present in the graft wall (**Figure 3**).

With the exception of highly porous grafts[13], endothelialization on vascular prostheses typically starts from the host arterial wall beyond the suture line. Endothelial coverage of the graft is limited due to endothelial cell aging. After approximately 70 cell divisions, it become difficult for endothelial cells to create new cells for the next generation[14]. They extend approximately 2 cm beyond the anastomosis. The remaining surface of the graft lumen is always covered with fresh thrombus[15]. In the BM-grafts, undifferentiated mesenchymal cells, stem cells, immature blood cells, and stromal cells with fibroblasts and endothelial cells in the bone marrow could potentially differentiate, migrate, proliferate and create a new blood vessel wall. This may be the reason why endothelialization of these grafts began simultaneously throughout the entire graft lumen within a short period of time after implantation.

Exogenic hemopoiesis on these vascular prostheses was unique. Bone marrow formation with capillary blood vessels surrounded with spongy bone was also a remarkable phenomenon although observed sporadically. During hemopoiesis, cytokines continued to be synthesized including bFGF[10,16,17] which could contribute to capillary ingrowth into the BM-grafts. Marrow cells need nutrition for their survival, raw materials for producing blood cells at hemopoiesis, and routes for shipping out their products, i.e. "blood cells". As a result, the capillary ingrowth was demanded. The requirements from the marrow cells might be translated by the cytokines and growth factors. Remarkable capillary ingrowth shortly after BM-graft implantation indicated the exsistence of strong angiogenic properties. Detection of bFGF in the transplanted marrow cells suggested that its production continued throughout the

graft wall as long as those cells existed. Recently, bFGF has gained attention due to its strong angiogenic property and accelerate capillary ingrowth to form collateral circulation for the ischemic heart[18]. The dark-red color came from the newly formed capillaries and numerous immature blood cells at hemopoiesis . The size of the capillaries autoregulatively decreased in parallel with the regression of the hemopoiesis and at that time the graft color turned into light pink. These four items, i.e., bFGF, capillary ingrowth, hemopoiesis, and graft color seem to be interrelated.

The sufficient dose of cytokine and growth factors for angiogenesis and graft healing might be small, but their continuous release would be essential for the stimulation of the cells to endothelialize the graft lumen, since their efficacy lasts a short time[17]. However, there appears to be an autoregulating system in the BM-graft, since endothelialization was complete within 3 weeks and was arrested without endothelial cell hyperplasia or hemangioma formation, while hemopoiesis continued for more than 6 months.

Active angiogenesis in the BM-graft and accelerated endothelialization throughout the graft lumen seemed to be the result of co-operation of the transplanted cells and cytokines secreted from them. The BM-graft appears to be an autocrine artificial organ producing growth factors for itself under an autologously controlled system. Based on these observations, similar phenomenon could be expected with bone marrow transplantation not only in the field of vascular prstheses, but in other fields as well, i.e., undifferentiated cells could differentiate into adequate functional cells with the guidance of environment, matrices and cytokines resulting in creation of new hybrid organs.

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Table and Figure LEGEND

Table 1 Post operative day of abdominal aortic graft removal

Figure 1 Photomicrograph of a cross-section of the BM-graft at 4 days. Many marrow cells with immunoreactively bFGF positive are observed near the adventitia of the graft wall. Immunohistochemical staining of bFGF. Original magnification: x200. The bar indicates 100 μm.

Figure 2 Photomicrographs of cross-sections of the BM-grafts (mid portion). The lumen was completely lined with a single layer of endothelial cells at 18 days (A). Erythroblastic islands (arrows' heads) were inside the graft wall at 91 days (B). Circumferentially arranged inner and longitudinally arranged outer multilayers of smooth muscle cells were underneath the endothelial cells at 182 days. Arrows indicate capillary blood vessels (C). Spongy bone with bone marrow was in the graft wall at 182 days. Arrows indicate capillary blood vessels (D). H.E. staining. Original magnification: A;x200, B;x200, C;x100, D;x100. The bars indicate 100 μm.

Figure 3 The number of capillaries in the BM- and control graft walls at 3 weeks, 3 and 6 months. Closed columns indicate the average of calculated number of capillaries in the BM-grafts and open columns indicate in the controls.

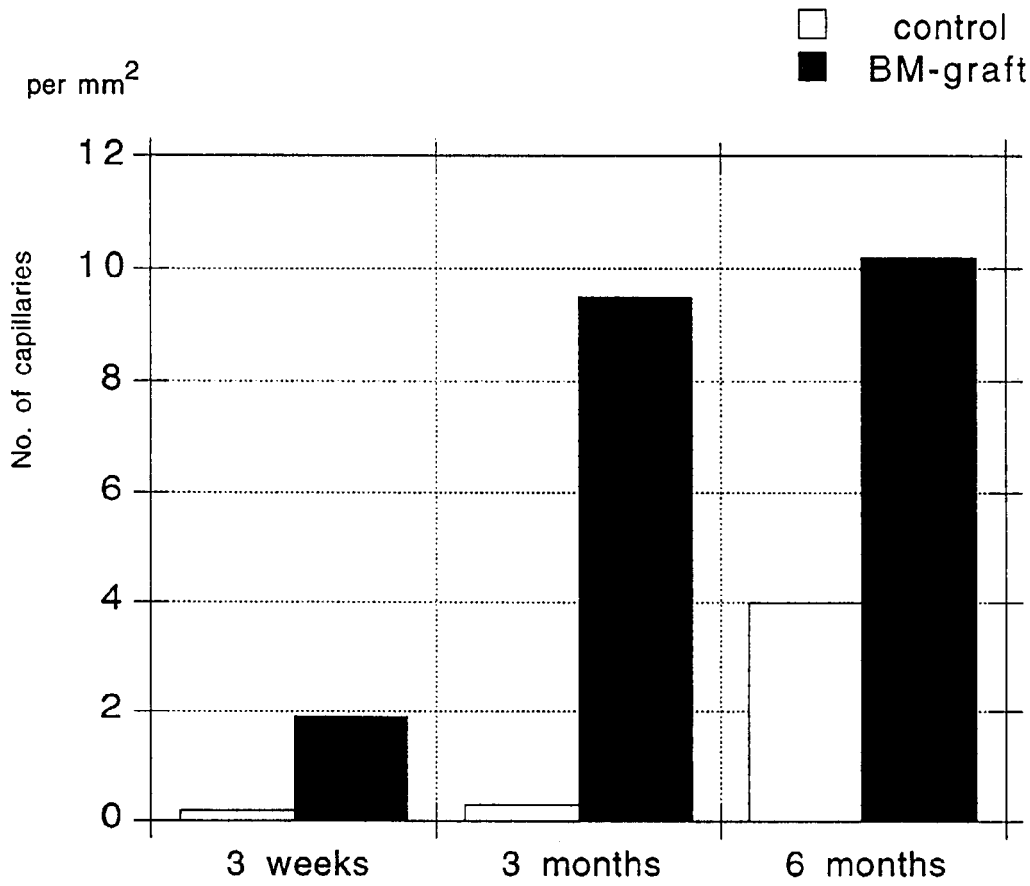
Table 1 Post operative day of the graft removal

BM-graft

Group I	1, 2, 3, 4, 5, 7, 14, 18, 20 and 21 days
Group II	60, 60, 91, 91, 91, 91, 91 and 91 days
Group III	182, 182, 182 and 192 days

Control graft

Group I	1, 3, 5, 7, 14, 15 and 27 days
Group II	83, 124 and 150 days
Group III	182, 182, 183 and 183 days


control
BM-graft
per mm2
No. of capillaries
12
10
8
6
4
2
0
3 weeks
3 months
6 months

第５章

生体組織利用型人工臓器材料

生体組織そのものを人工臓器用素材として活用する方法を我々は考案した。例えば自己の骨髄組織を人工血管壁上に播種すると骨髄組織はそこに生着して栄養を要求し毛細血管を呼び込む。またさらに骨髄細胞はその本能的性質として血液細胞を作る。この造血活動にはそれなりの素材が必要なため、毛細血管が引き寄せられて物質輸送を担う。そしてさらに、造血による産物を送り出すためのルートとしての毛細血管も呼び寄せられる。このようなことから骨髄組織を移植することにより自然に毛細血管豊富な状況を作ることが可能である。そして人工血管は短期間のうちに完全治癒を来す。このような生体のもつ本能的性質を引き出す型の人工臓器が次世代には出現すると期待される。

この度の研究では骨髄を皮下組織内に注入し、そのサイトカイン発現を in vivo で評価したこと、および骨髄を虚血心筋内に注入し、その発現を観察したので報告する。

1. 骨髄自家移植による　Angiogenesis　の誘導

骨髄組織の特性を利用して、その自家組織移植によりAngiogenesisの誘導を行った。骨髄細胞は原始的で幼若な細胞であるため、自家移植すると生着しやすいという特色を持つ。そして生着により栄養を要求し、毛細血管新生を促す。生着後は骨髄細胞はその本能的性質として造血を開始する。そしてそのための素材を要求し、毛細血管新生を促す。そしてさらにその産物である血球を送り出すルートである毛細血管の侵入をも促す。これらの要求は骨髄細胞の産生するサイトカインによって伝達される。この38特性を利用すれば血管新生を促すサイトカインを持続的に産生させるシステムを得ることができる。この度はこの仮説にもとずき、成犬皮下に骨髄細胞および対照として末梢血を自家移植し、1,3,7および10日目に採取したところ、予期した通りの無数の毛細血管の新生、およびそれを促すサイトカインの一種であるbFGFの産生を認めたことから、前述の仮説を立証できたと考えられる。

はじめに

ハイブリッド型人工臓器の設計においても、創傷治癒促進においても血管新生は重要な意義をもつ(1,2,3)。しかるにこれまでの外科的処置においては、それは患者自身の自然な治癒力にまかせた状態であった。我々はこの点を改善するため積極的に血管新生の誘導を試みてきた。この度は骨髄を自家移植することにより、骨髄細胞の本能的性質を利用した血管新生促進を動物の皮下組織内で発現させる試みを行ったので報告する。

材料と方法

6頭の成犬において全身麻酔下に腸骨を露出させ、腸骨稜を約1 cm切除し、この部分より骨髄穿刺針を刺入し、骨髄液を2 cc採取した。次に背部皮下組織内の2ヶ所に1 ccずつ採取した骨髄を注射針を用いて注入することにより自家移植を行った。対照としては、末梢血を静脈より2 cc採取し、これを1 ccずつ同様に皮下組織内に場所を変えて注入した。このような処置を行った動物より処置後1, 3, 7および10日目に注入部の組織を周囲組織を含めて採取し、肉眼的、光顕的に観察した。なお切片の染色はH.E.染色のほか強力な毛細血管産生誘導因子の一つであるbFGFをそのモノクロナール抗体を用いて染色する特殊染色、および内皮細胞の証明のため、Factor eight染出し用にPeroxidase anti-peroxidase method （PAP法）(4)による染色を行った。

結果

肉眼的観察では骨髄移植例において皮下組織内注入部に1日目には血腫状、3日目の例では注入した骨髄が集積している様子がみられたが、7日目にはそれが拡散している状態がうかがわれた。そして10日目には暗赤色のドーナツ状の拡がりがみられ、中央部分は淡白色となり、そこには硬結状態の結合組織塊が形成されていた。一方、対照の末梢血注入部では3日目に皮下血腫形成していたが、7, 10日と時間の経過とともに拡散し、退色しつつあった。

光顕的観察では骨髄移植例において、1日目には皮下脂肪組織間隙に多数の赤血球を含む骨髄細胞が散在していた。3日目には組織網の中に補足されるような形で存在していた。これらの細胞はbFGFのモノクロナール抗体を用いた染色で陽性を示していた。

注入7日目にはドーナツ状の拡散部分が赤血球の皮下組織内への拡がりであることが判明した。この部分では骨髄細胞を認めることはできなかった。しかし、ドーナツ状の中央部分に形成された硬結部分では線維芽細胞が脂肪組織内に増殖し、肉芽組織を形成し、その中には少数ながら骨髄細胞が残存していた。10日目にはドーナツ状の拡散部分で赤血球の拡がりは、さらに著明となるほか、赤血球に混じって多核白血球が多く認められるようになった。一方硬結部分においては、10日目で著しい線維芽細胞の増殖、毛細血管の増成、侵入が認められた。bFGFのモノクロナール抗体による染色では、この硬結部分のあちこちに、その陽性を示す細胞の存在を認めた。しかし、これらの細胞が骨髄細胞であるという証拠はbFGF染色では得られなかった。骨髄細胞は一般のH. E. 染色でも毛細血管、線維芽細胞等に埋もれて、その存在を証明することはできなかった。なお、骨髄細胞の間葉系細胞への分化を示す所見は見られなかったが、毛細血管および線維芽細胞の異常なほどの増殖は顕著なものがみられた。また、PAP法による染色では毛細血管は陽性に染色され、内皮細胞にfactor eight産生力のあることを示していた。

一方、末梢血を注入した部分では、3, 7, 10日と赤血球を多数含む皮下出血様の様相を示したが時間の経過とともにその濃度は薄くなり、分布は広がっていき、骨髄例でのドーナツ部分とみられたと同様に多核白血球がみられるようになり、赤血球を貪食したマクロファージも認められるようになった。しかしbFGFの存在を示す染色では、10日目の例において散在的にその陽性を示す細胞がみられた。なお、毛細血管もごく少数認められたが、これが同部で新生したものか、周囲より侵入したものかは不明であった。

考察

結果より明らかなように骨髄を自家移植すると、骨髄細胞は生着し、その部分でbFGFなどのサイトカインを産生しつつ、細胞活動を開始する。骨髄細胞はその特性として造血活動のほか(5)、細胞分化にも関与し、間葉系のあらゆる細胞へと分化しうる能力があるが(6)、この度の観察ではそれを示す明らかな所見は得られなかった。しかしながら、骨髄液を注入することにより、その部で間葉系細胞の代表的な細胞の一つである線維芽細胞の異常なまでの増殖を認めたことを考えると、すでに存在していた線維芽細胞の単純増殖のほか、骨髄細胞の分化および増殖も否定できるものではない。

これに比べ、多数の毛細血管の侵入および増成は顕著であった。周囲の皮下脂肪組織内に余り毛細血管のないことを考えると、これは明らかにこの部分で刺激を受けて増殖したものと考えられる。またbFGFの強い陽性を示す細胞が多数存在していたことも、その作用によって毛細血管の増殖が進行したことが骨髄細胞の働きによるものであることを示している。そして、これらの毛細血管壁を構成する細胞がPAP法で陽性に染色されていることから、機能をもつ内皮細胞が管腔形成し、同部に集合していることを示している。

骨髄細胞は生体内において、高齢においても活動を続けている特異的な細胞であり、いくつかの特有の本能的性質を持っている(5,6)。例えば骨髄細胞は本来、幼若で原始的な細胞であるため、自家移植をすると、異なった環境下においても生着する特徴がある。そして生着により必要な栄養を要求し、毛細血管の新生を促す。生着後、骨髄細胞はそ

の本能的性質として造血を開始する。そしてそのための素材を要求し、そのための毛細血管の侵入を促す。そしてさらにその産物である血球を送り出すためのルートとしての毛細血管をも要求する。これらの要求は骨髄細胞の産生するサイトカインにより伝達される。この度の皮下組織内における移植では、本来、毛細血管新生の必要性の高くない場において、そのような活発な新生が生じたことから、そしてbFGF産生が強く示されていることから、骨髄細胞の移植による影響が顕著に出ていると考えられる。この事実は、骨髄移植をすることによって任意的に毛細血管新生を促させることが可能であり、任意的 Angiogenesis の誘導に成功したものと考えられよう。そうなると、この手段を用いて全身のあらゆる部位における治癒促進の治療手段として本方法は活用されるのみならず、血管新生があると好ましいと考えられるハイブリッド型人工臓器を設計する上においても有用な補助手段となるであろう。

対照例の全体において、および骨髄移植のドーナツ状に拡散した赤血球を多く含む部分に於いて、毛細血管新生は著明ではなかった。しかし限局的にbFGF産生がみとめられた。これは血腫形成による機械的刺激によることも考えられる。一般に皮下出血を来した場合、無菌性の炎症とそれに引き続く毛細血管の新生は、このような事が生じていると推測される。それは赤血球の吸収による機械的刺激の減少によると推測され、bFGF産生が一時的なものであったと思われる。赤血球は一般の細胞とは異なり、平均して120日間、身体内のあらゆる狭い所をもすり抜けて循環し、全身に酸素を供給する働きをもつ。そのため、膜表面に積極的なアンカリングサイトを一切持っていない(7,8)。そのため本研究のように、皮下組織内に注入されても徐々に拡散してゆく。この過程においてbFGF産生を軽度ながらも機械的刺激で促していたことが考えられる。ものと思われる。骨髄注入例においても骨髄液中に混入した末梢血の赤血球が同様の拡散傾向を示したため、肉眼的にそれがドナーツ型の形態を来したものと思われる。しかし、中央の硬結部分では骨髄細胞がアンカリングサイトを持つことから、局所に残存し、その部で活動を継続したものと思われる。

骨髄移植例においては、7日以上経過すると移植した骨髄細胞の存在が明らかでなくなったことは、それが散在したこと、もしくは活性化され増殖してゆく線維芽細胞、毛細血管などが骨髄細胞コロニー内にて活発に増殖し、結果的には分散させられたという推測が立つ。人工血管における骨髄移植例では人工血管壁という特殊な環境において、骨髄細胞は90日以上生着していた(9)。これは、その場における環境が骨髄細胞の集落維持をを支配しているものと思われる。10日目でもbFGF陽性細胞が散在的ではあるが多く認められたことからも、皮下組織内では骨髄細胞はどこかで生着し続け、bFGFなどのサイトカインを産生し続けていると思われる。マーカーを付着した骨髄細胞を使用すれば、これは明らかとなるであろう。いづれにしても、bFGFを持続的に発揮させ得ることがこれで可能となった。

おわりに

骨髄細胞を自家移植することにより、任意的にAngiogenic factorを産生し続けるシステムを得るという仮説を立証した。この方法は今日の外科治癒においても人工臓器開発においても広く利用されているものとして期待される。

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2. 骨髄組織の自家移植による血管新生の誘導

1) 研究目的

医学、特に治療医学の実践については、4000年以上の昔まで逆のぼることができる。その長い歴史の中にあって、人工臓器の臨床応用の歴史はわずか40年である。しかし、この歴史の新しさにもかかわらず人工臓器は従来困難視されてきた多くの疾患の治療に大きく貢献してきた。そして今日、人工臓器には新たな、しかも大きな期待が寄せられており、21世紀における治療医学を支える大きな柱の一つになるのではないかと予測されている。

このような期待を背に、人工臓器研究者は心機能人工臓器の開発に取り組んで来た。これまでの研究では、新素材の開発、あるいはすでに完成した素材と新素材との組み合わせによる複合効果などを用いて従来存在しなかった、もしくは従来の方法では限界のあった領域での人工臓器の機能発揮に取り組んできた。しかしながら、生体機能には微妙な特殊高度機能が多く、これらを人工物で代用することがきわめて困難な事が多いのも実状である。確かに一部の領域においては、人工的に作成した機能の方が天然物より、つまり臓器や器官よりも機能的に、例えば強度面などで凌篤することもあるが、多くの場合、総合的にみても個々の機能においても、人工物のみで天然器官、天然臓器の機能を代替えする事は難しい。そこで生体の機能をもつ細胞と人工物との組み合わせにより、個々の特性を生かした、いわゆる「ハイブリット型人工臓器」の創造が過去10年間ほどの間に行われた。

我々も人工臓器領域を中心として、ハイブリット型人工臓器の開発に取り組んできたが、この度の研究プロジェクトにおいて、ハイブリット型人工臓器に用いる細胞の工夫として、細胞のもつ特殊機能を十二分に発揮させ、機能的な拡がりをもつ人工臓器の創成を目指した研究を行い、昨年度の研究成果として骨髄移植人工血管をその一例として報告した。今年度はその実績をもとに、その長期例の結果を報告するとともに、機能をもつ特殊細胞、例えば骨髄細胞を用いるとどのように治療効果が拡がるかについて考察を加え、新たな治療法開発への糸口をさがすことを研究の目的とした。

2) 研究成果

昨年報告した骨髄自家移植人工血管の成果を主として以下に示すが、今年度はその中において観察期間を6ヶ月としたことから、その6ヶ月目の採取試料の結果の解析を加えて報告する。

研究の背景

人工血管の新生血管壁形成は著しく遅延するのが常であり、剖検時においてみられる人工血管では、吻合部から約1cm程度の内皮細胞による被覆は認められても、他の部分においては内皮細胞は被覆せず、長期間経過しても血栓が付着したままの状態である。我々はこの現象を「血管壁における難治性潰瘍」であると見なした。そうなると一般に皮膚における難治性潰瘍の治療に皮膚組織の細切片を移植することで治癒の促進をはかっているように、あるいは遷延性骨折の治療に骨の細切片を自家移植して治癒を早めてい

るように、血管壁においても自家組織細切片移植が有効的手段であろうと推測される。

以上の推測のもとに我々は自家静脈片を細切して布製人工血管壁に移植し、予期した通りの新生内膜早期治癒を得た。そこで次に我々は静脈片を必要十分量得られない場合を考慮して、皮下脂肪組織を細切して自家移植する方法を考案し、動物実験を行ったところ、静脈片を使用した場合とほぼ同等の結果を得ることができた。

この結果を受けて我々は臨床への応用を行ない、四肢の末梢動脈閉塞性患者に対し、皮下脂肪組織細切片を播種した人工血管の植え込みを行なった。その結果、36例中33本開存という良好な成果を得た。しかしながら皮下脂肪組織は年令、性別、栄養状態、健康状態、糖尿病などの合併症の有無などにより、個々の例で著しい差違のあることが判明した。そのため我々はこれら以外の組織を用いることの必要性にせまられていた。

この度の研究におけるコンセプト

前述の状況下にあり、我々は新しいコンセプトとして次の条件を考慮した。

1、高齢者においても、病的状態の人からでも、活発な分裂能を有する細胞を含む組織を利用できるよう、採取組織の種類を正しく選択する。

2、移植された組織自身が各種サイトカインやgrowth factorsを産生し、組織治癒を促進させることのできる「オートクリン型の器官形成」を得る。

3、内皮細胞を人工血管に播種するという発想を捨て、その代りに未分化の細胞を用い、環境に応じてその細胞が必要とされる成熟細胞へと分化するよう、そしてそのことによって新しい器官を形成するように材料と環境を操作して誘導するという、新しい概念の「細胞分化誘導型人工臓器」にのっとったハイブリッド型人工血管を創る。

このコンセプトを満足させるため、我々は骨髄をその自家組織として選び、動物実験を開始した。骨髄は常に若い細胞を産生し続けており、その幹細胞はあらゆる間葉系細胞の源となりうる細胞であって、環境に応じて分化する。また骨髄は活発な造血能を有し、この造血過程において多量の、かつ多種類のサイトカイン、growth factorsを産生することが知られている。例えば骨髄細胞が造血するためには多量のエネルギーとなる栄養が必要であるため、その付近への毛細血管の侵入が要求される。また造血によって産出される血球成分を搬出するための経路としても毛細血管が必要とされるため、さらに毛細血管の侵入が要求される。これらをサイトカイン、growth factorが伝える。このようなangiogenesisは新生血管壁形成過程において、とても望ましい状態である。

人工血管の作成

このコンセプトが正しいか否かの確認のため、我々はE-PTFE graftを人工血管基材として選んだ。なぜならば、一般にE-PTFE graftは吻合部にのみ内膜が形成され、他の部分には内膜が形成されないといわれている。従って骨髄細胞から産生されるサイトカイン、growth factorの効果、および細胞分化能の有無と内膜形成との関係がE-PTFE表面上で明らかにされると期待できる。但し、市販のE-PTFE graftはfibril lengthが30μmであり、個々のfibrilの間隙は数ミクロン以下であることから骨髄組織を捕捉させるには狭すぎることもあり、fibril length 90μmというE-PTFE-graftをBaxter Health Care

Corporation, Irvine, CA, USA に特注した。

人工血管への播種方法としては、骨髄を腸骨もしくは胸骨より穿刺にて約 0.5ml 得て、これをラクテイトリンゲル液 20ml 中に入れて骨髄組織浮遊液を作成した。次に閉鎖回路を用い、骨髄組織浮遊液を三方括栓を経由して人工血管内に圧注入した。人工血管壁を通過した液はコネクティブチューブを通って注射器内に吸引され、再び人工血管内に圧注入された。この操作を数回繰り返すことにより、骨髄組織播種人工血管（M-graft）を得た。対照としては骨髄組織を絡ませていない 90μm の fibril length をもつ基材の人工血管（C-graft）を用いた。

実験動物への植え込み

16 頭の雑種成犬（7～12kg）を全身麻酔下に腹部正中切開にて開腹し、腹部大動脈を約 5 cm 切除し、作成した人工血管（M-graft, 内径 6 mm, 長さ 6 cm）を 5－0 ポリエステル糸にて連続縫合により端々吻合した。植え込まれた人工血管は術後 3 週間および 6 ヶ月で採取した。対照としては C-graft を 12 頭の成犬に同様の手技で植え込み、同じ期間の経過後採取した。また術中には抗生物質の腹腔内投与を行なったが、抗凝固薬は一切使用しなかった。なお得られた各試料は肉眼的光顕的に観察した。

結果 1、肉眼的所見

M-graft の植え込みを受けた動物のうち、1 頭が慢性イレウス症状を示していた。この動物は 3 週間目に屠殺し試料を得た。3 週間目にはこの例を含め 4 本の M-graft を採取したが、そのうち 1 本は末梢側吻合部に器質化組織があり、人工血管内は血栓により閉塞していた。他の 3 本は開存していた。残りのうち 6 本は 3 ヶ月目に、さらに 6 本を 6 ヶ月目に採取した。これらはすべて開存していた。これらの人工血管の内面は植え込み 3 週間目で内表面には全く血栓の付着はなく、光沢のある平滑面を示したが、その色調は暗赤色であった。人工血管壁は柔軟で外膜側には瘢痕組織形成などの異常所見はみられなかった。3 ヶ月目の試料においても血栓は認められず光沢のある内面を示したが、色調は相変わらず赤色を呈していた。さらに 6 ヶ月目の試料においても同様の所見を呈していた。

一方対照の C-graft においては 3 週間目、3 ヶ月目および 6 ヶ月目に採取した 12 本すべての人工血管は開存していた。しかしすべての人工血管の内面に薄い血栓の付着を認めた。

植え込み 3 ヶ月目および 6 ヶ月目の例においては赤味は減少し、吻合部付近は白色であったが人工血管中央部分は血栓が薄く付着していた。

結果 2、光顕的所見

植え込み前の M-graft 壁には多数の骨髄細胞が捕捉されていた。しかしこの中での骨髄幹細胞の同定はできなかった。各細胞は単独で存在したり、集団となっている所もあった。

植え込み 3 週間目では、全内表面において一層の連続した内皮細胞による完全な被覆

を認めた。人工血管壁内部には形質細胞、大食細胞、細網細胞、組織球、線維芽細胞などを多量に認めたほか、無数の毛細血管が認められた。これらは特に外膜側の組織内に多く認められたが人工血管壁内にも多く認められた。

人工血管壁内および外膜側の組織内には赤芽球の集団（赤芽球島）が多く認められ、その他、リンパ球、リンパ芽球、巨核球、骨髄球、網状赤血球などが多く認められた。これらの細胞の出現は3ヶ月目には減少していたが、それでもまだ赤芽球島が人工血管壁内および外膜組織内に認められた。またこのような細胞のみられる所には必ず多数の毛細血管が侵入していた。3ヶ月目の内面はやはり内皮細胞が覆っていたが、内皮細胞層下には数十層の平滑筋細胞層による内膜形成がみられた。この層は3週間目の試料においては認められなかったものである。このような所見は6ヶ月目においても同様にみとめられた。

一方対照のC-graftの3週間目においては内面は完全に赤血球を多く含むフィブリン層に覆われていて、内皮細胞は吻合部に約1 mm認められる程度であった。人工血管壁内部においては線維芽細胞、大食細胞、形質細胞などが認められたほか、フィブリンが多量に残存していた。なお、毛細血管の人工血管壁内への侵入は認められなかった。植え込み3ヶ月目においてもフィブリン層は外膜側に厚さ約100ミクロンの層となって認められており、ここへの各種細胞や毛細血管の侵入は認められなかった。またこのようなフィブリン層の認められない所においては線維芽細胞と膠原線維が主体の細胞線維性組織を形成していたが、毛細血管の侵入はまれであった。一方内腔面においては、吻合部から約1.5 cmまで内皮細胞が宿主血管内面より縫合線を越えて人工血管内面に伸展していた。しかし、人工血管中央部分では内皮細胞はみられず、厚さ約200ミクロンのフィブリンを主体とした血栓層によって覆われていた。また6ヶ月目においても同様の所見がみられ、対照例においては長期間かけても治癒が進行していないことが判明した。

考察1、オートクリン型器官形成

実験結果から明らかな通り、骨髄組織を播種したM-graftの方が対照のC-graftに比べ明らかに内皮細胞の被覆が急速であり、しかも吻合部、中央部という部位による治癒の速度の差違は認められなかった。この結果、我々の当初目指していた自家組織片移植による人工血管内膜形成の促進は骨髄組織を用いてでも静脈片と同等に効果のあることが判明した。

その治癒過程で肉眼的に特徴的であった点は色調の赤さである。一般に内皮細胞の被覆した新生内膜は白色で光沢がある。しかし、本実験では血栓が付着しておらず、光沢があるにもかかわらず、赤色を呈していた。この理由は壁内部の異常なほどに増生した毛細血管の侵入と骨髄の異所性造血による赤血球、赤芽球などの赤色に由来するものと思われる。骨髄の異所性造血はまれに認められる現象であるが、このような形で人工血管壁内にみられたのは世界でも初めてのことであろう。それにも増して、毛細血管の増生、侵入には著しいものがあった。これは本研究のコンセプトの所で述べ、骨髄を使用することの長所の点で期待した現象、つまり人工血管壁内部でangiogenic factorが働き続けていることによると思われる。つまり人工血管壁内にgrowth factorを固定化しなく

とも、それを産出す細胞を植え込んだがために、それを産生し続けたことによってこのような毛細血管の侵入、そしてそれに引き続く早期の内膜形成という結果が出たと思われる。すなわち、「オートクリン型の器官形成」がここに誕生したことを実証しているといえよう。

考察２、細胞分化誘導型人工臓器

この骨髄細胞の活躍により活発なサイトカイン産生、growth factor の分泌が行われたことは、これらをまだ検出してはいないが、現象面では明らかであった。しかし一方、骨髄内の幹細胞は他の細胞へと分化して成熟し、人工血管壁の一部でも形成するのに貢献したであろうか。この点については試料不足で同定できなかった。例えば新生血管壁を形成した内皮細胞の由来はどこであったのか、これは３週間目に採取したすべてのM-graft の内面がすでに内皮細胞によって覆われていたため討論できるだけの試料がないので判断しかねるのが実状である。もっと早期の試料において、マークした骨髄細胞と内皮細胞を使用するとか、詳細な幹細胞の追跡が必要であろう。それに毛細血管の増生が外膜側に著しく認められたことも周囲組織から侵入した毛細血管の内皮細胞が内腔面に至ってコロニーを作ったと、充分に推測できる情報となっている。しかし、最も内腔面に近い所に置かれた幹細胞はその置かれた環境下でどのような態度を取ったのであろうか。幹細胞が環境に応じていかなる間葉系の細胞にもなりうるという特別な分化能を持つことが知られているだけに興味深い所である。この「細胞分化誘導型人工臓器」の芽がここに誕生していたことを実証するために、詳細な研究をさらに続けてゆきたいと考えている。

考察3. オートクリン型人工臓器の考え方を利用した新しい治療法

骨髄組織を自家移植することにより、血管新生因子を持続的に産生させるシステムを人工物に組み込ませることが可能となった。このことはあらゆる人工臓器において、血管新生を必要とする要件があればこのシステムを使いうることを示している。そしてさらにまた人工臓器に限らず、天然の臓器においてすら血管新生を必要とするような病的状態であればこのシステムを使うことが可能であることを示している。

例えば心臓において血流量の減少した状態、すなわち疎血性、虚血性心疾患などでは、現在は手術的に、あるいはカテーテルを用いて Inter ventional に冠血流量の増加を試みている。しかしながら、このような治療法を用いても、なかなか血流量の望めない心筋に対しては最近遺伝子導入により血管新生因子を産生するように操作した細胞を用いて、あるいは遺伝子導入により血管新生因子を産生させて、その因子を用いて、心筋内への血流量を増加させようとする研究が行われてきている。しかしながら、このような研究は世界中の主な研究所、大学で多大の時間と費用、人材をつぎ込んで行われているにもかかわらず、良好な成果は出していない。

我々はこのような状況を解決するため、オートクリン型人工臓器の考え方より派生した血管新生促進システムを虚血心筋へ転用する試みを行った。その成果の一部を以下に示す。

実験モデルとしては、成犬の冠動脈左前下行枝、左廻施枝の一部を結紮することにより、心尖部に心筋虚血部分を作成した。次に腸骨稜より穿刺にて骨髄を約0.5ml採取し、これをそのまますでに作成している心筋虚血部分に注射針を用いて筋肉内注射を行った。この処置後、3週間まで経時的に移植された骨髄細胞の挙動および心筋の変化を観察した。

その結果、骨髄を筋肉内注射していない対照群では心筋細胞は徐々に壊死におちいり、瘢痕性の組織となったが、骨髄を注入した心筋では筋細胞の壊死はおきるものの、その部に無数の毛細血管を含む肉芽様の組織が形成された。移植された骨髄細胞は移植後1週間まではその存在を認めることができたが、それより長期例では消失したのか、他の細胞へと分化したのか、骨髄細胞の形態としては存在を認めることはできなかった。また血管新生因子の存在を示すbFGFのモノクロナール染色では骨髄移植心筋で強陽性に染色されていることが判明した。

以上の結果より、血管新生産生システムがハイブリット型人工臓器のみならず、一般の天然臓器においても機能を発揮し、心筋内でも毛細血管新生に大きく貢献していることが明らかとなった。従ってハイブリット型人工臓器の研究は、人工臓器の機能向上のみならず、広く一般の治療法の改善とか、新しい治療法の開発に役立つ事が明らかとなった。

結語

ハイブリット型人工臓器を設計する上で過去10年間、細胞をいかに快適な状態に置くか、そのためのマトリックスの立体構造はいかにあるべきか、化学構造をどのように改良すれば良いかといったことに注意が払われてきた。そして、それに用いる細胞も直接的に役に立つ高度に分化した細胞が用いられてきた。従って人工血管の場合は、細胞が生着しやすいようにコラーゲンゲルやデルマタン硫酸などを用い、使用細胞も内皮細胞が用いられてきた。しかし、この度の研究ではさらに細胞の特殊機能を発揮させるための高度に分化した細胞より、むしろ未分化の細胞、具体的には骨髄細胞を用いることで、その細胞が生体内の環境に応じて特殊細胞へと分化したり、さらにまた、その過程において種々のサイトカインを出すことによって、前述した「オートクリン型人工臓器」および「細胞分化誘導型人工臓器」という概念を打ち立て、それの一つのモデルを示すことに成功した。またさらにこのような考え方を用いて人工臓器のみならず一般の臓器においても、この基本的考え方を転用した新しい治療法を開発することができるという可能性を示すことができた。

このようなことから「ハイブリット型人工臓器」の開発研究はこのプロジェクト研究を境に新たな段階に入るのではないかと予測される。

THERMAL CROSSLINKING FOR BIOLOGICAL DEGRADABLE MATERIALS

-----PRELIMINARY REPORT-----

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Running Head

Themal crosslinking for biological materials

Key Words:

Thermal Crosslinking, Esterification, Collagen, Blood vessel prostheses, Glutaraldehyde, Biomaterials, Dehydration, Foreign body reaction

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In order to diminish undesirable side effects of chemical crosslinking for biodegradable materials, we developed a thermal crosslinking method which involved esterification by dehydration under dry conditions. The optimal condition for crosslinking was the heating at 130 °C to 140 °C, for 40 hours. Efficacy of the crosslinking was evaluated using enzymatic digestion of 0.01% protease in phosphate buffer at pH 7.4. Collagenous materials without crosslinking were digested completely within 30 minutes. With the thermal crosslinking, however, it required 7 days for digestion. The capacity for holding water was also tested. Water content decreased when the crosslink density was increased. As an in vivo experiment, 6 succinylated collagen sealed vascular grafts treated with the thermal crosslinking were implanted in the abdominal aortae of dogs and removed 21 days later. These grafts showed no foreign body reaction at all. The collagen layer was almost completely absorbed. A collagen sealed graft crosslinked with formaldehyde (Hemashield, Meadox Co.) used as a control showed strong foreign body reaction. These results suggest that the physical crosslinking method was suitable for biodegradable biological materials such as collagenous materials without the undesirable side effects of chemical crosslinking reagents.

Introduction

Collagen and collagenous materials have been recognized as suitable biocompatible materials[1,2]. Those used for medical implants are crosslinked by chemical reagents such as glutaraldehyde except when they are used autologously. Without crosslinking, several problems are encountered including immune reactions problems, weak mechanical properties, cracking, and detachment of the materials resulting in embolism in the case of vascular prostheses. To overcome these problems, chemical crosslinkers are widely used [3,4]. Vascular prostheses sealed with collagenous materials can also be expected to have biocompatible properties. Collagen is a unique protein which contains hydroxyl, carboxyl, and amino groups[5]. Modification of collagen to introduce intermolecular crosslinking can be done either by esterification of the cardoxyl groups[6,7] or by amide formation of the amino groups with chemical reagents[8]. But chemical crosslinking of collagenous materials produces undesirable side effects[9-12].

In the present study, we tested a thermal crosslinking method for biodegradable materials. We also discuss the relationship between crosslinking density and water content in

the crosslinked materials. A vascular prosthesis collagen coated by the thermal crosslinking was implanted in the abdominal aorta of dogs and compared with a control (Hemashield, Meadox Co.) in order to evaluate the foreign body reaction against the thermal crosslinked materials in vivo.

Materials and methods

1. sealing substances and framework for the vascular prosthesis

As coating, succinylated collagen (Koken Co. Ltd., Tokyo, Japan) and gelatin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used. Heparin sodium and chondroitin sulfate C sodium salt (Wako Pure Chemical industries, Ltd., Osaka, Japan) were used without purification.

As a framework for the vascular prosthesis, a knitted Dacron fabric prosthesis (MICRON, InterVascular Co. Ltd., Clearwater, Florida, U.S.A.) was adopted. As a control vascular graft, a Hemashield graft (Meadox Co. Ltd, Oakland, NJ, U.S.A.) was used.

2. Collagen sponge used for evaluation

Succinylated collagen powder was dispersed to create a collagen suspension at a concentration from 1 to 15%. The suspension was poured into a tray, and frozen at -80°C, and then lyophilized overnight. The dried sponges were placed in an oven and heated for 20 and 40 hours at each of the following temperatures: 100°C, 120°C, 130°C, 140°C, and 150°C . The following four types of materials were crosslinking in this way and tested: 1) succinylated collagen; 2) succinylated collagen containing 5% heparin sodium; 3) succinylated collagen containing 6% chondroitin sulfate C sodium salt; and 4) gelatin.

3. Protease assay

The resistance of crosslinked materials to biodegradation was assayed by immersion in protease (obtained from bovine pancreas Type 1, Sigma Chemical Co., StLouis, MO, U.S.A.) phosphate buffer solution[11]. The protease solution containing 0.8 units/ml was prepared by adding 80 units of protease (1 unit protease will hydrolyse casein to produce color equvalent to 1.0 micromole of tyrosine per minute at pH 7.4, at 37°C) to 100ml of phosphate buffer, pH 7.4. Approximately 0.04 to 0.1 gram of each specimen was added to 2ml enzyme solution and put

on a vortex mixer for 30 sec., then incubated at 37°C. Each sample was weighted after removal from incubator and dried. Calculation of degradation percentage D was as follows.

$$D=\frac{Wo-Wi}{Wo} \times 100\%$$

Wo: dry weight of sample before digestion.
Wi : dry weight of sample after digestion.

4. Water content determination

Dried samples were weighed (dry weight) and placed into a tube, then immersed in a distilled water vortex for 30 sec. The tubes were left at room temperature. After 30 min, the sample was placed between two dry pieces of tissue paper to absorb surface water. The sample was then weighed and the wet weight recorded. Water content (C) was calculated as follows:

$$C=\frac{Ww-Wd}{Ww} \times 100\%$$

Ww: Weight of sample after absorbtion of water (wet weight)
Wd: Weight of sample before swelling (dry weight)

5. In vivo experiments

1) Preparation of vascular prostheses:

A 3% succinylated collagen solution was injected into a vascular prosthesis several times under pressure with our closed circuit system[12]. A Teflon rod (Sanplatec Corp., Osaka, Japan) was inserted into the seal prosthesis to preserve a smooth surface in the prosthesis lumen. The prosthesis was frozen at -20°C and lyophilized, and then baked in an oven at 130°C for 40 hours. Thus the prosthesis was thermally crosslinked and sterilized simultaneously instead of using chemical or physical sterilization.

2) Implantation of the prepared prosthesis and the control

The vascular prostheses (internal diameter 6mm, length 6cm) were implantedin the abdominal aorta of 12 dogs. Six of the dogs were used for the prepared grafts, and the other six for the control grafts which had been treated with formaldehyde vapor.

Under anesthesia, 5 cm of the aorta was resected and replaced by the prosthesis (6 cm in length). Cephalosporin 1mg was administered into the peritoneal cavity before closing the abdomen, but no anticoagulants were used at any time.

All animal care was in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Cares and Use

of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institues of Health (NIH publication No. 80-23, revised 1985).

3) Explantation of prostheses

The specimens were removed from the animals at 3 weeks after implantation. Before harvesting, heparin sodium (100IU/kg) was administered intravenously to prevent clotting. All the specimens were examined microscopically.

4) Histological examination

For light microscopic observation the explanted grafts were fixed with 1% glutaraldehyde in PBS, pH 7.4, dehydrated in a graded series of alchohol, and embedded in hydrophilic resin. Sections were stained with hematoxylin and eosin.

Results

1. Prepared sponges

The prepared sponges were slightly hard when dry, but soft when wet. The swelling speed of the sponges was slower than in non-treated samples at the primary stage as shown in Figure 1. Swelling speed was decreased with increasing crosslink density. Water absorption decreased with increasing crosslink density.

Non-crosslinked sponge were dissolved in water at room temperature after 30 min., because there was no intermolecular covalent crosslinking and the molecules were not compacted tightly enough to have strong non-covalent intermolecular bonding, but all the thermally crosslinked sponges were intact in water even after one week at room temperature.

Sponges containing polysaccharides such as heparin or chondroitin sulfate have a higher capacity of water absorption as shown in Figure 2. These results indicate that mucopolysaccharides which had hydroxyl and carboxyl groups were condensed with collagen by thermal dehydration.

2. Protease assay

The resistance to degradation by protease was assayed for each sample treated under different conditions. Figure 3 shows that the samples treated at 130°C for 40 hours resisted significantly for longer time than those treated at 100°C for 40 hours. The resistance to protease increases with increased crosslink density. Thus, the crosslink density obtained by

thermal dehydration at 130°C to 140°C for a longer time was significantly higher than below 120°C or over 150°C for a shorter time.

3. In vivo analysis

All the grafts were patent at retrieval. At 3 weeks, the outer surfaces of the grafts with thermal crosslinking were enclosed by loose, thin connective tissue containing adipose tissue like natural arterial adventitial tissue (Fig. 4A). The graft was easily separated with scissors from the neighbouring organs through the connective tissue at the adventitia.

Most areas of the luminal surface at 3 weeks were white without red thrombus, but approximately 20 % of the surface had thin red thrombus (Fig. 5A). In light microscopy, a complete endothelial-like cell lining with many layers of smooth muscle cells underneath could be seen in the white areas throughout the graft surface. In the interstices of Dacron fibers, numerous fibroblasts and some capillaries were also present. Most of the impregnated collagen was already absorbed. There were no giant cells, plasma cells or lymphocyte infiltration around the collagen (Fig. 6A).

The outer surface of the control grafts was enveloped by granulation tissue (Fig. 4B). The areas near the anastomotic sites were covered with endothelial cells. Other areas were covered with fresh thrombus (Fig. 5B). Most areas of the graft had the crosslinked collagen remaining under the thick thrombus. Around the collagen and inside the graft wall, multiple macrophages, giant cells, and lymphocytes were present (Fig. 6B). There were no neutrophiles around the collagen and no fibroblast infiltration.

Discussion

1. Thermal crosslinking

In the protease assay, the materials without thermal crosslinking were completely digested within 30 minutes. But the thermal crosslinking made the material insoluble for over 1 hour, it required 7 days for complete digestion by the protease. This showed the efficacy of the thermal crosslinking.

After complete removal of water from the materials, condensation reaction can cause the equilibrium concentrations to shift irreversibly towards the formation of intermolecular crosslinking as follows.

$$\text{protein-OH} + \text{HOOC-protein'} \underset{+H2O}{\overset{-H2O}{\rightleftharpoons}} \text{protein-}\overset{\overset{O}{\|}}{\text{OC}}\text{-protein'}$$

esterification by dehydration

In thermal crosslinking, intermolecular crosslinking is achieved by esterification of the cardoxyl groups and hydroxyl groups[7]. Succinylated collagen proved to have desirable properties with high water content. The hydrous materials containing water trapped in the intermolecular spaces by the negative charge induced by the succinylation showed non-thrombogenic properties and host cell affinity[13]. Succinylated collagen contains hydroxyl and carboxyl groups, but no amino groups. In the present study, succinylated collagen was crosslinked by condensation of hydroxyl groups with carboxyl groups without amino groups.

Most chemical crosslinkers can react to amino groups easily. Without amino groups, chemical crosslinking requires special conditions such as low or high pH with accelerators and catalysts. Crosslinking by glutaraldehyde or formaldehyde with amino groups[8] forms a methylene bridge between molecules which confers resistance to degradation in vivo.

2. Control of the crosslinking

Crosslinking density was controllable as shown in Figs. 1 and 2. The higher temperature and longer duration of the heating produced high density crosslinking. However, raw materials will be destroyed by extremely high temperatures. Therefore, the optimam condition was heating at 130 °C to 140 °C for 40 hours. Though we did not use any other supporting method, dehydration in a vacuum could theoretically accelerate the crosslinking.

3. Undesirable side effects caused by chemical crosslinking reagents

As shown in the results, the vascular prostheses with thermal crosslinking did not show any foreign body reactions. Numerous fibroblasts migrated from the host adventitial tissue into the interstices of the Dacron fibers sealed with the thermally crosslinked collagen. In the control grafts, numerous giant cells were seen around the graft. There was no fibroblast migration inside the graft wall. The foreign body reaction without fibroblast migration and fibroblast migration without foreign body reaction are completely different from each other. During the foreign body reaction, the original collagen crosslinked with chemical reagents will be dissolved, followed by absorption of the sealant, so that the sealed graft becomes unsealed. Therefore, the graft will be in an unstable state.

4. Absorption of the crosslinked materials and tissue repair

In the thermal crosslinking, fibroblasts migrated without any disturbance. These cells created a natural collagen network around them resulting in stable cellulofibrous tissue. On the prosthesis wall, natural cell activity coused rapid neointima formation resulting in the creation of a strong and stable vascular wall.

The thermally crosslinked collagen layer was almost completely absorbed at 21 days in vivo. These results suggested that the thermal dehydration caused partial intermolecular crosslinking. The speed of the biodegradation was controllable, since the degree of the crosslinking could be control led easily by varying the thermal treatment conditions. It will be possible to adjust the absorption of the biodegradable materials to the speed of tissue repair with host fibroblast migration without foreign body reaction.

Conclusion

With the thermal treatment, we could obtain a desirable crosslinked materials free of the side effects of chemical crosslinkers. The method was suitable for crosslinking of biological materials, especially biodegradable materials. An additional advantage of the method was sterilization. The graft can be sterilized simultaneously with the crosslinking instead of using chemical or physical sterilization. The method will be applicable not only in the vascular field, but also in various other biomaterials.

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Figure legends:

Figure 1. Swelling speed and water absorption of succinylated collagen on different thermal treatment conditions. A: Without crosslinking. B: Thermal crosslinked at 130 °C for 20 hours. C: Thermal crosslinked at 130°C for 40 hours

Figure 2. Water content of the following substances crosslinked at 130°C for 40 hours. A: Gelatin . B: Succinylated collagen. C: Succinylated collagen containing 5% Heparin sodium. D: Succinylated collagen containing 6% chondroitin sulfate C sodium.

Figure 3. Resistance to degradation of thermal crosslinked materials determined by protease digestion assay. A: Gelatin without crosslinked. B: Succinylated collagen crosslinked at 100°C for 40 hours. C: Succinylated collagen crosslinked at 140°C for 40 hours.

Figure 4. Gross appearance of outer surface of implanted grafts at three weeks. A: Succinylated collagen coated graft (SC-graft) crosslinked at 130°C for 40 hours. B: Control Hemashield graft crosslinked by formaldehyde.

Figure 5. Gross appearance of luminal surface of implanted grafts at three weeks. A: SC-graft thermal crosslinked has almost no thrombus. B: Luminal surface of the contral graft is covered with fresh and red thrombus.

Figure 6. Photomicrographs of cross section of implanted grafts. A: SC-graft thermal crosslinked has no foreign body reaction. The luminal surface is lined with endothelial cells. B: In the control, multiple macrophages, giant cells or lymphocytes are present. No fibroblasts are seen inside the graft wall. H.E. staining, X 200.

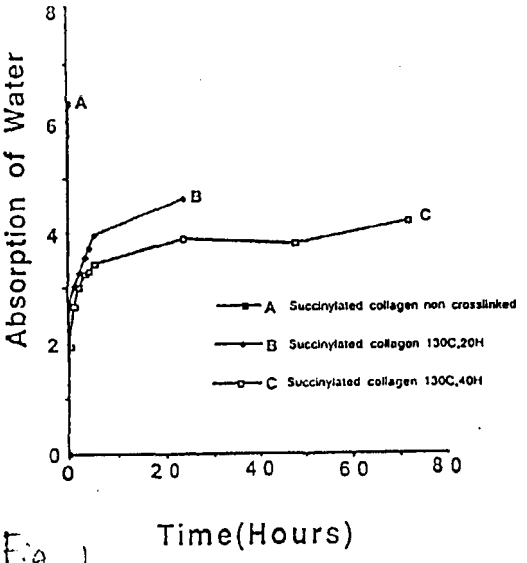

Fig. 1

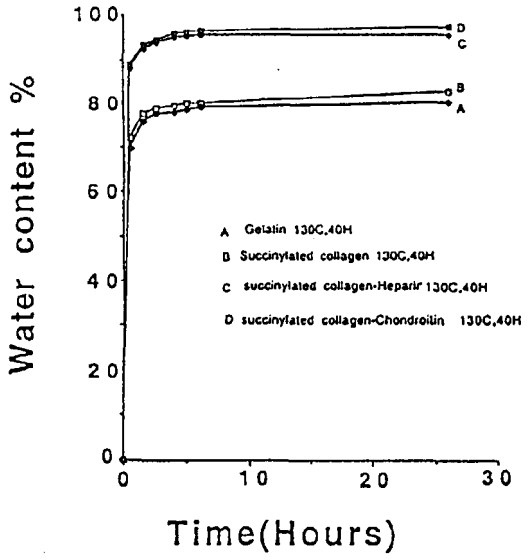

Fig 2

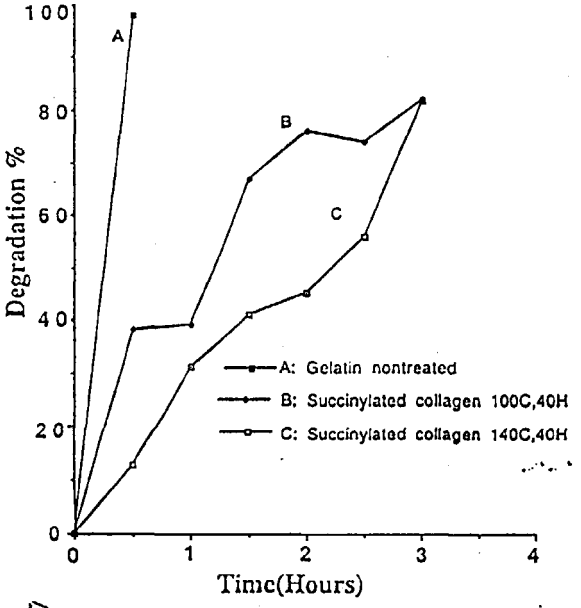

Fig 3

まとめ

本研究で明らかなように、骨髄を自家移植すると骨髄組織の特性により、血管新生を促すサイトカインが産生されることが明らかとなった。すなわち、骨髄の自家移植はサイトカイン産生システムの移植であると言っても過言ではない。その結果、生体内のあらゆる所にサイトカインを局所で持続的に発生させることが可能となった。この技術は広い範囲での応用が可能である。例えば人工腹壁、人工胸壁、皮膚移植、痔瘻処理、消化管潰瘍などの治療の補助療法としても使用できる。

また、この度の研究の延長線上として我々はこの技法を人工血管に使用した。その成果は今年 Nature Medicine に掲載されることとなった。この参考論文を後に添付するので参考にしていただきたい。

結論および今後の予定

この度の研究プロジェクトの結果より、同一素材でも形態をマイクロ化、マイクロ加工化することにより、細胞親和性を高め高機能かつ安全な人工臓器開発の一端を改善、促進させうることが明らかとなった。また生体由来材料も化学処理方法を改良するだけで、現在抱えているいくつかの問題点を改良することも可能となった。さらに患者自身のもつ治癒促進能力を骨髄組織自家移植などの方法により活用できる。すなわち治癒促進能力が可能となった。これらはそれぞれについて人工臓器開発の基礎的改良点のキーポイントを指しているとともに、それらを組み合わすことによって相乗効果が発揮されることが推測されることから、今後の人工臓器の設計において組み込むべき成果であると思われる。

最近、マイクロマシーンが研究課題として取り上げられ、いくつかの成果が話題となっている中において、細胞、蛋白レベルにおいてもマイクロ化、マイクロ加工化などの形態学的手法によって、また細胞成長因子などを組み込むことによって生物化学的な手法によって細胞挙動を誘導することが明らかとなったことから、その成果の産業への応用、そしてその成果を用いた人工臓器開発が望まれる。

今後の予定としては、我々は本研究成果が生物学上の普遍的な特性であることから、前述したように、その成果を人工臓器のような医療関係への応用を推進することはもとより、他の領域にもこのような現象が利用できるのか否かについて、異分野研究者とともに広く一般的な領域へも広げてゆきたいと考えている。

幸いにして我々はすでに以下に示すような実績をこのプロジェクトではなくて他のプロジェクトにおいてではあるが、我々の共同研究者とともに医学の研究が他領域への波及効果をもつことを示すことのできる実績を二つもっている。

その一つは、ファイブロネクチンという細胞接着性の蛋白である。細胞が周囲の繊維や材料に付着するとき、フアイブロネクチンという蛋白を分泌して接着するための足場を用意する。そこで細胞接着因子の研究が医学領域において活発となり、細胞を接着させるための工夫として、フアイブロネクチンを材料表面に塗布しておくことで細胞増殖を確実なものとし、促進させる手法が確立した。この成果を我々の共同研究者の一人は、真珠の養殖に転用した。すなわち、真珠の養殖にはアコヤ貝の中に貝の破片を投入することにより、それを核として細胞が付着し、無機質を分泌して球状の無機物質構造を作り上げる。この場合、破片に細胞が付着しなければ破片は貝の中より排泄されて真珠の形成は行われない。一般に自然な状態では、この成功率は数パーセントであった。ここにフアイブロネクチンと細胞接着に良好な足場を提供することのできるコラーゲンを混在させて、貝の破片に被覆させて貝の中に投入すると細胞の付着が飛躍的に向上し、90％以上の回収率を得るようになった。

またもう一つの例は、DDSと呼ばれる薬の徐放性制御システムである。これは薬剤の至適濃度を維持させるために、薬剤をゼラチンや多糖類に混ぜて、徐々に溶けるようにし、長期間効果が持続するようにしたものである。この研究は一大ブームとなり、現在全世界の主な医学、薬学の研究施設、大学で、その研究が進められている。我々の共同

研究者の一人は、この手法を農薬に転用し、農薬にカニの甲羅から得られるキチンなどの無害な多糖類に混ぜることにより、農薬を畑の植物の根元に長時間留めておくことに成功した。そのことにより、降雨時や散水時に限って必要最少限な微量な農薬が根元にのみ溶出して効果的に植物の育成に貢献することとなった。従来は農薬が散布してもすぐ流れてしまうため、たびたび散布する必要があった。そのための労力を過剰に使用し、これが不必要な場所に流れ出るため公害にまで発展することもあった。しかし、医学知識の農業への転用で多くの面での改善を得ることができた。この手法は、特に砂漠などの乾燥地において効果を発揮することから、日本のこの技術が海外でも役立つこととなっている。

以上のような実績を横目にみて、我々のこの度の成果は、共同研究の輪を異分野に拡大することによって、広く、多くの人々の生活に貢献してゆくよう努力するつもりである。

成果発表、特許等の状況

この度は平成8年1月16日より3月31日までの76日間の研究期間であったが、それまでにすでに研究の準備を進めてきた関係もあり、一応の成果を出すことができた。その成果の報告は、第42回アメリカ人工臓器学会（1996年5月2日～4日、於ワシントンDC）、および第5回国際バイオマテリアル会議（1996年5月29日～6月2日、於カナダ、トロント市）にて報告した。またさらにヨーロッパ生化学第24回年会（1996年7月7日～12日、スペイン、バルセロナ市）、国際生化学、分子生物学会第4回会議（1996年7月14日～17日、イギリス、エディンバラ市）で報告するとともに、Dr. Tomas Ekstrom（スエーデン、ストックフオルム大学）やDr. Victar Dzao（アメリカ、スタンフォード大学）とも共同討議をする予定にしている。

発表の抄録を以下に添付します。

NATURAL CYTOKINE SYNTHESIS FOR VASCULAR PROSTHESES INDUCED FROM AUTOLOGOUSLY TRANSPLANTED BONE MARROW

Natural cytokines synthesized from autologously transplanted bone marrow resulted in rapid neointima formation on long-fibril ePTFE vascular prostheses (LFEPTFE grafts). Another advantage of bone marrow transplantation is that bone marrow contains many undifferentiated young cells which can differentiate into various kinds of mesenchymal cells including fibroblasts, smooth muscle cells, and endothelial cells. A LFEPTFE graft (internodular length; 90 to 120 microns, internal diameter; 6mm, length; 6cm) was implanted into the abdominal aorta of 24 dogs. Half of the grafts had autologous bone marrow transplanted into the graft wall and the other half were used as controls without such treatment. These grafts were retrieved from 1 day to 6 months after implantation. After 3 weeks, the luminal surface of the treated graft was completely lined with endothelial cells. Colonies of erythroblasts (hemopoiesis) were observed frequently. Marrow cells and surrounding cells with capillary blood vessels were immunohistochemically bFGF reactive. The graft wall contained many capillary blood vessels. In the control grafts, the endothelialization was limited to the vicinity of the anastomotic sites. The center area of the graft was still covered with fresh red thrombus without endothelial cell lining at 4 months, and required 6 months for complete endothelialization. Differentiation of the transplanted marrow cells into various kinds of mesenchymal cells was not seen. These results indicated that bone marrow transplantation is effective for the acceleration of neointima formation on LFEPTFE grafts in dogs.

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A PUNCTURABLE COLLAGEN COATED FABRIC VASCULAR PROSTHESIS FOR AN A-V SHUNT GRAFT

We have developed a collagen coated fabric vascular prosthesis which shows non-thrombogenicity on its luminal surface and rapid neointima formation without intimal hyperplasia at anastomotic sites. The prosthesis is extremely hydrous since it is actually sealed with water which is absorbed into the intermolecular spaces of negatively charged collagen. As framework for the graft, Micron (InterVascular) was used, since it is the cleanest fabric graft commercially available. Succinylated collagen suspension, which is negatively charged, was injected under pressure into the graft wall to become entangled in the Dacron network. Then the graft was lyophilized and thermally crosslinked. Water leakage from needle holes created by a 16 G needle puncture in vitro under water pressure of 120 mmHg was measured at 6 puncture sites. The result was an average of 34.5 ml/min in the graft and 169.9 ml/min in a control ePTFE graft such is used as an A-V shunt graft for hemodyalysis. Hemostatic time at the puncture sites of the graft implanted in the abdominal aorta of dogs was measured at 6 puncture sites. After a puncture, a finger was applied to the puncture hole, and removed once a minute to observe blood coagulation at the hole. Hemostatic time was an average of 9.6 min at the graft and 34.2 min at the control. These results suggest that it is possible to reduce blood leakage from puncture sites when the newly developed graft is used as an A-V shunt graft.

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THERMAL CROSSLINKING FOR BIOLOGICAL DEGRADABLE MATERIALS

Biological materials for medical implants are crosslinked by chemical reagents such as glutaraldehyde except when they are used autologously, but chemical crosslinking has undesirable side effects. To overcome this problem, we developed a thermal crosslinking method. The method produces dehydration using hydroxy groups with carboxylic groups under dry conditions, and no amine groups are used. Therefore, crosslinking is also possible with materials having no amine groups, such as completely succinylated collagen. As examples, six each of succinylated and non-succinylated collagen coated fabric vascular prostheses were thermally crosslinked at 130°C for 20 hours. As controls, coated prostheses of each type without thermal crosslinking were used. The prostheses were digested in 0.1 % protease in phosphate buffer, pH 7.4, for 7 days. The controls without thermal treatment were completely digested in 1 day, but both of the thermally crosslinked groups showed resistance to digestion, and complete digestion required 7 days. As an in vivo experiment, 6 succinylated and thermally chrosslinked collagen grafts were implanted in the abdominal aorta of dogs and removed from one to 21 days after implantation. These grafts showed no foreign body reaction at all. The collagen layer was almost absorbed at 21 days. A collagen coated graft (Hemashield, Meadox Co.) used as a control showed foreign body reaction. These results suggest that the thermal treatment causes partial intermolecular crosslinking, and the method is suitable for biodegradable biological materials such as collagnous materials. Another advantage of the method is sterilization. The graft can be sterilized simultaneously with the crossslinking instead of using chemical or physical sterilization.

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APPLICATION OF A COLORIMETRIC METHOD TO DETERMINE HEPARIN CONTENT IN INSOLUBLE COLLAGENOUS MATERIALS

A quantitative method was tested for heparin measurement in insoluble collagenous implants. The metachromatic dye o-toluidine blue, which has long been used as a colorimetric indicator of heparin concentration in solution, can bind to heparin in a complex state. The method depends on dye depletion in the supernatant at an ultraviolet wavelength of 631nm as toluidine blue is absorbed onto the heparin in an immobilized state. The absorbance was measured with a Perkin Elmer Hitachi 200 spectrophotometer. As test material, a collagen coated and heparinized fabric vascular prosthesis was used. Solutions containing 10 % modified collagen with 0.1, 0.3, 0.5, or 1.0 % heparin were prepared. The solutions were injected into a porous fabric vascular prosthesis (Micron, InterVascular Co.) so as to become entangled in the interstices of Dacron fibers of the prosthesis wall. Then the prosthesis was lyophilized and thermally crosslinked at 130°C for 20 hours. Each piece of the prosthesis was measured by this method. The measurements were made by comparison with a standard curve previously determined. The amount of heparin in the prostheses was 0.7, 2.3, 3.4, and 4.7 mg/cm2 at 0.1, 0.3, 0.5 and 1.0 % heparin, respectively. These results showed that the amount of heparin in the insoluble collagen can be detected precisely, and that the method can be used for the determination of heparin concentration in various collagenous medical implants.

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HEPARINIZATION OF BIOLOGICAL MATERIALS WITHOUT CHEMICAL REAGENTS AND ITS APPLICATION TO A LONG-FIBRIL E-PTFE GRAFT

In general, heparinization of biological materials is perfomed using chemical reagents such as glutaraldehyde for covalent bounds and protamine sulfate for ionic bounds. But these chemical reagents have undesirable side effects. To overcome this problem, we developed a heparinization method by thermal crosslinking. Since heparin is a bioactive substance, it is believed to be destroyed functionally at high temperatures. However, we found that heparin was stable under dry conditions at 130°C for 24 hours, as during thermal crosslinking of collagenous materials. After the thermal treatment, reduction of the biological activity of heparin was less than 9 %. A long-fibril ePTFE graft (fibril length: 90 to 120 mm) was used as a framework. Ten % gelatin solution containing 0.2 % heparin was injected with pressure into the graft wall to become entangled in the PTFE fibrils. Then the graft was lyophilized and thermally crosslinked under the same conditions. The amount of heparin in the graft wall was 0.427mg/cm^2. Eight grafts were implanted in the left carotid artery of dogs and eight ordinary ePTFE grafts without heparinization were implanted in the right carotid artery as controls. Four each were removed after one hour, and remaining four grafts were removed one week after implantation. Control grafts showed thrombus adhesion, but the test grafts showed almost no thrombus after one hour and less thrombus after one week than the controls. Under light microscopy, the grafts did not show foreign body reaction. These results indicate that the method can be used a simple heparinization procedure for bioabsorbable materials without inducing any side effects.

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DEVELOPMENT OF A NEW SEALING METHOD WITHOUT FOREIGN BODY REACTION FOR POROUS VASCULAR PROSTHESES

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INTRODUCTION

Recently collagen coated vascular prostheses were used in clinic, however, side effects of the coating have been reported. We developed a method to seal a graft without these side effects.

MATERIALS AND METHODS

Purified fiber collagen was obtained from Achilles tendon of bovine. To prepare negatively charged collagen, it was succinylated. Most clean fabric vascular prosthesis (MICRON, InterVascular SA, Clearwater, Florida) was used for the graft framework. Collagen suspension was sieved through the prosthetic wall. Then the prosthesis is lyophilized and thermally cross linked at 130 °C for 20 hours, simultaneously sterilizing the graft (SC-graft). A collagen coated prosthesis cross linked by formaldehyde (Hemashield graft, Meadox Co. Ltd., Oakland, NJ, U.S.A.) was used as a control.

Thirty adult mongrel dogs were used for implantation of the grafts (6 cm in length) in the abdominal aorta. The grafts were retrieved from 1 hour to 3 weeks after implantation.

RESULTS

The SC-graft was slightly rigid, white with velour like collagen fibrils, and looked porous under dry condition. However, once it was dipped into saline solution, it absorbed water quickly and become soft and pliable. Water permeability was 0.1 ml/cm^2 at 120 mmHg. Collagen fibers were present in both inner and outer surfaces and even in the interstices of Dacron fibers, however, the graft wall was porous.

Amount of collagen in the graft wall was calculated as 4.0 mg/cm^2 in average, and water content was 64.0 mg /cm^2. While in the control, amount of collagen in the graft wall was 5.5 mg/cm^2 and water content was 30.0 mg/cm^2

One hour after implantation, both luminal and outer surfaces of the SC-graft were red, however, there was no thick thrombus deposition on the wall. While in the control, luminal surface was completely covered with thick red, and irregularly protruding thrombus. At three weeks after implantation, the SC-grafts were surrounded with a loose and thin connective tissue containing adipose tissue like a natural arterial adventitia tissue. Most areas of luminal surface at 3 weeks were white without red thrombus. By means of light microscopy, endothelial-like cell lining were present at the white areas throughout the graft surface, not only near the anastomotic sites, but also at the center areas of the graft. These cells were stained positively by PAP method, suggesting they are endothelial cells. Numerous fibroblasts infiltration with capillary ingrowth from the adventitia was observed. There were no giant cells, plasma cells and lymphocytes around the collagen, suggested no foreign body reaction.

While in the control, outer surface were surrounded with a granulation tissue, which formed a tumor of encapsulation around the graft. Luminal surface of the control graft were covered with fresh, red and irregular surfaced thrombus layer. There were some white areas of approximately 3 mm from the suture line. The white areas had endothelial cell lining. Other areas were covered with fibrin or red thrombus layer. Most parts of the luminal surface had collagen remaining under the thick thrombus. Around the remaining collagen and inside the graft wall, multiple macrophages, giant cells and lymphocytes are present.

DISCUSSION

Amount of collagen enmeshed in the SC-graft wall was small, however, the prosthesis can retain a high amount of water inside the graft wall, resulting in complete sealing. Under wet condition, carboxyl groups of the succinylated collagen tend to separate each other due to repulsion of the same negative charge, which results in aggressive absorption of water. Therefore, the graft looked porous under dry condition, but once it was wet, water permeability became extremely low.

The control showed non-infectious inflammation around the graft wall, i.e., foreign body reaction. Similar phenomena were reported previously. These side effects of the coated grafts were due to contaminated collagen, immunogenic reactions against animal collagen, and toxicity of cross-linkers such as glutaraldehyde and formaldehyde. These problems could be overcome by reducing coating substances, using a clean fabric graft and adopting thermal cross linking.

CONCLUSION

In this experiment, we sealed a prosthesis with small amount of clean, negatively charged collagen and large amount of water instead of sealing with chemically treated collagen. The heat cross linking was useful to insolubilize collagen in alternative to chemical crosslinkers.

BONE MARROW TRANSPLANTED VASCULAR PROSTHESIS FOR RAPID ENDOTHELIALIZATION

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Introduction

Complete endothelialization of vascular prostheses has been strongly desired for small diameter vascular prostheses. We found that there is high possibility of rapid endothelialization in e-PTFE vascular prostheses with bone marrow transplantation, since transplanted marrow cells can synthesize angiogenic growth factors. Continuous release of these growth factors form the transplanted cells may accelerate neointima formation. With this hypothesis, we designed an animal experiment in the current communication.

Materials and Methods

Autologous bone marrow was obtained from experimental animals and was enmeshed into an e-PTFE graft (Diameter: 6 mm, length: 6 cm, fibril length: 90 mm) pores. Thirty-four mongrel dogs, twenty for the BM-grafts, and fourteen for the control, were used. Under general anesthesia, a 5 cm segment of the abdominal aorta was replaced with the prepared graft. All animal care was in compliance with the "Guide for the Care and Use of Laboratory Animals" . All grafts were retrieved on a predetermined postoperative day (one to 192 days) and no animal died during observation. Those specimens were microscopically observed and immunohistochemical staining was performed for the distribution of bFGF.

Results

Macroscopically, the BM-graft lumen was red with a fibrin layer within 14 days, turned to glistening dark red around 3 weeks without thrombus adhesion, became glisting pink between 2 and 3 months, then finally became white. In the controls, the graft lumen was light red with a thin thrombus at 7 days. A pannus infiltrated over the anastomoses at 25 days. A thrombus remained in the center at 3 months. The graft lumen was white at 6 months.

Microscopically, the BM-graft wall contained clumps of marrow cells including marrow stromal cells, polychromatophilic erythroblasts, myelocytes. Stem cells were not identified. The graft wall at 3 days was occupied with erythrocytes and many marrow cells, which were immunoreactively bFGF positive. Colonies of endothelial-like cell on the lumen were Factor VIII positive at 7 days and more than half of the lumen was covered with endothelial cells at 14 days. Heterophilic myelocyte in mitoses, colonies of erythroblasts (erythroblastic islands) were always noticed with numerous capillaries. Complete endothelialization on the graft lumen was observed at 18 days. The number of erythroblastic islands decreased at 2 months but heterophilic myelocyte mitoses were still common at 3 months. They were immunohistochemically bFGF reactive. A bone marrow tissue and capillaries in a spongy bone was observed among PTFE nodes in 2 out of 6 six-month grafts. No intimal hyperplasia at distal anastomosis was noticed. In the controls, calcification without bone marrow tissue, rare capillary ingrowth and no hemopoiesis were observed in the graft wall. Complete endothelialization on the lumen at 6 months but not 3 was noticed.

Discussion

Rapid endothelialization due to angiogenesis including hemopoietic ability and the reactivity of basic fibroblst growth factor was unique in the bone marrow transplanted vascular prostheses. The evidences of survived transplanted marrow cells, continuous exogenous hemopoiesis with growth factor synthesis up to 6 months, and possible differentiation into endothelial cells may work as an autocrine system for endothelialization of a vascular prosthesis.

MOLECULAR AND FUNCTIONAL ANALYSES OF THE GLUTAMATE RECEPTOR CHANNEL SUBUNITS EXPRESSED IN BACULOVIRUS AND HERPESVIRUS VECTOR SYSTEMS

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Glutamate receptor (GluR) channels have been shown to play crucial roles in many central nervous system functions, including synaptic plasticity, nervous system development, and neuronal cell death. Excessive activation of glutamate receptor channels causes neuronal damage or death, and involves in pathogenesis of neurological diseases. In order to elucidate the neurotoxicity mechanism via GluR, which is one of final goals of our research projects, we have expressed and characterized AMPA-selective GluR channel α-family subunits and an NMDA receptor channel ζ-family subunit in a baculovirus vector system (1, 2). In the present study, we newly developed recombinant baculoviruses of ε- and δ-family subunits. Biochemical properties, including *N*-glycosylation and phosphorylation, and ligand binding properties, including site-directed mutagenesis studies in the putative ligand binding region, of the recombinant baculovirus-expressed GluR channel subunit proteins, have been investigated. Further, we also constructed defective herpes simplex virus type 1 (HSV-1) vectors of GluR channel subunits, and have analysed the molecular and functional properties of the recombinant receptors expressed in Vero and CHO cells. These defective HSV vectors will be used in *in vivo* experiments to study the functional roles of GluR in excitotoxicity or neuronal death. (1) J. Neurochem. 64: 1258 (1995) (2) Mol. Brain Res. 30: 137 (1995)

国際生化学、分子生物学第4回会議演題要旨（和訳）

バキュロウイルス及びヘルペスウイルスベクター系で発現させたグルタミン酸受容体チャネルサブユニットの分子的、機能的解析

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グルタミン酸受容体（GluR）チャネルはシナプス可塑性、神経系発達、神経細胞死等、多くの中枢神経機能で重要な役割を果たしていることが示されてきた。グルタミン酸受容体チャネルの過度の興奮が神経細胞障害・死の原因となり、神経系疾病の病理に関連している。GluRによる神経毒性の機構を明らかにすることが、我々の研究の最終ゴールの1つであり、これまでバキュロウイルスベクター系を用いてAMPA型GluRチャネルαファミリーサブユニットやNMDA型受容体ζファミリーサブユニットを発現させ、性質を調べてきた。本研究では、新たにεやδファミリーサブユニットの組換型バキュロウイルスを作成した。そして、バキュロウイルスを用いて発現させた組換型GluRチャネルサブユニットタンパク質のN-グリコシル化やリン酸化反応を含む生化学的性質やリガンド結合想定部位の部位特異的変異的研究等により、リガンド結合の性質を調べた。更に、我々は欠損型ヘルペスウイルス（HSV-1）ベクターを構築し、Vero細胞、CHO細胞に発現させた組換型受容体の分子的、機能的性質を解析した。これらのHSVベクターを興奮毒性や神経死におけるGluRの機能、役割を研究する目的で、動物個体における実験に用いる予定である。

EXPRESSION OF THE NMDA RECEPTOR CHANNEL SUBUNITS USING A BACULOVIRUS VECTOR AND A DEFECTIVE HERPES SIMPLEX VIRUS VECTOR

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The ζ1 subunit is an essential component of NMDA receptor channels in the central nervous system, and the ε subunits provide the molecular basis of the functional diversity of the NMDA receptor channels. Using a baculovirus vector system, the ζ1 (1) and ε family subunits have been expressed in Sf21 insect cells and biochemical properties, including *N*-glycosylation and phosphorylation, and ligand-binding properties have been investigated. Further, we also constructed defective herpes simplex virus type 1 (HSV-1) vectors of NMDA receptor channel subunits. The subunit cDNAs were inserted downstream of the CMV promoter in the amplicon plasmid vector to yield the recombinant HSV-1 vectors. We have analyzed the molecular properties of the recombinant receptors expressed in Vero cells and processed in *in vivo* experiments.

(1) Kawamoto, S. et al. (1995) Mol. Brain Res. 30: 137

ヨーロッパ生化学会第２４回年会演題要旨（和訳）

バキュロウイルスウイルスベクター及び欠損型ヘルペスウイルスベクターを用いたNMDA受容体チャネルサブユニットの発現

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ζ1サブユニットは中枢神経系におけるNMDA受容体チャネルに必須な成分であり、εサブユニットはNMDA受容体チャネルの機能の相違を決めている分子的基盤である。バキュロウイルスベクター系を用いて、ζ1及びεファミリーサブユニットをSf21昆虫細胞に発現させ、*N*-グリコシル化、リン酸化反応を含む生化学的な性質やリガンド結合活性を調べつつある。更に、NMDA受容体チャネルサブユニットの欠損型ヘルペスウイルス (HSV-1) ベクターを構築した。サブユニットcDNAをアンプリコンプラスミドのCMVプロモーターの下流に挿入し、組換型HSV-1ベクターを得た。Vero細胞に発現させた組換型受容体の分子的性質を解析し、動物個体における実験を進めつつある。

Protection of capillary blood vessels from ischemia by bone marrow transplantation into myocardium

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Running head: bone marrow transplantation into ischemic myocardium

Key Words: Ischemic myocardium, Bone marrow transplantation, bFGF, Angiogenesis, Revascularization,

Back ground Bone marrow transplanted into a synthetic vascular prosthesis has showed continuous synthesis of angiogenic growth factors resulting in rapid neointima formation on the prosthesis after implantation. We expected a similar angiogenic phenomenon to occur if bone marrow was transplanted into ischemic myocardium.

Methods and results Autologous bone marrow was injected intramuscularly into ischemic myocardium created in the left ventricle cardiac wall of dogs. Control operations were performed without bone marrow. On days 3 and 7, marrow cells, their adjacent cells, and their surrounding extrecellular matrix were immunohistochemically bFGF and VEGF reactive. At three weeks, no marrow cells could be identified. Myocytes disappeared but a capillary blood vessel network remained. With some exceptions, these capillaries did not contain blood cell components. In the controls, scar tissue with a very small number of capillaries was formed.

Conclusions Marrow cells survived for a short period of time after transplantation, and continued synthesis of angiogenic growth factors, which were effective in protecting endothelial cells from ischemia, but not myocytes. However, the capillaries without blood cell components suggested that there are limitations in the various treatments of ischemic myocardium using angiogenic growth factors alone.

Introduction

Various methods for revascularization of ischemic myocardium have long been studied[1,2,3]. How to supply blood to ischemic regions in which coronary artery bypass grafting and percutaneous coronary artery angioplasty were unable to function has been intensely discussed[4,5]. Transmyocardial revascularization by laser was also tried[6,7]. Recently recombinant angiogenic growth factors such as the fibroblast growth factor (FGF) family and vascular

endothelium growth factor (VEGF) were utilized for acceleration of collateral blood vessel formation in ischemic sites[8,9,10].

For the continuous release of natural angiogenic growth factors, we have developed a bone marrow transplantation method for vascular prostheses[11]. Marrow cells transplanted into a vascular graft wall continued exogenous hemopoiesis with growth factor synthesis. In general, endothelialization of synthetic vascular prostheses is extremely slow[12]. With bone marrow transplantation, however, neointima formation with complete endothelial cell lining and capillary blood vessel ingrowth in the graft was achieved within a short period of time[11]. We expected a similar continuous expression of angiogenic growth factors to take place if bone marrow was transplanted into ischemic myocardium. In order to test this hypothesis, autologous bone marrow was injected into ischemic myocardium created in the left ventricle wall of dogs.

Materials and methods

Creation of ischemic myocardium

Forty dogs weighing 8 to 18 kg were used. Under general anesthesia, the chest was entered via a left lateral thoracotomy at the fifth intercostal space. The pericardium was opened. Appropriate parts of the left descending branch, the left circumflex branch, and the first diagonal branch of the coronary arteries were repeatedly clamped and unclamped several times with a bulldog hemostat while carefully observing the ECG. When a stable condition without arrhythmia was obtained, the coronary arteries at the sites were ligated with 3-0 polyester sutures to create ischemic myocardium approximately 3cm square at the apex and the left ventricle anterior wall.

Bone marrow transplantation

Bone marrow was obtained by needle puncture from a small edge bite with an osteotribe on the iliac bone of the experimental animals. Approximately 0.5 ml of

bone marrow was slowly injected here and there into the ischemic myocardium of 22 animals for uniform scattering of the marrow cells (Fig. 1). As controls 18 animals were left without bone marrow treatment. The chest was closed temporarily and antibiotics were injected locally. After a predetermined period of time, the animals were anesthetized again while observing the ECG. The chest was opened and the heart was exposed for macroscopical observation. The heart was resected at 3, 7, and 21 days after surgery from 6, 8, and 8 of the tested animals, and 6, 6, and 6 of the controls, respectively. All animal care was in compliance with the "Guide for the Care and Use of Laboratory Animals"[13].

Histological examination

The apex of the ischemic area and the normal left ventricle myocardium without coronary arterial ligation were resected, and fixed with 10% formaldehyde in PBS. Each specimen was cut into three 5-mm segments, which were embedded in paraffin. Cross sections were cut and stained with hematoxylin and eosin, the Mason and May Giemsa method, the peroxidase anti-peroxidase (PAP) method, and Von Kossa's stain. Immunohistochemical staining for the detection of bFGF and VEGF was also done after freezing the specimens by means of a cryostat microtome with anti bFGF and anti VEGF antigens (Daco Co, Glostrup, Denmark) using a modified method of previously described[14].

Numbers of capillary blood vessels

The number of capillaries per 0.1mm^2 (0.5mm by 0.2 mm) in ten fields randomly chosen from the middle of each specimen was counted on photomicrographs and averaged. The number of capillaries which contained blood cell components was also counted in each field.

Results

Ligation of the coronary arteries and injection of bone marrow

After ligation of the coronary arteries, the color of the apex area of the heart muscle turned dark red. Muscle movement of the region was reduced. During the ligating procedure using repeated clamping, the electrocardiogram showed tall, peaked T waves, followed by symmetrically inverted T waves. After complete ligation, elevation of ST segments occurred.

Bone marrow was injected without problems. A small amount of bleeding was seen at the injection point, but it stopped spontaneously. No remarkable changes in ECG were noticed during the procedure.

At the time of heart explantation on days 3 and 7, the ischemic sites were dark red without adhesion. After 3 weeks, part of the pericardium or the lung adhered to the site, which was whitish with slight shrinkage. The movement of the myocardium was reduced at the site. However, there was no difference in the degree of color change or myocardial movement between the bone marrow transplanted hearts and the controls.

Histological studies

In the control group, normal myocardium tissue was present in the unligated areas. Myocytes have many capillary blood vessels around them. Ordinarily these capillary blood vessels can not be identified by H.E. staining. But they were recognized by the presence of erythrocytes as shown in Figs. 1a and 1b. Capillary blood vessels were tightly compressed by bundles of myocytes, but they contained numerous erythrocytes inside. Therefore endothelial cells of capillary blood vessles were not demonstrated, but the presence of the capillary blood vessels was recognized in these photomicrographs. On day 3, however, myocytes became atrophied and thin, leaving sufficient room for capillary blood vessels. But the sections parallel to the muscle bundles could not demonstrate the presence of capillary blood vessels. Using the PAP method to stain factor eight, capillary blood vessels could be identified as shown in Fig. 1d. They were among the atrophied myocyte bundles and were empty of blood cell components. On day 7,

the size and number of muscle cells decreased and fibroblasts became dominant in the area. At three weeks, most of the myocytes disappeared. Numerous fibroblasts with collagenous extracellular matrixes were the major component of the area (Fig. 1e). A small number of capillary blood vessels were present containing some blood cell components. Macrophages were present in the scar tissue and they were bFGF reactive (Fig. 1f). However, VEGF was not detected.

In the bone marrow group 3 days after injection, marrow cells with numerous peripheral blood cells such as erythrocytes infiltrated the interstices of muscle bundles. These muscle bundles became atrophied. Capillary blood vessels were present along the atrophied myocyte bundles. On day 7, numerous endothelial cells of capillary blood vessels were seen prominantly among the atrophied myocytes. Marrow cells including megakalyocytes were still alive among the myocyte bundles (Fig. 2a). Marrow cells, their adjacent cells and capillary blood vessels in the surrounding loose connective tissue were bFGF reactive (Fig. 2b). VEGF was also detected at the surrounding loose connective tissue. Inside the clumps of transplanted marrow cells, many capillary blood vessels containing erythrocytes were created as shown in Fig. 2c. On the other hand, many large capillary blood vessels composed of enlarged endothelial cells were recognized among the atrophied myocytes, but they had no blood cell components inside (Fig. 2d). At three weeks, the clumps of marrow cells were no longer seen. Some capillary blood vessels remained at the sites of the marrow cell clumps. Most of the atrophied myocytes also disappeared, but some still remained with fibroblasts in the loose connective tissue, in which capillary blood vessels were the major component (Fig. 2e). Most of the capillary blood vessels ran in parallel rows. Their arrangement was observed in both cross sectional and parallel sectional views as shown in Figs. 2e and 2f. These capillaries were stained positively by the PAP method, suggesting that these cells were endothelial cells, as shown in Fig. 2f These arrangements looked similar to the original

capillary network of the normal myocardium. Some capillaries had connections with each other. Their diameter was from 5 to 10 microns. The spaces between the capillaries were 10 to 30 microns. With a few exceptions, the capillaries did not contain blood cell components (Fig. 2f). Therefore, empty capillary blood vessels were the major component of the area at 3 weeks.

Number of capillary blood vessels

The number of capillaries and those having blood cell components per 0.1 mm^2 in ten fields randomly chosen from the middle of each specimen at 3 weeks were counted and averaged. The average numbers of capillaries and those with blood cell components were counted in specimens obtained from the intact, i.e., undamaged, areas without coronary artery ligation, the areas of ischemia with bone marrow transplantation, and the areas of ischemia without bone marrow transplantation obtained from the control group. The averages were 150 $\pm$1.5 and 120 $\pm$1.5, 120 $\pm$1.5 and 20 $\pm$1.5, and 20 $\pm$1.5 and 10 $\pm$1.5, respectively. The number of capillary blood vessels in the ischemic areas with bone marrow transplantation was significantly ($P < .05$) higher than in the areas without bone marrow transplantation, but capillaries containing blood cell components were rare. These results are shown in figure 3.

Discussion

1, Transplanted marrow cells in the ischemic myocardium

From the results, it was obvious that transplanted bone marrow survived for a short period of time and synthesized bFGF and VEGF in the ischemic myocardium. Marrow cells were seen at 1 week, but not at 3 weeks. We did not clarified whether these marrow cells differentiated into other cells, migrated out from the heart or died out. We were interested in the differentiation of transplanted marrow cells into myocytes. If the marrow cells could differentiate into myocardial cells spontaneously in the environment of ischemic myocardium, they would act as

myoblasts in the site naturally, since young cells can act and become newly organized according to the environment. Recently, myocardial cells immunologically modified by gene technology were transplanted into the heart myocardium[15], but the efficacy of the transplantation has not yet been clarified. In our experiment, however, we could not find any evidence of the differentiation of transplanted marrow cells into myocytes.

We expected that the transplanted marrow cells would survive in the ischemic myocardium like those in vascular prostheses[11], because marrow cells are young, primitive, and have the power to survive even in a poor environment in vivo. But transplanted bone marrow disappeared within 3 weeks. We speculated that during this time they tried to replicate. They would try to make blood cells because of their natural hemopoiesis. After these efforts under ischemic conditions, however, they became dystrophied due to poor nutrition and hypoxia. During the process, they synthesized large amounts of angiogenic growth factors such as bFGF and VEGF because of the natural behavior of damaged cells[16,17] and the special properties of bone marrow cells[18,19].

2, Vascular network in the ischemic myocardium

Compared with the control groups, bone marrow transplanted ischemic heart had atrophied myocardium with capillary blood vessels at one week, and numerous capillary blood vessels network at three weeks. These capillary blood vessels ran in parallel similarly to those in the undamaged region. As shown in the Fig 3, number of the capillaries did not increase with the bone marrow transplantation compared with that at the undamaged region. Anatomically undamaged myocardium has a fine capillary network along each myocyte bundle[20], as shown in Figs. 1a and 1b. Therefore, the capillary network in the ischemic region with bone marrow was not a newly developed one, but the original one maintained in spite of the poor environment. This phenomenon could not be observed in the control. Therefore, the phenomenon could be caused by the transplanted bone

marrow, and we speculated that transplanted marrow cells could not prevent myocardial damage from ischemia, but could rescue endothelial cells from the ischemic stress.

3, Growth factors from the transplanted bone marrow

We did not identify what kinds of growth factors and cytokins were synthesized during the time by the bone marrow, because marrow cells can synthesize many kinds of growth factors and cytokins[21,22]. At least, bFGF and VEGF were synthesized in the ischemic area. Numerous capillary blood vessels observed in the clumps of the transplanted marrow cells may be the result of direct stimulation of the angiogenic growth factors synthesized from them. bFGF and VEGF may also affect endothelial cells in the ischemic region. Angiogenic growth factors have been suggested to be involved in maintenance of vasculature in tumors and in normal tissue[23]. VEGF and bFGF are already known to be effective in facilitating endothelial repair and endothelial cell replication[24]. They are also known to work as survival factors during at hypoxic stress in various tissues such as nerve cells, epithelial cells, and endothelial cells[25,26,27]. A recent study suggested that VEGF and bFGF have synergistic effects on the induction of angiogenesis in vitro and in vivo[28]. We expected that marrow cells implanted in an ischemic region would synthesize many kinds of cytokins including VEGF and bFGF, and that the synergistic effects of them all would aid angiogenesis at the site. Growth factors such as bFGF and VEGF synthesized by bone marrow may contribute to endothelial cell survival in the ischemic myocardium.

4, Growth factors for revascularization

Recently recombinant bFGF has become popular for the acceleration of capillary ingrowth. A single direct injection of bFGF into an ischemic myocardium showed effective results experimentally[29]. Revascularization by collateral capillary ingrowth into the ischemic regions requires a certain period

of time[30,31]. But it is already known that the dosage for effective angiogenesis of these growth factors is small, and the duration of its efficacy is short. Continuous release of these factors is essential for the stimulation of endothelial cells involved in persistent capillary ingrowth[32]. Therefore, some kind of continuous supply system or slow release system is needed. Capillary growth into the heart from the aorta using a growth factor mixed in fibrin for slow release has been reported[33]. Some reports on gene transfer described angiogenesis resulting in increased number of capillaries[34,35,36]. Successful intracoronary gene transfer of bFGF in pig ischemic myocardium is an example of the continuous release system[37]. The gene transfer in vivo has, however, unclarified side effects such as virus infection[38,39]. On the other hand, transplanted bone marrow synthesized growth factors continuously without undesirable side effects in the current experiments.

5, Revascularization for ischemic myocardium

Although the myocytes could not be protected from ischemia by the bone marrow transplantation, we found two characteristic phenomena. The first was the efficacy of the continuous-synthesizing system of natural angiogenic factors in protection of the capillary network from ischemia. The second was that the numerous capillary blood vessels were empty, i.e., they contained no blood cell components. Ischemic regions of the controls did not show active capillary ingrowth or bFGF synthesis. These two observations can aid us in developing future strategies for treatment of ischemic myocardium.

It was surprising that the capillary blood vessel network did not function. Although we could thus maintain the capillary blood vessel network in the ischemic region with the help of growth factors, improvement of myocardial function was not obtained. Even if capillary blood vessels were created, improvement of the ischemic myocardium would require supplying blood components to the site by some other method. These observations gave us

important information for treatment of ischemic myocardium with recombinant growth factors and gene transfer technologies alone, since they increase demand for blood supply, but actual blood supply always lags far behind if the ischemic region is wide. If we could supply blood to the ischemic region using some method such as Vineberg's operation[4,40] and transmyocardial laser revascularization[41], the empty capillaries could play a role of their own, resulting in improvement of ischemic myocardium.

Conclusion

Transplanted marrow cells showed synthesis of natural angiogenic growth factors in the ischemic myocardium. Bone marrow transplantation could not protect myocardium from ischemia, but was effective in preserving the capillary blood vessel network. However, it also showed the limitation of treatment of the ischemic myocardium with angiogenic growth factors alone. Since ectopically transplanted bone marrow can synthesize natural angiogenic growth factors, the transplantation method should be applicable not only in the cardiovascular field but also in various other fields as well.

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Figure Legends

Figure. 1. Photomicrographs of cross sections of the control heart muscle. 1a: Before ligation of coronary arteries. Section parallel to the row of muscle bundles. Capillary blood vessels are present among the muscle bundles. They are recognized by the blood cell components they contain. H.E. X 100. 1b: Before ligation. Cross section at right angles to the muscle bundles. Capillary

blood vessels are recognized by the red blood cells inside. H.E. X 100. 1c: Three days after ligation. Capillary blood vessels are not seen. Interstices of the muscle bundles have opened due to muscle bundle atrophy. H.E. X 200. 1d: Cross section of a muscle bundle 7 days after ligation. Empty capillary blood vessels are recognized by the factor VIII staining. PAP method, X 200. 1e: At 3 weeks, muscle bundles have disappeared. Scar tissue with a few capillary blood vessels is present. H.E., X 200. f: With bFGF staining, macrophages are stained brown. bFGF immunohistochemical staining, X 100.

Figure 2. Photomicrographs of bone marrow transplanted heart muscles. a: at 7 days, bone marrow cells are recognized among myocyte bundles. Megakaryocytes are also seen (arrows). H.E. staining. X 200. b: At 3 days, there are bone marrow cells in the interstices of muscles and surrounding cells, and capillary blood vessels are bFGF reactive. bFGF staining, X 200. c: At 7 days, numerous capillary blood vessels have been created in the sites of bone marrow transplantation. H.E., X 200. d: At 7 days, endothelial cells are prominently observed in the interstices of muscle cell bundles, H.E., X 200. e: At 3 weeks, loose connective tissue containing numerous capillary networks is seen. H.E. staining, X 50. f: High magnification of the area at 3 weeks. Empty capillary blood vessels are the major components of the tissue. H.E., X 200.

Figure 3 Capillary numbers in the heart muscles of the intact area, the ischemic area and the bone marrow transplanted area. Average numbers are plotted and compared with the capillaries containing blood cell components.

Natural tissue engineering in vivo on vascular prosthesis wall

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Neointima formation of synthetic fabric vascular prostheses is extremely delayed. Most of prostheses implanted in humans are not healed for long period of time after implantation. Our hypothesis of the delayed healing is as follows; Fabric prostheses are lacking of extracellular matrices (ECM) for adsorption and release of cytokines such as bFGF and VEGF which play an important role for the wound healing, i.e., a kind of tissue engineering in vivo. During the prosthesis implantation, many cells are injured or destroyed by surgical procedure resulting in cytokine synthesis, but they are washed out. If synthetic prostheses were given a suitable ECM, natural neointima formation will be expected. In order to test this hypothesis, a fabric vascular prostheses containing collagen fibers and chondroitin sulfate was implanted in abdominal aorta of a dog. The prosthesis adsorbed bFGF naturally. Numerous fibroblasts and macrophages with capillaries infiltrated into the prosthesis wall resulted in rapid formation of neointima composed of smooth muscle cell layers and lined with endothelial cells. These

results indicate that a natural tissue engineering in vivo is realized with the aid of a suitable ECM.

Various methods have been attempted for the acceleralarion of neointima formation of vascular prostheses[1-10], but in humans, neointima is only formed on anastomotic sites and other areas are not healed for long period of time after implantation[11,12]. The formed neointima at anastomotic sites is composed of multilayers of smooth muscle cells lined with a monolayer of endothelial cells and collagenous tissue with fibroblasts around polyester fibers[13]. Therefore, natural neointima formation throughout the luminal surface is a kind of tissue engineering in vivo[14].

Recently, some experiments with growth factors and gene transfered cells were used on vascular prostheses[15]. But the procedure was not easy and not yet established. Endothelial cell seeding methods were also applied for the last two decades[16,17,18]. Complete neointima formation in vitro using three dimentional cell culture technique was also adopted[19]. In practice, however, these methods require specialists, special facilities, special instruments, and certain periods of time for the prosthesis preparation, and are not available for emergency use.

In a natural tissue injury, however, many cells are damaged or destroyed at the wound site, resulting in cytokine synthesis[20,21,22] such as basic fibroblast growth factor (bFGF), vascular endothelium growth factor (VEGF), hepatocyte growth factor (HGF), and tissue morphogenic factor β (TGF-β) which act important roles for wound healing. These cytokines are trapped by an extrecellular matrix (ECM) around the wound and released slowly for the following cell migration[23]. Their dosage for wound healing and angiogenesis might be small and the duration of their efficacy short. Therefore its continuous release from the ECM is essential for the stimulation of cells involved in rapid and smooth wound healing[24,25].

For the natural tissue engineering on vascular prostheses, principal cells, cytokines and a suitable ECM are required. However, synthetic prostheses are lacking of them, especially ECM as an anchring site for cell migration, and as an adsorption and slow release system for various cytokines. Therefore, these cytokines are washed out and are not used for the following neointima formation. In case of vascular surgery, however, major cellular components, i.e., fibroblasts, smooth muscle cells, and endothelial cells from capillary blood vessles are present around the prosthesis. Furthermore many cells are injured or damaged by surgical procedure resulting in cytokine synthesis[20,21,22]. Therefore, if the prosthesis were given with a suitable ECM for the tissue engineering, neointima formation in vivo will be expected naturally.

In order to test this hypothesis, a fabric vascular prosthesis containing collagen and chondroitin sulfate as majour components of ECM was provided. For natural introduction of cell migration, they were thermally crosslinked instead of chemical crosslinking of glutalardehyde and formaldehyde in order to diminish cytotoxicity of these chemical crosslinkers[26]. Succinylated collagen was used in order to make non-thrombogenic property by hydrous luminal surface[27,28], because succinylated collagen fibers tend to separate each other due to repulsion of the same negative charge in their molecular structure when they are in wet[29,30]. The hydrous condition induced by succinylated collagen in wet provides a non-thrombogenic property on the luminal surface of vascular prostheses. Succinylated collagen network is also expected as an adequate framework for host cell migration. Chondroitin sulfate was used in order to stabilize the cytokines such as bFGF and VEGF. Heparin has the stabilizing function for these cytokines[31] and antithrombogenic property for prevention of thrombus on the luminal surface, but control of the dosage of heparin is not easy to prevent excessive bleeding through the suture hole of the prosthesis at implantation. Mucopolysacchariudes such as chondroitin sulfate has the similar

function for these cytokines[32] and has no warring for the bleeding. Antithrombogenic property of chondroitin sulfate is not powerful compared with that of heparin, but was more convenient to use without bleeding problem.

An adsorption experiment using radioisotope-rabeled bFGF showed high amount of adsorption (14.3 ng/g) of bFGF to the succinylated collagen and chondroitin sulfate sponge and slow release (53%, 7.7ng/g release for 7 days). A sponge using heparin instead of chondroitin sulfate as a control showed a similar result (12.2ng/g adsorption and 65%, 6.9ng/g release). Dacron fibers adsorbed bFGF, but the amounts of adsorprion and release were small (2.9ng/g adsorption and 20%, 0.58ng/g release).

An in vivo insertion test of the prepared prosthesis using rabbit subcutaneous layer revealed that the ECM in the prosthesis wall was under way to be dissolved at 7th day after insertion, and immunohistochemically reactive to bFGF. Numerous fibroblasts, macrophage infiltration accompanied with many capillary blood vessels were present around and interstices of ECM. No foreign body reactions such as giant cell infiltration were present.

The ECM in the prosthesis wall implanted in the abdominal aortic position were also reactive to bFGF at 7 days. Numerous macrophages, fibroblasts and many capillary blood vessels were present in the interstices of the ECM. The prostheses at 3 weeks were completely healed throughout the luminal surface and the ECM was almost dissolved. The luminal surface was completely lined with endothelial-like cells, and Multi layers of smooth muscle cells were underneath them. Capillary ingrowth were remarkable in the prosthesis wall. They created neointima and neomedia. These findings were equally present not only near the anastomotic sites, but also at the center areas, far from the suture lines. The endothelial-like cells were stained with PAP method to confirm the production of factor VIII, indicating they are endothelial cells. From these findings, it was

clarified that the prepared prosthesis with the ECM could obtain a naturally tissue-engineered neointima with the aid of the ECM.

In the stand point of tissue engineering, major disadvantages of synthetic prostheses compared with natural tissues are lack of cell components, ECM, and cytokines. Synthetic vascular prostheses are not a suitable site for the tissue engineering. However, if we could give a desirable ECM into the prosthesis wall along our hypothesis, cytokines would be adsorbed and cells would be introduced with the activities of the cytokines as shown in the implantation experiment. Natural bFGF adsorption in vivo and the rabeled bFGF experiment in vitro gave the seal to our hypothesis.

Previously we obtained unique neointima formation using bone marrow transplantation on a synthetic vascular prosthesis[33]. Clumps of marrow cells survived inside the prosthesis wall and continued exogenous hemopoiesis for up to observed 6 months. Transplanted marrow cells and surrounded cells were immunohistochemically bFGF reactive and numerous capillary blood vessels were induced into the prosthesis wall. Therefore, the treated prosthesis had cells, cytokins and a suitable ECM for tissue engineering in vivo. In general, implantable artificial organs such as vascular prostheses are composed of biologically inert substances[34]. These polymeric scaffolds are not enough to induce fine natural cell activities. Multiple trees and shrubs on a mountain can keep and release water continuously resulting in contribution for natural plant growth and animal lives without flood when raining. Multiple complecated substances or absorbents in soil can adsorbe and release small amount of agricaltural chemicals for long period of time and contribute plant growth without envilonmental pollution. In like manner, as a scaffold for cell migration and a cytokine control system, the ECM is very important for in vivo tissue engineering.

Until now, several kinds of vascualr prostheses coated with collagen and gelatine have been developed[35,36,37]. They are useful in clinic without bleeding problem, since the collagen was used for a sealant of porous fabric striucture. Most of collagen was crosslinked by chemical crosslinkers, such as glutaraldehyde and formaldehyde, which are cytotoxic[38]. Some of collagen coated prostheses were pointed out to have endotoxin in the coating substances[39]. These coated prostheses are not designed for cytokine adsorption and release. However, if they are modified for this purpose, they will be completely regenerated into full of tenderness prostheses and will contribute for surgeries of poor risk patients such as aged or nutritious disorder patients and infants. (One experiment using cell culture technology was reported related to ECM with successful neointima formation. The aother did not described cytokine adsorption, but the prostheses should be re-examined for the detection of cytokines such as bFGF and VEGF.)

In conclusion, we obtained natural tissue engineered neointima using an ECM within short period of time after prosthesis implantation. we recognized how important the ECM was in this field. The basic idea described in this paper will be applicable not only in the field of vascular prostheses, but also in all surgical treatments in general.

Methods

Fiber collagen was obtained from a cow Achilles tendon. Succinylation of the collagen fibers was performed by adding 2% succinic anhydride in acetone to collagen suspension at pH 9.0. Succinylated collagen was precipitated at pH 4.2 and purified by reprecipitation. Approximately 0.5 % of the succinylated collage suspension with 0.5 % chondroitin sulfate at pH 7.4 was obtained and enmeshed into a Dacron fabric vascular prosthesis (MICRON, InterVascular Co. Ltd., Clearwater, FL, U.S.A.). Teflon mandrill was inserted into the prosthesis and it was freeze-dried. Then it was thermally crosslinked at 130°C for 7 to 10 hours under negative pressure. It was rinsed to remove uncrosslinked substances and then

lyophilized and EOG sterilized. As a control, a collagen impregnated prosthesis with heparin was prepared. As an another control, a plain Dacron fabric prosthesis was adopted. For an in vitro experiment, RI raveled bFGF solution was prepared and these specimens were dipped for one hour. Then they were rinsed in distilled water and immersed in high amount of daily renewed saline solution for 10 days. The amount of adsorbed bFGF and the releasing behavior was measured. As a subcutaneous experiment, four each specimens were inserted aseptically in the subcutaneous layer of four rabbits. At 7 days, they were removed and examined macroscopically and microscopically. The prepared prostheses with the controls were implanted into the abdominal aortae of 35 dogs, 20 for the prepared prosthesis, and 15 for the controls. Animals were anesthetized with an initial dose of 40 mg of Kitamin hydrochloride (Sankyo Pharmacy Co. Ltd., Osaka, Japan) intramuscularly and 8 ml of pentobarbital (2.5% solution) intravenously. Supplemental doses were given when required. The dogs were intubated and supported with a respirator at 20 respirations per minute and with a tidal volume of 150 ml of 20 % of oxygen. A 5-cm segment of the abdominal aorta was replaced with the grafts. All animal care was in compliance with Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals. All grafts were retrieved on a predetermined postoperative day (1 to 192 days) and no animal died during observation. For microscopy, the specimens were stained with hematoxylin and eosin (H & E), the Masson and May Giemsa method, the peroxidase-antiperoxidase (PAP) method and Von Kossa's stain. Immunohistochemical staining was performed for the distribution of bFGF along a method previously descrived[40].

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購入機器一覧

品　名	設置場所	主な使用者
パーキンエルマ　PCR 9600	横浜市立大学細菌学教室	川本　進
タイテック　エアーシェーカー BR - 40 LF	横浜市立大学細菌学教室	川本　進
ニコン LS - 1000 マック用	横浜市立大学第二内科	石井當男

あとがき

ここにおいて、我々は本来の医学領域において一つの壁に突き当たっている。これは、この度のプロジェクトに示したように、この技術は細胞と材料との対話とも言うべく、生体内で生じる微妙な挙動を我々の知恵で目的とする方向に誘導するものであって、生体内での反応が主な現象である。すなわち、この効果を用いることによって、植え込み用人工臓器の機能を飛躍的に高めようとするものである。従って、この成果を世の人々のために役立ってもらうようにするには、是非とも企業の方々の協力が不可欠である。我々は、ほんの一つの真実、現象を見つけ、これを医療、人工臓器に応用する方法を開発した。これを一般化するには企業の力であって、企業はいわば巨大な増幅機的機能を持つ。しかしながら、我が国の企業のほとんどすべては植え込み用の人工臓器の開発には消極的である。特に大企業といわれている企業は全くこの方面の事業も研究をもしようとしない。それは 1993 年 1 月 15 日の DuPout 社の声明以来、明確となったことである。それまでは日本の企業は大企業を含め、人工臓器など医療関係機器が最も高い成長率の望める分野として研究、開発に力を入れてきた。

しかし、ダラコーニング社のシリコーンを用いた豊乳術用のデバイスが発癌性などの副作用有りとして訴えられ、巨額の医療訴訟となった。最終的には、ダラコーニング社は次々と敗訴し、ついに会社更生法を申請することとなった。このことが引き金となり、DuPout 社が生体内に植え込む可能性のある人工臓器もしくは、生体内に 30 日以上入れておく（カテーテルなど）可能性のあるデバイスに使用するものの素材は一切出荷しないと声明を出した。従って、現在世界中の 95 %以上を占めている人工血管の素材であるダクロン繊維、テフロンなどは医療分野から姿を消すこととなった。

このことを受けて、それまで世界最高の性能を誇る新しい人工血管を製造しはじめた東レ株式会社は、その製造を中止し、販売も 2 年後に中止すると決定し、現在はその供給がとだえている。そして、日本の主な企業は植え込み用人工臓器の製造のみならず、次世代へ向けた研究開発をも中止し、今日に至っている。そのため、現在は人工腎臓や人工肺などの体外で一時的に使用する人工臓器もしくは、一時間以内の体内使用のようなカテーテル類にのみ、日本の企業はたずさわり、他のもの全ては、外国からの輸入品に頼る現状となっている。

この分野における日本の基礎研究レベルは非常に高く、永年の日本の研究者の努力の結果、知識の蓄積は世界に誇るものがある。しかし、これが現在国民の医療に生かせない状態にある。しかも最近の輸入品の中には、副作用の発生するものがかなり多く認められている。この副作用の原因について、海外のメーカーに問い合わせてみても、いずれの会社も＂そのような事実はない、そのような原因は見あたらない＂と否定するのみである。今日、大企業がこの分野から撤退し、名も知れぬ小さな企業が、浅い知識のもとで生産し、これを我が国の輸入業者が購入し、厚生省の認可を何とか取って、国民の医療に使用している。この時、厚生省の認可を取るにあたっての臨床治験が、最近の薬の臨床治験でも明らかになっているように、不可思議なこともあって、我々専門家から見ると、当然おかしいと思われるものまで認可されている。このようなことでは将来の

日本はどのようになるのだろうか心配でならない。

薬害エイズの問題でも、日本で独自にいち早く血液製剤を作っていたら、これほどまでひどくならずにすんだはずである。この二の舞を人工臓器分野は進もうとしている。数年前、米の不作があり、外圧もあって食料安保論が盛んに新聞をにぎわしたことがある。しかし、今日の人工臓器関係では、食料安保論以上に国民の健康を維持するための力としては、日本は自立できないし、今後さらにこの傾向は進むものと思われる。このことを企業も厚生省も通産省も真剣に受けとめ、認識して、今から手を打たないと21世紀における国民の医療を政府が保障するなど夢物語となりそうである。高齢者社会に向けての論議も大切であるが、さらに大切なことが見捨てられている。

付録（添付資料、参考文献等）

この度の研究関連の成果として、短い期間中であるが、研究成果として挙げられる論文として、"Autocrine angiogenic vascular prosthesis with bone marrow transplantation" がある。これはイギリスの科学雑誌 Nature Medicine に掲載されたものである。

以下にその別刷りを添付する。

Autocrine angiogenic vascular prosthesis with bone marrow transplantation

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& Akihiko Matsumoto

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18 days (Fig. 2*b*) and was maintained for the entire six-month observation period. The number of erythroblastic islands decreased at two months, but heterophilic myelocyte mitoses were still common at three months (Fig. 2*c*). These cells with the surrounding cells and capillary blood vessels were immunohistochemically bFGF reactive. Bone marrow tissue and capillaries in spongy bone were observed among PTFE filaments in two out of seven six-month grafts (Fig. 2*d*). No intimal hyperplasia at the distal anastomosis was seen. In the controls, endothelial cells were present at the anastomotic sites of the grafts for more than 25 days. Complete endothelialization of the lumen occurred in three out of seven grafts at six months, but three were occluded and one was still covered with a fresh thrombus. No hemopoiesis was seen. Calcification without bone marrow tissue and occasional capillary growth were observed in the graft wall.

In the BM grafts, as shown in Fig. 2*b*, exogenic hemopoiesis was remarkable. bFGF was always detected in capillary blood vessels, marrow cells and cells adjacent to the hemopoiesis. Thus during the hemopoiesis, these cells continuously synthesized cytokines such as bFGF, which has strong angiogenic properties[15–17], contributing to capillary growth[18] in the BM grafts. Marrow cells need nutrition for their survival, raw materials for producing blood cells and routes for shipping out their products, namely, blood cells. As a result, capillary growth was required. The lumen of the BM graft appeared red or pink because of numerous capillary blood vessels, with red cells both outside and inside the BM graft wall. These three items, hemopoiesis, bFGF and capillary growth, appeared to interrelate in the BM graft.

Some endothelial cells on the lumen may have originated from marrow tissue, because endothelialization began even before capillaries reached the graft lumen (Fig. 2*b*). Bone marrow contains some mature endothelial cells and many immature cells that can differentiate to many kinds of mesenchymal cells including endothelial cells. Bone marrow tissue also contains stromal cells, fibroblasts, endothelial cells and adipocytes, and these cells can proliferate and act as feeders. Cell transplantation with feeder cells has been used effectively to accelerate a skin healing process[19]. The endothelialization was complete at three weeks without intimal hyperplasia. Growth factors were released during hemopoiesis. The dosage of bFGF for angiogenesis might be small and the duration of its efficacy short, but its continuous release is essential for the stimulation of cells involved in rapid neointima formation.

From this evidence, the reasons for success with the BM graft are considered to be as follows. Marrow cells contain various kinds of young and primitive cells. Mixed cells can grow *in vivo* easily without mutual suppression. Cells must be in clumps to survive. For example, in cancer, there must be a clump of more than 50 cells to form a new metastasis colony. Mixture of different kinds of cells can work together as feeder cells to each other. Differentiation of marrow cells and synthesis of endothelial growth factors may also accelerate endothelialization, but the most important factor would be the combination of all of the following that play a role during the creation of a new vascular wall, that is, proliferating and differentiating young cells, continuous synthesis of growth factors, cooperation of different kinds of cells and the environment of the vascular wall, such as easy penetration of nutrition, oxygen tension and tensile stress. The BM graft appears to be an organism-controlled autocrine artificial organ.

Methods

Approximately 0.5 ml of autologous bone marrow was obtained from the iliac bone of dogs and infiltrated into e-PTFE graft material (diameter, 6 mm; length, 6 cm; fibril length, 90 μm; Baxter Healthcare). ePTFE grafts commonly used in human vascular prostheses have numerous slitlike fissures that make the graft flexible. These fissures are approximately 30 μm in size and are thus too small and complicatedly ramified to trap the bone marrow tissue inside the graft wall. In the current experiment, we used an expanded ePTFE with 90-μm fissures that pass through the graft wall without ramification. Forty-four mongrel dogs were used, 24 for the BM grafts and 20 for control grafts. The dogs were anesthetized with an initial dose of 40 mg of ketamin hydrochloride (Sankyo Pharmacy Co. Ltd., Osaka, Japan) intramuscularly and 8 ml of pentobarbital (2.5% solution) intravenously. Supplemental doses were given when required. The dogs were intubated and supported with a respirator at 20 respirations per minute and with a tidal volume of 150 ml of 20% of oxygen. A 5-cm segment of the abdominal aorta was replaced with the graft. All animal care was in compliance with Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals[20]. All grafts were retrieved on a predetermined postoperative day (1 to 192 days) and no animal died during observation. For microscopy, the specimens were stained with hematoxylin and eosin (H&E), the Masson and May Giemsa method, the peroxidase-antiperoxidase (PAP) method and Von Kossa's stain. Immunohistochemical staining was performed for the distribution of bFGF (ref. 21).

The number of capillaries per mm^2 (2 mm by 0.5 mm) in six fields randomly chosen from the middle of each graft wall was counted on 3-week, 3-month and 6-month explants. The average number of capillaries was calculated for each implant duration as shown in Fig. 3.

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Development of a Growable Vascular Graft

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A vascular graft that can grow with the growth of its recipient was developed. The graft implanted in the thoracic descending aorta grew slowly to the expected size within a year after implantation in puppies. Human saphenous vein was used as the substrate material. It was dipped into distilled water and sonicated, resulting in cell destruction, and followed by cross-linking with a polyepoxy compound to give both controlled biodegradability, hydrophilicity, and antithrombogenic properties. Four millimeter inner diameter (ID) grafts, enveloped with polyester mesh tubes of 10 mm ID, were implanted in 11 puppies. The diameter of the grafts grew to 9.5 mm from their original 4 mm. After 1 year, the graft walls that were reinforced with polyester mesh were covered with endothelial cells. The following requirements were provided in a growable graft: 1) antithrombogenicity in a small caliber graft; 2) ability to grow as well as to terminate growth. The polyester mesh tube, which was larger than the graft, caused arrest of growth at the expected diameter, whereas the growth rate was controlled by the degree of graft cross-linking. With this method, any size graft can be made by changing the size of the original graft and the polyester mesh tube around it. ***ASAIO Transactions*** **1988; 34: 308–313.**

Recently, cardiovascular surgery has been performed safely in infants, with the use of numerous artificial organs such as heart valves and blood vessel prostheses. Most of them work very well, but some display problems. In the case of heart valves made of biologic materials, dysfunction resulting from degenerative changes such as calcification have been reported, whereas with vascular prostheses, small diameter grafts easily occlude after implantation, making antithrombogenic treatment necessary. The side effects of anticoagulation cause other problems, and, even if the graft remains patent, it does not grow as the patient grows, requiring patients to have reoperation to expand the site after a certain period of time.[1,2] These repeat procedures are usually very troublesome because of adhesions from the primary surgery, and patients sometimes become poor risks because of bleeding during preparation of the site. To overcome these difficulties, a small diameter vascular graft that has the potential for growth as the child grows has been needed for a long time. In this communication, a "growable" vascular graft is described and its growth confirmed in an animal study.

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Materials and Methods

Preparation of the Growable Graft

Human saphenous veins were obtained aseptically at the time of resection of varicose veins of the lower extremities. A segment of vein with an internal diameter (ID) of 4 mm and without deformity was selected. It was immersed in distilled water for 1 hr to swell the cells inside by osmotic pressure and was then sonicated at 28 kc for 20 sec in order to cause cell destruction. Cell debris was then removed by washing with distilled water. In this way, a natural tissue tube composed of collagen and elastic lamina was obtained.

The graft was inflated with air to a pressure of 30 to 40 mmHg and was treated with a 2% polyethylene glycol diglycidyl ether (PEGDGE) solution in 5% ethanol, 0.1 wt% catalyst 2,4,6,-tris(dymethylaminomethyl)phenol (TDAMP), and 0.007 wt% accelerator salicylic acid at pH 10.0 for 5 hr to cross-link the graft wall. It was then washed with distilled water.

The graft was then preserved and sterilized in a 70% ethanol solution.

Before implantation, the graft was soaked and washed several times in physiologic saline solution to completely remove the ethanol.

A highly porous fabric vascular prosthesis made of ultrafine polyester fiber (water porosity: 3,650 ml/cm^2, 120 mmHg, H_2O) with a 10 mm internal diameter was provided to envelop the graft for reinforcement **(Figure 1).**

Determination of Reacted ϵ-NH_2 Groups of the Graft with PEGDGE

The degree of the reacted ϵ-NH_2 groups of the graft with the hydrophilic cross-linking reagent was measured by a modification of the method of Kakade and Liener.[3]

Implantation of the Graft

Eleven puppies weighing 3.5 to 7.0 kg (average weight 5.2 kg) were used in this study. The left pleural cavity was entered through an incision in the sixth intercostal space with

RAPID ENDOTHELIALIZATION OF VASCULAR PROSTHESES BY SEEDING AUTOLOGOUS VENOUS TISSUE FRAGMENTS

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Rapid endothelialization of vascular prostheses by seeding autologous venous tissue fragments

A method was developed to obtain rapid endothelialization of a fabric vascular prosthesis by seeding autologous venous tissue fragments into its wall. In an animal study, complete endothelialization was observed in the entire inner surface of the prosthesis within 2 weeks after implantation. A piece of peripheral vein was minced with scissors and then stirred into saline to create a tissue suspension. This suspension was enmeshed into the wall of a highly porous fabric vascular prosthesis by repeated pressurized injections with a syringe. The prostheses (7 mm inside diameter and 5.7 cm in length), seeded with tissue fragments, were implanted into the descending thoracic aorta of 25 dogs, and they were removed from 1 hour to 2 months after implantation. Twenty-five prostheses, preclotted with fresh blood, were used as control prostheses. In the seeded graft, a thin fibrin layer covered the inner surface just after implantation, but countless numbers of endothelial cells migrated from the fragments and came up to the luminal surface like multiple "mushrooms" under the fibrin layer. Smooth muscle cells made multiple layers underneath the endothelial cell layer. The healing proceeded equally at every part. By this active migration and proliferation, the inner surface was completely healed within 2 weeks. (J THORAC CARDIOVASC SURG 1992;104:770-8)

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Endothelial cell lining of vascular grafts is considered important for the antithrombogenicity of vascular prostheses. Endothelial cell seeding on grafts has been used to achieve this goal.[1,2] However, these attempts have not been successful thus far because, after implantation, the seeded cells are washed off from the surface by the bloodstream.[3] Improving cell adherence to the graft surface by using more hydrophilic material[4] or coating with fibronectin, for example,[5,6] has not overcome this problem because local inflammatory reaction with leukocytes[7] causes desquamation of the endothelial cells.[8] Since we observed that seeding of smooth muscle cells enhanced endothelialization of vascular grafts,[9] we anticipate that physiologic cell interaction may be needed to consolidate an endothelial lining. To improve the anchoring effect and survival rate of the seeded cells, we have developed a method to impregnate tissue fragments into a fabric prosthesis.[10] In the present study we present a modification of the original method, which allows seeding of homogenated vein tissue in porous prostheses.

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Materials and methods

Preparation of the graft. A canine left jugular vein about 8 cm in length and weighing approximately 0.2 gm was resected and minced with scissors and then stirred into 20 ml of physiologic saline solution to create a venous tissue fragment suspension. This suspension was sieved through the wall of a highly

reaction in the tissue fragments). The next group, 11 animals, were killed from 8 to 31 days after grafting each at 8, 10, and 14 days and two at 21 days, respectively (to observe the healing process of the neointima). The next group, four animals, were killed at 1 month (actually, two animals each at 28 and 31 days to observe the absorption of the original tissue fragments). The last animal was killed at 61 days to observe degenerative changes of the neointima.

In the control graft, 6 animals died because of the bleeding. The surviving 19 animals were divided into four groups. The first eight were killed from 1 hour to 7 days after grafting (one animal per day to observe the bleeding from the graft wall). The next five animals were killed at 10, 14, 16, 18, and 21 days after grafting so that the initial healing process of the neointima could be observed. The next four animals were killed at 25, 38, 39, and 46 days after grafting to observe the healing process of the neointima. The last two animals were killed at 56 and 63 days, respectively, so that we could observe degenerative changes of the neointima.

Macroscopic appearance. In the control grafts, the initial red thrombus seen after 1 hour changed into a white, nonshining layer within 3 days. This white inner surface remained unchanged after 7 days; at the tenth day, however, many thick (1 to 3 mm), red thrombi appeared over the entire inner surface. They increased in size gradually. During 14 to 21 days after implantation, they fused into a large, red, thick thrombotic area. By the twenty-fifth day most of the inner surface except the anastomotic lines, was covered with a thick layer of red thrombus (Fig. 2, *E*). On the thirty-eighth day the layer of red thrombus had decreased in size, thickness, and redness and changed into a white, nonshining area that increased in size. At the sixty-third day, the red, thrombotic area was limited only on the center area of the graft (Fig. 2, *F*). Other areas turned white but were shiny only within 1 cm from the anastomotic lines.

In the seeded grafts the following macroscopic differences compared with the control grafts were noticed. The initial red thrombus seen after 1 hour was more obvious (Fig. 2, *A*), but this changed with time. On the second day, predominantly white, nonshining multiple spots, 1 to 3 mm in size, were spread over the prosthetic surface, with areas of fresh red thrombus in between. These white spots were thinner than the surrounding red, thrombotic areas (Fig. 2, *B*). On the fifth day these white spots increased in size and number. At the seventh day the red, thrombotic areas decreased in size and thickness and turned into pink granulation-like tissue. On the tenth day the white multiple spots fused into a confluent, smooth area covering the entire inner surface. On the fourteenth day the white, shiny areas had spread and completely covered the luminal surface (Fig. 2, *C*). No further macroscopic changes were observed during a 2-month observation period (Fig. 2, *D*). Macroscopic examination of the kidneys resected from all the animals implanted with the seeded grafts and the control grafts showed no fresh, trapped microemboli in their cut surfaces.

Light microscopic appearance. The control grafts showed blood coagulum filling all the pores because of the preclotting procedure. The inner surface was also completely covered with a fresh, thin blood coagulum. On the second and third days this layer became thinner, and blood cell components, such as erythrocytes, disappeared from the layer. Occasionally the layer was composed of dense fibrin network. Some leukocytes were noticed in the fibrin layer at the outside of the graft fabrics. On the fifth day some transparent areas were noticed inside the fibrin network. In these areas some erythrocytes were noticed. On the seventh day numerous fibroblasts were observed at the adventitial side of the graft. On the tenth day a great number of fibroblasts proliferated at the adventitial side; however, few were observed in the fibrin layer on the luminal side of the fabric. On the fourteenth day endothelial cells from the host aortic wall surface had started to creep over the fibrin layer at the anastomotic sites.

By the sixteenth day smooth muscle cells followed underneath the endothelial cells. With migration of the smooth muscle cells, the fibrin layer disappeared. On the eighteenth day the neointimal formation of endothelial cell lining with multilayer formation of smooth muscle cells extended about 2 mm from the anastomotic lines. The migration of smooth muscle cells from the anastomosis was always preceded by the endothelial cells, but the areas away from the anastomotic lines remained covered with a fibrin layer without fibroblast migration. In the fibrin layer fresh blood infiltration and clotting formation were observed. Consequently, multiple thick thrombus formations on the fibrin layer made a rough, protruded polypoid surface. On the eighteenth day some of the newly formed thrombus layers became thick. From 3 weeks to 2 months after implantation, new thrombus formations were observed in the center area of the graft. With time the fresh thrombus formation decreased, and a remaining thin fibrin layer became dominant. On the sixty-third day most of the inner surface was covered with this thin fibrin layer without endothelialization. In the center areas fresh, thick spots of thrombus remained on the surface (Fig. 3, *H*). At the anastomoses, neointimal formation with endothelial cell lining was observed, but this was limited to about 5 to 8 mm from the suture lines.

In the seeded grafts, the sieving resulted predominantly in accumulation of tissue fragments at the luminal surface and in pores of the interstices of the fabric (Fig. 3, *A*), but also some small tissue fragments could be seen in the pores at the outer surface of the graft. After implantation, fresh thrombus formed over the inner surface, making a smooth layer at the inner surface, incorporating the tissue fragments. On the third day the fibrin layer became thin, with some of the tissue fragments facing directly to the lumen. Erythrocytes in the fibrin layer disappeared, and some leukocytes infiltrated from the outside. From the tissue fragments, obvious migration into the fibrin layer of numerous fibroblasts, smooth muscle cells, and endothelium-like cells was noticed. Some of them reached the luminal surface. On the fourth day some areas were lined with the endothelium-like cells (Fig. 3, *C*). After the lining, no platelet or fibrin adhered to)urface. In deep areas of the fibrin layer, endothelium-like cells migrated and proliferated actively and formed many capillary-like structures without blood components. Some of them were coming up toward the luminal surface (Fig. 3, *A* and *B*). Colonies of these cells were observed sporadically on the luminal fibrin layer. Cell segmentation of them was frequently noticed.

On the fifth day numerous smooth muscle cells proliferated in the fibrin layer. Fibrin was no longer seen around the cells, which indicated fibrinolysis. On the sixth day most of the fibrin layer was occupied with these proliferating cells and endothelial cells spread over the luminal surface. Smooth muscle cells followed the endothelium-like cells and made multiple layers underneath the endothelium-like cell layer. By the seventh to eighth days most of the luminal fibrin layer was covered with endothelium-like cells (Fig. 3, *D*). Original tissue fragments were still observed in the fabric, but cell components were no longer noticed inside the fragments. The active cell migration fr( )he tissue fragments resulted in a residual noncellular matrix that decreased with time. By the tenth day there was almost no fibrin in the neointima of the grafts. On the fourteenth day, many vasa vasorum were noticed opening to the luminal surface (Fig. 3, *E*). Endothelium-like cells lined the entire luminal surface and the surface underneath multiple-layered smooth muscle cells. Fibroblasts with collagen fibrils formed the outer layer of the neoarterial wall of the graft. The formation of a neointima was virtually completed at 2 weeks. Staining by PAP method showed that these endothelium-like cells contained factor VIII (Fig. 3, *G*), suggesting that they were endothelial cells. The original tissue fragments were still noticed in the graft at the twenty-first day, but they had almost disappeared in the specimens at the twenty-eighth and thirty-first days and were no longer observed by the sixty-first day. The neointima was completely healed and showed no changes. There were no degenerative changes, and no calcifications were present in the neointima (Fig. 3, *F*).

Scanning electron microscopic appearance. In the control grafts endothelial cells migrated only from the anastomotic sites after more than 2 weeks. Far from the anastomotic sites, the surface was covered with fresh thrombus or with a fibrin layer. In the seeded grafts, on the first day, the inner surface of the grafts showed a fibrin network with erythrocytes and platelets. However, some cell components were already seen on the surface on the third day. On the fourth day numerous flat cells were noticed on the luminal surface (Fig. 4, *A*). They made their colonies on the fibrin layer. On the colony surface, neither platelet adhesion nor fibrin precipitation was observed. On the fifth and sixth days many small colonies of these flat cells connected to each other were noticed over the entire graft surface. On the seventh day continuous endothelium-like cell creepings were noticed from the fibrin layer. On the eighth day these "mushroom-like sproutings" were observed all over the inner surface (Fig. 4, *B*). Continuous endothelium-like cell creepings were also noticed from the fibrin layer (Fig. 4, *C*). Large, confluent colonies of these cells were also noticed sporadically. On the tenth day most of the inner surface was covered with a continuous layer of endothelium-like cells. On the fourteenth day the inner surface was completely lined with endothelium-like cells. They showed the typical cobblestone morphology without adherence of platelets (Fig. 4, *D*). During the observation period of up to 61 days, there was no thrombus formation on the endothelialized surface of the grafts.

Discussion

Advantages of the method. By this method we could obtain a rapid and reliable endothelialization of the neointima. The method was simple and easy to do, and it can be made available in any operating room without special techniques, instruments, or facilities. Another advantage of this method was that the prostheses with tissue fragments sieved in the pores prevented bleeding during and after the operation. As indicated in the control experiment, fatal bleeding developed in some of the highly porous vascular prostheses as a result of fibrinolysis after implantation. The hemostatic capacity might be due to the presence in the tissue fragments of high amounts of collagen fibrils, which cause platelet aggregations. Consequently there was no bleeding at the anastomotic sites. However, this increased thrombogenicity has

lem we had developed a new technique with tissue fragment seeding with intraluminal suction.[17] In the current experiment we sieved the tissue fragments from the luminal side to the outside by injecting the tissue suspension. The tissue fragments were located at the luminal side of the graft. Therefore the "mushroomlike sproutings" could be noticed in this experiment all over the inner surface at an early stage after implantation. Successful transplantation of autologous tissue fragments has already been used in orthopedic and plastic surgery. Osteoblasts migrate rapidly from the cut edges of transplanted bone fragments and regenerate new bone structure, and epidermal cells from skin fragments quickly regenerate new skin.[18, 19] Multiple tiny fragments are composed of a large area of cut edges from which cells can migrate and will proliferate quickly under physiologic conditions.

)tionship of endothelial cells with smooth muscle cells and fibroblasts. In this experiment three kinds of cells (fibroblasts, smooth muscle cells, and endothelial cells) migrated and proliferated from the fragments at the same time. This is a unique phenomenon that is not observed in cell culture; when fibroblasts and endothelial cells are cultured in a Petri dish, endothelial cells are suppressed and fibroblasts are proliferated to form a confluent layer. In this in vivo experiment, however, we noticed that these cells migrated and proliferated in conjunction. Endothelial cells rose to the inner surface of the graft to face the bloodstream. Accordingly, smooth muscle cells made multiple layers underneath the endothelial cells, and fibroblasts crawled down under the smooth muscle cell layer around the Dacron fibers. This phenomenon suggested that growth of these cells was controlled by nature and their interactions in a physiologic environment. On the other hand, the interaction of endothelial with smooth muscle cells might be of crucial importance. In)case of an artificial skin graft, the new skin could not be produced with cultured epidermal cells alone. However, with cultured fibroblasts underneath the epidermal cell layer, a skin-equivalent graft was produced.[20] Fibroblasts are considered to participate as feeder cells for the epidermal cells. Fibroblasts do not suppress the epidermal cell growth in vivo. Combination use of different cell types was important for organ reconstruction. We already found that seeding of smooth muscle cells enhanced endothelialization of vascular grafts.[9] In this experiment endothelial cells and smooth muscle cells appeared to have a relationship like that of the epidermal cells and the fibroblasts in the artificial skin graft.

In conclusion, seeding of tissue fragments of venous tissue within a fabric porous vascular prosthesis was introduced to obtain rapid arterialization. Within 14 days complete endothelialization of the entire vascular surface occurred. This autologous tissue fragment transplantation method will be applicable not only in the field of vascular grafts but also in many hybrid artificial organs.

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Development of a Small Caliber Vascular Graft by a New Crosslinking Method Incorporating Slow Heparin Release Collagen and Natural Tissue Compliance

Y. NOISHIKI,* T. MIYATA,† AND K. KODAIRA†

There is a great demand for a usable small caliber vascular graft, but no satisfactory graft for long-term use has as yet been developed. Numerous pilot studies have been performed, but in only a few grafts made of either synthetic polymers such as segmented polyurethane, or biological materials, has some improvement been seen. It is, however, very difficult to give permanent antithrombogenic and compliant properties to graft materials. For example, any prosthetic surface, which has excellent antithrombogenicity in an artificial heart circumstance, becomes covered with biological substances such as plasma proteins when used in a vascular substitute. Because of this response, good initial results in vascular grafts are often short-lived. In order to solve this problem, we have previously developed a new heparinized vascular graft made of hydrophilic polyurethane.[1] Our current interests, however, have been focused on a graft made of biological material. To adapt biological materials for medical use, one needs to perform some modification, except when the tissues are autologous. Until now, only glutaraldehyde (GA) has been used for this purpose, but GA treatment completely changes the native properties of the materials. The heparinized canine carotid artery graft we recently developed was treated with GA, yet showed excellent antithrombogenicity. Despite this, we were dissatisfied with the graft because of its poor compliance. Because of this, a new cross-linking method for biological materials was developed, and the previously reported carotid artery graft was made soft and pliable by use of polyepoxy compounds. Consequently, the graft had both antithrombogenicity from slow heparin release, and natural tissue compliance.

Materials and Methods

Crosslinking Reagent Used

The new crosslinking reagents currently available are Polyepoxy Compounds (PC) such as Polyethylene Glycol Diglycidyl Ether, Glycerol Polyglycidyl Ether, Polyglycerol Polyglycidyl Ether, and Sorbitol Polyglycidyl Ether (Nagase Chemical, Ltd). In this study, Polyglycerol Polyglycidyl Ether (PGPGE) was used. Its representative molecular structures are illustrated in **Figure 1.** The crosslinking reaction can be performed at room temperature; specimens crosslinked with PGPGE become hydrophilic.

Basic Graft Material

Fresh carotid artery with an ID of 2.5 to 3.0 mm was obtained from a dog. It was soaked in distilled water for 1 hr and sonicated at 28 kc for 20 sec to cause cell destruction. Cell debris was then removed by washing with distilled water. In this way a natural tissue tube composed of collagen and elastic lamina was obtained.

Heparinization Method

1) A 2% protamine sulphate solution at pH 5.9 was poured into the natural tissue tube graft lumen, and the graft was inflated with air at a pressure of 80 to 100 mmHg for 30 minutes to force the protamine into the graft wall.

2) After inflation the graft was treated with a 5% PGPGE solution in 50% ethanol and 0.1 M Na_2CO_3 at pH 10.0 for 5 hr to crosslink the tissues and covalently immobilize protamine impregnated into the wall. The graft was then washed with distilled water.

3) The graft was soaked in a 1% heparin solution at pH 7.0 for 5 hr at 45°C and repeatedly washed with distilled water.

4) The graft was then preserved and sterilized in a 70% ethanol solution (**Figure 2a**).

The Control Graft

For the control experiment, unheparinized grafts treated with either GA and PGPGE were prepared. A carotid artery obtained from a dog was crosslinked with 1% GA solution, inflated at 80 to 100 mmHg air pressure for 5 hr after sonication to remove endothelial cells, and used for mechanical property measurements and animal study. The PGPGE treated control was prepared by the method described in III (heparinization method) without using protamine and heparin. This control was used for mechanical property measurements. The ϵ-NH_2 group of the graft collagen that reacted with the reagents was analyzed by the TNBS method.[2]

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the heparin is slowly released following implantation. This slow release of heparin can prevent fibrin formation on the graft surface. As the heparin is gradually absorbed, the graft becomes naturally antithrombogenic because endothelial cells advantageously cover the graft surface. Consequently, the graft can remain permanently antithrombogenic by endothelialization. Animal experiments reveal that this method produced antithrombogenicity in small caliber artery grafts. In previous preparation of this heparinized graft, the protamine impregnated into the graft wall was crosslinked with GA under condition of graft inflation, and although the graft showed no thrombus formation on its surface following implantation, the graft became less pliable and yellow with time. These changes occurred because when the graft was treated with GA to crosslink the protamine to its wall, it also crosslinked the protamine to the collagen molecules inside the graft wall. While GA crosslinking makes the materials less biodegradable, insoluble, and less antigenic, it makes the materials less flexible. Recently, other adverse effects of GA treatment have been reported.

To overcome these difficulties, a new crosslinking method was introduced. A remarkable difference between materials treated with GA and PC is their color; GA treatment makes the materials yellow, but PC makes them white. There is also a remarkable difference in their softness and elasticity, which are reflected in their mechanical properties. In the long-term animal experiment the grafts treated with PC kept their elasticity. This natural compliance during implantation seems to be very important in obtaining permanent patency of small caliber vascular grafts. One of the characteristics of PC treatment is the hydrophilic property imparted to the material which is important for affinity with host tissue, and makes the graft more nonthrombogenic.

The strength of the graft treated with PC is also important since despite its softness, it has sufficient strength to withstand mechanical stress, as supported by an examination of its mechanical properties and the animal studies done. The absence of aneurysmal dilatation in any graft following long-term implantation indicates the stability of the graft *in vivo* and the finding of no foreign body reaction around the graft wall also indicates that this crosslinking method is quite safe for implantable biological materials. Reconstruction of an arterial wall with the graft was most successful, with fibroblasts infiltrating the graft wall, but no migration of smooth-muscle-like cells as are seen on fabric vascular prostheses occurred. We observed the healing process of the implanted vascular graft and learned that the smooth muscle cells which infiltrate the neointima of the graft surface and arranged parallel to the direction of the tensile stress placed upon the graft wall.[8] If tensile stress is not present, smooth muscle cells seldom appear. The appearance of such cells in the graft treated with PC suggests that there is enough compliance of the graft to induce the migration of these cells during implantation.

Infection was the most likely cause of graft occlusion in this study since only one graft became occluded if no infection process was present. In this case, the anastomotic area had scar tissue around the graft, suggesting the presence of microinflammation around the anastomotic line. Therefore, if infection can be eliminated, graft patency rate will be very high.

From these results, we concluded that the combination of antithrombogenicity and compliance in these grafts were the major reasons for its success as a small caliber vascular graft, applicable to A-C bypass or vascular surgery of the leg below the knee.

Acknowledgment

Dr. Hayashi, National Cardiovascular Center, Japan, offered valuable suggestions and assistance in examining the graft softness and elasticity.

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Acceleration of neointima formation in vascular prostheses by transplantation of autologous venous tissue fragments

Application to small-diameter grafts

We have previously demonstrated rapid and complete endothelialization in synthetic fabric vascular prostheses that have been pretreated with autologous venous tissue fragments. However, significant thrombogenicity has been a major problem when this method has been applied to small-diameter grafts. By masking the positively charged collagen fibrils in the tissue fragments with negatively charged heparin, we were able to overcome this problem. A canine jugular vein was resected, minced into tissue fragments, and suspended. This mixture was sieved through the wall of a highly porous vascular prosthesis with a water porosity value of 4,000 ml/cm^2 per minute by pressurized injection, which caused the tissue fragments to be trapped in the graft wall. Tissue-fragmented grafts (7 mm inside diameter, 5.7 cm long) were implanted into the thoracic aorta of 35 dogs. In addition, tissue-fragmented grafts of small diameter (4 mm inside diameter, 3.5 cm long) were pretreated with heparin and implanted into the carotid arteries of 16 dogs (32 grafts). Preclotted grafts without tissue fragmentation were implanted into the thoracic aorta (25 dogs) and carotid arteries (6 dogs, 12 grafts) as controls. Grafts were explanted from 1 to 495 days after implantation. New arterial wall formation was complete throughout the tissue-fragmented grafts within 2 weeks; however, in the control grafts, neointima formation was limited to the anastomotic sites even after 2 months. Twenty small-caliber tissue-fragmented grafts that were pretreated with heparin in the carotid position were patent, but all the control grafts were occluded within 1 week. These results demonstrate that neointima formation can be enhanced in synthetic fabric prostheses; furthermore, long-term patency of vascular grafts of small caliber is possible in dogs with this tissue-fragmentation technique. (J THORAC CARDIOVASC SURG 1993;105:796-804)

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During the state of protracted healing of the vascular wall, endothelialization of vascular prostheses is extremely delayed.[1] Transplantation of autologous tissue fragments to delayed healing areas may accelerate the healing process, as does transplantation of autologous skin fragments for protracted skin ulcers[2] or bone fragment transplantation for intractable bone fractures.[3] Endothelial cell aging may play an important role in this process. In vitro, endothelial cell experiments have demonstrated that cell division is limited to 70 cycles[4-6]; therefore, new endothelial cell resources are required for complete

Table I. *Postoperative day of descending aortic explantation*

Control		*TF graft*	
Animal No.	*Postop day*	*Animal No.*	*Postop. day*
1	1	1	1
2	1	2	1
3	3	3	3
4	3	4	3
5	7	5	4
6	7	6	5
7	10	7	6
8	10	8	7
9	14	9	7
10	14	10	7
11	18	11	8
12	21	12	9
13	25	13	10
14	35	14	10
15	38	15	10
16	39	16	14
17	46	17	14
18	56	18	15
19	63	19	20
20	90	20	21
21	98	21	21
22	140	22	28
23	217	23	30
24	734	24	30
25	739	25	50
		26	50
		27	61
		28	62
		29	62
		30	79
		31	167
		32	167
		33	172
		34	339
		35	495

TF, Tissue-fragmented.

Table II. *Postoperative day of carotid artery explantation*

Control		*TFH graft*	
Animal No.	*Postop. day*	*Animal No.*	*Postop. day*
1	1	1	1
2	1	2	1
3	1	3	14
4	7	4	14
5	7	5	14
6	7	6	30
		7	30
		8	30
		9	34
		10	42
		11	42
		12	42
		13	62
		14	65
		15	67
		16	400

TFH, Tissue-fragmented (graft) with heparin.

endothelialization of graft surfaces that are distant from anastomotic sites. In addition, endothelial cell proliferation has been shown to improve with a base of feeder cells, including smooth muscle cells and fibroblasts.[7,8] On the basis of these findings, we transplanted venous tissue fragments that contained both endothelial cells and feeder cells onto a fabric vascular prosthesis to enhance healing based on these findings.

In preliminary studies, we observed rapid endothelialization of tissue-fragmented grafts (TF grafts) implanted into the descending aortic position,[9-11] but thrombogenicity became a problem when we implanted these grafts into arteries of small diameter. We believe the increased thrombogenicity is due to positively charged collagen fibrils at the edges of the tissue fragments. We have therefore developed a method to reduce this thrombogenicity by ionically bonding heparin with the collagen fibrils.[12] This article reports the application of this technique in small-diameter grafts and the healing process of TF grafts that are implanted in the descending thoracic aorta and the carotid artery of a canine model.

Materials and methods

Preparation of the TF graft. A canine left jugular vein and its surrounding connective tissue (10 cm length; 2 gm weight) was resected and minced with scissors into tiny (less than 0.2 mm) tissue fragments, then stirred into 20 ml of normal salin solution containing 10,000 international units (IU) penicillin. The resulting venous tissue fragment suspension was injected with pressure into a highly porous fabric Dacron vascular prosthesis (Microknit), Golaski Laboratories Inc., Philadelphia, Pa.) with a water porosity value of 4,000 ml/cm^2 per minute at 120 mm Hg inside diameter (ID) of 7 mm as previously described.[11] After several pressurized injections, the fragments were trapped in the prosthesis wall, creating a TF graft. This graft was then implanted into the animal from which the jugular vein was resected. The 7 mm ID control graft was a fabric vascular prosthesis (Microknit), preclotted with fresh blood by means of the four-stage Sauvage preclotting technique.[13]

Preparation of the graft for an artery of small diameter. The tissue suspension used for the small-caliber grafts was produced by the addition of minced canine jugular vein with connective tissue to 20 ml of normal saline solution that contained 1,000 IU heparin and 10,000 IU penicillin. To create the tissue-fragmented, heparinized graft (TFH graft), a 4 mm ID Microknit fabric prosthesis was first invaginated, enveloped by

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Development of a Soft, Pliable, Slow Heparin Release Venous Graft

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To prevent their collapse, a certain amount of stiffness is generally required for prosthetic venous grafts, so EPTFE grafts have been used. However, the native vein is pliable without any stiffness. We developed a soft and pliable graft that can maintain patency of the lumen because of its compliance. Fresh porcine ureter was incubated in a ficin solution to remove cell components and noncollagenous proteins. One percent protamine sulfate solution was injected into the ureter lumen to impregnate the inner surface. The ureter was then crosslinked with a 1% glutaraldehyde solution, dipped into a 1% heparin solution for 5 hours, and rinsed with distilled water. This procedure made the ureter very soft and pliable, and also conferred antithrombogenicity to the graft by heparinization. The grafts were implanted into the posterior vena cavae of 20 dogs and were removed from 1 to 878 days after implantation. Eighteen grafts were patent, but two grafts were occluded at the anastomotic site at 218 and 107 days, respectively. As a control experiment, nonheparinized grafts were implanted into 15 dogs; all were occluded with fresh thrombi. All the patent grafts kept their original elasticity, which allowed them to heave in unison with the heartbeat, and were similar in appearance to the native vena cava. Heparinization was effective in preventing thrombus formation. These results indicate that this type of graft is an ideal prosthesis as a venous graft, having physiologic properties such as compliance and antithrombogenicity. *ASAIO Transactions* 1990; 36: M343–M346.

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Another advantage of the graft was compliance. Except in cases in which scarring had formed at the anastomotic sites, the grafts maintained their patency without any structural stiffness. Although the negative pressure of the pleural cavity might help the grafts to keep their patency, the elasticity of the grafts could have aided in maintaining their physiologic function since it allowed them to heave in unison with the heartbeat. The graft maintained its softness over a long period of time, but further experiments would be required to explain the specific mechanism responsible for this finding. One weakness of the graft was that it was not resistant to the constriction caused by scarring at the anastomotic sites. Therefore, the sources of scar formation, such as local infection and hematoma, should be eliminated.

In conclusion, the grafts kept their patency during the slow release of heparin the early post implant stage, and because of partial endothelialization over the long-term. Natural physiologic properties, such as compliance, may have helped in maintaining the patency of these venous grafts.

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9. A small-caliber vascular graft for aortocoronary artery graft with temporarily artificial and permanently natural antithrombogenicity and natural vessel compliance

Yasuharu Noishiki[1], Teruo Miyata[2], Chisato Nojiri[3], and Hitoshi Koyanagi[3]

Summary. A small-caliber vascular graft was developed and evaluated as a carotid artery replacement and aortocoronary artery graft in animal experiments. Canine carotid arteries were obtained, soaked in distilled water, and then sonicated bring about cell destruction. In this way, a natural tissue tube composed of collagen and elastic laminae was obtained. The tube with a protamine sulfate solution inside was cross-linked with polyepoxy compounds, which made the graft white, hydrophilic, soft, and pliable. The graft was dipped into a heparin solution, which allowed heparin to bind ionically. Eighty grafts (3 mm inner diameter, 6 cm in length) were implanted in the carotid arteries of 40 dogs and resected 1–389 days after implantation. Three grafts were occluded by graft infection and 77 were patent (96% patency). Sixteen control grafts, in which the tissue tube was cross-linked with glutaraldehyde but was not heparinized, were implanted in eight dogs. All of them were occluded within 1 week. Aortocoronary bypass grafting was also performed using the heparinized graft in eight dogs for periods up to 4 months. All the grafts were confirmed to be patent by autopsy or graft angiography. Measurement of the heparin content revealed that about 90% of it was released within 1 month. After the release of heparin, the surface of the graft was completely covered with endothelial cells. The graft wall kept its soft and pliable properties even after the long-term implantation. These results indicate that the antithrombogenicity produced by the combination of the slow release of heparin and endothelialization, together with the natural vessel compliance in the graft, were the major reasons for its success as a small-caliber graft.

Key words: Small-caliber vascular graft—Polyepoxy compounds — Heparinization — Endothelialization — Aortocoronary bypass grafting

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Recently, large-caliber vascular grafts have shown satisfactory clinical results. However, middle-sized grafts have some problems and no satisfactory small grafts for long-term use have as yet been developed. For small-caliber vascular grafts less than 5 mm, both the antithrombogenic and compliant properties are essential to the long-term patency. Numerous pilot studies have been performed, but in only a few grafts made of either synthetic polymers, such as segmented polyurethane, or biological materials has some improvement been seen. It is, however, very difficult to give permanent antithrombogenic and compliant properties to graft materials. For example, prosthetic surfaces, which seem to have excellent antithrombogenicity in an artificial heart application, become covered with biological substances such as plasma proteins when used as vascular substitutes. Because of this response, good initial results in vascular grafts are often short-lived. To solve this problem, we developed a new heparinized vascular graft made of a hydrophilic polyurethane [1] and discussed its advantages and disadvantages. Our current interests have been focused on a graft made of biological material. In general, biological materials have an affinity to the host tissue. To adapt the biological materials for medical use, one needs to perform some modification, except when the tissues are used autologously. Until now, only glutaraldehyde (GA) has been used for this purpose, but GA treatment completely changes the native properties of the material. A heparinized canine carotid artery graft [2] was treated with GA, yet showed excellent antithrombogenicity. Despite this, we were dissatisfied with the graft because of its poor compliance. Because of this, a new cross-linking method for biological materials was developed, and the previously reported carotid artery graft was made soft and pliable by the use of polyepoxy compounds. Consequently, the graft had both antithrombogenicity from slow heparin release and natural vessel compliance. The graft was evaluated as a carotid artery replacement and a coronary bypass graft in experimental animals.

Materials and methods

Cross-linking reagent used

The cross-linking reagents used were polyepoxy compounds (PC), such as polyethylene glycol diglycidyl ether, glycerol polyglycidyl ether, polyglycerol polyglycidyl ether, and sorbitol polyglycidyl ether (Nagase Chemical, Ltd., Osaka, Japan). In this study, polyglycerol polyglycidyl either (PGPGE) was used. Its molecular structure and cross-linking reaction with collagen molecules are illustrated in Fig. 9.1. The cross-linking reaction can be performed at room temperature, and the specimens cross-linked with PCs become hydrophilic.

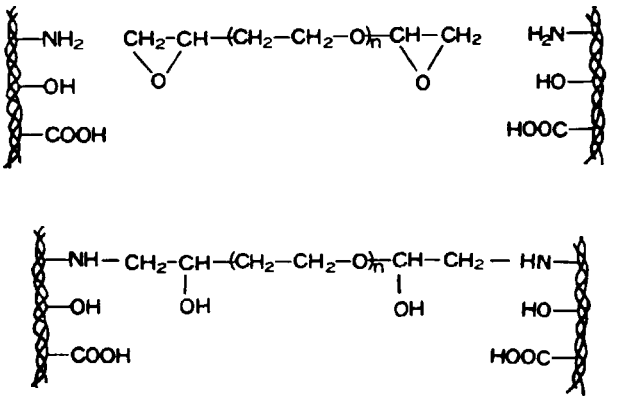

Fig. 9.1. Molecular structure of polyethyleneglycol diglycidyl ether (PC) and cross-linking reaction of PC with ε-NH2 groups of collagen molecules

Basic material of graft

A fresh carotid artery with an inner diameter of 2.5–3.0 mm was obtained from dogs. The artery was soaked in distilled water for 1 h and sonicated at 28 kilocycles for 20 s to produce cell destruction. Cell debris was then removed by washing with distilled water. In this way, a natural tissue tube composed of collagen and elastic laminae was obtained.

Heparinization method

A 2% protamine sulfate solution at pH 5.9 was poured into the natural tissue tube graft lumen, and the graft was inflated with air at a pressure of 80–100 mmHg for 30 min to force the protamine into the graft wall.

The graft inflated with air pressure was treated with a 5% PC solution in 50% ethanol and 0.1 *M* Na_2CO_3 at pH 10.0 for 5 h to cross-link the tissue and covalently immobilize protamine impregnated into the wall. The graft was then washed with distilled water.

The graft was soaked in a 1% heparin solution at pH 7.0 for 5 h at 45°C and repeatedly washed with distilled water.

The graft was then preserved and sterilized in a 70% ethanol solution (Fig. 9.2a).

Control graft

For the control experiment, nonheparinized grafts treated with either GA or PC were prepared. Carotid arteries obtained from dogs were cross-linked with 1% GA solution, inflated at 80–100 mmHg air pressure for 5 h after sonication to remove the cell components and used for mechanical property measurements and animal studies. The PC-treated control was prepared by the method described in the preceding section without using protamine or heparin. This control was used for mechanical property measurements.

The number of ε-NH_2 groups of the collagen molecules in each graft which had reacted with the reagent was analyzed using the trinitro-benzene sulfonic acid (TNBS) methd [3].

Mechanical properties

Strength

Fresh canine GA cross-linked, PC cross-linked, PC cross-linked, and heparinized carotid arteries were used. Cylindrical specimens were fixed longitudinally and tensile strength measurements were performed. The elongation and tensile strength were measured on each specimen.

Stiffness and elastic behavior

Each cylindrically shaped specimen was placed in an evaluation system developed by Hayashi et al. [4]. The relation between the intraluminal pressure and external radius of each specimen was plotted as the logarithm of pressure ratio versus distension ratio.

In vivo experiments

Two kinds of in vivo experiments were peformed using the prepared graft.

Carotid artery replacement

Fifty-six mongrel dogs weighing 8–12 kg were used for the experiment. About 6 cm of both carotid arteries was harvested and a 6-cm long by 2.5- to 3.0-mm internal diameter segment of heparinized graft was implanted end to end. Penicillin (500 mg) was given, but no anticoagulants were used at any time. Eighty heparinized grafts were implanted, as well as 16 GA-treated control grafts. The experimental animals had

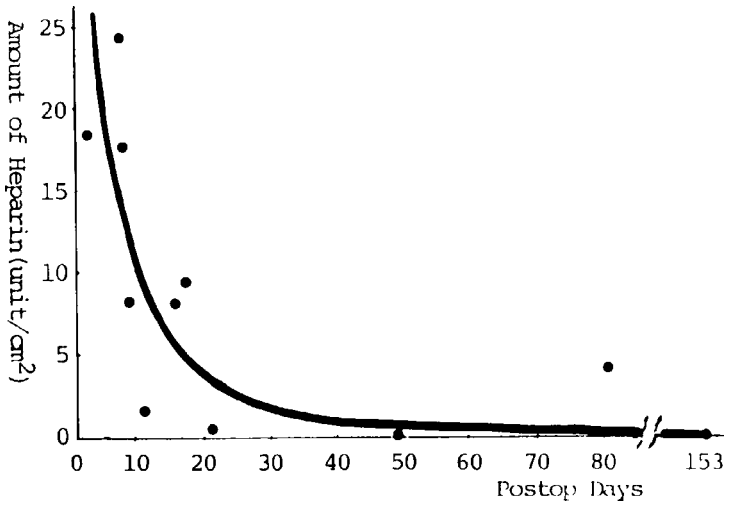

Fig. 9.10. Amount of heparin remaining in the grafts as a function of implantation time

endothelialization. Animal experiments reveal that this method produces stable antithrombogenicity in small-caliber arterial grafts. In previous preparations of this heparinized graft, the protamine impregnated into the graft wall was cross-linked with GA under conditions of graft inflation, and although the graft showed no thrombus formation on its surface following implantation, the graft became less pliable and yellow with time. These changes occurred because when the graft was treated with GA to cross-link the protamine to its wall, it also cross-linked the protamine to the collagen molecules inside the graft wall. While GA cross-linking makes the materials less biodegradable, insoluble, and less antigenic, it makes the materials less flexible. Recently, other adverse effects of GA treatment have been reported.

To overcome these difficulties, a new cross-linking method was introduced. A remarkable difference in appearance between materials treated with GA and PC is their color: GA treatment makes the materials yellow, but PC makes them white. There are also remarkable differences in their softness and elasticity. In long-term animal experiments, the grafts treated with PC maintained their elasticity. This natural compliance during implantation seems to be very important in obtaining permanent patency of small-caliber vascular grafts. Another characteristic of the PC treatment is the hydrophilic property imparted to the material, which is important for its affinity with the host tissue and makes the graft more nonthrombogenic.

The strength of the graft treated with PC is also noteworthy because despite its softness it has sufficient strength to withstand mechanical stresses associated with vascular graft applications. The absence of aneurysmal dilatation in any graft following long-term implantation indicates the stability of the graft in vivo. The finding of no foreign body reaction around the graft wall also indicates that this cross-linking method is quite safe for implant applications.

Reconstruction of an arterial wall with the graft was most successful with smooth muscle-like cells infiltrating the graft wall. We observed the healing process of the implanted vascular graft and noted that the smooth muscle cells infiltrated the neointima of the graft and were arranged parallel to the direction of the tensile stress placed upon the graft wall [8]. If the tensile stress is not present, smooth muscle cells seldom appear. The appearance of such cells in the graft treated with PC suggested that there is enough compliance of the graft to induce the migration of these cells. In the case of GA-treated grafts, the environmental condition in the graft wall is considered to be insufficient for the infiltration of the smooth muscle-like cells. The grafts cross-linked with GA become yellowish and lose their elastic characteristics. By contrast, PC cross-linked grafts maintain their natural vessel compliance and are stronger than the original vessels, thus providing excellent suturability and compliance match. Futhermore, the PC cross-linked grafts are hydrophilic because of hydroxyls in the molecular structure (Fig. 1), while the GA cross-linked grafts are hydrophobic. In this respect, the PC cross-linked graft has superior antithrombogenic characteristics because the high hydrophilicity may give the material antithrombogenicity [9]. Mori et al. [9] clarified that materials which were given hyperhydrophilic properties with polyethylene glycol became antithrombogenic. Our graft cross-linked with PC, which contains polyethyleneglycol in its molecular structure, has high hydrophilic properties. It has another merit with regard to antithrombogenicity in that it became weakly negatively charged after the cross-linking. This was because ε-NH_2 groups in the collagen molecules were used for the cross-linking, and these increased the carboxy groups relatively. The weak charge in negativity

contributes to the antithrombogenicity of the materials because of the prevention of platelet aggregation on the negatively charged surface. A basic study we made on a graft cross-linked with PC without heparinization showed antithrombogenicity on the surface. This experiment will be reported in the near future. Therefore, in terms of antithrombogenicity, as well as compliance match, PC cross-linked grafts are superior to GA cross-linked grafts when applied as small-caliber vascular substitutes.

Aortocoronary bypass grafting with this graft resulted in 100% patency, although the graft was handicapped with a low-flow rate and flow turbulence induced by the side-to-end anastomosis. By comparison, in carotid replacements, the flow rate was higher and the anastomosis was end-to-end.

It has been reported that the patency rate of human aortocoronary saphenous vein bypass grafts dropped dramatically during the 1st year. In 30% of cases, occlusion occurred within the 1st month because of mural thrombosis overlying areas without an endothelial cell lining, which were damaged by intraoperative manipulation and distension pressures above 100 mmHg [10, 11]. Furthermore, late graft failure related to progressive intimal hyperplasia of the vein wall may be due to the organization of early intimal thrombi after the initial endothelial damage [12]. Therefore, the anticoagulant or antiplatelet therapy is usually instituted after aortocoronary bypass grafting [13]. However, bleeding complications are not rare and sometimes they are fatal. The present graft has the advantage that no anticoagulation or antiplatelet therapy is necessary after the operation. From the results of aortocoronary bypass grafting to the right coronary artery, in which flow within the graft was very low because of the hypoplastic right coronary artery of dogs, it appears that the graft can be applied to a coronary artery having poor run-off.

Endothelialization was delayed because heparin inhibits cell adhesion and fibrin deposition on the luminal surface. However, in these preliminary animal experiments, the PC cross-linked heparinized grafts showed excellent patency, suggesting that the graft will be a potentially promising graft applicable to aortocoronary bypass or vascular surgery below the knee.

Infection was the most likely cause of graft occlusion in this study because only one graft became occluded in the absence of infection. In this case, the anastomotic area had a scar around the graft, suggesting the presence of microinflammation around the anastomotic line. Therefore, if infection can be eliminated, the graft patency rate should be very high.

From these results, we can conclude that the combination of short-term antithrombogenicity of slowly released heparin followed by the more permanent antithrombogenicity of endothelialization, together with the natural tissue compliance of these grafts, were the major reasons for their success as small-caliber vascular grafts.

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Long-term Evaluation of a Growable Graft

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The safety of a vascular graft that can grow with the growth of its recipients was evaluated during long-term implantation in experimental animals. An acellular matrix made of human saphenous vein was cross-linked with a polyepoxy compound to give controlled biodegradability, hydrophilicity, and antithrombogenic properties. Four millimeter internal diameter (ID) grafts, enveloped with a polyester mesh tube of 10 mm ID were implanted in 15 puppies. The diameter of the graft grew to 9.5 mm after 1 year from the original 4 mm, and remained stable for 2 years and 7

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hydrophilic reagent, which is less toxic than glutaraldehyde.[5] Moreover, grafts was moderately cross-linked, because the molecular weight of the cross-linking reagent was larger than that of glutaraldehyde, producing a lower cross-linking rate. Results of this experiment showed that numerous fibroblasts infiltrated the graft wall, indicating that the graft had high affinity for host cells. Migrating fibroblasts generally, produce collagenase, which digests old collagen fibers; highly cross-linked collagen fibers may not be digested, while moderately cross-linked ones are digested slowly. Our previous study revealed the cross-linking rate of the collagen ϵ-amino groups in the graft to be about 78%.[1] Therefore, collagenase produced by the fibroblasts migrating inside the graft matrix may gradually digest the original collagen. Along with this digestion, fibroblasts make a new collagen matrix, under the tensile stress provided by the blood pressure during reconstruction. With the combined effects of destruction, reconstruction and tensile stress, the graft will grow slowly.

Safety of the Graft

A growable graft has to be provided with some important properties, such as the ability to terminate growth at a predetermined size. Our previous experiment showed excellent graft growth and arrest at the expected size. However, the graft must have an even more important property, because it is made of a biologic material, and will be implanted in infants. In general, biologic implantable materials, such as heart valves[6] possess the serious problem of degenerative changes, *i.e.*, calcification, when they are used in babies. In this experiment, the original vein graft was absorbed, and the newly formed neointima was constructed with host cells as a completely autologous organ, resulting in no calcification in the graft wall.

Polyester fibers[7] used for reinforcement have proved to be a reliable material for implantable artificial organs. Therefore, growable grafts can permanently maintain their final size because of the reinforcing polyester mesh. As to immunogenicity of the graft, we clarified that cross-linking by hydrophilic reagents made the materials less antigenic, just as are those treated by glutaraldehyde. For these reasons, there should be no major problems in using these grafts for clinical purposes.

Acknowledgment

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Evaluation of a New Vascular Graft Prosthesis Fabricated from Ultrafine Polyester Fiber

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The major important properties of vascular grafts are compliance, suturability, and healing.

A high-porosity graft that is loosely woven or knitted satisfies the above described properties[1] but has the problem of severe blood leakage through its wall, especially during the administration of anticoagulants.[2] For this reason, the high-porosity graft is not acceptable as a graft for the thoracic aorta or as a composite graft with a heart valve. Lately, low-porosity grafts have become widely used for these purposes. In the low-porosity grafts developed so far, however, there are problems such as difficulty in anastomosis because the surgical needle cannot penetrate the rigid, tightly woven, or knitted structure. Another serious problem with low-porosity grafts is delayed and uneven endothelialization, because its dense texture does not provide enough sites at which this can occur. In order to eliminate these drawbacks, we have developed a new low-porosity graft (SS-G) using ultrafine polyester fibers (UFPF) with a diameter of 3 μm or less.

In this study, we report the preparation, mechanical properties, and animal implantation testing of the SS-G.

Materials and Methods

Preparation of the SS-G

The SS-G was prepared by the following processes: 1) Tube formation: UFPF (thickness: about 3 μm) and ordinary polyester fibers (OPF) (thickness: about 16 μm) were used as the front and back yarns in the weft backed woven method. The preparation and fabrication of UFPF have been reported in detail elsewhere.[3] 2) Napping of the UFPF: The front surface of the tube was napped by a nap-raising machine. 3) Ravel-proofing: The front surface of the tube was treated by a water jet machine. 4) Exchange of the front (outer) and back (inner) surfaces: The tube was turned inside out and crimped. After these procedures, the SS-G has an inner surface composed of napped UFPF.

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Measurements of Physical Properties

Compression behavior of the grafts was measured both in a tube configuration and as fabric using a RHEOROBBOT (Kyowa Company, Ltd.).

Water permeability was measured by standard methods,[1] and resistance to raveling was examined by cutting the end of the grafts on the bias.

In Vivo *Experiments*

Forty-five healthy mongrel dogs of both sexes, weighing 8 to 12 kg, were used as test animals. The SS-Gs (8 mm in inside diameter and 5.7 cm in length) were implanted in the thoracic descending aorta of 30 dogs for up to 375 days. As controls, Cooley Veri-Soft grafts (Cooley graft, Meadox; indicated water permeability, less than 130 ml/min/cm^2) were implanted in the remaining 15 dogs in the same manner.

All specimens excised were submitted to light and scanning electron microscopy.

The specimens for light microscopy were fixed with 1% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, and embedded in a hydrophilic resin (JB-4, Polyscience, Inc.). The cross sections were examined with hematoxylin and eosin stain, as well as Weigert's elastic fiber stain. Specimens for scanning electron microscopic observation were fixed with 1% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, and stained with a 1% OsO_4 solution. Specimens were dehydrated in a graded series of ethanols and in amylacetate, then critical-point dried with carbon dioxide and spattered with gold palladium. The examination was performed with a JSM-50A scanning electron microscope (JEOL, accelerating voltage: 15 kV).

Results

Preparation of the Grafts

SEM observations of the cross-sectional and the inner surfaces of the SS-G and Cooley graft are shown in **Figures 1*A* and *B***, respectively. It should be noted that the difference in fiber diameter between UFPF and OPF is very large. In the SS-G, the napped and entangled UFPF's were seen on the inner surface, while in cross section, the tightly woven and entangled UFPF and OPF for reinforcement on the outer side were observed. The cut end shows no raveling. On the other hand, the Cooley graft was constituted of tightly woven

to cause much speedier healing of the high-porosity graft. The color change in the initial clot layer from red to white at 3 days after implantation of the SS-G in **Figures 4*A* and *B***, which indicates the change of the clot to a fibrin layer, took place as promptly in the high-porosity graft, but in the SS-G the initial clot formation remained in the brushed layer, and no further growth was noticed. Consequently, the clot layer of the SS-G was very thin (10 to 100 μm). In the Cooley graft, however, the color change from red to white was significantly delayed, and overgrowth of the initial clot always occurred. These findings suggest that the extremely pliable UFPF on the inner surface of the SS-G effectively prevents overgrowth of the initial clot, leading to rapid endothelialization. However, the mechanism has not as yet been clarified.

In the Cooley grafts, endothelialization took place only in the vicinity of the anastomotic line but did not move to the central area, even after long-term implantation (360 days), as shown in **Figure 5*F***, while in the SS-Gs endothelialization was noticed in the middle part of the grafts early on (42 days), as shown in **Figures 6*A* and *B***. In low-porosity grafts like these, the tightly woven structure is said to prevent the formation and extension of colonies of endothelial cells, derived from the capillaries, infiltrating from the outside of the graft. Therefore, we suspect that the early central endothelialization of SS-Gs occurs as follows: At the anastomotic region, the endothelial cells actively proliferate and, carried by the blood flow,[6] adhere to the middle surface of the SS-G, because the thin and even fibrin layer formed on its surface provides good scaffolding. This colony formation significantly accelerates the healing process of the SS-G.

Another conspicuous characteristic of the SS-G is shown in **Figure 5*A***, in which numerous fibroblasts can be seen infiltrating into the fibrin layer over the brushed UFPF, something not seen without UFPF. This finding suggests that UFPF provides a suitable microenvironment for the infiltration and proliferation of these cells, which accompany capillary formation on the graft surface, leading to induction of endothelial cell colonies. The reddish surface at 7 to 35 days (**Figure 4*C***) shows this capillary formation. In addition, it seems that formation of a neointima, *i.e.*, infiltration and proliferation of fibroblasts and other cells, significantly accelerates the proliferation of endothelial cells by releasing cell growth factors. On the other hand, in conventional low-porosity grafts, such healing was markedly delayed, even after long-term implantation, as shown in **Figures 5*E* and *F***. This poor healing is considered to be caused by overgrowth of the initial clot, delayed change to a thin fibrin layer, and no infiltration and no proliferation of cells on the inside surface of the graft. It is therefore concluded that the extremely fine fiber used in the SS-G improves the softness, pliability and the healing seen in conventional low-porosity grafts. Furthermore, as shown in **Figure 5*D***, vasa vasorum was clearly seen in the neointima of the SS-G surface implanted for 375 days. Such formation is thought to prevent degenerative changes such as calcification, hyalinization, and arteriosclerosis and leads, as well, to long-term stabilization of the neointima.[8,9] It seems clear, therefore, that the healing process strongly depends on the thickness of the fiber. Studies on the correlation between the cross-sectional shape of the fiber and the healing process are in progress.

These findings suggest that fine fibers, which provide a suede-like fabric, are a promising material applicable to not only vascular grafts, but to a variety of artificial organs as well.

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Discussion

DR. BUTT: Let me just ask you what suture material you used for sewing these grafts?

DR. NOISHIKI: Usually we use 5–0 Tevdek.

DR. NGHIEM: Could you comment about the compliance, the elasticity of this graft as compared to other vascular prostheses?

DR. NOISHIKI: The graft is very compliant as compared to the Cooley graft, it is about eight times softer and expandable.

The participants in the discussion are identified as follows: Khalid M. R. Butt, MD, State University of New York, Downstate Medical, Brooklyn, New York, and Dai M. Nghiem, MD, The University of Iowa Hospitals and Clinics, Iowa City, Iowa.

NEW CONCEPTS AND DEVELOPMENT OF VASCULAR GRAFT PROSTHESES

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INTRODUCTION

There are several kinds of vascular graft prostheses, such as fabric Dacron prostheses, biological grafts, EPTFE grafts, cell seeding grafts, chemically treated connective tissue tube grafts, etc. They have been used safely in clinic, although with advantages and disadvantages. The biggest disadvantages are the poor healing ability of the neointima and lack of antithrombogenicity. Therefore, they cannot be used as venous and small diameter arterial grafts. For example, fabric Dacron prostheses have no natural antithrombogenicity. The surface is covered with fresh thrombi for a long period of time after implantation. Endothelialization of the grafts is limited to areas near the anastomotic sites. Conversely, EPTFE grafts have been expected to prevent thrombus formation. The grafts are hydrophobic and have less adhesive property. They can prevent the hydrophilic substances, but, hydrophobic substances can adhere to it followed by thrombi. Biological grafts are also not antithrombogenic and have a thrombus layer on the lumenal surface. If these grafts have an excellent healing ability of the neointima, they can maintain their patency for long periods of time with a powerful anticoagulant therapy just after implantation.

To accelerate the endothelialization, or to maintain the antithrombogenicity of the grafts before the endothelialization, we developed our own methods which showed satisfactory results in animal studies. In this communication, four types of vascular prostheses will be displayed. One is a heparinized hydrophilic polymer graft which has an antithrombogenic property[1]. The graft showed problems in long-term implantation. Other three are new types of grafts, i.e., a temporally antithrombogenic biological graft which can be reconstructed by host cells[2], a fabric prostheses

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transplanted with autologous tissue fragments[3], and a fabric prosthesis fabricated with ultrafine polyester fibers[4].

VASCULAR PROSTHESIS MADE OF AN ANTITHROMBOGENIC POLYMER

We have developed a heparinized hydrophilic polymer utilizing an ionically-bound heparin in order to render excellent antithrombogenicity for a long period[5]. This material has been successfully applied to medical devices, such as catheters, drainage tubes, ascites tubes and chambers of blood pumps[6-8]. As previously reported, its thromboresistance is caused by the continuous release of a certain amount of heparin from the surface into the blood stream. In this experiment we have applied this heparinized polymer to vascular prostheses by making it porous in order to render pliability and ease of suturing.

Materials and methods

Preparation of heparinized hydrophilic polyurethane (H-USD): The cationic hydrophilic polyurethane (USD) was synthesized by blending N,N-dimethylacetamide (DMAC) solutions of segmented polyurethane (Lyra T-127, DuPont, polymer concentration: 14 wt%) and DMAC solution of a cationic copolymer (SD^+) was synthesized by radical copolymerization of methoxypolyethyleneglycol methacrylate (SM) of 60 wt% and N,N-dimethylaminoethyl methacrylate (DAEM) of 40 wt% in DMAC at 45°C for 30 hours using azodimethylvaleronitrile as an initiator and subsequently by quarternizing dimethylaminoethyl groups at 55°C for two hours using ethylbromide. Heparinization was achieved by ionically binding heparin to the quarternized nitrogen groups of USD polymer after fabrication.

Preparation of the vascular prosthesis: The cylindrical glass mandrils, ranging from 3 to 8 mm in diameter, were dipped into the DAMC solution of USD polymer (polymer concentration: 1 wt%), dried under varying conditions and subsequently dipped into the N,N-dimethylformamide (DMF) solution of the segmented polyurethane (polymer concentration: 10 wt%). For the purpose of reinforcement, polyester mesh of fiber was incorporated in the coating layer of the segmented polyurethane. Without drying the coated segmented polyurethane layer, the mandril was immediately soaked in distilled water to precipitate the polymer by substitution of water for the solvents (DMF and DMAC) involved in the polymer matrix. Then the polymer coating layer was slipped over the mandril and soaked in distilled water at room temperature for one day in order to extract the residual solvents and unreacted monomers. Heparinization of the USD coating layer was carried out in 3 wt% sodium heparin aqueous solution at 60°C for two days. The average heparin concentration in the H-USD layer measured by electron probe x-ray microanalyzer (EMX) was 15 wt%.

In vivo experiments: Seventy-three healthy mongrel dogs of both sexes weighing 6 to 12 kg were used as the test animals. Forty prostheses were implanted in the aortas of 40 dogs. A 5.5 cm segment of the thoracic aorta was resected and replaced by the prosthesis (8 mm in internal diameter and 5.7 cm in length). The implantation periods ranged from one to 575 days.

Twenty-eight prostheses were implanted in both the external iliac arteries of 14

dogs. The segments of both the external iliac arteries (3 to 5 cm long) were excised and replaced by the prostheses (3 mm in internal diameter and 4 to 7 cm in length). The implantation periods ranged from one to 98 days.

Nineteen prostheses were implanted in the inferior vena cavae of 19 dogs. A 2 cm segment of the inferior vena cava was resected and replaced by the prosthesis (8 mm in internal diameter and one to 3 mm in length). The implantation periods ranged from one to 309 days. In all experiments, antibiotics were used at the time of operation, but no anticoagulants were used at any time.

Angiographical examination: In the thoracic descending aorta and the inferior vena cava, the occlusion of the implanted prosthesis was confirmed by death of the animal. In the external iliac artery, however, the occlusion of the implanted prosthesis did not cause death, so that translumbar aortographic studies were performed in the animals with the prostheses implanted for more than one month in order to assess the patency.

Observations: The specimens for light microscopy were fixed with 10% formaldehyde aqueous solution and then embedded in paraffin and examined using Weigert elastic fiber stain, as well as hematoxylin and eosin. The specimens for scanning electron microscopy were fixed with 2.5% glutaraldehyde in phosphate buffered saline solution (PBS) and stained with 1.0% osmium tetroxide aqueous solution at 4°C. The fixed specimens were dehydrated in a graded series of ethylalcohol and in amyl acetate, then critical-point dried with carbon dioxide, and coated with gold palladium. The examination was performed with a JSM-50A scanning electron microscope. The specimens for transmission electron microscopy were dehydrated in a graded series of ethyalcohol and propylene oxide and then embedded in epoxy resin (Epon 812). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. These stained sections were examined with a JSM-100C electron microscope.

Results

All the prostheses implanted in the thoracic descending aorta were patent at the time of removal. The average implantation period was 146 days. Twenty-one prostheses out of those implanted in the external iliac arteries were patent and the residual seven prostheses were occluded. The patency rate and the average implantation period were 75% and 33 days respectively. In 19 prostheses implanted in the inferior vena cavae, 14 prostheses were patent and five prostheses were occluded. The patency rate and the average implantation period were 74% and 56 days, respectively.

Autopsy findings demonstrated that the occlusion of three prostheses implanted in the external iliac arteries for 28, 60 and 98 days was caused by pannus formation in the anastomotic lines. In addition, in the prostheses implanted in the inferior vena cavae for 23, 72 and 111 days, the occlusion was caused by the similar pannus formation. The occlusion was observed at the early stage (5 to 7 day-implantation) in the external iliac arteries; in the inferior vena cavae failure was due to thrombi formed in the anastomotic lines by technical failure.

One translumbar aortographic observation of a prostheses implanted in both external iliac arteries for 98 days, indicated that the right prosthesis was occluded and the left prosthesis was patent. At the distal area of the anastomotic site a small pannus was noticed in the photography.

Antithrombogenic polymer prevents not only the thrombus deposition but also pannus adhesion. The graft cannot control the pannus growth. Therefore, small diameter graft and venous graft will be occluded easily with the pannus growth.

TEMPORALLY ANTITHROMBOGENIC BIOLOGICAL GRAFT WHICH CAN BE RECONSTRUCTED BY HOST CELLS

Biological materials have unique, fine structural and mechanical properties which cannot be simulated by any current technologies. For example, arteries have ideal hemodynamic shaped ramifications. Inside the arteries, they have uniquely and fine structures specially suitable for cell inhabitation. They also have suitable mechanical properties to accept and to pass the pulsatile blood pressure and flow. If we could use these special properties and structures for biomedical materials, we could make excellent artificial organs. One of the problems is antigenicity except for those of autologous origin. Biodegradability of the materials is also one of the problems. To reduce the biodegradability and antigenicity of the materials, chemical modifications by glutaraldehyde, dialdehyde starch, formaldehyde, and hexamethylene diisocianate have been used; however, these treatments make the materials hydrophobic and stiff. Glutaraldehyde is the most frequently used treatment, but it has cytotoxicity and prevents cell infiltration inside the graft wall. To overcome these problems, we introduced a new crosslinking reagent[9]. Another technology we have developed is the heparinization of biological materials[10]. Collagen is one of the major components of the biological materials. It has unique properties for the host cells migration and proliferation, and also for platelets adhesion and accumulation[11]. Therefore, this biological material is thrombogenic if used for cardiovascular artificial organs. To reduce the thrombogenicity, we developed a heparinization method. With the combined use of heparinization and the crosslinking method, a small diameter biological graft was developed.

Materials and Methods

Cross-linking reagent used: The cross-linking reagents used were polyepoxy compounds (PC), such as polyethylene glycol diglycidyl ether, glycerol polyglycidyl ether, polyglycerol polyglycidyl ether, and sorbitol polyglycidyl ether (Nagase Chemical, Ltd., Osaka, Japan). In this study, polyglycerol polyglycidyl ether (PGPGE) was used. Its molecular structure and cross-linking reaction with collagen molecules are illustrated in Fig. 3. The cross-linking reaction can be performed at room temperature, and the specimens cross-linked with PCs become hydrophilic.

Preparation of the graft: A fresh carotid artery with an inner diameter of 2.5 to 3.0 mm was obtained from dogs. The artery was soaked in distilled water for one hour and submitted to ultrasonic waves of 28 kilocycles for 20 seconds to produce cell destruction. Cell debris was then removed by washing with distilled water. In this way, a natural tissue tube composed of collagen and elastic laminae was obtained. A 2% protamine sulfate solution at pH 5.9 was poured into the natural tissue tube graft lumen, and the graft was inflated with air at a pressure of 80 to 100 mmHg for 30 min to force the protamine into the graft wall. The graft inflated with air pressure was treated with a 5% PC solution in 50% ethanol and 0.1 M Na_2CO_3 at pH 10.0 for five hours to cross-link the tissue and covalently immobilize protamine impregnated into

Results

Mechanical properties of the graft: Using the method of Hayashi et al., quantitative comprehensive wall stiffness of each specimen was calculated. From these results, the vascular compliance of each specimen at 90 mmHg was also calculated. The strength and elongation of each specimen was measured. The compliance of native, GA treated and PC treated artery were 2.33, 0.80, 1.70 percent radial change per mmHg x 10^{-3}, respectively. Elongation rates were 11.6, 57, 127 %, respectively. Tensile strengths were 207, 127, and 199 g/mm^2, respectively.

Animal experiment: Heparinized grafts were white, pliable, and more elastic than the yellowish controls. The inner surfaces of both grafts were shiny and smooth, but the heparinized grafts were easier to suture and match to the host arterial wall. There was no blood leakage through the grafts wall on implantation, and no kinking occurred even when the grafts were bent.

Implanted grafts: All the grafts were patent at the time of the angiographic examination. The inner surface of the grafts was smooth throughout its length, and no stenosis or aneurysmal dilatation was observed in any of the grafts. At the graft)xplantation, the dogs were anesthetized with intravenous sodium pentobarbital, and heparin 2 mg/kg was given intravenously to prevent clot formation. In the heparinized graft, 79 were patent and three were occluded. In one dog, killed at 172 days after implantation, a graft implanted in the right carotid artery was patent, but the graft in the left artery was occluded. The occluded graft was soft and white, but anastomotic lines were hard. An angiographic examination of the dogs performed at 40 days after implantation, however, showed both grafts to be patent. Consequently, it was considered that the graft occluded a certain period of time after the angiogram. In another dog, killed at 11 days, the cervical wound was infected and the grafts implanted in both carotid arteries were occluded. As these were the only grafts occluded, the patency rate of the heparinized graft was 96%. All the patent grafts were still as soft and pliable as the native artery. Within 100 days after implantation, the inner surfaces were completely free from thrombus deposit. The surfaces were as shiny, white, smooth, and glistening as those of the host arterial intima. In the case of those grafts that remained in place for more than 100 days, slightly yellowish and semitransparent small spots were sporadically observed on the surface.

The control grafts were occluded within one week after implantation, and they were very hard and dark brown in color.

Scanning electron microscopic observations: Scanning electron microscopy (SEM))evealed the inner surface of the heparinized grafts before implantation not to be smooth,but to have a naked elastic lamina with many holes and wrinkles on the surface. No endothelial cells were seen. After implantation,the surface was covered with a layer of protein that was so thin that the structure of the elastic lamina could be observed throughout it. On the surface of the grafts removed at less than 100 days, there was neither fibrin deposition nor platelet aggregates, and the surfaces were rough due to the wrinkles in the elastic lamina. At the anastomotic line, pannus was first observed at 37 days and was noticed at each anastomotic line in all the grafts left in place for more than 37 days. The size of the pannus was not longer than 1 mm beyond the anastomotic line. The pannus was completely covered with endothelial cells and adhered on the graft surface. After 106 days, there were small fibrin deposits on the graft surface at the lines formed by the elastic lamina. Endothelial cells were not observed on the inner surface of the center areas of any grafts after periods as long as 172 days. However, in the cases of the grafts which remained in situ for 389 and 429 days, the whole entire surfaces were covered with endothelial cells.

Microscopic observations: Microscopic observations confirmed that there was neither thrombus nor fibrin deposit on any graft before 81 days. The luminal surface was composed of the internal elastic membrane, with no endothelial cells on the surface. At the early stage, there was no foreign body reaction such as giant cell infiltration on the outer surface of the graft. A small number of plasma cells were observed a short period of time after implantation. At the anastomotic line of the grafts implanted for more than 37 days, there were small panni covered with endothelial cells seen by SEM to be adherent to the graft surface. The size of the pannus grew with time. At more than 30 days, some macrophages were noticed in the inter elastic luminal spaces near the luminal surface. Before implantation, these spaces were occupied by disrupted smooth muscles cells, collagen, and elastic fibrils. After implantation, macrophages gradually phagocytized the smooth muscle cells. After more than two months, spaces containing only collagen and elastic fibrils were observed. After more than six months, smooth muscle-like cells infiltrated these spaces from the adventitial sides (Fig. 4). In the wall of long-term grafts, cells filled spaces completely. In 75% of the spaces near the luminal surface, these elongated cells were arranged circumferentially. The rest were oriented longitudinally. After 389 days, the central part of the inner surface was covered with endothelial cells, which impinged directly on the surface of the elastic lamina. The structure of the graft following long-term implantation closely resembled that of the native arterial wall, and near the anastomotic line at 389 days a thick layer of pannus with an endothelial cell lining covered the surface. The pannus adhered on the graft surface. The thickness of the pannus was about 30 μm. There was no foreign body reaction in the long-term specimens and no degenerative changes such as hyalinization, calcification, or arteriosclerosis.

Concentration of heparin in graft: Before and after the implantation, the total amount of heparin in the graft was measured. The results indicated that the amount before implantation was about 7.0 units/cm^2, but in specimens in place for more than 80 days, there was no heparin in the graft wall. This was confirmed for grafts in place for 106, 153, 172 and 389 days.

Discussion

Antithrombogenicity and compliance are both important factors in small-caliber vascular grafts. We previously developed a method that would afford antithrombogenic properties to collagenous biomaterials, such as vascular grafts made from carotid arteries[2] and ureters[13]. This method was very effective in preventing thrombus formation for both small caliber arteries and large vein vascular grafts. The mechanism is as follows. Heparin is bound ionically to protamine that has been previously covalently linked to the materials, so that the heparin is slowly released following implantation. This slow release of heparin can prevent fibrin formation on the graft surface. As the heparin is gradually desorbed, the graft becomes naturally antithrombogenic because endothelial cells begin to cover the graft surface. Consequently, the graft can remain permanently antithrombogenic by endothelialization. Animal experiments revealed that this method produces stable antithrombogenicity in small-caliber arterial grafts. In previous preparations of this heparinized graft, the protamine impregnated into the graft wall was cross-linked with GA under conditions of graft inflation, and although the graft showed no thrombus formation on its surface following implantation, the graft became less pliable and

yellow with time. These changes occurred because when the graft was treated with GA to cross-link the protamine to its wall, it also cross-linked the protamine to the collagen molecules inside the graft wall. While GA cross-linking makes the materials less biodegradable, insoluble, and less antigenic, it makes the materials less flexible. Recently, other adverse effects of GA treatment have been reported.

To overcome these difficulties, a new cross-linking method was introduced. A marked difference in appearance between materials treated with GA and PC is their color: GA treatment makes the materials yellow, but PC makes them white. There are also marked differences in their softness and elasticity. In long-term animal experiments, those treated with PC maintained their elasticity. This natural compliance during implantation seems to be very important in obtaining permanent patency of small-caliber vascular grafts. Another characteristics of the PC treatment is the hydrophilic property imparted to the material, which is important for its affinity with the host tissue and makes the graft more nonthrombogenic.

Reconstruction of an arterial wall with the graft was most successful with smooth muscle-like cells infiltrating the graft wall. We observed the healing process of an implanted fabric Dacron vascular prosthesis and note that the smooth muscle cells infiltrated the neointima of the graft and were arranged parallel to the direction of the tensile stress upon the graft wall[14]. If the tensile stress is not present, smooth muscle cells seldom appear. The appearance of such cells in the graft treated with PC suggested that there is enough compliance of the graft to induce the migration of these cells. In case of GA-treated grafts, the environmental condition in the graft wall is considered to be insufficient for the infiltration of the smooth muscle-like cells. The grafts cross-linked with GA become yellowish and lose their elastic characteristics. By contrast, PC cross-linked grafts maintain their natural vessel compliance and are stronger than the original vessel, thus providing excellent suturability and compliance match. Furthermore, the PC cross-linked grafts are hydrophilic because of hydroxyls in the molecular structure, while the GA cross-linked grafts are hydrophobic. In this report, the PC cross-linked graft has superior antithrombogenic characteristics because the high hydrophilicity may give the material antithrombogenicity[15]. It has another merit with regard to antithrombogenicity in that it becomes weak-negatively charged after the cross-linking. This was because e-NH_2 groups in the collagen molecules were used for the cross-linking, and these increased the carboxyl groups relatively. The weak charge in negativity contributes to the antithrombogenicity of the materials because of the prevention of platelet aggregation on the negatively charged surface.

From these results, we can conclude that the combination of short-term antithrombogenicity of slow released heparin followed by the permanent antithrombogenicity of endothelialization, together with the natural tissue compliance of these grafts, were the major reasons for their success as small caliber vascular grafts.

A FABRIC VASCULAR PROSTHESIS TRANSPLANTED WITH VENOUS TISSUE FRAGMENTS

Endothelialization of vascular grafts in human is extremely delayed[16]. Most of the grafts implanted are not endothelialized, and are covered with fresh thrombi for a long time after implantation except for those areas near anastomotic sites. Endothelial cell seeding methods have been attempted for the past decade[17]. Some

of them have produced satisfactory experimental results, but they remain unavailable for general use, as they require special cell culture techniques and facilities. They are also not available for emergency use, since the cell culture requires an extended period of time. Recently, we developed a new method to seed tissue fragments which contained endothelial cells, smooth muscle cells and fibroblasts[18]. This mixed cells seeding was very effective in making a new arterial wall in vivo. With this method, we made a fabric vascular prosthesis transplanted with autologous venous tissue fragments into the wall.

Materials and methods

Preparation of the graft: A highly porous fabric vascular prosthesis (Microknit, Golaski Laboratories, Inc., Philadelphia, PA; Water porosity: 4,000 ml) was used as the framework of the graft. The prosthesis was connected with a syringe through a three way stopcock, and was enveloped by a transparent bag connected to the three way stopcock through a tube. A piece of peripheral vein weighing about 0.3 g was obtained, cut into tiny fragments, and stirred into 20 ml of saline, thereby creating a tissue suspension. This suspension was sieved through the wall of the prosthesis by strong injection with a syringe. The residual suspension that passed through the prosthetic wall was suctioned again through the connective tube. The prosthesis was then implanted as a vascular graft into the same dog from which the vein used had been resected.

Implantation of the prostheses: Forty adult mongrel dogs, weighing 8-12 kg were used. The seeded grafts, 7 mm ID and 5.7 cm in length, were implanted into the descending aortae of 25 dogs. Fifteen preclotted prostheses (Microknit) were also implanted as controls.

Graft harvesting: Implanted grafts were harvested at from 1 to 61 days after implantation. Before harvesting, sodium heparin (100 IU/kg) was intravenously administrated to prevent clotting. After removing the prostheses, the kidneys of each animal were resected and examined for fresh, trapped microemboli.

Histologic examination: The specimens were fixed with 1% glutaraldehyde in phosphate buffer, pH 7.4, and embedded in hydrophilic resin (JB-4, Polyscience Inc., Warrington, PA). Sections were stained with hematoxylin and eosin, PAP method, and Van Kossa stains.

Results

Implantation of the graft: Grafts seeded with tissue fragments were soft and pliable (Fig. 5), and there were no bleeding complications postoperatively, while in the control grafts, problems occurred after surgery. Three of 15 control animals (20%) bled into the pleural cavity and died within 24 hours after implantation.

Graft harvesting: Both the seeded and control grafts were patent at the time of harvesting. In the tissue fragments graft, fresh red thrombi adhered to the luminal surface just after implantation. The next day, the luminal surface became white with small spotted thrombi. On the third to fifth day, the luminal surface became whiter, and at 14 days, the surface was completely white, without any thrombus. No macroscopic changes were observed up to the two months observation period. In the control graft, the luminal surface was covered with fresh thrombi, except at anastomotic sites, at two weeks after implantation. After two months, the center area

In this experiment, three kinds of cells - fibroblasts, smooth muscle cells, and endothelial cells - migrated and proliferated at the same time from the fragments. This is a very unique phenomenon, that is not observed previously in cell culture. When fibroblasts and endothelial cells are culture in a petri dish, endothelial cells are suppressed and fibroblasts proliferated to form a confluent layer. However, in this in vivo experiment, we noticed the three cells migrated and proliferated together. Endothelial cells produced capillaries, and rose to the inner surface of the graft to face the blood stream. Accordingly, smooth muscle cells made a multilayer beneath the endothelial cells, and fibroblasts crawled down under the smooth muscle cells layer around the polyester fibers. This phenomenon suggests that growth of these cells was controlled by their physiological environment.

Mixed cell culture of different types have been reported to behave uniquely. For example, in the case of an experiment of a skin equivalent substitute[19], epidermal cells cannot make a satisfactory membrane by themselves. Fibroblasts are necessary as a feeder cells underneath the epidermal cells. In case of the neointima, endothelial cells cannot produce a stable neointima by themselves. With the smooth muscle cells or fibroblasts underneath, endothelial cells can maintain a more stable condition. Also, smooth muscle cells and fibroblasts cannot maintain stability without the protection of the endothelial cells.

These results in this experiment show the efficacy of transplantation of venous tissue fragments into vascular prostheses, and the possibility of overcoming delayed neointimal healing in humans.

FABRIC PROSTHESIS WITH HIGH CELL AFFINITY FABRICATED WITH ULTRAFINE POLYESTER FIBERS

After the detailed animal and clinical experiments of Wesolowski, a highly porous fabric vascular prosthesis is recommended because of the high healing ability of the neointima[20,21]. For this purpose, many preclotting methods have been developed to seal the highly porous grafts before implantation[22-24]. In clinic situations however, such a highly porous graft cannot be used due to bleeding through the graft wall[25]. Even after a perfect seal using these reliable preclotting techniques, the grafts have the possibility of bleeding due to the fibrinolysis. In the clinic, middle or low porous fabric grafts have been used to prevent postoperative bleeding. But these grafts show little endothelialization. Special methods such as an immobilization of growth factors have been tried, without success. For these reasons, a fabric vascular prosthesis with the high healing capability of the neointima has been desired. Recently we developed a new graft fabricated from ultrafine polyester fibers[4]. Our animal experiment revealed that fibroblasts adhere onto the ultrafine polyester fibers very avidly. We believe that these phenomena are from the basic affinity of the cells to the graft, i.e., the cells want to adhere to the sharp edge or fine fibers. We term this behavior "contact guidance." The vascular prosthesis fabricated from ultrafine polyester fibers can accumulate many host cells, which will require their nutrition. Therefore after migration and proliferation of these cells, capillary blood vessels will follow, resulting in a natural angiogenicity. Using the behavior of these cells, we developed a special vascular prosthesis.

Materials and methods

Preparation of the graft: The graft was prepared by the following procedures:

1) Tube formation: Ultrafine polyester fibers (UFPF), thickness: about 3 μm and ordinary polyester fibers (OFP), thickness: about 16 μm were used as the front and back yarn in the weft-backed woven method. The preparation and fabrication of UFPF have been reported in detail elsewhere[26]. Napping of the UFPF: The front surface of the tube was napped by a nap-raising machine. 3) Ravel proofing: The front surface of the tube was treated by a water jet machine. 4) Exchanges of the front (outer) and back (inner) surfaces: The tube was turned inside out and crimped. After these procedures, the graft has an inner surface composed of napped UFPF.

In vivo experiments: Forty-five healthy mongrel dogs of both sexes, weighing 8 to 12 kg were used as test animals. The grafts (8 mm in internal diameter, and 5.7 cm in length) were implanted in the thoracic descending aortae of 30 dogs for up to 375 days. As controls, Cooley Veri-Soft grafts (Cooley graft, Meadox Inc., indicated water porosity: less than 130 ml) were implanted in the remaining 15 dogs in the same manner. All specimens excised were submitted to light and scanning electron microscopy. The specimens for light microscopy were fixed with 1% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, and embedded in a hydrophilic resin (JB-4, Polyscience, Inc.). The cross sections were examined with hematoxylin and eosin stain, as well as Weigert's elastic fiber stain. Specimens for scanning electron microscopic observation were fixed with 1% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, and stained with a 1% OsO_4 solution. Specimens were dehydrated in a graded series of ethanols and in amilacetate, then critical point dried with carbon dioxide and spattered with gold palladium. The examination was performed with a JSM-50-A scanning electron microscope (JEOL, accerelating voltage:15kV).

Results

Preparation of the graft: SEM observations of the cross-sectional and the inner surfaces of the graft and control Cooley graft are shown in Fig. 7a and 7b, respectively. It should be noted that the difference in fiber diameter between UFPF and OPF is very large. In the graft, the napped and entangled UFPF's were seen on the inner surface, while in cross section, the tightly woven and entangled UFPF and OFP for reinforcement on the outer side were observed. The cut edge shows no raveling. On the other hand, the Cooley graft was constituted of tightly woven plain OPF alone, without napped fibers which ravels easily. Water permeabilitys of the graft and Cooley graft were 92.6 and 134 ml/min./cm^2, respectively.

In vivo experiment: Because the graft was soft and pliable, fitting and suturing it to the aortic wall became significantly easier. Although no preclotting was performed, blood leakage through the wall of the graft was minimal because of the dense structure. Blood infiltrated no further than into the whole cloth, which turned reddish but did not leak. On the other hand, the rigidity of the control graft caused difficulties in fitting and suturing and, in several cases, the surgical needle became bent. All the grafts were patent at the time of harvesting. The following healing processes were macroscopically noted in the graft. The red color was seen two hours after implantation, but the surface turned white in three days. during a period of seven to 37 days after implantation, the suture turned gradually reddish again, followed by a final change to yellow. After 42 days of implantation, white, glistening areas developed as in growths of the host vessel at both ends of the graft, and occasionally similar areas were noticed more centrally. These areas gradually grew to cover the whole of the surface in three months. The surface remained white and glistening for a long period of time. In the control grafts, however, severe clotting occurred immediately after implantation. At the anastomotic sites, healing of the

Discussion

Ultrafine polyester fibers (UFPF, about 3 μm in thickness) are used for making the suede-like fabric "Ultrasuede" (U.S.A.), "Alcantare" (Europe), "Ecsaine" (Japan) produced by Toray Industries, Inc. The fabric is brushed to provide a velvetlike feel to the surface. Accordingly, the vascular grafts fabricated from such very fine fibers are soft and pliable, regardless of their dense textures, and have a low porosity. Permeability of the grafts (water permeability: about 93 ml) is much lower than that of conventional high-porosity grafts (water permeability: 1,200 to 2,00 ml) made from ordinary polyester fibers (OPF, about 16 μm in thickness). Therefore, the grafts are expected to solve some of the problems found with implantation of conventional low-porosity grafts. For example: 1) Difficulty in fitting and suturing to an aorta which is stiffened due to arteriosclerosis and calcification, which can lead to severe bleeding at the anastomotic line. 2) Low-porosity grafts which ravel easily at their cut ends because of their plain woven structure and can be brushed to prevent raveling as a result of its fiber entanglement created thickness. 3) Formation and stabilization, or the healing process is significantly delayed compared with that of high-porosity grafts because the tightly woven structure reduces the number of interstices among the fibers, which play an important role as scaffording for cell adhesion and infiltration.

The most important property of a vascular graft is rapid and uniform vascularization, which closely correlates with initial clot formation, and subsequent adhesion and infiltration by endothelial and other cells. In the high-porosity grafts, the quick change of initial clot to a fibrin layer occurs by the elution of erythrocytes. This rapid shift is considered to cause much speedier healing of the high-porosity graft. The color change in the initial clot layer from red to white at three days after implantation of the graft, which indicated the change of the clot to a fibrin layer, took place as promptly as in the high-porosity graft, but in the graft the initial clot formation remained in the brush layer, and no further growth was noticed. Consequently, the clot layer of the graft was very thin (10 to 100 μm). In the control graft, however, the color change from red to white was significantly delayed, and overgrowth of the initial clot always occurred. These findings suggest that the extremely pliable UFPF on the inner surface of the graft effectively prevents overgrowth of the initial clot, leading to rapid endothelialization. However, the mechanism has not as yet been clarified. In the control grafts, endothelialization took place only in the vicinity of the anastomotic line but did not move to the central area, even after long-term implantation, while in the grafts endothelialization was noticed in the middle part of the grafts early on (42 days).

A conspicuous characteristic of the graft is shown in Fig. 8a, in which numerous fibroblasts can be seen infiltrating into the fibrin layer over the brushed UFPF, something not seen without UFPF. This finding suggests that UFPF provides a suitable microenvironment for the infiltration and proliferation of these cells, which accompany capillary formation on the graft surface, leading to induction of endothelial cell colonies. The reddish surface at 7 to 35 days shows this capillary formation. In addition, it seems that formation of a neointima, i.e., infiltration and proliferation of fibroblasts and other cells, significantly accelerate the proliferation of endothelial cells by releasing cell growth factors. On the other hand, in conventional low-porosity grafts, such healing was markedly delayed, even after long-term implantation. It is therefore concluded that the extremely fine fiber used in the graft improves the softness, pliability and the healing seen in conventional low-porosity grafts.

Furthermore, vasa vasorum was clearly seen in the neointima of the graft surface implanted for 375 days. Such formation is thought to prevent degenerative changes such as calcification, hyalinization, and arteriosclerosis and leads, as well, to long-term stabilization of the neointima. It seems clear, therefore, that the healing process strongly depends on the thickness of the fiber. Studies on the correlation between the cross-sectional shape of the fiber and the healing process are in progress.

These findings suggest that fine fibers, which provide a suede-like fabric, are a promising material applicable to not only vascular grafts, but to a variety of artificial organs as well.

SUMMARY

Four kinds of vascular graft prostheses were demonstrated in this communication. One is a graft with an artificial antithrombogenic property. The other three have a common concept, i.e., natural antithrombogenicity of endothelialization on their luminal surfaces. For a small diameter vascular graft, antithroambogenicity is essentially required. During the past two decades, antithrombogenic synthetic polymer prostheses like a heparinized polymer graft displayed above have been studied. Some of them showed marked ability to prevent platelets adhesion and fibrin deposit, however, the properties were not long-term. The polymer materials were covered with some blood components, such as proteins or cells. The materials were attacked by these cells and enzymes. It has been very difficult to maintain the initial property against the blood stream, because most of the materials cannot face the blood stream directly after implantation. After the covering with such substances, the materials have to act as the matrix for cell migration and proliferation, however, many of the antithrombogenic polymer materials were not designed for this purpose and the cells cannot obtain a stable anchoring on the surface. The floating pannus observed in the antithrombogenic graft will be developed in this way.

Except as vascular prostheses for temporary use, most of the prostheses have to be implanted for a very long-term, sometimes, more than 30 years. In the implantation period, the initial stage just after implantation is very short. Therefore, the vascular prostheses have to be designed to have a stable condition for the long-term. To maintain the antithrombogenicity of vascular prostheses, it will be very difficult with the properties of the synthetic polymer materials. Permanent antithrombogenicity of endothelial cells is the best and natural method for this purpose.

In this communication, three grafts were demonstrated to have stability of neointima, which were covered with endothelial cells. Artificial antithroambogenicity have to be effective within only very short period of time after implantation. Heparinization in the biological materials is one example of a temporally used antithrombogenicity. After the release of heparin, the graft has to provide the best condition in structural and mechanical properties for cell migration and proliferation. EPTFE graft is one of the samples with its incomplete structure. It had been believed to have an antithrombogenic property on the luminal surface. PTFE is a very hydrophobic material with poorly adhesive properties. Expanding gives it a flexible property and microporous structure, which was expected to contribute the cell anchoring. Ideally, antithrombogenic properties will prevent the cell adhesion, while microporous structure will help the cell adhesion.

Ultrafine polyester fibers accelerate the cell migration and proliferation, resulting in a rapid neointima formation with endothelial cell lining. The biological graft crosslinked with polyepoxy compounds can give a natural anchoring site for cell habitation. The tissue fragments transplantation technology will contribute to rapid neointima formation on the prosthesis. These technologies will be useful for the development of vascular prostheses in future.

CONCLUSION

There are two concepts of the design of vascular prostheses with respect to antithrombogenicity. One is artificial and another is natural. Our experimental data showed that the former is suitable for the short-term use and the latter is for permanent use. Some temporary antithrombogenicity will be helpful for the very short period of time after implantation to maintain early stage patency before endothelialization, however, basic structure of the vascular prostheses have to be designed as a best matrix for the host cell migration and proliferations.

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Natural Antithrombogenic Surface Created In Vivo for an Artificial Heart

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Summary. Thromboses and embolisms arising from the prosthesis surface are among the most significant problems in artificial hearts. Although antithrombogenic polymer materials have been developed to prevent thrombus formation, they have problems in long-term applications. While the natural antithrombogenicity of endothelial cells is reliable, development of the cell lining requires long periods after implantation. To overcome this problem, we developed a method to obtain rapid endothelialization by seeding autologous venous tissue fragments. The rate of endothelialization and the antithrombogenicity were evaluated in a small-diameter vascular graft treated by this method. A canine jugular vein was minced and suspended with heparin. This was sieved through the wall of a fabric prosthesis by pressurized injection, causing tissue fragments to be trapped in the graft wall. Twenty out of 32 grafts were patent up to 400 days, while all 12 control fabric grafts with preclotting were occluded. The luminal surface at 1 h showed no thrombus deposition. At 1 month, complete endothelialization was noted. There were no degenerative changes in any neointimae of the explanted grafts. These results indicated that heparin reduces the thrombogenicity of collagen by electrostatic binding during endothelialization, and that a natural antithrombogenic surface can be obtained by this method within a short period.

Key words: Antithrombogenicity — Endothelialization — Neointima — Small-diameter vascular prosthesis — Tissue fragment transplantation

Introduction

The luminal surface of the artificial heart faces the blood-stream, and it thus requires antithrombogenic properties to prevent thrombus formation on the surface. For this purpose, some synthetic polymers with antithrombogenic properties, such as segmented polyurethanes [1–3], rigid titanium [4], and biological materials like gelatin [5] have been adopted. While they showed adequate results over short periods, some problems still remain for long-term application. After long periods, the surfaces of these polymers are covered with certain host substances, such as serum proteins [6], and they cannot, therefore, function in their original state, since they cannot directly face the bloodstream. i.e., they are in a situation which will not allow them to display their original antithrombogenic properties by direct contact with the bloodstream.

There is another way of obtaining antithrombogenic properties, i.e., via the natural antithrombogenicity of the endothelial cell lining [7]. Here we introduce our new method of acquiring natural antithrombogenicity on a fabric prostheses within a short period after implantation.

Materials and Methods

To evaluate the antithrombogenicity of the material treated by our new method, we adopted a test system involving small-diameter vascular graft implantation in the carotid arteries of dogs. Small-diameter vascular prostheses require perfect antithrombogenicity to maintain their patency over long periods.

Fabric Vascular Prosthesis Adopted with the New Method

For this evaluation, we made a small-diameter vascular prosthesis transplanted with autologous tissue fragments, in accordance with a method described previously [8,9]. A highly porous Dacron fabric prosthesis (Microknit; Golaski, Philadelphia, Pa.; water porosity, 4000 ml/cm^2 per min at 120 mmHg) was used as the framework of the graft.

A canine left jugular vein with its surrounding connective tissue (10-cm-long; 2-gm weight) was resected and minced with scissors into tiny (less than

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the graft acquired the natural and permanent antithrombogenicity of endothelial cells.

One application of our new method has already been reported to be successful, in a pilot study with an artificial heart [19]. In that study, the luminal surface with tissue fragments seeded in the artificial heart showed a uniform smooth surface with complete endothelialization. As shown in this experiment and in the results for the artificial heart, our new method could be applicable not only to coating technology for the artificial heart, but also in many artificial organs which face the bloodstream for long periods.

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Endothelialization of Vascular Prostheses by Transplantation of Venous Tissue Fragments

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SHINICHI SATOH,§ AND CHARLES R. H. WILDEVUUR‖

A method to accelerate the endothelialization of vascular prostheses by seeding venous tissue fragments was developed. A piece of peripheral vein was obtained, chopped into small fragments, and stirred into 20 ml of saline, making a tissue suspension. This suspension was sieved through the wall of a highly porous vascular prosthesis (water porosity: 3,600–4,000). The prostheses, (7 mm ID and 5.7 cm in length) seeded with tissue fragments, were implanted into the thoracic descending aortae of 20 dogs, and were removed from 1 to 371 days after implantation. Ten prostheses, preclotted with fresh blood, were used as controls. In the seeded grafts, an infinite number of endothelial cells migrated and proliferated from the fragments. These had produced numerous capillaries by 5 days after implantation that had reached and opened onto the luminal surface of the prosthesis. From these openings, numerous endothelial cells spread out and formed colonies. With the increase in the size of the colonies, the inner surface was completely endothelialized within 5 weeks. This quick neointimal formation by seeding venous tissue fragments might be applicable to several artificial organs. *ASAIO Transactions* 1990; 36: M346–M348.

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Antithrombogenic vascular prostheses will not become covered with thrombus or neointima.[1] After implantation of thrombogenic vascular prostheses, such as fabric Dacron grafts, their inner surfaces will be covered with a thin thrombus layer. Following this, fibroblasts and smooth muscle cells migrate inside, and endothelial cells cover the inner surface, forming a permanent and natural antithrombogenic surface.[2,3] This neointima formation is observed in animal experiments, but in humans endothelialization is delayed with the surface remaining thrombogenic long after implantation.[3] To overcome this problem, we evaluated a method to accelerate endothelialization by transplantation of venous tissue fragments into a fabric vascular graft wall.

were soft and pliable. Consequently, fitting and suturing to the aortic wall was very easy, and almost no leakage of blood was noticed at the suture lines. After implantation, there was no sign of separation of the seeded tissue fragments from the graft wall.

Resected Specimens From Animals

Both the seeded and control grafts were patent at the time of harvesting. There were no differences in gross appearance of the outer surfaces; however, significant differences were seen on the inner surfaces of the grafts left in place for more than 5 days. Just after implantation, the inner surfaces of the grafts were covered with a fresh thrombus layer, and colored red. By the next day, the redness was reduced, and on the third day, the color of the inner surface turned white. Inner surfaces of the seeded grafts thereafter remained white and smooth, and subsequently became shiny. There were no degenerative changes in the long-term specimens. In contrast, the inner surfaces of the control grafts at 7 days had numerous micropolypoid, rough areas, which became thick and red colored. At 10 days, these areas increased their size and thickness, and after 20 days, they showed a red confluent granulation-like tissue covered with fresh thrombus. Only the anastomotic sites remained white. These white areas gradually spread out from the anastomotic line, with a 52 day specimen revealing a 7 by 2 mm white area from the proximal and distal anastomotic lines, respectively; at 122 days, they were about 2.5 and 2 cm, respectively, but the middle of the graft still showed granulation-like tissue with fresh thrombus.

Microscopic Examinations

By means of light microscopy, the graft wall showed many fragments from the venous tissue seeding. After 3 days, numerous fibroblasts migrated from the fragments, and after 5 days endothelial cells migrated and proliferated inside the graft wall, forming capillaries that became confluent. There were no erythrocytes in these capillaries. At 7 days, some reached the inner surface and opened to the lumen of the graft, producing colonies of endothelial cells on the surface. These capillaries contained erythrocytes. After 14 days, much of the inner surfaces were covered with a layer of endothelial cells, and at 35 days, this layer was complete. Many smooth muscle cells were noticed under the endothelial cells, producing a multilayer. Fibroblasts located under the smooth muscle cell layer surrounded the polyester fibers with collagen fibrils. Neointima formation was already completed in this 5 week specimen. Upon long-term observation, there were no degenerative changes, such as ulceration or calcification, on the neointimae. In contrast, in the control grafts, endothelial cells were noticed only at the anastomotic sites at 14 days, where endothelialization began. In the 54 day specimen, only areas near the anastomoses were endothelialized, and at 122 days, a fresh thrombus adhered to the center area of the graft, without any endothelialization.

Discussion

To obtain endothelialization of fabric vascular prostheses, endothelial cell seeding techniques have been investigated over the last decade.[5] Some of them produced nice results, but they remain unavailable for general use, as they require special cell culture techniques and facilities. They are also not available for emergency use, since the cell culture requires an extended period of time. To overcome this problem, we developed a new technique to accelerate endothelialization with an autologous tissue seeding method. This was simple and easy to do, and can be available in any operating room without special techniques, instruments, or facilities. In this experiment, our results showed excellent efficacy in seeding of the tissue fragments, and provision of complete endothelialization within a short period of time, with no negative side effects in the long-term specimens.

Transplantation of autologous tissue fragments has already been used in orthopedic and plastic surgery, as osteoblasts migrate very rapidly from the cut edges of transplanted bone fragments, and epidermal cells from skin fragments. Multiple tiny fragments have large areas of cut edges from which cells can migrate and proliferate very quickly under physiologic conditions.

In this experiment, three kinds of cells—fibroblasts, smooth muscle cells, and endothelial cells—migrated and proliferated at the same time from the fragments. This is a very unique phenomenon, that is not observed in cell culture; when fibroblasts and endothelial cells are cultured in petri dishes, endothelial cells are suppressed and fibroblasts proliferated to form a confluent layer. However, in this *in vivo* experiment, we noticed these cells migrated and proliferated together. Endothelial cells produced capillaries, and rose to the inner surface of the graft to face the blood stream. Accordingly, smooth muscle cells made a multilayer beneath the endothelial cells, and fibroblasts crawled down under the smooth muscle cell layer around the polyester fibers. This phenomenon suggests that growth of these cells was controlled by their physiologic environment.

By this *in vivo* cell culture technique, we could obtain rapid and reliable neointima formation on the fabric vascular prosthesis. This technique will be applicable not only in the field of vascular grafts, but also in many artificial organs of the hybrid type.

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A New Cardiac Wall Substitute with High Affinity for Fibroblasts that Can Induce an Endothelial Cell Lining

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A new cardiac wall substitute (PC graft) was developed using equine pericardium cross-linked with a polyepoxy compound. Compared with glutaraldehyde cross-linked pericardium (GA graft), the PC graft showed an approximately 10 times higher affinity for fibroblasts as measured by our *in vitro* cell migration and proliferation test. Six PC grafts (5 × 3 cm) were implanted into the right ventricular-pulmonary outflow tract position as a cardiac wall patch. Three GA grafts were used as controls. The PC grafts showed excellent handling during surgery because of their softness and elasticity. These grafts were explanted at 2 and 7–8 weeks after implantation. All PC grafts showed a white and glistening surface without any thrombus formation except in one case where thrombus deposition was observed in the center of the graft. In the GA grafts, thrombus adhered to the luminal surface. Light microscopic observation showed that the PC graft surface was covered with a connective tissue layer and significant fibroblast infiltration. Approximately 60% of the area infiltrated by these fibroblasts was endothelialized, whereas in the GA graft, endothelialization was limited to within 2–5 mm of the suture line. Other areas were covered with a thrombus layer without any endothelial cells or fibroblast infiltration. PC cross-linking can maintain the biologic and mechanical properties of the original materials. The PC graft offered excellent affinity for fibroblast migration and proliferation, which induced an endothelial cell lining on the surface. The results of this experiment indicated that the PC graft, which obtained the natural antithrombogenic property, was superior to a GA graft in terms of safety as well as mechanical, physiologic, and biologic properties as a cardiac wall substitute. *ASAIO Journal* 1994;40:M751–M756.

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Rapid endothelialization is required on cardiac wall substitutes, because thrombus deposition and its potential detachment from the graft can lead to dangerous consequences. A newly developed cardiac wall substitute could overcome these problems. An expanded polytetrafluoroethylene (EPTFE) patch,[1,2] fabric patch,[3,4] and biologic membrane[5–7] were used in clinical situations; however, they have parallel advantages and disadvantages. PTFE is nonadhesive for various substances and was expected to show nonthrombogenicity, but it has no active antithrombogenic property.[8] Thrombus can be produced on the EPTFE graft surface, but anchoring of the PTFE surface to the thrombus is poor. Therefore, the thrombus can easily detach from the surface, resulting in embolization in the peripheral arteries. Anchoring of a pannus at the anastomotic sites was also poor, and the detached pannus disturbs the blood stream, resulting in thrombus formation at the anastomotic sites. In the case of a fabric patch, neointima formation was observed more frequently, without pannus detachment; however, peeling of the neointima from the low porosity graft surface was another problem, whereas tightly woven grafts showed cut edge fraying and poor handling because of stiffness. Glutaraldehyde crosslinked biologic membranes were sometimes selected for cardiac wall substitutes because of their elasticity and excellent handling. Despite this, we were dissatisfied with the GA grafts because of their poor affinity for host cells,[9] producing rare neointima formation.[10] In this experiment, we report a new cardiac wall substitute that showed a high affinity for fibroblasts. After fibroblast migration, an endothelial cell lining is expected to form on the surface.

Materials and Methods

Preparation of the Graft

Fresh equine pericardium was harvested, crosslinked with a 4% polyepoxy compound (Denacol, Nagase Chemical. Osaka, Japan) solution at room temperature for 3 days, washed with distilled water, and treated with a glycine solution to minimize the residual free epoxy groups. After rinsing

with distilled water, the membrane (PC graft) was used for this series of experiments.

For the controls, glutaraldehyde crosslinked equine pericardium (GA graft, Xenomedica, Baxter Healthcare Corp, Irvine, CA) was used.

Mechanical Property of the Grafts

Tensile strength of both the PC and GA grafts was measured by the standard tearing test system formulated by Japanese Industrial Standards. The fixed size, 37 mm wide specimens of PC graft, GA graft, and original nontreated equine pericardium were put on a special instrument and were pulled at a cross head speed of 500 mm/min until the membrane was torn. The tearing was recorded at a chart speed of 500 mm/min. The mean value was calculated and compensation allowed for thickness of each membrane.

In Vitro *Cell Affinity Test*

Our own *in vitro* cell culture method was used to evaluate the cell affinity of the grafts. Bovine aortic endothelial cells (BAECs) were used, with confirmed presence of Factor XIII antigen by immunohistochemical staining. Bovine fibroblasts (Fbs) were also used. These cells were a gift from the Japanese Cancer Research Resources Bank, Tokyo, Japan. Six 1 × 1 cm PC and GA grafts was used for this experiment. They were rinsed with saline solution and then with Hank's buffered saline solution. After incubation in cell culture medium with 20% fetal bovine serum (Sigma Chemical, St. Louis, MO), 2.0×10^5 BAECs and FBs were suspended onto the membranes. On the 2nd and 7th days of incubation, cell proliferation on the membranes was observed by light microscopy. Morphologic observation of the seeded cells on these membranes was performed by scanning electron microscopy (JOEL 15-s, Tokyo, Japan) at an accelerating voltage of 20 kV. Specimens for scanning electron microscopy were fixed with 1% glutaraldehyde buffered physiologic saline, and treated with 1% osmium tetraoxide. They then were dried with a critical point drier and evaporated with gold paladium for observation.

Implantation of the Grafts

Nine mongrel dogs (8.5–17.5 kg) were used for graft implantation. Animals were anesthetized with 9–18 ml intravenous pentobarbital sodium (2.5% solution) and intubated. The animals were mechanically ventilated. The left pleural cavity was entered through an incision in the fifth intercostal space, the anterior part of the right ventricular outflow tract and proximal main pulmonary artery were longitudinally clamped, and a 4 to 5 cm incision was made. The PC and GA grafts, 3 × 5 cm in size, were sutured in place as patches. Antibiotics were instilled into the pleural cavity during surgery, but no anticoagulant was used.

Graft Removal

All the implanted grafts were removed at 2 and 7–8 weeks after implantation. Before harvesting, sodium heparin (100 IU/kg) was administrated intravenously to prevent clotting during graft retrieval. The animals were exsanguinated under general anesthesia. All specimens were rinsed with saline solution to remove excess blood on the graft surface and then were inspected macroscopically.

Histologic Examination

For observation by light microscopy, the removed grafts were cut longitudinally into four pieces that were approximately 3 mm in width, from the proximal anastomotic site at the right ventricular wall to the distal anastomotic site at the pulmonary artery. The specimens were fixed with 1% glutaraldehyde in phosphate buffer 0.2 mol/l, pH 7.4, then embedded in hydrophilic resin (Technovit 7,100, Kulzer & Co. GmbH, Friedrichshsdorf, Germany). Sections were stained with hematoxylin and eosin, by the peroxidase (PAP) method to stain for Factor VIII in the endothelial cells, and with von Kossa's stain to detect calcification of the neoi. tima.

Specimens for scanning electron microscopic observation were treated with the same procedure as that used for specimens obtained from tissue culture.

Results

Prepared PC Graft

The PC graft was white, soft, and pliable. The graft contained a certain amount of water and was very slightly transparent. The control GA graft, which was slightly yellow, was also pliable, but the PC graft was softer and more flexible **(Figure 1)**.

Results of mechanical property measurement showed that there was almost no differences between the tensile strength of the PC graft and that of the GA graft. Results from the PC graft, the GA graft, and nontreated original equine pericardium were approximately 1.02 kg, 0.8 kg, and 1.2 kg, respectively.

Cell Affinity Tests

Under *in vitro* cell culture on the second day, there was no fibroblast or endothelial cell proliferation on the GA graft. There was, however, good cell attachment and cell proliferation of both fibroblasts and endothelial cells on the PC graft **(Figure 2)**. Most of the surface of the PC graft was covered with fibroblasts during the 2 days. These cells were flat. Endothelial cells also adhered; however, fewer cells were on the surface. The shape of these cells was not completely flat. At the 7th day, endothelial cells were well anchored on the pC graft, but they did not yet show confluent growth, whereas fibroblasts did. WIth GA grafts, a few fibroblasts adhered to the graft surface at 2 days; this was not changed at the seventh day. There were almost no flat cells on the GA graft, and although a small number of endothelial cells were noticed, their shape was round, not flattened, and they showed

to overcoming the problems we face with cardiac wall substitutes in the clinical setting.

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Sealing of Highly Porous Fabric Vascular Prostheses by Adipose Connective Tissue Fragments Instead of Preclotting with Fresh Blood

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Abstract *Preclotting is an essential procedure for porous fabric vascular prostheses, but fatal bleeding due to fibrinolysis after implantation can occur in some cases. To overcome this problem, a method was developed to seal highly porous fabric vascular prostheses with adipose connective tissue fragments. A piece of subcutaneous adipose connective tissue weighing approximately 1 g was minced with scissors and stirred into 20 mL of saline, thereby creating a tissue suspension. This was sieved through the wall of a highly porous fabric prosthesis from the outside to the inside by intraluminal suction. The prostheses were implanted in the thoracic descending aortae of 20 dogs and removed from 1 h to 1 year after implantation. Twelve preclotted prostheses were used as controls. None of the treated grafts experienced bleeding complications postoperatively. In the controls, the chest wall was closed after the bleeding stopped from the suture line and through the prosthesis wall, but problems occurred after surgery. Three out of 12 control animals (25%) bled in the pleural cavity within 24 h. In the tissue-sealed grafts, smooth neointima formation without any degenerative changes was observed during a 1-year observation period.*

Keywords Vascular prosthesis, preclotting, porosity, adipose tissue fragments, autologous tissue sealing.

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prosthetic wall was suctioned again (Fig. 1). By repeating this sieving procedure several times, the pores of the fabric were completely sealed with the tissue fragments. The prosthesis was rinsed with a saline solution to remove free, untrapped fragments.

A Teflon rod was inserted in the prosthesis to preserve a smooth surface by further manipulation. The excess solution on the outside of the prosthesis was removed with a dry sponge. The prosthesis was poured with fresh blood to cement the tissue fragments within the prosthesis wall. This procedure was not for sealing by preclotting, but for the fixation of the tissue fragments by fibrin network to prevent entanglement of the suture during anastomosis. The Teflon rod was then withdrawn. The prepared prosthesis was implanted as a vascular graft in the same dog from which the connective tissue was resected. The control prostheses (Milliknit) were only preclotted with fresh blood. The procedure of the preclotting was done throughout the four stages of the Sauvage preclotting technique without heparin in step 4.

Implantation of the Prostheses

Mongrel dogs of both sexes, weighing 8–12 kg, were used to implant the vascular prostheses (ID: 7 mm; length: 5.7 cm). Twenty tissue-sealed vascular prostheses were implanted in the descending aortae of dogs along the following series. Animals were anesthetized with an initial intravenous dose of 8–12 mL pentobarbital (2.5% solution). Supplemental doses were given when required. The dogs were intubated and put on a respirator at 18 respirations per minute and with a tidal volume of 250 cm^3 room air.

The chest was entered via a left thoracotomy at the sixth intercostal space. The descending aorta was exposed and mobilized by sacrifying five pairs of intercostal arteries. The aorta was then clamped both proximally and distally, and a 5-cm segment of the aorta was resected and replaced by the prosthesis. A continuous suturing technique with 5-O Tevdeck was used. After suturing was completed, the two vascular clamps occluding the aorta were removed. Two chest tubes were placed and the thoracic cavity was closed in a normal manner. When the animal was able to breathe spontaneously, mechanical ventilation was discontinued and the dogs were extubated. Twelve preclotted fabric prostheses were also implanted in the descending aortae of dogs in a control group.

During surgery, antibiotics were administered (1 g penicillin) into the pleural cavity, and no anticoagulants were used at any time. All animal care was in compliance with the *Principles of Laboratory Animal Care* formulated by the National Academy of Sciences and published by National Institutes of Health (NIH publication No. 80-23, revised 1978).

Explantation of the Prostheses

The specimens from the animals were removed from 1 h to 351 days in the tissue-sealed prostheses, and from 1 to 340 days in the controls. Before harvesting, sodium heparin (100 IU/kg) was intravenously administrated to prevent clotting. All the specimens were rinsed with saline solution to remove excess intraluminal blood, inspected macroscopically, and prepared for histological examinations. After removal of the prostheses, the kidneys of each animals were resected and examined for fresh, trapped microemboli.

Histological Examinations

For light microscopical observation, 4-mm-wide sections of the prostheses were cut along the longitudinal direction from the proximal to distal anastomoses. Each sample was cut into seven pieces. The specimens were fixed with 1% glutaraldehyde in 0.2 mol/L phos-

Discussion

Preclotting is one of the most important procedures for implantation of porous fabric vascular prostheses.[4–6] A porous fabric vascular prostheses cannot be used without preclotting. Wesolowski showed that highly porous prostheses could obtain excellent endothelial cell lining.[1] The role of the pores of vascular prostheses has already been recognized. Highly porous prostheses have the serious disadvantage of delayed hemorrhage.[2,3] But some recent vascular surgeries required many anticoagulants to be administered during and after the operations. Under the influence of high amounts of anticoagulants, highly porous fabric vascular prostheses will result in dangerous uncontrolled bleeding after surgery. Even if bleeding stops during surgery, fibrinolysis after surgery might bring on bleeding. The fibrin layer, which sealed the pores of the fabric prosthesis, has always had the possibility of being dissolved by fibrinolysis. Therefore, we could not use a large dosage of anticoagulants, such as urokynase, even if the implanted prosthesis was occluding. In the treated grafts in this experiment, all the interstices of the fabric were occupied with tissue fragments, which were not dissolved by the fibrinolysis. Moreover, these tissue fragments contained an abundance of collagen fibrils, which were thrombogenic to accumulate platelet aggregation.

The thick thrombus at the 1st day indicated high thrombogenicity. But, no microembolisms infarctions occurred in the kidneys resected from these animals. The reason was as follows: All tissue fragments were outside the graft and interstices of the fabrics due to the intraluminal suction. Therefore, the thrombus on the luminal surface and the tissue fragments in the graft wall were not washed off by the blood stream at the luminal surface. The fact that no embolization or infarct occurred in the kidneys showed the safety of this method. The safety and efficacy of this procedure can be also determined by the fact that there was no bleeding during and after implantation in the short term and no degenerative changes in the long term. The original tissue fragments had been absorbed with time. Since these tissue fragments were autologous tissue, there were no problems of antigenicity or foreign body reaction with these fragments.

Recently, some highly porous fabric vascular prosthesis coated with biodegradable materials, such as gelatin, collagen or albumin, have been used.[7–9] The sealing materials were treated by heating them or with chemical crosslinking. Adequate results were reported in some studies, but not in others. Our tissue fragments are autologous, which have the highest affinity with the host. Therefore, our new method would have the most natural healing process after implantation compared with that of any other coated fabric vascular prostheses. From these tissue fragments, migration of fibroblasts and endothelial cells from capillaries is expected into the inner surface of the neointima. In this experiment, the tissue-sealed graft showed quick neointima formation compared with that of the control. This was due to the autologous tissue transplantation in the fabric vascular prosthesis. Autologous tissue fragment impregnation into cardiovascular prostheses was initiated by Ghidoni et al. in 1968.[10,11] They forced tissue fragments by a tightly fitting piston onto the luminal surface of impermeable vascular prostheses and diaphragms of the left ventricular bypass pump to produce a cellular blood–prosthesis interface.

We used tissue fragments for sealing a highly porous prosthesis to overcome the bleeding problem during and after implantation, and could obtain the merits of both autologous tissue fragment transplantation and a highly porous fabric prosthesis described by Wesolowski. We are now working to clarify the healing process of the neointima with cell migration and proliferation from the tissue fragments in the prosthetic wall.

Acknowledgment

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Rapid Neointima Formation with Elastic Laminae Similar to the Natural Arterial Wall on an Adipose Tissue Fragmented Vascular Prosthesis

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A vascular prosthesis that can induce a neointima similar to a natural arterial wall is reported. The authors have developed a sealing method using autologous tissue fragments. The sealed graft showed many advantages, with characteristic neointima formation in an animal study. The grafts were implanted in the thoracic descending aortae of 40 dogs and were removed from 1 hour to 608 days after implantation. Another 40 dogs, used as controls, had a fabric graft implanted using the preclotting method. The luminal surface of the sealed graft was completely endothelialized and the original adipose tissue fragments were absorbed within 1 month. Smooth muscle cells infiltrated and proliferated at the same time as endothelialization took place. Most of the smooth muscle cells were arranged in parallel rows and oriented circumferentially within the graft. At 1 month, elastic fibers appeared around the smooth muscle cells near the anastomotic sites. In the long-term specimens, these elastic fibers constituted a fine lamina in the neointima. Intimal hyperplasia and degenerative changes in the neointima were not observed. These results indicated that the sealing method could induce a very stable neointima with a smooth muscle cell layer and elastic laminae similar to a natural arterial wall within a short period of time throughout the graft wall, with maintenance of the neointima for a long period of time after implantation. *ASAIO Journal* 1994;40:M267–M272.

Several kinds of sealed vascular prostheses have become commercially available[1–3]; however, some adverse effects have been reported with these devices.[4,5] We believe that the most reliable sealant is autologous tissues. Until now, however, there has been no convenient method to seal fabric prostheses with autologous tissues. To overcome this problem, we developed a sealing method using autologous adipose tissue fragments.[6,7] The graft showed characteristic neointima formation in an animal study. In this communication, the healing process of the neointima is investigated, focusing on endothelialization, the pattern of smooth muscle cell arrangement, and elastic fiber formation.

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Materials and Methods

Preparation of the Graft

Canine subcutaneous adipose connective tissue (2 g weight) was resected and chopped with scissors into tiny tissue fragments, then stirred into 20 ml of saline containing 10,000 IU penicillin. To obtain a smooth, tissue fragmented luminal surface, a highly porous Dacron fabric vascular prosthesis (Microknit, 7 mm ID, Golaski Laboratories Inc., Philadelphia, PA; water porosity: 4,000 ml/cm^2/min, at 120 mmHg) was invaginated, and the resulting adipose tissue fragment suspension was injected several times under pressure into the graft, until fragments were trapped in the prosthesis wall. The sealed graft was reinvaginated to yield a completed adipose tissue fragmented (ATF) graft with a smooth luminal surface and rough outer surface. The excess saline solution on the prosthesis was removed with a dry sponge. The prosthesis was covered with fresh blood to cement the tissue fragments within the prosthesis wall. This procedure was not for sealing by preclotting, but for fixation of the tissue fragments by a fibrin network. The graft was implanted into the same animal from which the adipose tissue was resected. The control graft was a fabric vascular prosthesis (Microknit, 7 mm ID) preclotted with fresh blood using the four stage Sauvage preclotting technique.

Implantation of the Grafts

Eighty mongrel dogs (8–12 kg) were used for thoracic aorta implantation (40 ATF grafts; 40 controls). Animals were anesthetized with 8–12 ml of intravenous pentobarbital

(2.5% solution) and intubated. The descending aorta was dissected and mobilized, sacrificing five pairs of intercostal arteries as described by Wesolowski.[8] After clamping the aorta proximally and distally, a 5 cm segment was resected and replaced with the designated prosthesis using a 5-0 Tevdek (Deknatel, NY) continuous suture. Antibiotics were administered (300,000 IU penicillin) into the pleural cavity, but no anticoagulants were given during or after surgery.

All animal care was in compliance with the "Principals of Laboratory Animal Care," formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Science and published by the National Institutes of Health (NIH publication No. 80-23, revised 1985).

Graft Removal

All grafts were explanted on a predetermined postoperative day. Before harvesting, sodium heparin (100 IU/kg) was administered to prevent clotting, and the animals were exanguinated under general anesthesia. All specimens were rinsed with saline solution to remove excess intraluminal blood and then inspected macroscopically. The kidneys also were examined for macroscopic thromboemboli.

Histologic Examinations

For light microscopic observation, the explanted grafts were cut longitudinally from the proximal to distal anastomosis into five 3 mm wide segments. The specimens were fixed with 1% glutaraldehyde in 0.2 mol/l phosphate buffer, pH 7.4, then embedded in hydrophilic resin (Technovite 7,100; Kulzer & Co. GmbH, Wehrheim, Germany). Sections were stained with 1) hematoxylin and eosin, 2) the peroxidase anti-peroxidase method for staining factor VIII in endothelial cells, 3) von Kossa's stain to detect calcification of the neointima, and 4) van Gieson's stain for the staining of elastic fibers.

Results

Preparation of the Graft

The sealing procedure was performed without difficulty, taking approximately 15 minutes at the operating table. After pressurized injections of the tissue suspension, the outside of the graft became very smooth and numerous tiny tissue fragments emerged from the interstices of the fabric. With reinvagination, the smooth surface was returned to the inside of the graft, whereas the outer surface was rough with multiple tissue fragments.

Implantation of Grafts

The ATF graft was soft and pliable and was easily handled during implantation. Fitting of the grafts to the aortae was excellent. Immediately after the aortic clamps were released the graft was inflated, but there was no blood leakage through the graft wall. There was minimal leakage at the suture lines, which stopped spontaneously within 1 minute of declamping. Two of 40 ATF grafted animals died from graft infection at 8 and 11 days. Grafts were explanted at from 1 hour to 608 days.

In the control group, no bleeding occurred through the graft wall or at the suture lines before chest closure; however, eight of these animals bled to death within 24 hours of surgery. The explanted grafts from those dogs were white, and the preclotted fibrin network had disappeared from the graft walls. Two dogs died from pneumonia at 30 and 33 days, but they had no sign of graft infection. The grafts were explanted at from 1 hour to 738 days.

Removal of the Grafts

All grafts were patent at the time of retrieval. The wall of the ATF graft was thin, soft, and pliable. The outer surface of the grafts at from 1 to 24 hours were without hematoma formation, and the grafts removed after more than 3 weeks were surrounded with a loose connective tissue that was free of any inflammatory reaction.

The luminal surface of the AFT graft at 1 hour was completely covered with thick red thrombus **(Figure 1A)**. No blood leakage through the graft wall was observed. At 1 day, the inner surface was predominantly covered with pink thrombus. There were numerous white tissue fragments under the thin thrombus layer, and red thrombus was sporadically present among the interstices of the tissue fragments. At 3 weeks the surface was glistening white, but small (< 2 mm) thrombi were still present sporadically on the luminal surface. The grafts obtained at 33, 36, and 37 days showed completely white luminal surfaces with no microthrombi among the tissue fragments **(Figure 1B)**. There was no difference in the whiteness of the surface between the center area and the areas near anastomotic sites. In the long-term grafts, the neointima was less than 0.5 mm thick, and the fabric of the prosthesis was transparent through the neointima. There was no scar formation or wall irregularity in the graft or around the graft wall **(Figure 1C)**.

In the control group, local hematoma formation was present around the grafts in three of six explanted at the early stage. At 1 hour, an initial thick red thrombus covered the luminal surface. At 7 to 10 days, the luminal surfaces of the grafts were red with fresh thrombus. At 21 days, there were white areas, free of red thrombus, near the anastomotic sites; however, in the center of the graft, fresh red thrombus adhered to the surface **(Figure 1D)**. At 25 days, the white areas were 5 to 15 mm from the anastomotic lines. Other areas in the midregion of the graft were red with thick thrombus adhesion. At 90 days, the luminal surface had thrombus deposition 10 mm wide in the center of the graft. At 217 days the graft was completely white, with a thin neointima. The grafts removed at 341, 412, 734, and 738 days demonstrated a white neointima without any stiffness of the graft wall.

No evidence of thromboemboli was present in the kidneys of either the ATF or control group.

Microscopic Examination

ATF Graft. Before implantation, each interstice of the ATF graft Dacron fibers was occupied by tissue fragments con-

riod of time in the sealed grafts. Elastic fibers were seen first around the smooth muscle cells, and they became multilayered with time. Complete elastic laminae formation would have been suggestive of creation of an arterial wall, but was never observed. In general, elastic fibers are synthesized by smooth muscle cells.[12,13] Very small fillaments of elastic fibers have already been recognized in neointimae of synthetic fabric vascular prostheses.[14-16] On long-term observation of the neointimae, regularly arranged elastic fibers around smooth muscle cells were demonstrated in fabric prosthesis.[17] In our experiment, however, elastic fibers were noticed at an early stage of neointimal formation, and multilayers of thick elastic fibers, similar to those of natural arterial wall, were seen in the long-term specimens.

In the long-term grafts, we observed no degenerative changes in the neointima. Maintenance of the neointima in a natural condition also will be helped by the physiologically arranged smooth muscle cells and elastic fibers.

With our new sealing method, we could provide a safely sealed graft that showed rapid endothelialization and stable neointima formation within a short period of time. The graft was instantaneously sealed with autologous tissue fragments, and looked like an autologous tissue tube reinforced by a Dacron fabric. The graft enjoyed the many advantages of autologous tissue.

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Synthesis and Function Control of Biofunctionality Materials
T. Tsuruta, M. Doyama, M. Seno, Y. Imanishi (editors)

Healing process of vascular prosthesis and a method to accelerate the neointima formation by transplantation of autologous tissue fragments

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In fabric vascular prostheses, endothelialization begins from the anastomotic sites, however, it is extremely delayed in the areas distant from the anastomotic sites. We regarded this delayed healing as a protracted ulcer of)lood vessel wall, and this protracted healing can be treated by transplantation of autologous tissue fragments as being treated by transplantation of autologous tissue fragments for skin ulcers or bone fractures. An animal study for a large caliber graft implanted in the descending aorta showed rapid endothelialization equally all over the luminal surface, but the graft was thrombogenic. In order to reduce the thrombogenicity for the application of the graft to a small diameter artery, heparin was used with the tissue fragments, since heparin can bind electrostatically with collagen flbrils. Such a graft in carotid arterial interposition in the canine model showed long-term patency without any degenerative changes of the graft wall.

1. INTRODUCTION

Vascular prostheses have been used safely in clinic, although with advantages and disadvantages. The two of the major disadvantages are the lack of antithrombogenicity and the poor healing characteristics of the neointima[1,2,3]. Therefore, these prostheses cannot be used as venous and small diameter arterial grafts. They are used only in arterial system of more than 6 mm in internal diameter, in which high pressure and high mean blood flow velocity reduce the possibility of large thrombus formation on the graft's surfaces. However, the luminal surfaces were not endothelialized for long period of time after implantation even if they maintain their patency[4]. Therefore, small diameter vascular grafts, require some antithrombogenic properties or some ability to induce a quick endothelialization[5,6]. For this purpose, we developed a new technology, "transplantation of autologous tissue fragments in the fabric vascular prostheses wall", which can accelerate the neointima formation and prevent the early thrombus deposition on the graft surfaces[7,8,9]. In this communication, usual healing process of the neointima on ordinary fabric prostheses and the new technology will be explained in detail.

2. HEALING PROCESS OF NEOINTIMA IN FABRIC VASCULAR PROSTHESES

Fabric vascular prostheses made of Dacron fibers have a porous structure by knitting or weaving of the fabrics. Therefore, these are sealed with fresh clot by a preclotting procedure before implantation [10,11,12]. Therefore, the neointima formation always begins from the fresh thrombus layer.

2.1. Early stage of the neointima formation

Just after implantation, the luminal surface was red in color with fresh thrombus deposition. Figure 1a showed the luminal surface of a fabric prosthesis implanted in the thoracic descending aorta and removed 1 hour after implantation. The graft wall was composed of Dacron fibers, fibrin network, numerous erythrocytes, platelets, and small number leucocytes. After 2 days of implantation, the luminal surface appears as white in color. Light microscopical observation showed that the luminal surface was covered with a thin layer of fibrin. No erythrocyte was noticed in the fibrin layer . At 5 to 7 days, red thrombus deposition was noticed again on the surface. Many erythrocytes reappeared in the fibrin layer. The redness on the graft surface increased at 10 to 20 days.

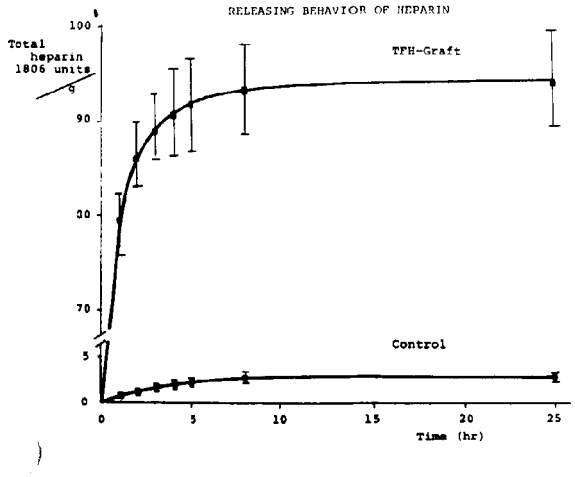

Figure 4. Heparin release from the TFH-grafts and the control non-heparin treated grafts into the rinsing saline. Reprint from Y. Noishiki et al, Trans. Am. Soc. Artif. Intern. Organs, 38,P. 160, 1992.

below the endothelial cells, and fibroblasts around the Dacron fibers At three weeks, the luminal surface was shiny with the same color as a native aortic wall, and it remained this way up to 495 days postimplantation. Light microscopical observation showed that the neointima healing was completed between two and four weeks. Following complete healing, no intimal hyperplasia at the anastomotic sites nor degenerative changes such as calcification in the neointima were observed in the explanted grafts up to 495 days(Figure 3d).

4.4. Special findings of the healing process of the TF-graft

We found that all of the grafts treated with autologous tissue fragments were endothelialized within a very short period of time. We saw no difference in healing between the anastomotic sites and other regions distant from the anastomoses, including the center of the graft. This was in contrast to untreated grafts, in which endothelialization occurred only at the anastomoses and was delayed. Endothelial cell division is limited to 70 cycles, and we believe that endothelialization limited to only the anastomotic sites of untreated grafts may be the result of cell aging.

5. APPLICATION OF THE NEW TECHNOLOGY TO A SMALL DIAMETER GRAFT

We showed rapid endothelialization in the TF-graft, however, significant thrombogenicity of the collagen fibrils on the grafts was the major problem when the technique was applied to a small diameter graft. Because collagen fibrils have a positively charge that aggregate negatively charged platelets[18,19]. Three dimensional structure of collagen molecule has also special affinity with platelets. We therefore propose that the graft's thrombogenicity would be decreased if we could mask the collagen molecule, especially positively charged areas of the collagen fibers[20]. Based on this hypothesis, we masked the fibrils electrostatically with heparin, which has a strong negatively charge.

5.1. Preparation of the tissue fragmented and heaporinized graft (TFH-graft)

The tissue suspension used for the small-caliber grafts was produced by adding minced canine jugular vein with connective tissue to 20 ml of normal saline containing 1,000 IU heparin. To create the tissue fragmented heparinized graft (TFH-graft), a fabric prosthesis (MICROKNIT, 4 mm ID) was first invaginated to turn inside out. After rein-vagination, the graft was implanted into both carotid arteries of the same animal from which the jugular vein was taken.

5.2. The patency and the healing process of the TFH-graft

Twenty out of 32 TFH-grafts were patent at the time of the graft removal. Twelve grafts were occluded and eight of them were due to the graft infection, but 4 grafts which occluded between 2 and 3 weeks after implantation without infection. The luminal surface of the TFH-graft at one hour was red in color without thrombus formation(Figure 5a). At two weeks, the redness markedly decreased(Figure 5b). Light microscopical observation showed the migration of numerous fibroblasts from the tissue fragments into the fibrin layer by two weeks. At four weeks, the luminal surface was white and glistening with no red thrombus. Light microscopy

including feeder cells in the suspension. For example, a single cancer cell separated from its main tumor cannot survive to create a metastasis unless it is associated with a group of cells[25,26,27,28]. In addition, endothelial cell proliferation is greatly improved with an underlining base of feeder cells. In the skin, fibroblasts act as feeder cells to epidermis cells[29]. In a similar experiment, neointima, smooth muscle cells and fibroblasts enhance endothelial cell growth[30,17]. In our study, we observed a very unique phenomena: Three different kinds of cells, smooth muscle cells, fibroblasts and endothelial cells simultaneously migrated and proliferated from the tissue fragments. It is impossible to observe such a phenomenon in vitro, since "strong Cells" such as fibroblasts grow and make a confluent layer suppressing "weaker " cells such as endothelial cells. However, in vivo, endothelial cells migrated to face the blood stream, and smooth muscle cells create multilayers beneath at the same time. Fibroblasts, on the other hand, migrated to the Dacron fibers to encapsulate the foreign body. We were able to develop a neointima with the cooperation of endothelial cells, smooth muscle cell, and fibroblasts in vivo.

7. MAINTENANCE OF THE GRAFT PATENCY

The advantages of these grafts were considered as follows: As a raw material, autologous tissue is ideal for implantable artificial organs. Both these grafts were made of autologous tissue reinforced with Dacron fibers, since the fabric used as the framework of the graft was very porous. Therefore, the neointima formed in both grafts were created by the host, and will maintain the neointima in good condition without any degenerative changes as observed in the long-term animal experiments.

8. CONCLUSION

In order to overcome the problems associated with implantation of fabric vascular prostheses, the new technology was introduced. With this method, we could create a new vascular wall on the fabric prosthesis. What we set importance was the method to induce the dynamic function of host cells under the physiological and natural environment, i.e., in vivo tissue culture technique. By this method, we could obtain special activities of various cells which cannot be observed in any in vitro experiments. This in vivo cell culture technology would be useful for the development of new artificial organs. The matrices used in the artificial organs could induce the natural activity of these cells into our desirable purpose for the development of our new artificial organs. The data shown in this communication is one of the samples for designs of new artificial organs .

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NEOINTIMA FORMATION OF FABRIC VASCULAR PROSTHESIS AND A METHOD TO ACCERELATE THE HEALING PROCESS BY TRANSPLANTATION OF VENOUS TISSUE FRAGMENTS

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ABSTRACT

Standard healing process of neointimae of fabric vascular prostheses implanted in animals was demonstrated, and a method to accerelate the endothelialization of the neointima using venous tissue fragments transplantation into the prosthesis wall was introduced. A piece of peripheral vein was obtained, chopped into small fragments, and stirred into 20 ml of saline, making a tissue suspension. This suspension was sieved through a highly porous prosthesis. The prostheses seeded with tissue fragments were implanted into the descending aortae of dogs and were removed from 1 to 371 days after implantation. Preclotted fabric prostheses were used as the control. In the seeded grafts, an infinit number of endothelial cells migrated and proliferated from the fragments. These had produced numerous capillaries by 5 days after implantation that had reached and opened onto the luminal surface of the prosthesis. From these openings, numerous endothelial cells spread out and formed colonies. With the increase in the size of the colonies, the inner surface was completely endothelialized within 5 weeks.

Keywords: Endothelial cells, fabric vascular prosthesis, neointima, tissue fragments transplantation, haemocompatibility.

INTRODUCTION

Fabric vascular prostheses can obtain a permanent haemocompatibility by endothelialization of the neointimae on their luminal surfaces. Therefore, neointima formation is essential for the graft stable patency.[1] However, it required a certain period of time to get an adequate neointimal formation with endothelial cells lining. In human, endothelialization is extremely delayed.[2] Most of the grafts implanted in human were not endothelialized and the luminal surfaces of them were thrombogenic long after implantation. To overcome this problem, endothelial cell seeding methods were developed and applied in clinic in the last decade,[3] but no satisfactry results were reported except some limited animal experiments. Recently, we developed a new

prosthesis wall. Then the Teflon rod was withdrawn. The prepared prosthesis was implanted as a vascular graft in the same dog from which the venous tissue was resected.

The control prostheses (MILLIKNIT) were only preclotted with fresh blood. The procedure of the preclotting was done throughout the four stages of the Sauvage preclotting technique without heparin in the No. 4 step.

2. Implantation of the prostheses

Forty adult mongrel dogs, weighing 8−12 kg were used in these experiments. The prepared grafts, 7 mm in internal diameter and 5.7 cm in length, were implanted into the descending aortae of 25 dogs along the following series. Animals were anesthetized with an initialintravenous dose of 8 to 12 ml pentobarbital (25 mg/kg, 2.5 % solution). Supplemental doses were given when required. The dogs were intubated and put on a respirator at 18 respirations per minute and a tidal volume of 250 cc room air. The chest was entered via a left thoracotomy at the sixth intercostal space. The descending aorta was exposed and mobilized by sacrifying five pairs of intercostal arteries. The aorta was clamped both proximally and distally, and a 5 cm segment of the aorta was resected and replaced by the prosthesis. Continuous suturing technique with 5−0 Tevdeck was used. After suturing was completed, the two vascular clamps occluding the aorta were removed. Two chest tubes were placed and then the thoracic cavity was closed in a normal manner. When the animal was able to breathe spontaneously, mechanical ventilation was discontinued, and the dog was extubated. Fifteen preclotted fabric prostheses were also implanted in the descending aortae of dogs as the controls. During the surgery, antibiotics were administrated (1 g penicillin) into the pleural cavity, and no anticoagulants were used at any time. All animal care was in compliance with the "Principles of Laboratory Animal Care" formulated by the National Academy of Science and published by National Institutes of Health (NIH publication No. 80−23 reviced 1978).

3. Graft harvesting.

The implanted grafts were harvested from 1 to 371 days after implantation. Before harvesting, sodium heparin (100 IU/kg) was intravenously administrated to prevent clotting. All the specimens were rinsed with saline solution to remove excess intraluminal blood, and inspected macroscopically and were served for the following histological examinations. After remove the prostheses, the kidneys of each animals were resected and examined for fresh, trapped microemboli.

4. Histological examinations.

For the light microscopical observation, 4 mm wide sections of the prostheses were cut along the longitudinal direction from the proximal to distal anastomoses. Each sample was cut into seven pieces. This series of specimens were treated in the following manner. The specimens were fixed with 1 % glutaraldehyde in 0.2 mol/L phosphate buffer, pH 7.4. They were embedded in hydrophilic resin (JB − 4, Polyscience Inc., Warrington, PA, U.S.A.). Sections were stained with hematoxylin and eosin, and Van Kossa stain.

experiment, no endothelialization was noticed whole over the luminal surface. In human, especially in old aged or poor risked patients, no endothelialization is noticed on the fabric prosthesis.[2]

To obtain endothelialization of fabric vascular prostheses, endothelial cell seeding techniques have been investigated over the last decade.[3] Some of them have produced nice results, but they remain unavailable for general use, as they require special cell culture techniques and facilities. They are also not available for emergency use, since the cell culture requires an extended period of time. To overcome this problem, we developed a new technique to accelerate endothelialization with an autologous tissue seeding method. This was simple and easy to do, and can be available in any operation room without special techiques, instruments or facilities. In this experiment, our results showed excellent efficacy in seeding of the tissue fragments, and provision of complete endothelialization within a short period of time, with no negative side effects in the long-term specimens.

Transplantation of autologous tissue fragments has already been used in orthopedic and plastic surgery, as osteoblasts migrate very rapidly from the cut edges of transplanted bone fragments, and epidermal cells from skin fragments. Multiple tiny fragmetns have large areas of cut edges from which cells can migrate and proliferate very quickly under physiological conditions.

In this experiemnt, three kinds of cells-fibroblasts, smooth muscle cells, and endothelial cells-migrated and proliferated at the same time from the fragments. This is a very unique phenomenon, that is not observed in cell culture in vitro; when fibroblasts and endothelial cells are cultured in a petridish, endothelial cells are suppressed and fibroblasts proliferated to form a confluent layer. However, in this in vivo experiment, we noticed these cells migrated and proliferated together. Endothelial cells produced capillaries, and rose to the inner surface of the graft to face the blood stream. Accordingly, smooth muscle cells made a multilayer beneath the endothelial cells, and fibroblasts crawled down under the smooth muscle cell layer around the polyester fibers. This phenomenon suggests that growth of these cells was controlled by their physiological environment.

By this in vivo cell culture technique, we could obtain rapid and reliable neointima formation on the fabric vascular prosthesis. This technique will be applicable not only in the field of vascular grafts, but also in many artificial organs of the hybrid type.

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HEALING PROCESS OF A VASCULAR PROSTHESIS TRANSPLANTED WITH VENOUS TISSUE FRAGMENTS

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INTRODUCTION

In general, endothelialization of fabric vascular prosthesis in human is extremely delayed (1). Most of the grafts implanted were not endothelialized, and the inner surface remained thrombogenic for a long time after implantation. To obtain the endothelialization, endothelial cell seeding on grafts has been initiated(2,3). Some of them produced favorable results,(4) but they remain unavailable for general use. To overcome this problem, we developed a new method to accelerate the endothelialization of the prosthesis by transplantation of autologous venous tissue fragments into the wall.

MATERIALS AND METHODS

A highly porous fabric Dacron vascular prosthesis (MICROKNIT, Golaski Lab, Inc., Water porosity:4,000) was used. A canine left juglar vein was resected, minced with scissors and then stirred into 20 ml of saline, to create a tissue suspension. This suspension was sieved through the wall of the prosthesis by repeated injection into the closed prosthesis for several times. Then the prosthesis was washed from the luminal side by saline solution to remove remnants of tissue fragments and was implanted as a vascular graft to the same dog from which the vein was resected.

The prepared grafts, 7 mm ID and 5.7 cm in length, were implanted into the descending aortae of 20 dogs. The control prostheses which were preclotted with fresh blood were implanted into 15 dogs. The specimens were explanted from 1 hour to 63 days after implantation.

RESULTS

Macroscopically, the inner surface was covered with fresh thrombus 1 hour after implantation, but after 2 days the thrombus decreased. Small island-like areas without thrombus were noticed sporadically on the surface. These areas increased their number and size very rapidly. After 10 days, fresh thrombus disappeared. All the inner surface was occupied by the white areas. Light microscopical observation revealed that these areas contained a lot of smooth muscle cells, fibroblasts and numerous capillary blood vessels. Most of them were covered with endothelial cells. Within 2 weeks, the inner surfaces were completely endothelialized. Beneath the

endothelial cells, multi-layers of smooth muscle cells were noticed. By the active migration and proliferation of these cells from the tissue fragments, neointima was completely healed within 2 weeks. The original tissue fragments were noticed at the 21st day, but it became thin in the prosthesis at 28 days and almost disappeared at 31 days. At 61 days, they were not observed. There was no degenerative changes such as necrosis of smooth muscle cells, ulceration nor calcification.

In the control grafts, endothelialization was limited only near the anastomotic sites. The center area was not endothelialized and covered with fresh thrombus during the 63 days of observation period.

DISCUSSION

Seeding tissue fragments of veins within a scaffold of highly porous vascular grafts a rapid arterialization was induced. Within 14 days, complete endothelialization of the whole vascular surface occured, while endothelialization was still incomplete in the control grafts, even after 2 months. In these control grafts, endothelial and smooth muscle cells migrated from the adjacent aortic tissue into the graft, which is a slow and even limited process. In contrast with seeding vein fragments over the vascular graft migration and proliferation of endothelial and smooth muscle cells as well as fibroblasts started to growth from cut edges of the multiple vein fragments and covered rapidly the whole graft surface.

In this experiment, three kinds of cells - fibroblasts, smooth miscle cells, and endothelial cells - migrated and proliferated at the same time from the fragments. This is a very unique phenomenon, that is not observed in cell culture; when fibroblasts and endothelial cells are cultured in a petridish, endothelial cells are suppressed and fibroblasts proliferated to form confluent layer. However, in this experiment, we noticed these cells migrated and proliferated in conjunction. Endothelial cells rose to the inner surface of the graft to face the blood stream. Accordingly, smooth muscle cells made a multilayer underneath the endothelial cells, and fibroblasts crawled doun the smooth muscle cell layer around the Dacron fibers. This phenomenon suggested that growth of these cells was controlled by their interactions in a physiological environment.

This autologous tissue fragments transplantation method will be applicable not only in the field of vascular grafts, but also in many hybrid artificial organs.

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A Functional Neointima with Regularly Arranged Smooth Muscle Cells in a Fabric Vascular Prosthesis Transplanted with Autologous Venous Tissue Fragments

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Regular arrangement of smooth muscle cells underneath an endothelial cell layer was observed in the neointima of a fabric vascular prosthesis treated with new technology to accelerate endothelialization, *i.e.*, transplantation of autologous venous tissue fragments in the graft wall. This finding indicated that the neointima has a vital function as the intima of the blood vessel. A canine left jugular vein was minced and stirred into 20 ml of saline containing 1,000 IU heparin. It was injected with pressure into a fabric prosthesis (4 mm inner diameter [ID], 3.5 cm in length, Water porosity: 4,000 ml) to create the tissue fragmented, heparinized graft. The graft was implanted into the same animal from which the jugular vein was taken. Forty tissue fragmented heparinized (TFH) grafts were implanted in both carotid arteries of 20 dogs and explanted from 1 hr to 400 days after implantation. In this study, the neointimae of the grafts implanted for more than 1 month are analyzed, with a focus on the arrangement of smooth muscle cells in the neointima. A circumferential arrangement of smooth muscle cells with a thin layer of longitudinally arranged cells underneath was seen in the neointimae, which resemble the arrangement of smooth muscle cells in the natural arterial wall. Some areas had a thin smooth muscle cell layer in the longitudinal direction just under the endothelial cell layer. At anastomotic sites, they ran in parallel rows in the longitudinal direction. The authors previously clarified that the smooth muscle cells arrange in parallel rows in the direction of strain caused by tensile stress. Thus, these smooth muscle cells were subjected to the circumferential tensile stress caused by factors such as blood pressure, longitudinal shear stress of the bloodstream, and tensile stress on the fabric wall at the anastomotic sites. These results indicate that the graft has a vital, functional neointima that will contribute to the maintenance of graft patency as a physiologic arterial wall. *ASAIO Journal* 1993; 39: M746–M749.

Neointima formation is delayed on fabric vascular prostheses.[1] To overcome this problem, we have developed a method to accelerate the neointima formation by transplantation of autologous tissue fragments onto the fabric graft wall.[2-4] With this method, we observed complete neointima formation with a single layer of endothelial cells and multilayers of smooth muscle cells within 1 month after implantation. In these neointimae of the grafts explanted from experimental animals, we found a unique arrangement of smooth muscle cells. In this study, special attention was focused on the arrangement in the neointimae of grafts explanted from animals after more than 1 month of implantation. The pattern of arrangement is discussed as a vital sign of the neointima of a new arterial wall.

Materials and Methods

Preparation of the Graft

Preparation of the graft was performed by the method described elsewhere[4]; the procedure was as follows: A canine left jugular vein and its surrounding connective tissue (10 cm length; 2 g weight) was resected and minced with scissors into tiny (smaller than 0.2 mm) tissue fragments, stirred into 20 ml of saline containing 10,000 IU penicillin and 1,000 IU heparin. To create the tissue fragmented heparinized graft (TFH graft), a 4 mm inner diameter [ID] fabric prosthesis (Microknit, Golaski Laboratories Inc., Philadelphia) was first invaginated, enveloped by a transparent vinyl chloride bag, and connected to a syringe through a three way stopcock to create a closed circuit system. The tissue suspension was injected with pressure into the prosthesis, causing the tissue fragments to seal the pores of the prosthesis completely. After re-invagination, the graft was implanted into the same animal from which the jugular vein was taken. The control graft was a fabric vascular prosthesis (Microknit, 4 mm ID)

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layer of endothelial cells, arranged in parallel rows in the circumferential direction as observed in **Figures 1** and **2.** Under this layer, another multilayer of smooth muscle cells was arranged in the direction of the graft axis. These two layers of smooth muscle cells resembled the direction of smooth muscle cells in the natural arterial wall. In general, smooth muscle cells participate in contraction. Thus, smooth muscle cells may have some vital function in a newly formed arterial wall.

Arrangement of smooth muscle cells in the neointimae of vascular grafts has been described in fabric prostheses[6] and in the structural and functional adaptation recognized as follows: they orient in parallel rows in the direction of strain of tensile stress caused by such factors as blood pressure, constriction of surrounding tissue, and contraction of the host arterial wall. In studies of repair processes of skin wounds and severed tendons, infiltrating cells from neighboring tissues grow in parallel rows, and the direction of these rows is determined by the direction of the tension to which they are subjected.[7] Thus, similar phenomena might be observed with other tissues, such as neointima, which is a kind of repair tissue in the arterial wall.[8-10] With this evidence, it could be concluded that the orientation of smooth muscle cells in the neointima was determined by the strain of tensile stress in the graft wall. It then becomes possible to determine the prevailing direction of tensile stress on the graft wall during implantation by examination of the explanted graft.

In the case of a cylindrical structure, tensile stress is seen in the circumferential direction when the stress is caused by intraluminal pressure, not in the longitudinal direction. Aortic aneurysms always have longitudinal rupture lines; they do not rupture in a circumferential direction, which indicates that the tensile stress is felt in the circumferential direction. The major arrangement of smooth muscle cells in the neointima in this experiment was circumferential, an arrangement that could be explained by this hypothesis of tension theory. Near the anastomotic sites, the cells arranged themselves in the longitudinal direction, but this area may have different stresses, possibly caused by the suture at the anastomosis. A thin layer of smooth muscle cells also was observed just underneath the single layer of endothelial cells. This would be attributable to shared stress with the bloodstream. This arrangement could be explained by this hypothesis, but the longitudinal arrangement of smooth muscle cells could not. However, inside the neointima, two kinds of strain could work in completely different directions. We are attempting to clarify the cause of these arrangements.

In the grafts prepared with our new technology, rapid endothelialization was seen within 1 month throughout the graft luminal surface. There were no differences in endothelialization between the areas near the anastomotic sites and the other areas, including the center of the graft. Underneath the smooth muscle cells, regularly arranged smooth muscle cell layers were observed on the fabric vascular graft wall.

From these observations, it can be concluded that our newly developed method of transplantation of autologous tissue fragments into fabric vascular prostheses could introduce a functional, vital neointima on the fabric graft wall. This functional neointima would contribute to the maintenance of graft patency as a natural arterial wall with various vital roles.

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Sealing of a Fabric Vascular Prosthesis with Autologous Adipose Tissue: A Preliminary Report of Its Clinical Application

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Abstract: A new method of sealing fabric vascular prostheses with autologous adipose tissue was clinically applied as an alternative to preclotting with fresh blood. Thirty-six patients with peripheral arterial occlusive disease were implanted with highly porous fabric prostheses. The prostheses were prepared by sealing the fabric pores with autologous adipose tissue that had been chopped up into small pieces and enmeshed in the fabric by forceful injection of the tissue suspension through a syringe. There was no complication related to the sealed graft such as graft bleeding after implantation. In-hospital mortality occurred in 4 patients: 1 case each of pneumonia, pulmonary infarction, sepsis, and acute myocardial infarction. During the period of 274 ± 190 days, 3 prostheses were found to be occluded. All the other grafts were patent. The overall patency rate was 91.4%. Postoperative angiography revealed neither intimal thickening at the anastomotic sites nor irregularity of the prosthetic surface. The method proved safe and useful for implantation of smaller caliber artificial grafts. **Key Words:** Vascular prostheses—Autologous tissue—Graft sealing—Intimal hyperplasis—Adipose tissue sealed graft.

It was 40 years ago when the first synthetic vascular graft was implanted in a patient. Since then a variety of modifications (1–3) and the introduction of newer materials (4,5) have resulted in a synthetic graft satisfactory for clinical use. This is especially true for grafts larger than 10 mm in diameter. However, grafts smaller than 6 mm in diameter still need further modifications to maintain long-term patency. Two major drawbacks of synthetic arterial prostheses are the resultant delay in tissue healing with endothelialization over the graft inner surface (6) and development of distal anastomotic intimal hyperplasia (DAIH), hump formation (7,8). In an attempt to overcome these problems, we devised a new method to accelerate the neointima formation of fabric prostheses using autologous tissue embedded in the pores of the grafts and have succeeded in producing a graft that may facilitate tissue healing and prevent late thrombotic occlusion (9,10). Various types of tissue were initially used as the autologous tissue (11,12), but the adipose tissue was selected because of its greater availability (13,14). We have previously documented a series of animal experimental results related to safety and reliability of the sealed graft. In this paper the first clinical trial of the autologous adipose tissue-sealed graft is reported.

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MATERIALS AND METHODS

Sealing of fabric vascular prostheses

Subcutaneous adipose tissue obtained from the lower abdominal subcutaneous layer of the patient was chopped up into small pieces and suspended into lactated multiple electrolyte Ringer's solution to create adipose tissue suspension according to our original method (13). A highly porous fabric vascular prostheses, Micron (water porosity: 1,200 ml/cm^2/min at 120 mm Hg; Intervascular, Clearwater, FL), MILLIKNIT (water porosity: 3,000 ml; Golasky Laboratories, Inc., Philadelphia, PA), and Sauvage externally supported graft (Sauvage EXS) (water porosity: 1,500 ml, C.R. Bard, Inc., Biller-

ica, MA) were used. Micron and MILLIKNIT were soft without reinforcement, they were invaginated, and the suspension of tissue fragments was forcefully injected by a syringe into the graft lumen several times until all the pores were filled with the fragments and no liquid leaked through the graft wall. Then the graft was everted again, returning the smooth surface to the inside of the graft and leaving the outer surface rough with multiple tissue fragments. Since Sauvage EXS, which was externally supported with polypropylene monofilament, cannot be everted, the graft preparation was performed without eversion. Injection of a suspension of tissue fragments was followed by fresh blood infusion into the graft and insertion of a Teflon rod whose diameter exactly equaled the inner diameter of the graft to cement the tissue fragments with a fibrin network to the graft luminal surface. This procedure made the inner surface smooth. Heparin was added to prevent early thrombotic occlusion due to the high thrombogenicity of adipose tissue. The size of the grafts ranged from 16-8 mm (Y graft) to 5 mm in internal diameter.

Patients

Thirty-six patients were randomly selected for this study from those diagnosed at the Yokohama City University Hospital and one of our affiliated hospitals as having peripheral artery occlusion or aneurysm. They consisted of 35 patients with arteriosclerosis and 1 patient with an aneurysm of the common iliac artery. The mean age was 71.4 ± 8.7 years, ranging from 44 to 90 years. The male:female ratio was 3:2. The anastomotic sites and diameters of the vascular grafts were preoperatively chosen based on angiographical findings. The grafts used included the Micron, 16-8 mm diameter (Y graft) in 8 cases, 10 mm diameter in 2 cases, 8 mm diameter in 4 cases, and 6 mm in 1 case; the Sauvage EXS, at 6 mm in 18 cases and 5 mm in 2 cases; and the MILLIKNIT of 6 mm diameter in 1 case. All the anastomoses were performed using the continuous suturing technique.

General inhalation anesthesia combined with epidural anesthesia was used in all cases. Approximately 30 g of adipose connective tissue was obtained from the subcutaneous layer of the lower abdomen with careful hemostasis, and the vascular prosthesis was prepared intraoperatively as described above. An atraumatic needle with polypropylene thread was used for the anastomosis. During the operation, systemic heparinization was performed (100 IU Heparin/kg) intravenously.

The patients were treated after the graft implantation with antiplatelet medication. The patency of the grafts was confirmed by Doppler echo sonography and x-ray angiography according to a predetermined schedule.

RESULTS

Preparation of the graft was easy. It took approximately 30 min on the operating table during the surgery. The surgery was started with the removal of the adipose tissue and then exposure of the occluded artery. Therefore, during the exposure the graft preparation had been completed. The graft was soft and pliable and the suturing was performed without any problems. Adaptation of the graft to the arterial wall was excellent. Just after the blood vessel clamps were released, bleeding through the graft wall and at the suture lines was observed, but it was controllable.

There were no bleeding problems at the sites of the adipose tissue removal. At first we took the adipose tissue from the femoral region in the case of femoropopliteal bypass surgery, but lymph edema appeared after the surgery at the site. It healed about 1 month later. From this experience, we decided to take the adipose tissue from the lower abdominal subcutaneous layer separated from the areas of the graft implantation. For safety during and after the surgery with anticoagulant therapy, careful hemostat was required. Therefore no problems with the adipose tissue retrieval were experienced.

After surgery, there was no embolism in the peripheral areas due to the detachment of tissue fragments nor bleeding problems due to the hyperfibrinolysis phenomenon or anticoagulant treatment. The patients with patent grafts showed satisfactory postoperative results of the bypass surgery. Reoperation was performed in 1 patient, a 72-year-old male with a partially infected graft, at 134 days after implantation, i.e., the previously implanted graft was removed and a new graft without the tissue fragmentation was implanted.

Operative or in-hospital death occurred in 4 cases: 1 case each of pneumonia, pulmonary infarction, sepsis, and acute myocardial infarction. During an average observation period of 274 ± 190 days, an occlusion of the graft occurred in 3 cases: 1 instance is the Micron of 6 mm diameter and 2 instances in the Sauvage EXS of 6 mm diameter due to the graft infection and poor run-off of the peripheral blood flow. The occlusion was not correlated to both the diameter and the type of the grafts.

Patency was confirmed in 87.5% of the Micron grafts of 16 mm diameter and in 100% of those of 10,

have a good healing of their neointimae. Some of them, coated with collagen or gelatin and cross-linked with glutaraldehyde or formaldehyde, showed unusual reactions such as fluid accumulation, bleeding, or seroma formation around the grafts (28,29). Antigenicity against the bovine collagen was pointed out as a cause of the unusual reactions (30); however, we had also pointed out endotoxin contamination in the graft sealant as one of the causes (31). Another problem might come from the cross-linker, glutaraldehyde (32). It has already been shown that the toxic effect of glutaraldehyde can be released very slowly after implantation. Some of these problems look inevitable at present.

By contrast, the sealing with autologous tissue is an ideal method. It has many advantages including viability, resistance to infection, good healing characteristics, and no foreign body reaction. In the case of adipose tissue, it is easily obtainable without any difficulties. With our own enmeshing procedure, sealing with very tightly anchored tissue fragments sealing is available within a short period of time. The prepared graft is an instantaneously produced, autologous tissue tube reinforced with Dacron fibers. Consequently, the graft safety, reliability, and healing would be expected for long-term implantation.

CONCLUSION

Autologous adipose tissue-sealed grafts were clinically implanted. The observation period is not long enough, but we have found some remarkable advantages related to graft healing and safety. We are now observing long-term graft performance and are continuing to implant the graft clinically. This sealing method will be suitable not only in vascular grafting but also for other implant applications such as a cardiac wall substitute. We are trying to expand further the applications of this technology.

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Transplantation of Autologous Tissue Fragments into an e-PTFE Graft with Long Fibrils

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Abstract: An expanded polytetrafluoroethylene (e-PTFE) graft with long fibrils transplanted with bone marrow showed rapid and uniform neointima formation in a dog study. The e-PTFE grafts (fibril length, 90 μm; 6 mm internal diameter; length, 6–8 cm) transplanted with autologous bone marrow were implanted in the abdominal aortae of 10 dogs and retrieved at 3 weeks and 3 months after implantation. Control e-PTFE grafts without bone marrow treatment were also implanted in the same manner in 8 dogs. Macroscopically the treated graft wall appeared red in color; however, there was no thrombus deposition on the surface. Light microscopic observation revealed that the treated grafts were completely lined with endothelial cells at 3 weeks. The neointima was uniform without intimal hyperplasia at the anastomotic sites. Inside the graft wall many capillary blood vessels were observed. At 3 months moderate intimal hyperplasia throughout the graft with complete endothelialization was observed. In the control grafts, endothelialization was observed at the anastomotic sites; however, half of the other areas were covered with a fibrin layer devoid of endothelial cells even in the 3-month grafts. These results indicated that neointima formation was effectively accelerated with the autologous bone marrow transplantation, but moderate intimal hyperplasia throughout the graft was inevitable in e-PTFE grafts even after complete endothelialization. **Key Words:** Bone marrow transplantation—Endothelial cell—e-PTFE graft with long fibrils—Vascular prosthesis—Neointima—Intimal hyperplasia.

Commercially available expanded polytetrafluoroethylene (e-PTFE) grafts with 30 μm of fibril length do not show neointima formation except at the anastomotic sites (1–3). Arterial cells at the anastomoses are the only source of intimal cells. Endothelial cells grow along the luminal surface from the suture line, followed by an underlining of smooth muscle cells (3). It was believed that these smooth muscle cells proliferate to produce an intimal hyperplasia with some stimulation such as platelet-deprived growth factor (PDGF) (4). With an e-PTFE graft with long fibrils, some possibilities exist for endothelialization with transmural capillaries originating from the surrounding granulation tissue (3,5); however, they were not always confirmed, and frequently these grafts occluded within 24 h (6,7). Accordingly most of the luminal surface of e-PTFE grafts was covered with fresh thrombus or with a thin layer of fibrin. These are the major problems with e-PTFE grafts. In this paper the e-PTFE graft with long fibrils transplanted with autologous bone marrow was evaluated as a graft that can overcome these problems.

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MATERIALS AND METHODS

Preparation of the graft

As the basic matrix, e-PTFE grafts with a fibril length of 60–150 μm (average 90 μm) were used as the basic matrix for this experiment. The graft was specially donated from the Vascular Group, Baxter Healthcare, Vascular Systems Division (Irvine, CA). Autologous bone marrow was obtained from the iliac bone of experimental dogs by a needle puncture kit under general anesthesia. To obtain sufficient bone marrow, a small sharp spoon was required to scrape off the spongy substances of the

proximal to the distal area to observe the entire length of the graft in sequence. These series of specimens were treated in the following manner. The specimens were fixed with 1% glutaraldehyde in phosphate buffer, 0.2 mol/L, pH 7.4. These tissues were embedded in a hydrophilic resin (Technovit 7,100, Kulzer & Co. GmbH, Friedrichsdorf, Germany). Sections were stained with hematoxylin and eosin, the peroxidase antiperoxidase (PAP) method for the staining of Factor VIII of endothelial cells, and Von Kossa's stain for detection of calcification of the neointima.

In vivo culture test

In order to examine the viability of the seeded bone marrow, cells in the tissue suspension after enmeshing were cultured in vivo using a standard cell culture system with 10% calf serum in a 5% CO_2 incubator. Cell migration and proliferation were observed for 10 days.

RESULTS

Preparation of the graft

The procedure to create the bone marrow suspension was simple. The e-PTFE graft used as the basic matrix of the treated graft was very soft and pliable due to the special mechanical characteristics created by the long-fibril lengthened framework. The procedure of the bone marrow sieving into the graft wall was also easy and took about 15 min on the surgical table. After several repeated injections of the suspension through the graft wall, multiple tiny tissues of the bone marrow were trapped in the e-PTFE graft wall. These trapped tissues were firmly fixed and could not be washed away by flushing the luminal surface of the prosthesis. After this treatment, the graft became reddish with the impregnated bone marrow, but the graft maintained the elasticity of the original e-PTFE graft.

Graft implantation

The mechanical properties and the handling of the treated grafts did not show any differences compared with those of the control grafts. Both were soft and pliable. They could be elongated easily in the longitudinal direction, but no tendency of graft dilatation was observed. The suturing with an atraumatic needle presented no difficulties. There was no bleeding problem through the suture holes of the treated grafts; however, in the case of the controls, a small amount of bleeding through the suture holes continued. It was stopped within 3 min by fingertip pressure.

Compared with the control, the treated graft was wet and looked like a hydrous autologous tissue tube containing bone marrow and blood components. The cut edge of the treated graft wall was also soft and pliable and approximately 0.6 mm in thickness, which was similar to that of the natural aortic wall of the experimental animal. Consequently, fitting and suturing to the aortic wall was easy, and almost no leakage of blood was noticed at the suture line or the suture holes. Immediately after the blood vessel clamps were released, the graft turned completely red, and a small amount of blood leaked through the graft wall. However, this bleeding stopped spontaneously within 1 min. There were no postoperative complications associated with the treated grafts.

In the control graft, there was a small amount of bleeding at the anastomotic line and the suture holes. But the bleeding was controllable. Through the control graft wall, a certain amount of bleeding continued. It required 5 min to repeat a procedure of clamping and declamping the aorta several times to stop the bleeding throughout the graft wall. Even after the bleeding stopped, a very small amount of blood "sweating" through the graft wall was seen continuously.

Graft removal

All of the animals with graft implantation looked healthy during the observation period; however, chronic ileus symptom with a distended abdomen due to intestinal adhesion was noticed in 1 animal with a treated graft. It survived during the observation period; however, for the treated grafts, 4 out of 10 were retrieved at 3 weeks after implantation. One of them was occluded, and the other 3 were patent. The other 6 grafts were explanted at 3 months. All of them were patent. As for the control graft, 3 grafts were removed at 3 weeks, and the other 5 grafts were retrieved at 3 months. All of them were patent.

Macroscopic appearance

The treated grafts removed at 3 weeks were dark red in color along the entire luminal surface, but there was no fresh thrombus observed (Fig. 1a). The graft wall was soft and pliable. There was no unusual adhesion, hematoma, seroma formation, inflammatory reaction, or scar tissue around the graft wall. One occluded graft was hard along the entire graft and dark brown in color. Its lumen was completely occupied with connective tissue, but there were no inflammatory reactions either inside or outside the graft. At 3 months, the luminal surfaces of the treated grafts were still red in color, but the degree of the redness decreased compared with

In vitro cell culture study

The bone marrow cells obtained from the suspension after the sieving procedure were cultured in vitro without any difficulties. They made some colonies of bone marrow tissue such as erythroblasts, granulocytes, endothelial cells, and fibroblasts. They grew without any suppression with each other for 10 days of the observation period.

DISCUSSION

Autologous tissue fragments transplantation

From the results it is obvious that the autologous bone marrow fragment transplantation in the e-PTFE graft with long fibrils accelerated the neointima formation effectively. Since an ordinary e-PTFE graft is not endothelialized except at anastomotic sites (1,2), this method had a remarkable advantage for endothelialization. Bone marrow cells were tough enough (9) and could survive during the sieving procedure and after the graft implantation and induced complete endothelialization throughout the graft within a short period of time.

We had developed an original method to accelerate the endothelialization of a fabric vascular prostheses by transplantation of chopped autologous tissue fragments (8,10), but the method was not applicable in e-PTFE grafts. Tissue fragments chopped with scissors were too large to impregnate the interstices of the grafts. Another problem we faced was a source of the autologous tissue fragments. First, we started the method with a piece of autologous venous tissue (10), but the source of the venous tissue was limited. Second, we used an adipose tissue (11,12), but the adipose tissue varied in each case, i.e., age, sex, nutritional condition, and other complications such as diabetes mellitus. Consequently, two problems exist. To obtain more tiny fragments at the cellular level and to obtain more active cells for transplantation even from an aged patient should satisfy the original method.

With bone marrow we could produce a cellular level tissue suspension without chopping, and it always contains active numerous undifferentiated mesenchymal cells (9,13) which can differentiate into various kinds of cells and can produce many kinds of cytokines at the location and environment where they are situated. These tissue fragments were entrapped in the interstices of the specially expanded-PTFE graft wall. The washing of the lumen to remove excessive free fragments eliminated the possibility of unexpected microemboli.

Bone marrow has already been applied to produce new organs in orthopedic surgery. Ohgushi and his associates (14,15) implanted marrow cells with porous hydroxyapatite and tricalcium phosphate into a rat subcutaneous connective tissue layer to induce ectopic bone formation. The hydroxyapatite showed osteogenic ability in the presence of marrow cells. Bone marrow tissue has the potential to create some new organs if they are transplanted in suitable conditions.

e-PTFE graft with long fibrils

In the 1970s, an optimal fibril length of e-PTFE grafts was discussed (6,7,16); however, reevaluation of the fibril length has recently become a controversial issue (3,5,17,18). Especially for the purpose of endothelial cell seeding onto e-PTFE graft (19,20), the currently available grafts of 30 μm have pores too small to anchor the cells. Basically, PTFE has a nonadhesive properties to prevent not only thrombus adhesion, but also cell adhesion (21). To give flexibility to the PTFE tube, it was expanded thereby giving numerous internodular spaces. If the fibril length is highly expanded, the total surface of PTFE area on the luminal surface of the graft will decrease, resulting in a reduction of the nonadhesive properties against thrombus. However, the graft will obtain a new advantage, i.e., neointima anchoring, since numerous large bundles of tissue penetrating through the graft wall can bind the neointima to the adventitial tissue (6). In the current experiment, an ~90 μm e-PTFE graft was used to give enough anchoring spaces for the bone marrow enmeshing; therefore, the nonthrombogenic property of the graft should be low compared with that of a 30 μm e-PTFE graft. However, some advantages of an e-PTFE graft with long fibrils were observed in the control graft without bone marrow transplantation. Even in the center area of the graft, some isolated endothelialized areas were noticed. We did not observe any capillary blood vessels penetrating through the graft wall; however, these endothelial cells might bring through the transmural capillaries originating from the adventitia side (3,5). Another advantage was the thickness of the fibrin layer. At 3 weeks the thickness ranged from 100–300 μm, but at 3 months it became thin (10–100 μm). The thin fibrin layer was ready to be endothelialized, and about half of these areas had been covered with endothelial cells.

Intimal hyperplasia at anastomosis

Hump formation (development of distal anastomotic intimal hyperplasia) (22) was not present in the treated grafts nor in the control grafts. We ob-

served pannus ingrowth from the host aortic wall beyond the suture line, but they were formed to make the luminal surface smooth between the host aorta and the graft. The observation period was too short to discuss the future hyperplasia at the anastomotic sites, but from these results, there were no differences between the anastomotic sites and the center areas related to the intimal hyperplasia.

We believe that one of the causes of the humps is the separation of the pannus from the e-PTFE graft surface. Compliance mismatch is also one of the causes (23). However, since PTFE has a tendency to separate from the surrounding tissue, detachment has a baneful influence upon the stability of the tissues on an e-PTFE graft. In case of a highly porous fabric graft, the anchoring of the pannus is strong enough to be separated from the graft surface. After implantation endothelial cells proliferate and connect each other to cover all the luminal surface that contacts the blood stream. It is a kind of mother nature of endothelial cells. Therefore, at the suture line, pannus covered with endothelial cells is always extending toward the graft surface. In an ordinary 30 μm e-PTFE graft, neointima formation is limited to the pannus at the anastomotic sites. Therefore, the intimal hyperplasia or pannus hyperplasia always means hump formation at the anastomotic sites. Since the anchoring of the pannus to the ordinary 30 μm e-PTFE graft surface is poor (6,7), the pannus always tends to detach, resulting in a thrombus formation in the space produced between the pannus and the graft surface. Smooth muscle cell migration from the host artery into the thrombus is quite possible due to platelets derived growth factor (PDGF) (4) in the thrombus layer. With the migration of smooth muscle cells, the thrombus layer was organized but was still in an unstable condition for the next detachment. Repeated thrombus formation in the detached area will always supply PDGF into the pannus even if the pannus were completely endothelialized to prevent platelet infiltration from the luminal surface, resulting in hump formation at the anastomotic site.

In the current experiment we used a 90 μm e-PTFE graft which offers enough anchoring area to the pannus. Even if PTFE tended to separate from the connective tissue (as shown in the results section), large bundles of tissue containing fibroblasts and collagen fibers penetrating through the interstices of the long-fibril lengthened PTFE graft wall can anchor the pannus to the graft and to the tissue in the adventitia. Fibroblasts, infiltrated into the interstices of the graft, produced collagen fibers, which were the strongest framework for the anchoring. This is the reason both the treated graft and the control did not show hump formation at the anastomosis.

Intimal hyperplasia throughout the graft

In this study thin but intimal hyperplasia was observed throughout the graft surface retrieved at 3 months. It was uniform and smooth. In the 1960s it was believed that a thick initial thrombus formation would lead to a thick neointima formation (21,24, 25). However, from our previous experiment, the initial thrombus became a thin fibrin layer within 2 days after implantation, and then another red thrombus was produced on the surface to become a thick thrombus formation (26). It was believed in general that after endothelialization, the thickness of the neointima decreased in time; however, in the current experiment, we could observe very rapid endothelialization followed by moderate intimal hyperplasia throughout the graft wall. This evidence refutes the hypothesis that the areas with endothelial cell injury or endothelial cell defect can accelerate macrophage adhesion and platelet infiltration resulting in the intimal hyperplasia on the arterial wall (27).

From our previous experience, a biological vascular prosthesis obtained from an animal artery and crosslinked with a polyepoxy compound graft did not show intimal hyperplasia, and the graft wall was soft and compliant (28). A graft made of polyurethane also showed a low rate of intimal hyperplasia. Miwa et al. (29) demonstrated less intimal hyperplasia at the anastomotic sites in cases of compliant polyurethane grafts, but we think his data also indicate less intimal hyperplasia throughout the graft. Moreover, an extremely porous fabric vascular prosthesis did not show any intimal hyperplasia, and the graft wall was also sufficiently compliant (30). For aortacoronary bypass grafting, vein grafts showed a high rate of intimal hyperplasia but less in the case of arterial grafts (31,32). These results are similar to those observed in the case of arteriosclerosis. In young people the arterial wall is soft and compliant, and the wall does not have any intimal hyperplasia, but in older people, the artery loses elasticity, and the intimal hyperplasia starts to accelerate arteriosclerosis.

From this evidence, it is speculated that the intimal hyperplasia throughout the graft wall was correlated with the compliance of the graft wall. Endothelial cells on the neointima of a less compliant graft wall might provide a greater opportunity for injury, resulting in production of a PDGF-like protein (33). Since the e-PTFE graft is flexible but less

compliant in the circumferential direction, moderate intimal hyperplasia throughout the graft wall may be inevitable in e-PTFE grafts even after complete endothelialization.

Further application of the method

The bone marrow transplantation was easy to perform without a special technique, instruments, or facilities. The graft was completely endothelialized within a short period of time. Although further investigation with long-term observation is warranted, intimal hyperplasia was minimal. The bone marrow transplantation into the e-PTFE graft with long fibrils might be applicable as a small diameter vascular prosthesis. The only problem in this stage will be the high thrombogenic property of the graft after enmeshing of the bone marrow. The fibril length was very long; therefore, the graft was thrombogenic itself. Moreover, bone marrow tissue is not antithrombogenic. During the first 3 weeks of the term of full endothelialization therefore, we need a temporally antithrombogenic graft and anticoagulant therapy to prevent the thrombus formation.

CONCLUSION

In conclusion the e-PTFE graft transplanted with bone marrow showed rapid and uniform neointima formation and maintained patency without intimal hyperplasia at the distal anastomotic sites, indicating that the method may overcome the problems that we are facing with the e-PTFE graft in general. However, minimal hyperplasia throughout the graft wall may be inevitable in e-PTFE grafts even after complete endothelialization.

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Guest Editorial

Progress and Problems in the Development of Vascular Prostheses

After Voorhees' use of a synthetic fabric tube as a vascular prosthesis in 1952 (1), various kinds of synthetic vascular prostheses have been produced. Creech et al. reported in 1957 that Dacron and Teflon are suitable raw materials for implantable vascular prostheses due to their biostability and nontoxicity (2). In the 1960s, Wesolowski, who proposed the "porosity hypothesis," and other earnest researchers clarified the healing process of vascular grafts using animal and clinical data (3). Wesolowski found that highly porous grafts will have complete and rapid neointima formation without degenerative changes. All the vascular prostheses produced thereafter have been influenced by this hypothesis. In 1972, e-PTFE grafts were introduced (4,5). In the 1970s, an autologous tissue tube was used (6,7). In 1986, a fabric graft made of ultrafine polyester fibers was introduced (8). Chemically treated biological grafts with glutaraldehyde, dialdehyde starch, or hydrophilic polyepoxy compounds (9) were also introduced in this field. Recently, fabric prostheses coated with biodegradable materials such as collagen and gelatin have been introduced in clinic.

After these activities, a variety of knitted and woven fabric grafts, e-PTFE grafts, coating grafts, and biological grafts have been developed and have become available for clinical use. Today, worldwide more than 20 companies are producing more than 50 kinds of vascular prostheses. Now we can choose any of them as the most appropriate one for each patient case. However, how we select one of them as the best vascular prosthesis for each case in clinic is a problem. With such a vast number of available grafts, there should be the best one, the second best, the third, . . . , and the worst one. Do we have enough data to understand the tremendous numbers of vascular grafts in detail perfectly for the appropriate selection?

In clinic, we select a certain one for each case from several available vascular prostheses, but it is always with very little information. Our individual knowledge is limited and different from each other. In the world, a large number of vascular prostheses are implanted with very little information on graft performance characteristics. It is feared that we might be evaluating the vascular prosthesis using patients like animal experiments: with a poorly organized database on a large number of prostheses and very low overall efficiency. Who would put together and publish this as one study? Someone should organize all the data related to the vascular grafts including their advantages, disadvantages, special properties, nonspecific characteristics, etc., in detail with an unbiased view for the user's decision. Public organizations such as the FDA are trying to accumulate such data. They set certain guidelines for safety and efficacy, but it is impossible for them to explain everything in detail.

We implant a large number of vascular prostheses in clinic, but we have not understood what is happening to the grafts in the patient's body after implantation. This is the second problem. Sometimes, we have opportunities to see the grafts at autopsy or in surgery in the case of reoperation for graft-related complications. These findings show some phases of the healing process, but they are not the entire healing process of each vascular graft. We need to have enough knowledge related to the condition of the graft after implantation in detail. In clinic, these findings are limited. Therefore, we have to obtain these real findings from animal experiments even though the healing process is different in each species (10). It is possible to obtain some information related to some advantages and special characteristics of grafts from their makers and on side effects and complications from clinical reports, but this information is fragmented insufficient. We are also unable to reorganize this information into a useful, clear, and concise format because of our poor previous, disorganized information.

Another problem with data obtained from animal experiments is that there is not sufficient information available that would predict the life-time performance of the vascular prosthesis. For example,

sometimes the animal results show unexpected phenomena. These unusual occurrences cannot be merely regarded as a fluctuation, but unfortunately researchers do not have enough time to search for the root cause because of a predetermined schedule. Animals never tell a lie. Due to a variety of reasons, such as high cost and limited resources, we cannot perform long-term animal experiments. We regret to say that we have insufficient knowledge to understand the real reason behind the apparently unusual phenomena in each experimental animal. As a result the clinician can only obtain limited data, and these unexpected phenomena can occur in clinic. There is a great need for an organized knowledge base related to vascular prostheses in general and in detail.

For example, it has been some time since the evidence pointed out that vascular prostheses are not healed except to the anastomotic sites (11). We still do not know the real reason the midsection areas are not endothelialized after such a long period of time. Other questions also remain unanswered. Why cannot small diameter vascular grafts maintain their patency even with powerful anticoagulant therapy? Why do recent biodegradable material-coated grafts show unexpected noninfectious reactions (12) such as fluid accumulation around the grafts? These grafts have been given FDA approval, yet these unusual reactions are observed in many cases (13,14). Some researchers explained that this is due to an immunological response against bovine collagen (15). The graft makers explain that they are quite rare and are negligible. But the rate of appearance of the unusual immunological response against purified bovine collagen is extremely low in cases of injectable collagen for cosmetic use (16).

In this special issue, we have tried to expand our knowledge related to the basic phenomena observed in vascular prostheses after implantation. First of all, I will explain the healing process of neointima of fabric vascular prostheses with vicious cycle (fresh red thrombus formation, increasing fibrin deposition, a decreasing fibrin layer due to fibrinolysis and its becoming low in density with multiple pores, infiltration of blood components into the holes of the fibrin network, and the resulting fresh thrombus formation again inside the fibrin network) on the luminal surface of the grafts in an animal experiment. This evidence will show the reader why the vascular grafts do not heal and why small diameter grafts occlude despite administration of powerful anticoagulant therapy. The thrombus is always fresh, and never stops producing the next new thrombus. Anticoagulant therapy will accelerate the vicious cycle. To arrest the vicious cycle, transplantation of autologous tissue fragments is the best method. The paper related to the transplantation of bone marrow in this issue will show the most effective method to overcome these problems. The sealing method of fabric vascular prosthesis with autologous adipose tissue fragments in the manuscript by Drs. Matsumoto and Kajiwara is one of the applications of autologous tissue transplantation. The readers will appreciate the effectiveness of this technology.

Dr. Satoh explains a clinical report of a fabric prosthesis made of ultrafine polyester fibers that shows no bleeding and is easy to handle without any biodegradable material coating. With the coming century, many say that the time of micromachining and ultrafine technology will arrive. Ultrafine fiber technology in the field of vascular prostheses will have a prosperous future. The ultrafine polyester fibers are soft, elastic, and strong and have a special property to induce host cell migration resulting in acceleration of the neointima. From the biological standpoint, host cells want to adhere along the edge of materials, and this phenomenon is well known as "contact guidance." It is natural for cells to adhere to the edge of materials. Therefore, the ultrafine fibers can induce the migration of cells onto the fibers very naturally. In future, the fibers will become one of the essential materials not only for vascular prostheses but also for various kinds of implantable artificial organs.

For example, in the case of intervention treatment, thin wall grafts made of ultrafine polyester fibers will become the essential prostheses. Dr. Nojiri will show the possibility of an antithrombogenic polymer graft without endothelialization. This technology also has great potential to create a permanent antithrombogenic surface with synthetic materials. Due to endothelial cell aging, it is impossible for a graft to be endothelialized from the anastomotic sites throughout the graft wall. Approximately 70 cell divisions make the cell old enough (17). Therefore, endothelialization from the anastomotic sites are actually impossible. We need a nonthrombogenic surface on a small diameter graft without endothelialization in the case of some synthetic grafts. Dr. Ninomiya discusses long-term patency of biological grafts in clinic and will explain how good the biological materials are. In general, biological materials obtained from animals have unique and special properties microstructurally, mechanically, hemodynamically, and biochemically that cannot be simulated by any currently available technology. Therefore, biological materials have a

great potential to be used as raw materials for implantable artificial organs. However, many biological materials have been treated with glutaraldehyde which is very cytotoxic. Host cells cannot migrate inside the graft wall. Dr. Ninomiya used the less toxic and hydrophilic cross-linker "polyepoxy compound" instead of glutaraldehyde for his group's graft preparations. Dr. Okoshi explains a new hypothesis related to the porosity of vascular prostheses using very small diameter grafts in a rat model. He explains how important the porous structure is in vascular grafts in general. His hypothesis is independent from that of Dr. Wesolowski (3). Dr. Tomizawa shows the vascular prostheses for aortocoronary bypass grafting and explains how difficult it is to create a new vascular prosthesis.

With these unique papers, I am sure the readers will obtain a limited but reorganized knowledge of vascular prostheses to make a judgment for selection of the best graft from a large number of choices. As our reorganized knowledge is increasing, some vascular prostheses currently commercialized will survive for clinical use, and some will disappear.

In the 21st century, we expect that newly produced vascular prostheses will have special properties that can induce a healing ability of host cells. Many cytokines, growth factors, tissue fragment transplantation technology, and gene technologies will be used to accelerate the neointima formation. Some high technologies in polymer science will be introduced to prepare the framework of vascular prostheses since special matrices made of unique polymers can change the environments of residence areas of host cells resulting in induction of new organ formation using the nature of these cells. All of these manipulations with materials, cytokines, and gene treatments will only be possible within the limitation of the provisions of nature, while taking advantages of the dispensation of nature, as ultrafine polyester fibers offer a suitable matrix for cell migration and proliferation resulting in acceleration of neointima formation.

Another great difference will be expected in the procedure of vascular prosthesis implantation. Interventional therapy (18) will become more popular. A very high percentage of graft implantations will be performed by this method. As in the case of cancer therapy, the effort of early diagnosis and early treatment has reduced cancer deaths markedly in the last two decades. In the case of vascular surgery, early diagnosis and early treatment with interventional or very simple and safe treatment will change the concept of the treatment of vascular disease.

I would like to thank Dr. Yukihiko Nosé, who offered me the opportunity to edit a special issue of *Artificial Organs* about vascular prostheses. Furthermore, I would like to thank Mrs. Joanne Elser for her help in editing this issue. I hope all of the readers can understand the most advanced information and create their own reorganized database for the selection of the best vascular prosthesis for each case in clinic, and I hope this issue will help to increase our knowledge about artificial organs for the coming century.

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The Vicious Cycle of Nonhealing Neointima in Fabric Vascular Prostheses

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Abstract: Delayed neointimal healing of a fabric vascular prosthesis was investigated in an animal study focusing on the relationship between red thrombus, fibrinolysis, and endothelialization on the luminal surface. Fabric vascular prostheses were implanted into the descending aortas of 72 dogs. Fifty-nine grafts were explanted from 1 h to 1,705 days after implantation. One hour after implantation, the graft wall was red in color due to fresh thrombus; however, at 1 day the luminal surface became white. Red thrombus reappeared at 1 week and remained present in the long-term. Microscopically the initial red thrombus contained numerous erythrocytes. The white thrombus at 1 day was composed of a dense fibrin network without erythrocytes. At 2 days numerous lacunae appeared in the fibrin layer, and at 3–5 days cavernae and low density fibrin areas were present secondary to fibrinolysis. These areas allowed the blood components to infiltrate into the fibrin layer, and as a result red thrombus reformed within it. The thrombi on the luminal surface in the long-term was always red in color and composed of complicated, multiple stages of thrombus formation, i.e., fresh thrombus with erythrocytes, dense fibrin without erythrocytes, low fibrin density areas, lacunae and cavernae in the fibrin layer, and blood component infiltration into these spaces. Thrombus was always newly formed and present, and involuted in parallel due to fibrinolysis, suggesting that these phenomena perpetuated in a vicious cycle. However, at the anastomoses fibrinolysis was present, but blood component infiltration was prevented by the endothelial cell lining. These results suggest that endothelialization may arrest the vicious cycle of nonhealing neointima in fabric vascular prostheses. **Key Words:** Vascular prosthesis—Neointima—Vicious cycle in the neointima formation—Fibrinolysis—Endothelial cell—Incomplete healing of vascular prosthesis.

Neointima formation of vascular prostheses in humans is extremely delayed (1). Most grafts never endothelialize (2), and their inner surface is covered with thrombus long after implantation (3–6). The cause of this delay has not been clarified, and even in animal studies, complete endothelialization along the entire luminal surface is not always present (7). Highly porous fabric vascular prostheses are expected to have rapid healing (8); however, neointimal formation is also delayed except at the anastomoses (9,10). This study was designed to assess the process of neointimal formation in an animal model, and we observed that the newly formed red thrombus on the graft surface was always present and involuted in parallel due to fibrinolysis. The relationship among thrombus formation, fibrinolysis, and the role of the endothelial cell lining was examined.

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MATERIALS AND METHODS

Vascular prosthesis

A highly porous fabric Dacron vascular prosthesis (MILLIKNIT, Golaski Lab., Inc., PA; Water porosity: 3,000 ml/cm^2/min at 120 mm Hg) was preclotted with fresh blood using a modification of the four-stage Sauvage technique (11) (heparin was not used during the fourth stage).

Implantation of the graft

Seventy-two adult mongrel dogs of both sexes (8–12 kg), underwent graft implantation (internal diameter, 7 mm; length, 5.7 cm) in the descending

aorta. Animals were anesthetized with 8–12 cc of intravenous pentobarbital (2.5% solution) and intubated. The chest was entered via a lateral thoracotomy in the fifth intercostal space. The descending aorta was exposed and mobilized sacrificing 5 pairs of intercostal arteries. A 5 cm segment of the aorta was resected and replaced by the preclotted fabric prosthesis without any temporary external bypass (12). A continuous suture technique with 5-0 Tevdeck (Deknatel of Pfized Products Group, Inc., Floral Park, NY) was used. During the operation, antibiotics (1 g of penicillin) were administered into the pleural cavity, but no anticoagulants were used at any time. Any dog that did not survive for 24 h was excluded from the study and replaced. Any dogs that died from unassociated diseases were excluded from the study. All grafts were explanted on a predetermined postoperative day (1 h to 1,705 days after surgery) as demonstrated in Table 1. All animal care was in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and Published by the National Institutes of Health (NIH publication No. 80-23, revised 1985).

Explantation of the graft

Animals were divided into 6 groups to observe neointima formation at various times. The first group was explanted between 1 h and 14 days to observe initial thrombus formation and its absorption. The second group was explanted between 15 days and 2 months to observe initial cell infiltration into the fibrin layer. The third group was explanted between 3 and 6 months to observe neointima formation. The fourth group was explanted between 7 and 12 months to observe the stable neointima. The fifth group was explanted between 1 and 3 years to observe the neointima over the long term. The last group was explanted later than 3 years to observe the degenerative changes of the neointima. Therefore, all grafts were explanted from 1 h to 1,705 days after implantation. Before harvesting, heparin sodium (100 IU/kg) was administered intravenously to prevent clotting. All the specimens were rinsed with saline solution to remove excess intraluminal blood and were inspected macroscopically. The kidneys of each animal were resected and examined for fresh trapped microemboli.

TABLE 1. *Animal number and the observation period of each group*

Group 1	1 h to 14 days (12 dogs) 1 h, 1 h, 1 day, 1, 2, 3, 5, 7, 8, 10, 12, and 14 days
Group 2	15 days to 2 months (12 dogs) 15, 16, 18, 20, 25, 30, 34, 44, 47, 56, 63, and 65 days
Group 3	3–6 months (10 dogs) 93, 94, 98, 103, 103, 104, 122, 141, 168, and 187 days
Group 4	7–12 months (11 dogs) 209, 216, 233, 249, 252, 261, 273, 291, 294, 297, and 340 days
Group 5	1–3 years (11 dogs) 373, 378, 416, 435, 498, 573, 671, 783, 828, 874, and 988 days
Group 6	more than 3 years (3 dogs) 1,188, 1,240, and 1,705 days

Histological examination

For light microscopic observation, 4 mm wide sections of the prostheses were cut longitudinally from the proximal to the distal anastomoses. Each sample was cut into 7 pieces, and identification marks were placed to observe the entire length of the graft in sequence. The specimens were fixed with 1% glutaraldehyde phosphate buffer (0.2 mol/L, pH 7.4) and embedded in a hydrophilic resin (JB-4, Polyscience, Inc., Warrangton, PA). Sections were stained with hematoxilin and eosin, PAP Method of Factor VIII of endothelial cells, and Von Kossa's stain for detecting calcification of the neointima.

RESULTS

Graft implantation

Graft implantation was performed without major complications. The occlusion time of the descending aorta was less than 15 min in all cases, and the average was 12 min. There were no bleeding complications prior to chest closure; however, 5 out of 72 animals (6.9%) bled to death into the pleural cavity within 24 h of implantation. These prostheses demonstrated red thrombus adhesion on their walls at implantation that disappeared from the graft wall at explantation. Therefore, 5 additional implantations were performed. No animal had a neurological complication following surgery. Four animals died within 2 weeks from graft infection, and 9 died at 40, 84, 103, 297, 616, 845, 1,229, and 1,956 days of unassociated diseases. The remaining 59 animals were evaluated for the study.

Macroscopic aspects

At the time of explantation, all the grafts were patent, and their walls were very soft and pliable. The grafts from the first group were obtained from 12 dogs (1 h–14 days). The inner surface of the graft at 1 h was covered with fresh red thrombus (Fig.

ties of erythrocytes would cause the graft's surface to change from red at 1 h to white by 24 h. From these observations, we can assume that a red thrombus containing numerous erythrocytes in an arterial graft is very fresh.

Multiple lacunae with a small number of leukocytes and macrophages were observed by the second day. The fibrin network was dissolved by protease secreted by these cells, resulting in lacunae formation with fibrinolysis. By 3–5 days, low density fibrin and fibrin-free areas (cavernae) appeared in addition to the small lacunae, and the fibrin network had obviously disappeared. These areas were created by fibrinolysis, starting from the lacunae, which allowed plasminogen to circulate easily and penetrate into the fibrin network. The activated plasmin dissolved fibrin to produce the low density, translucent areas and the cavernae. At this time, both the thickness and density of the fibrin layer had remarkably decreased on the lumen. Fibrinolysis is a physiologic phenomenon occurring in any thrombus in the native arterial system; however, it causes the thrombi on fabric prostheses to develop the very complicated features observed in this study.

Protracted healing started during the second week, during which various appearances of the thrombi were observed, i.e., fresh thrombus formation with erythrocytes, erythrocytes disappearance from the fibrin network, a dense fibrin network without erythrocyte, low density areas in the fibrin layer, cavernous formation with blood component infiltration into these spaces, and the formation of new thrombus in the fibrin layer. These various stages of the healing process were concurrently present within the same graft due to continued fibrinolysis. The spaces produced by fibrinolysis allowed the infiltration of blood components within the fibrin layer due to the high blood pressure of the arterial system; new bleeding occurred within the graft wall, and new thrombus began to form. This sequence was always observed within all thrombi in the specimens obtained after 2 weeks. This phenomenon created a vicious cycle as the cavernae created by the active fibrinolysis were formed in parallel with fresh intrawall bleeding.

Fresh intrawall bleeding brought the graft back to the starting point of neointimal healing. As observed at 10 days in Fig. 3f, the fibrin network on the graft wall partially disappeared, and blood components infiltrated into the interstices of the Dacron fibers creating a fresh thrombus similar to that seen following preclotting. Neointima formation did not occur away from the anastomoses after 10 days. Crack formation within the thrombus layer created a medium for intrawall bleeding, and as the cracks spread within the thrombus, the bleeding would result in detachment of the thrombus layer. Thrombus detachment would then stimulate further thrombus formation and distal microemboli. (While thrombus detachment was obvious from the graft's appearance, we saw no microemboli in the kidneys of these animals.) The macroscopic appearance of the graft surfaces correlated with these microscopic observations. The thrombus layer on the graft surface became thick and rough over time as shown in Figs. 1 and 2. In this vicious cycle, neointima formation cannot occur. The vicious cycle started on the second day with the infiltration of leukocytes into the fibrin layer and was followed by fibrinolysis at 3–5 days.

Anticoagulant or antithrombogenic therapy may accelerate fibrinolysis and increase intrawall bleeding, thereby perpetuating the vicious cycle. In patients, some grafts demonstrate excellent short-term patency, but occlude 1–4 weeks after implantation even with a powerful anticoagulant. These grafts might be experiencing the vicious cycle we observed in this study.

At the anastomoses, endothelial cells migrated from the host aortic wall, creating a white surface free of bleeding into the fibrin layer even after fibrinolysis occurred as shown in Fig. 5e. The endothelial cell lining prevents blood leakage into the wall, and under the protection of the endothelial cells, fibrinolysis occurred naturally. With this fibrinolysis, the healed neointima became thin compared with the thick fibrin layer present in the early stages. In eliminating the vicious cycle, rapid endothelialization on the luminal surface was most effective.

Endothelial cells are derived from host blood vessels either from the suture line (common) or from capillary ingrowth (rare) (8,9). In the specimens obtained at 44 and 47 days, the isolated white areas in the middle of thrombus might have been introduced by capillary ingrowth. When numerous fibroblasts infiltrate into the luminal fibrin layer of an implanted graft, capillary blood vessels follow (natural angiogenesis). However, it takes a significant amount of time for fibroblasts to migrate into the luminal fibrin layer through the Dacron fiber interstices.

Endothelial cell division is limited due to cell aging (20–22). The cells extending from the suture line cannot produce enough cells to cover the entire surface of the prosthesis so the endothelial lining is limited to the anastomoses. As a result most of the

graft surface remains covered with a complicated thrombus layer. In this study the entire luminal surface of one graft at 56 days was completely endothelialized, but many others did not endothelialize even after 3 years. The grafts at 1,240 and 1,705 days showed complete endothelialization without any degenerative changes; however, it is uncommon to see complete endothelialization even after 5 years in humans. Even with highly porous prostheses (8), complete endothelialization is rare.

Natural healing with endothelialization can only occur after the vicious cycle has been eliminated, and to eliminate the vicious cycle, introduction of endothelial cells into the graft wall is essential. Endothelial cell seeding methods have been reported during the last decade (23–25). These attempts have met with limited success thus far because the seeded cells frequently wash off due to high arterial flow following implantation. Some studies have demonstrated favorable results (26), but most remain inappropriate for general use due to special cell culture techniques and facilities. These techniques are also not available for emergency use since the cell cultures require an extended period of time to grow. Complete endothelialization on a fabric vascular prosthesis 2 weeks after implantation in a canine study was reported using a method to accelerate endothelialization by the transplantation of autologous venous tissue fragments into the graft wall (27). All the tissue fragments survived to produce multiple colonies of endothelial cells, smooth muscle cells, and fibroblasts rapidly forming a stable neointima. This tissue fragment method may be the best way to eliminate the vicious cycle of neointimal healing and to obtain complete endothelialization on a fabric vascular prosthesis.

CONCLUSION

The delayed healing of the neointima on fabric vascular prostheses was examined in a canine study. Thrombus on the delayed healing areas showed a complicated structure containing a fibrin network with numerous erythrocytes, a dense fibrin network without erythrocytes, and fibrinolysis with recurrent thrombus formation in the spaces produced by the fibrinolysis. These appearances were observed in all cases with red thrombus formation. The thrombus was always involuted due to fibrinolysis and was perpetuated by intrawall bleeding into the spaces produced by the fibrinolysis thereby creating the vicious cycle of nonneointimal healing. This phenomenon causes protracted healing of the neointima on fabric vascular prostheses, which may be prevented by early endothelialization of the grafts.

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Clinical Use of Low Porosity Woven Ultrafine Polyester Fiber Grafts

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Abstract: A woven fabric graft made of ultrafine polyester fibers (UFPF) (Toray Graft, water porosity: 100 ml/min/cm^2:120 mm Hg H_2O) was clinically applied in 81 cases (28 thoracic aortic aneurysms, 6 thoracoabdominal aortic aneurysms, 42 abdominal aortic aneurysms, and 5 atherosclerotic obstructions of the peripheral arteries). Eight patients died after surgery due to causes unrelated to the graft. The other 73 patients were in good condition after surgery. For operations requiring extracorporeal circulation, the graft was presealed with human albumin. For the abdominal aortic aneurysms, the graft was preclotted in situ with nonheparinized autoblood after the completion of the proximal anastomosis. It took about 2 min to complete the preclotting. A nonsealed graft was used for the reconstruction of peripheral arteries for the intraaortic balloon pumping procedure. The graft was easy to handle. There was no cut edge fraying problem with the graft in any direction of cutting. Even after presealing, the graft was soft and pliable enough to enable easy adaptation and anastomosis. Just after implantation, bleeding was minimal from the graft wall, anastomotic sites, and suture pores, and it stopped spontaneously. These clinical data showed that the woven UFPF graft exhibited both easy handling despite in spite of low porosity and safe application in the reconstruction of arterial systems even under totally heparinized conditions during extracorporeal circulation. **Key Words:** Ultrafine polyester fibers (UFPF)—Woven structure—Low water porosity—Toray graft—Presealing—Extracorporeal circulation.

It has been emphasized that ultrafine polyester fibers (UFPF) of around 3 μm in thickness exhibit higher affinity with cells; that is, both in vitro and in vivo studies have revealed that UFPF induced pronounced proliferation of fibroblasts on their surfaces in comparison with conventional polyester fibers of around 12 μm in diameter (1,2). On this basis a vascular graft fabricated with woven UFPF has been developed (Toray Graft, Toray Co., Tokyo, Japan). The graft was tightly woven with special UFPF, which was veloured both inside the graft wall and on its inner and outer surfaces and entangled with each other very densely (3). Therefore, the graft had low porosity but was still soft and pliable like a highly porous knitted fabric prosthesis. In 1989, the graft became available for clinical use with the approval of the FDA (Food and Drug Administration) in the U.S.A. and the Welfare Ministry of Japan. The graft enables easy handling owing to the soft and pliable characteristics of the UFPF and accelerates tissue regeneration by host cell migration inside the graft wall as shown in a preliminary study with animal experiments (4–7). The graft has enough durability for aortic reconstruction and a low water permeability of 100 ml/min/cm^2:120 mm Hg H_2O and basically does not need pretreatment to seal the interstices of the fabrics to avoid profuse hemorrhage. In our department, the prostheses have been applied for clinical use since 1990 after a preclinical study using an in vitro circuit model (8) that showed that the amount of blood leakage through the graft wall was not significantly greater than that of a conventional low porosity woven Dacron prosthesis. This report will describe our clinical results with the graft and its further application.

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nificant clefts on the graft walls. The weak structure of this textile fabric should not be ignored. We have to take significant risks during use of knitted grafts for long periods of implantation.

Recently, another problem was reported with the presealed fabric prostheses with collagen and gelatin (16,17). These prostheses showed unusual inflammatory responses without any graft infection. High amounts of endotoxin were detected from the sealants of these prostheses. These unexpected reactions prevented the neointima formation on the prostheses walls, accelerated the peel phenomenon, and induced bleeding problems after implantation. Collagen or gelatin were believed to have special characteristics to arrange a suitable scaffold for cell migration and proliferation in general (18,19). However, once they were contaminated with some microorganisms, which grow very rapidly in number inside the sealants during the manufacturing process, high amounts of endotoxin were exhausted inside the prostheses wall when they were killed during the prostheses sterilization (20).

Another type is the low porosity woven Dacron prosthesis. The structure of the low porous woven prosthesis is firm enough for long-term implantation. Few cases with formation of pseudoaneurysms were reported. In addition, even under extracorporeal circulation, the leakage of totally heparinized blood from its wall is minimal. However, the woven Dacron prostheses are very rigid and hard to handle. Moreover, low water porosity of these prostheses interferes with tissue regeneration. A woven Dacron prosthesis of middle porosity with collagen coating has been introduced recently in this field to minimize the graft stiffness and blood leakage through the graft wall; however, the sealant of the prosthesis results in the same situation as that of the knitted fabric Dacron prostheses mentioned above. During our clinic experience described in this paper, the UFPF graft was soft like a highly porous knitted prosthesis, although it has a low porous woven structure, showed no bleeding problems, and required no troublesome sealing materials.

As described above the ideal aortic prosthesis should have high durability, easy handling, and high affinity with living tissues. To achieve high durability by avoiding dilatation and shortening of the grafts after long-term implantation, woven structure is though to be indispensible. We have developed a low porosity woven graft made of UFPF of 3 μm in fiber thickness, which contribute softness and pliability providing an excellent manipulative quality with no cut edge fraying in comparison with those made of conventional Dacron fibers 12 μm in diameter. It has been emphasized that UFPF has a high affinity with living cells. The prosthesis made of UFPF also exhibits excellent affinity with living tissues. The grafts implanted into experimental animals exhibited a rapid healing process despite the rather low water permeability (100 ml/min/cm^2) (1). These phenomena have been explained by the following hypothesis. Cells in general have a special affinity with very fine fibers or some sharp edges. Cells want to adhere along these fine fibers or sharp edges. This phenomenon is known as "contact guidance." It is a kind of mother nature of cells. In the case of fibroblast cell cultures in vitro, we have observed this phenomenon frequently.

In the case of blood filters made of another type of UFPF (21), it has been reported that leucocytes accumulated around the UFPF selectively due to this phenomenon of contact guidance. Leucocytes in the blood actively adhered to the UFPF by themselves. Therefore, we could collect the leucocytes selectively from the blood (21). While in the vascular graft made of the UFPF, fibroblasts want to adhere to the UFPF actively resulting in a great number of fibroblasts migrating along the UFPF inside the graft wall. These migrated and proliferated cells require nutrition. Therefore, capillary blood vessels follow the fibroblast migration. With this sequence the graft wall can acquire natural angiogenesis. Therefore, the mechanism of rapid healing observed in the UFPF grafts is mainly due to the UFPF nature itself and not explained by the hypothesis of water porosity advocated by Wesolowski (22). In this study we had no opportunity to obtain the grafts from the patients, but we are expecting improved healing of the neointima in the UFPF grafts that have been implanted in patients, compared with that of the ordinary Dacron prostheses.

Though the grafts are made of UFPF, their durability was enhanced by using polyester fibers (12 μm) of conventional thickness concurrently. Therefore, mechanical strength is well preserved even after being subjected to three billion load cycles in a fatigue test (3). Moreover, using fiber entanglement and double velour structure, suture retention of UFPF grafts is rather high compared with that of conventional low porosity ~~Dacron~~ grafts (Cooley Low Porosity Woven Dacron Graft, 50 ml/min/cm^2, Meadox Medicals Inc., NJ) (3). These results show that the UFPF graft will exhibit enough durability upon long-term implantation into high pressure pulsatile circulation systems.

Prior to the clinical application of the UFPF graft,

we have investigated time-dependent changes of the blood leakage from the graft wall in comparison with that of a conventional Cooley graft (8). Both grafts of 26 mm ID and 5 cm in length with no pretreatments were assembled into an in vitro circuit model filled with totally heparinized (more than 1,000 s in ACT) human blood diluted with phosphate buffered Ringer's solution (20% in hematocrit, donated from volunteers) with a luminal pressure of 100 mm Hg. The results showed that the UFPF graft exhibited a significantly larger volume of leakage than that of the Cooly Grafts for the first min ($p < 0.05$). However, 2 min later, the leakage of both groups decreased rapidly, and there was no significant difference between them (Fig. 5). Therefore, we concluded that the UFPF graft is applicable for arterial reconstruction even under totally heparinized extracorporeal circulation. Based on this result, we applied presealing in a precise manner suitable for each case prior to implantation in order to avoid the first 1 min of profuse hemorrhage as described above. Actually, as shown in this communication, application of suitable treatments resulted in little hemorrhage from the graft wall even under totally heparinized extracorporeal circulation of ultrahypothermia as well as in little deterioration in its excellent handling performance.

CONCLUSION

In conclusion, the UFPF graft demonstrated safety related to the blood leakage resistance without any use of sealants and no cut edge fraying, and it also exhibited easy handling due to its softness and elasticity. The graft is also expected to have an excellent affinity with host cells due to its ability to induce cell migration into the graft wall using the nature of cells, eventually leading to endothelialization with natural angiogenesis. The graft could be used safely with patients with hemorrhagic diathesis, poor risk, old age, poor nutrition, and a low ability of wound healing.

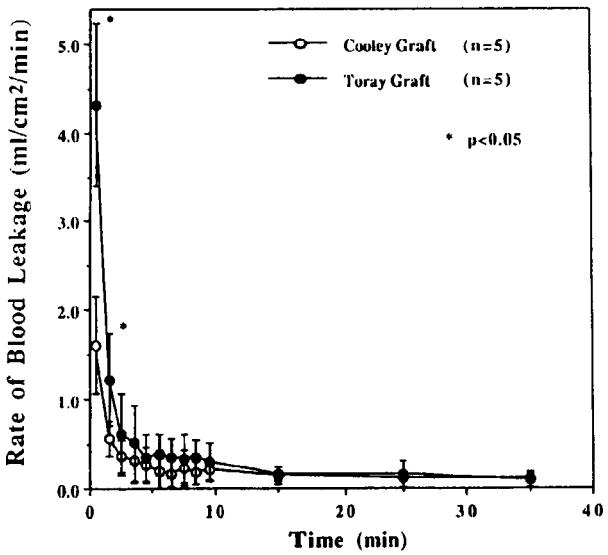

FIG. 5. Time-dependent change of the totally heparinized human blood leakage from the graft walls with no presealing, which was measured using an in vitro circuit system, is shown.

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●解説

超極細繊維の医用材料への応用

野一色泰晴

I. はじめに

生体の材料に対する反応は繊細微妙である．それは分子レベル，細胞レベル，細胞集団レベル，個体レベルで反応様式が異なり，材料側の条件によっても大きく変わる．しかし，この複雑な反応の中にも法則性があり，その根本は細胞の本能的性質や，生体を構成する諸分子等の物理化学的性質に基づいている．人工臓器の設計にはこの法則性を十分に理解しておくことが不可欠である．

最近の知見としては，細胞の本能的性質の一つとして，細胞は材料の化学的，物理的な一般的変化のみならず，サイズの変化にも微妙な反応を示すことが明らかにされてきた．たとえば，微粒子（マイクロスフィアー）に対する顆粒球や血小板の反応では，そのサイズの変化によって貪食能が変わっていく[1,2]．これは細胞が材料のサイズを識別していることを示している．細胞のこのような本能的性質に研究者の目が向けられるようになってきた．

たとえば西村ら[3]，梅香家ら[4]による超極細繊維を用いたリンパ球除去フィルターの開発はその良い例である．フィルターに用いる繊維を徐々に細くしていくと，3.0 μm以下のところでリンパ球が高い親和性を繊維に対して示す．この特異的反応を用いてリンパ球を選択的に取り除くというものである．

われわれもこれまで超極細ポリエステル繊維（ultra-fine polyester fiber, 以下UFPFと略す）に対する細胞の特異的反応を利用して新しい人工臓器を試作した．この度はそれらを通じて得た知識のいくつかを紹介したい．

II. 超極細繊維

繊維材料は手術衣，白衣といった一般医療用，人工臓器のような機能性医療用等，広く医療に貢献している．繊維材料のうち，綿や絹等の動植物由来繊維はその太さが定まっているが，ポリエステルやポリプロピレン等の合成高分子繊維は紡糸段階において繊維径を変化させうる．しかし，溶融紡糸，エマルジオン紡糸といった一般の方法では3 μm程度の細さが限界である．そこで，さらに細い繊維を作る工夫が試みられた．現在工業的に行なわれている方法には，紡糸の最終段階において溶融ポリマーを口金から吐出させる際に，高圧高温空気やポリマーと溶媒とを共存させて，一気に噴出させることにより，あるいは強い電界をかけて引き出すことによって，きわめて細い繊維のネット状構造物を得る方法がある．この方法で作った場合，長い繊維はできず，不織布状となるため，力学的強度はもたせられないが，単位重量当りきわめて表面積の広い構造物が得られ，フィルターや特殊物質の固定化の基材として，あるいは細胞培養用基材として等，有用である．

超極細繊維を得る他の方法として高分子配列体繊維法[5]がある．具体的にいえば，多数の連続した主繊維を，溶解しやすいポリマーとともに束にして，従来の方法にそって口金から引き出して細くし，そののち束ねていたポリマーを溶解して細い主繊維のみを残すものである．口金から出す1本の繊維の細さには限りがあるが，束にして引くと，その断面は金太郎飴のごとき状態となっており，細く引き伸ばしても断面は細いまま相似形を保つため，束となったまま細く，細く引き伸ばされてゆく．そして周囲を束ねていたポリマーを溶かすことによってきわめて細い繊維の集合体を得る（図1）．この方法だと長い連続した繊維が得られることから，丈夫な人工臓器の素材として使用可能である．以下に述べる著者らの研究はこの方法によって得た繊維の「細さ」の特性を生かしたものである．

III. 低有孔性，柔軟性，高治癒性，耐ほつれ性をもつ人工血管

一般に低有孔性人工血管は硬くて縫合しにくく，新生内膜の形成不全や脱落，潰瘍化が生じやすいが，UFPFを用いるとこれらの欠点を解消できるので[6]，ここに紹介する．

図2に作成した人工血管（ss-graft）および市販の低有孔性人工血管（woven graft）を示す．いずれも斜方向に切れ目を入れ，切断端でのほつれ程度を示しているが，市販の人工血管は平織りであって，ほつれやすい．しかし

Application of Ultra-Fine Synthetic Fibers to Biomedical Materials. **Keywords**: ultra-fine polyester fiber, vascular prosthesis, cardiovascular substitute, autologous connective tissue tube graft, cell culture matrix

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効率化はどうか等，一つ一つ考え直す必要がある．またさらに，これまで数多くの物質が人工臓器用素材を目指して開発されてきて，そして優れた特徴がないものとして忘れ去られているが，これら一つ一つにも，細くすることによって，思いがけぬ特性が期待できるかもしれない．

本項で示した例はわれわれの取り扱ってきた UFPF に関する研究の一部にすぎないが，このような考え方を人工臓器全般にわたって広めていくと，新しい人工臓器が次々と開発されていくように思われる．一つの素材が工夫しだいでこの領域では全体をも変えていく可能性をもっている．エレクトロニクスの世界でも，真空管からトランジスター，そしていまでは超 LSI の時代に変わってきたように，人工臓器領域においても，超極細繊維の出現は次世代の人工臓器に大変革を来す可能性を秘めている．

本稿をまとめるにあたり，京都府立医科大学第二外科・佐藤伸一先生，東京女子医科大学・小柳 仁先生，大越隆文先生，富沢康子先生，東レ基礎研究所・車谷 元先生，東レ繊維研究所・渡辺幸二先生，東京農工大学工学部・赤池敏宏先生，旭メディカル大分工場開発研究所・梅香家鎮先生，日赤中央血液センター・宮本正樹先生，通産省工業技術院繊維高分子材料研究所・一條久夫先生に情報のご提供を受けたことを記し ここに感謝の意を表します．なお本研究の一部は，文部省科学研究費重点領域研究 試験研究，一般研究C等の費用によって行なわれた．

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皮下脂肪組織細切片によりシールされた高有孔性人工血管

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高有孔性布製人工血管は種々の優れた性質があるが、術中、術後の出血の問題があり臨床では余り使用されない。我々はこの原因が Preclotting にあるとして、Preclotting に変わるシール方法を開発した。方法としては、自家皮下脂肪組織を細切し、人工血管繊維間隙に圧注入して絡め、Preclotting の代わりのシールとするもので動物実験の結果、出血問題はまったく生じなかったばかりか、人工血管全面にわたる良好な内膜治癒が得られた。対照とした Preclotting を行った人工血管では２１％に術後の出血死がみられたほか、出血死しないまでも人工血管周囲の血腫がみられた。皮下脂肪組織は動物種、年齢、栄養状態等によっても異なるため、この結果を直ちにヒトへの臨床応用へ結び付けることは出来ないが、自家組織による人工血管のシール法の道を開くものである。

Noishiki Y, Yamane Y, Kajiwara H, Hoshino K, Ishii M, Ichikawa Y, Suzuki S, Kosuge T, Mo M, Imoto K, Tomiyama I, Yamazaki I, Kondo J, Matsumoto A. **A VASCULAR PROSTHESIS SEALED WITH AUTOLOGOUS ADIPOSE TISSUE FRAGMENTS INSTEAD OF PRECLOTTING BY FRESH BLOOD.** A highly porous fabric vascular prosthesis sealed with autologous adipose tissue fragments into the wall was evaluated in an animal study. A piece of subcutaneous adipose connective tissue approximately weighing 2 g was minced with scissors and stirred into 20ml of saline, thereby creating a tissue suspension. A fabric vascular prosthesis was turned inside out. The suspension was poured into the closed prosthesis by repeated pressurized injections using a syringe. Then, the prosthesis was turned inside out again. Consequently, the tissue fragments were squeezed into the prosthesis wall and sealed all the pores of the prosthesis. The treated grafts were implanted into the thoracic descending aortae of 24 mongrel dogs. No bleeding was noticed with the graft during and after implantation. Neointima with complete endotheloalozation was observed in all the grafts implanted for more than 30 days. Twenty-four preclotted prostheses were used as the control. Although the chest wall was closed after confieming that the bleeding from the suture line and through the prosthesis had stopped, problems occurred after surgery in the control grafts. Five out of 24 control animals (21 %) bled in the pleural cavity and died within 24 hours after implantation. Neointima formation in the control grafts was limited near anastomotic sites in all cases up to 39 days of obserzation period. These results indicated that the sealing method was an ideal procedure of fabric vascular prostheses for cardiac wall substitutes. The results from an animal experiment could be expected in human if the property of the adipose tissue from human has a similar ability.**KEYWORDS:** Vascular prosthesis, Preclotting, Autologous tissue sealing, Neointima, Fibrinolysis
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はじめに

高有孔布製人工血管が植え込み後良好な内膜治癒を得ることは Wesolowski らの一連の研究で明らかにされており[1)2)]、臨床的にも、高有孔性人工血管の使用が勧められ、そのための Preclotting 法[3,4,5)]が報告されてきた。しかし現実には Wesolowski 等の言う高有孔性人工血管は出血の危険性が高く、たとえ術中に出血しなくても術後に高頻度で出血を生じている[6)]。我々はこの問題の解決のため Preclotting に代わり、出血問題のないシール方法の工夫を行ってきた。このたびは自家の皮下脂肪結合組織で人工血管繊維網を目詰まりさせシールする方法を開発したので報告する。

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材料と方法

Ⅰ．人工血管のシール方法

が遅れたり、線維素溶解現象が急激に生じると高有孔性人工血管の編み目を充分に置き代われずに出血することがある。この危険性を防止するため、アルブミンやコラーゲン、ゼラチンなどで人工血管をシールする方法がこれまで多く報告され、現在も臨床で広く用いられている[7,8,9,10]。これらの報告では良好な抗出血効果が示され、その内膜治癒も良好であると報告されている。しかし我々の用いた方法は自家組織であることから、自家組織の移植材料における有利性が期待されると思われる。

我々は、すでに自家静脈片を人工血管に播種する方法を報告した[11]。これによると、播種された、静脈の細切片より多数の内皮細胞、平滑筋細胞、線維芽細胞が遊走増殖し、急速に新生血管壁を形成した。このたびの脂肪組織片では、静脈片を用いた場合ほどの急速治癒は得られなかったが、それでも自家組織片移植の有利性は明らかで、植え込み後1カ月で新生内膜は完成し、しかもその治癒は吻合部中央部などの部位に関係なく一様に進行したことから、自家組織移植による治癒促進がみられたと思われる。

一般に自家組織移植法は、整形外科、形成外科領域に置いても既に使用されており、遷延する骨折や難治性皮膚潰瘍の治療に骨や皮膚の細切片が使用されている[12,13]。また、心血管系においても、無孔性の人工心臓壁やコネクターに自家組織片を圧入した報告もある[14,15]。我々はこのような利点も利用し、新しく高有孔性布製人工血管のシール法として、これを開発することが出来た。

脂肪組織は動物種、年齢によっても異なるため、本研究の成果より人への外挿はできないが、条件さえ得られると、臨床応用も可能な方法ではないかと考えている。

おわりに

高有孔性人工血管のシール法を開発し、Preclotting法に代わる安全な処置であることを動物実験で確認した。この原理を用い、他の採取しやすい組織を用いたシール法も今後追求してゆく予定である。

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人工臓器 21(1), 290-294 (1992)

自家静脈片播種型人工血管の新生血管壁形成過程とその長期例の検討

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自家静脈片を細切し，布製人工血管に播種して植え込むと内膜治癒が急速に進行する．これまでの基礎研究でこれを明らかにしたことから次の問題としてこの型の人工血管における新生血管壁の特徴とその長期例での壁の変性，石灰化，破裂，自家組織片による弊害について検討した．方法として成犬頸静脈を採取し，細切して20ccの生理的食塩水にいれて組織細切片浮遊液を作り，これを高有孔性人工血管内に圧注入することにより，組織片を人工血管壁に絡めた．次にPreclotting 操作によって網目をシールし，頸静脈を採取した同一犬の胸部大動脈へ自家移植の形で植え込み，35頭の動物から植え込み直後より 495日に至るまでの試料を採取し対照のPreclotting のみを行った25頭の成犬から得られた試料を対比した．その結果内膜治癒は良好であるばかりか，光顕的にも変性像は認められず長期間にわたって安全であることが確認された．

Noishiki Y., Yamane Y., Kajiwara H., Hoshino K., Ishii M., Suzuki S., Kosuge T., Mo M., Imoto K., Tomiyama I., Yamazaki I., Ichikawa Y., Kondo J., Matsumoto A.: HEALING PROCESS AND LONG-TERM EVALUATION OF FABRIC VASCULAR PROSTHESES TRANSPLANTED WITH AUTOLOGOUS VENOUS TISSUE FRAGMENTS　Fabric vascular prostheses transplanted with autologous venous tissue fragments showed extremely rapid healing of neointima in our previous study.　In this communication, stability of the neointima in long-term was evaluated in an animal study.　Highly porous fabric vascular prostheses (water porosity:4,000) were implanted into the descending aortae of 35 dogs, and were explanted from 1 hour to 495 days after implantation.　A highly porous graft with preclotting was used as the control after the sealing by the original preclotting technique in 25 dogs (1 hour to 783 days).　The neointima with the transplantation of autologous venous tissue fragments showed no degenerative changes such as calcification nor ulceration in the neointima. Control prostheses showed also no degenerative changes, however, in 9 out of 25 cases, hemothorax or perigraft hematomae were noticed.　From these observations, stability of the neointima of fabric graft with autologous tissue fragments transplantation was confirmed clearly in this animal study.　KEY WORDS:　Autologous tissue fragments transplantation,　Neointima,　Degenerative changes,　Calcification,　Fabric vascular prosthesis.
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はじめに

新生血管壁形成は臨床においては著しく遅延することが報告されている[1]．動物実験においても高有孔性人工血管以外，内膜治癒は吻合部付近には認めるも人工血管中央部分は内皮細胞による被覆が認められず，長期間にわたって血栓が付着し，人工血管は閉塞の危険性が高い状態にさらされている．この問題を解決するため我々は自家静脈細切片を布製人工血管に播種することで内膜治癒を促進させる方法を開発し予期した成果を得た[2,3]．そこでこの度は新生血管壁形成過程を示すとともに，このようにして急速に形成された新生内膜がはたして長期間安定して存在し続けるものか，新生内膜に変性，石灰化などが生じることはないか等の検討を行い，総合的に長期の安全性について解析をしたので報告する．

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材料と方法

Ⅰ．人工血管の作成

成犬頸静脈脈10cm，重さ0.3gを採取し，これを剪刀にて細切し，20ccの生理食塩水にいれて組織細切片浮遊液を作成した．次に高有孔性ダクロン布製人工血管（MICROKNIT，Golaski Lab. Inc, Water Porosity :4000cc）を用い透明の塩化ビニール袋にいれ，その一端を三方活栓を介して組織浮遊液を入れた注射器に結び，他端を止

変性が生じなかったとも考えられる．我々の過去の研究[4]やWesolovskiらの研究[5,6]においても人工血管に形成される新生内膜が変性に陥り，石灰化が認められる．しかしWesolovskiの主張するように高有孔性人工血管においては石灰化は生じにくいのかもしれない[5,6]．本研究において，使用した人工血管が Water Porosity 4,000cc ときわめて高いためPorosity の理論から言えばPreclotting 人工血管，静脈片人工血管とも，Preclotting や細切片播種といった操作に関係なく変性を生じないと推測される．　一般に高有孔性人工血管にはこのような利点があるが，術後の出血の危険性があって[7]，広く臨床に使用されるに至っていない．多くのPreclotting 法が報告[8,9,10]されているがそれでも出血の危険が指摘されている．本研究で用いた静脈片播種法は，それらに比べて，出血例が全くなかった．これは組織片が高有孔性人工血管に強く絡まって繊維素溶解現象によるフィブリン溶解に対しても，人工血管が全く裸になるようなことがなかったからであろうと推測される．対照例で出血した例はすべて，人工血管線維が露出しており，Preclotting の血栓が完全に消失していたことを考えると，我々の方法は短期間の結果を見ても安全な方法といえよう．また，内膜形成促進についてはこれまで報告されてきた内皮細胞を播種する他の方法[11,12,13]に比べても我々の方法が確実である点はすでに報告した通りであることから，この度の長期例の安全性格確認と合わせて本研究で示した方法は臨床応用に大きな期待がもてることであろう．

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人工臓器 21(3), 1207-1211 (1992)

In vivo の特性を生かした組織培養技術応用人工臓器

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In vivo における組織培養では、In vitro においては予測のつかなかった様な細胞の活性、細胞特有の働き、さらに細胞相互の関係がみられる。これらの作用を利用すれば、新しい人工臓器を作らせる可能性があることを人工血管の例を用いて説明したい。具体的な参考例としては、自家静脈片を細切し、布製人工血管壁に播種したところ、In vitro では線維芽細胞のみが増殖し、内皮細胞や平滑筋細胞等の増殖が抑制されるはずであるのに、In vivo ではこれらの諸細胞がすべて同時に活発に増殖し、それぞれの住み分けをした結果、新しい血管壁を作ることができた。異種細胞が共存すると、互いに特殊な関係をもち、影響を及ぼしあって独自の機能を発揮することが知られているが、本実験ではIn vivo において、この現象を容易に得られることから、新しい人工臓器をIn vivo 培養することで形成させることが可能となることを示していると思われる。

Noishiki Y, Yamane Y, Kajiwara H, Hoshino K, Ichikawa Y, Ishii M, Kosuge T, Mo M, Imoto K, Yamazaki K, Kondo J, and Matsumoto A. A NEW HYBRID ARTIFICIAL ORGAN WITH A CHARACTERISTIC ADVANTAGE OF IN VIVO TISSUE CULTURE TECHNOLOGY An applicability of a new technology of tissue culture in vivo on a hybrid type artificial organ was discussed with data of an animal experiment of vascular prosthesis which showed a rapid healing of neointima. The prosthesis has transplantation of autologous venous tissue fragments in its wall. After implantation of the graft in vivo, endothelial cells, smooth muscle cells as well as fibroblasts migrated and proliferated from the tissue fragments at the same time, resulting to form a neointima. This was a very unique phenomenon that is not observed in cell culture in vitro; when fibroblasts and endothelial cells are cultured in a petridish, endothelial cells are suppressed and fibroblasts proliferated to form a confluent layer. However, in this experiment, endothelial cells rose up to the inner surface of the graft to face the blood stream. Accordingly, smooth muscle cells made a multilayer underneath the endothelial cells, and fibroblasts crawled down under the smooth muscle cells layer around the Dacron fibers to encapsulate them. These different kinds of cells made a new organ with their cooperation. This phenomenon suggested that the active but controlled growth of these cells was regulated by nature and their interactions in a physiological environment. This natural activity of cells in vivo will be applicable to form new artificial organs of hybrid type in various fields. KEYWARDS: Hybrid artificial organ, in vivo cell culture, vascular prosthesis, neointima, tissue fragments transplantation.
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はじめに

人工臓器が広く臨床に用いられるようになった今日、さらに高機能を持つ人工臓器の開発が望まれるようになったが、このような機能を発揮させる一つの方法として、細胞や組織を組み込んだ、いわゆるハイブリット型人工臓器が考えられている[1)2)3)]。そのひとつの手段として、一般的には、特殊機能を持つ細胞をIn vitro で培養し、合成高分子材料との組み合わせにより、細胞の機能を引き出そうとしている。しかし、In vitro での細胞培養、細胞と材料との関係などにおいて、不明な点、問題点等もあり、予期した成果が出ていない。

我々はこのような現状を打破する一つの方法として組織のIn vivo 培養による器官形成を行わせることによって、新しい人工臓器を造る可能性を追求してきた[4)5)]。このたびは、人工血管を例にとり末梢静脈細切片の布製人工血管壁への播種による新生内膜形成を示し、考察を進めてゆきたい、この為、その討論のための資料を以下に示す。

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材料と方法

1．高分子材料への生体組織の播種

成犬頸静脈約10cm、重さ0.3gを採取し、これを剪刀にて細切し、20ccの生理的食塩水中にいれて組織細

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成長できる人工血管の長期例の解析

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患児の成長に合わせて徐々に口径が太くなり，成長してゆく人工血管の開発を行ない，動物実験にて長期例の経過を観察し，臨床応用への可能性を検討した。素材にヒト大伏在静脈を用い，浸透圧と超音波処理して膠原線維および弾性線維からなる管を作成した。これを親水性エポキシ化合物（デナコール）で架橋し，外側を太い口径のポリエステルメッシュチューブで覆った。15頭の小犬胸部下行大動脈に内径4㎜，長さ3.5～4㎝の人工血管を植え込み，1年後に内径9.5㎜に成長するのを確認した。成長後の長期例8頭の観察では，2年11ヶ月後も期待した口径を維持し，動脈瘤様変化が生じていないこと，および石灰化などの，乳幼児へ植え込んだ生体由来材料を用いた人工臓器に生じやすい変性が全く生じていないこと等を明らかにした。この結果臨床応用上からみて問題がないことが明らかとなった。

Noishiki, Y., Yamane, Y., Satoh, S., Niu, S., Murayama, U., Tomizawa, Y., Okoshi, T., and Koyanagi, H. LONG-TERM OBSERVATIONS OF A GROWABLE VASCULAR GRAFT INAN ANIMAL STUDY Long-term animal study of a vascular graft that can grow with the growth of its recepient was performed. Human saphenous vein was used as the substrate material. It was dipped into distilled water and sonicated, resulting in cell destruction, and followed by cross-linking with polyepoxy compound. Four milimeter inner diameter (ID) grafts, enveloped with polyester mesh tubes of 10 ㎜ ID, were implanted in the thoracic descending aortae of 15 puppies. The diameter of the grafts grew to 9.5 ㎜ from original 4 ㎜ after 1 year. Eight animals were used for the long-term observation of more than 300 days till 2 years and 11 months. The grafts maintained their expected size after their full growth. There was no degenerative changes such as calcification of the graft wall. The light microscopical observations revealed that the original saphenous vein graft was absorved after 1 year. Neoarterial wall with full endothelialization was formed with polyester mesh reinforcement. These results supported that the graft has high fesicability of clinical use without any problem. KEYWORDS: Growable vascular graft, Small diameter vascular graft, Denacol, Biodegradable graft, Calcification.
Jpn J Artif Organs 19(3), 1292-1296 (1990)

はじめに

患児の成長にともなって口径が太くなってゆくように設計された「成長できる人工血管」の開発を我々は行なって，これまでに成長経過や治癒像などを報告してきた[1,2,3]。その原理は生体内で劣化してゆく材料で人工血管を作ることにより，次第に動脈瘤様の拡張してゆく現象を利用し，目的とした太さに至ったとき膨らみを停止させるよう，あらかじめその太さのポリエステルメッシュ管で外側を覆っておくものである。成長速度は材料の化学処理程度で制御できる。この度はこのような設計の人工血管が長期間体内にあっても安全に人工血管としての役割りを果たすことができるか否かについて検討し，臨床応用の可能性を考察したので報告する。

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材料と方法

I 人工血管の作成

ヒト大伏在静脈を蒸留水に1時間以上浸漬して細胞成分を浸透圧によって膨満させたあと，超音波処理してそれを破壊し，主として膠原線維と弾性線維からなる管（脱細胞管）を得た。次にこれを2％デナコールEX861（ナガセ化成，大阪）で架橋処理し，人工血管とした[2,3]。これとは別に外側を覆う補強材として超極細ポリエステル繊維[4,5]で作成したメッシュチューブ内径10㎜を用意し，EOG滅菌した。これらの組み合せを図1に示す。

II 植え込み実験

15頭の小犬（体重3.5～7.0㎏，平均4.9㎏）の胸部下行大動脈を3㎝にわたり切除し，ここに作成した人工血管（内径4㎜，長さ3.5～4㎝）を端々に連続縫合した。次にその外側をポリエステルメッシュチューブで覆い，その両端は人工血管と宿主血管との吻合部を越えた宿主血管壁に結節縫合固定した。なお手術時には胸腔内

認めた。形質細胞，巨細胞，リンパ球等の侵入はみられなかった。157日目の例では内面は内皮細胞に覆われるとともに，人工血管壁全層を通じて宿主の線維芽細胞が侵入していた。339日目の所見では，末梢側吻合部付近でオリジナル人工血管壁内部に無数の平滑筋様細胞がみられ，それらは人工血管の輪状方向にその長軸を平行にして並んでいた。静脈片構造は消失していた。523日目および600日目の試料では，全体にわたって静脈片構造は消失しており，線維芽細胞を主体とし，表面を内皮細胞で覆った新生血管壁が完成していた。なお，内膜の脱落，潰瘍化などの変性はみられなかった。新生血管壁の石灰沈着を観察する目的で行ったフォンコッサ染色では，植え込み後339，523，600，600日目の各試料において，陽性に染まる部分はみられなかった（図4）。

考　　察

最近の外科手術手技および術前後の患者管理の進歩によって，乳幼児に対する手術は良い成績を納めるようになったとともに，さらに進んだ手術も行われるようになった。これらの手術のうちのいくつかは当面の状況を改善し，現状維持を計ることを目的としており，患児の成長に伴う変化に合わないものがある。例えば，血管の狭窄閉塞に対するダクロン製人工血管使用の手術では，手術時に適切であっても患児の成長によって人工血管部分は次第に狭窄を生じるようになる。このような不便さを解消するため，我々は「成長できる人工血管」の開発を行ない，動物実験を積み重ねてきた[2,3]。本研究の結果から明らかなように，作成した人工血管は予期した通り1年間かかって徐々に膨らみ，1年半後に完全に停止し，その後2年11ヶ月経ても過剰な膨らみは生じておらず，期待した太さを維持していた。光顕的観察では植え込み時に用いた静脈片は1年かかって消失し，宿主細胞が新しい血管壁を形成していた。

以上の結果から，周径方向の成長は予期した通りであり，その停止も確実であった。そしてさらに結果を詳細に観察すると，長軸方向にも人工血管が変化していることが判断できる。3.5～4 cmの人工血管を植え込んだため，縫いしろ部分を差し引いて，実際は3.3～3.8 cmである。事実，植え込み47日目では3.9 cmであったのが，523日目の試料で5.0 cmとなっている。そして外側に覆ったポリエステルメッシュの蛇腹構造が徐々に伸びてきており，339日目の試料では人工血管の消失部分は完全に伸び，末消失部分はまだ伸びきっていないことが判る。523日目の試料では人工血管は完全に消失し，蛇腹構造も消えている。このような事実から，この人工血管は長軸方向にも伸びる，すなわち「成長」し，そして停止し，その後その状態を維持していることが明らかである。

このような過程にあって，人工血管としての基本的条件である「血液を凝固させることなく流す」ための内面の抗血栓性については，植え込み12時間後の例で明らかなように，内面には血栓は付着していない事実より，人工血管にある程度の抗血栓性があることがわかる。この人工血管は親水性架橋剤で化学処理されており，高含水性となっているため，軽度の抗血栓性を得ている[6]ものと我々は考えている。そしてその後は内皮細胞が内面を覆い，永久的，天然の抗血栓性を期待したのであるが，47日目の試料で明らかなように，すでに全内表面は内皮細胞によって覆われていたことより，我々の期待した通りになっており，将来にわたって血栓は付着しないと思われる。この内皮細胞の被覆速度は一般の布製人工血管における治癒過程[7]に比べると著しく速い。これは本研究で若い動物を用いたこと以外，材料が細胞親和性に富んでいたことが考えられる。使用した親水性架橋剤は従来から用いられているグルタールアルデヒドに比べ，処理後の材料の細胞毒性は低く，しかも親水性で，細胞親和性が良いことが指摘されている[8]。このようなことから，元来細胞親和性の良いコラーゲンマトリックスを用い，それを細胞親和性の良い状態を保つべく化学処理しているため，自然な細胞の動きによる治癒が人工血管表面で得られたことと考えている。

本人工血管は結果でも明らかなように，オリジナルの人工血管の破壊と，新生血管壁の新生とが併行して同一場所で行われている。これらはバランス良く，互いに補いつつ行われることが望ましい。しかし157日目の試料にみられるように，壁の一部に血栓が付着し，新生血管壁形成が良好には行われていないことを示している。これは破壊が一時的にしろ急激に進み，壁の形成が追いつかなかったことによると思われる。オリジナルの人工血管壁は徐々に吸収されてゆくにしても，血圧が常にかかっているため，ある程度まで力学的強度を保って，形態を維持していたのが，ある時期に持ちこたえられなくなり，大きな亀裂が入ったものと思われる。このような力学的作用の場では，連続した現象は得にくく，時には地層のズレが地震を起こすように，連続した力が波状的現象となって現われてくるものと思われ，本人工血管においても，前述の2つの動きが必ずしも予期した通りバランス良く進行しているものではないことを示している。

成長できる人工血管の条件として，①小口径人工血管としての抗血栓性を有すること，②徐々に成長できること，③成長が予定した時期に予定した太さ，長さで停止すること，④形成される新生血管壁が長期間にわたって安定していること，等が挙げられる。本研究では①～③は確実であることが実証されたが，④においても300日以上経過例をみる限り，最長2年11ヶ月まで④の条件は満足されているように思われる。すなわち，肉眼的にみて，動脈瘤形成や狭窄といった変形は生じていないし，光学顕微鏡的にみても，壁の石灰化像はみられていない。成長できる人工血管の需要は当然乳幼児においてであり，乳幼児が生体由来材料の植え込み術を受けると，材料が短期間のうちに高率に石灰化することが報告されている[9,10]。従って，本人工血管においても石灰化の危険性が高いことが考えられるが実際には石灰化は認められてい

ない。この理由として我々は以下の2つを考えている。すなわち，①材料を親水性エポキシ化合物で架橋していること，②その材料は生体内で吸収されてしまうこと，である。これまで親水性エポキシ材料で架橋された人工血管の長期植え込み例や，皮下に埋入された心臓弁片で石灰化が生じていないこと，グルタールアルデヒド処理したものに比べ石灰化率が著しく低いこと等が報告されている[7,11]。これらのことを考えると，本研究に用いた架橋剤が石灰化を防ぐ意味でも優れていたと考えられる。次に②の項目であるが，本研究ではオリジナルの材料は消失し，代って宿主自身の作った新生血管壁が残ることが判っている。すなわち，材料の石灰化が進むのに，その材料が吸収されてしまうため，石灰化をする場がないこととなる。また，残るのは自分自身の組織，すなわち自家組織であり，一般に自家組織の石灰化はまれである。このようなことから石灰化がさらに生じにくかったものと思われる。

以上のすべての結果を考えると，ヒトと動物とは状況は異なるけれど，動物実験結果すべてをまとめた上でヒトへの外挿線を引いた場合，現在のデータからみる限り，本人工血管の臨床への応用は安全であると思われ，また期待した成長も生じると思われ，さらに，乳幼児に用い，長期間にわたる植え込みを行っても安全であろうと予測される。

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小口径人工血管

野一色泰晴

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人工臓器 17(2), 578-581 (1988)

柔軟性をもつ抗癒着性人工心膜

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ヘパリンを徐放する柔軟な構造の抗癒着性人工心膜を作成し、動物実験を行った。その結果、8例中5例に癒着はなく、1例に炎症性癒着を認め、残り2例は長期安全性試験のため、管理中である。対照群としてグルタールアルデヒド処理心膜を用いたが、5例中4例に癒着は認められず、1例は術後失った。採取した膜は対照群に比べて柔軟であり、膜の接する心臓表面へ与える影響は柔軟性に比例して、対照群より軽度であった。ヘパリンのフィブリン析出阻止作用は人工血管内面への抗血栓性賦与のためすでに利用された方法であるが、癒着に先行して創面にフィブリンが析出すること、およびフィブリン析出を阻止すれば癒着組織は形成されないことの理由から、ヘパリン徐放性癒着防止膜は腸管の癒着阻止用にすでにその効果が実証されている。本研究ではそれを人工心膜に応用したこと、および膜を柔軟にすることで心臓表面へ与える機械的刺激を軽減させたこと、そしてヘパリン放出後の人工心膜表面を天然の抗癒着性をもつ漿膜細胞により覆わせたこと等が成功のかぎであった。

Noishiki, Y., Miyata, T., Furuse, M., and Yamane, Y. HEPARIN SLOW RELEASE ANTIADHESIVE PERICARDIUM WITH NATURAL TISSUE ELASTICITY. Antiadhesive pericardium with heparin slow release was newly develcped. Fresh porcine pericardium was soaked in distilled water and sonicated in order to cause cell destruction. In this way, a natural tissue membrane composed of collagen was obtained. The membrane was cross-linked by a hydrophilic epoxy compound instead of glutaraldehyde to maintain the natural tissue elasticity and hydrophilicity, and heparinized ionically using protamine as a binding site. Heparin prevents the precipitation of fibrin, which leads to cellulofibrous adhesive tissue. Therefore, the membrane with heparin slow release can prevent the adhesion in its early stage. Animal experiment revealed that the membrane had excellent antiadhesive property. The pericardium in the long term experiment was covered with a layer of mesothelial cells which has a natural and permanent antiadhesive property. KEYWORDS, Pericardium, Antiadhesive membrane, Heparin, Slow release of heparin, Polyepoxy compounds.
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はじめに

近年の心臓外科手術の進歩は目覚ましく、従来では絶望視されていたいくつかの疾患もその恩恵をこうむるようになってきて、手術適応疾患数および症例数が急速に増加している。これらの手術件数の増加に伴い、最近では再手術、再々手術の件数も増加している。ところが、心臓における再手術、再々手術は初回手術と同等もしくはそれ以上の危険性、困難性を伴うことがある。その原因の1つに心膜面への周囲組織の癒着が挙げられる。その癒着剝離には多大の時間と労力とを要し、思わぬ合併症発生をまねくことがある。これらの困難点を解消するため我々は抗癒着性人工心膜を試作し、動物実験にてその有用性を明らかにしたので報告する。

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材料と方法

I 人工心膜の作成

新鮮ブタ心膜を蒸留水中に1時間浸漬し、超音波処理(28KC、20秒間)して細胞成分を破壊したあと、よく水洗し、細胞破壊片を流すことにより、主としてコラーゲンからなる生体由来の膜（脱細胞膜）を得た。次にこれを凍結乾燥したのち、5％プロタミン液中に浸してコラーゲン線維間隙にプロタミンを含浸させ、膜を膨潤させたあと、2％親水性エポキシ化合物（デナコールEX－313、ナガセ化成）50％エタノール液（pH 11.0、室温、24時間）で架橋処理し、その後十分に水洗し、未結合のプロタミンおよび余剰のエポキシ化合物を洗い流した。次に1％ヘパリン液（pH 5.0）に浸漬してヘパリンを膜内のプロタミンにイオン結合させる[1]ことによってヘパリン化したあと、十分に水洗し、結合していないヘパリンを流した。このようにして得られた膜は70％エタノール内に保存し

人工臓器 15(1), 331-334 (1986)

超極細繊維製人工血管の特徴

野一色 泰 晴, 山 根 義 久※, 森 有 一※※

低有孔性であるが柔軟性に富み，しかも植え込み後形成される新生内膜の治癒が急速で良好であるという人工血管を開発し，その特徴を動物実験で明らかにした。超極細ポリエステル繊維（0.12デニール，繊維断面直径3.0ミクロン，東レ開発の鹿革感触の人工皮革，エクセーヌ用原糸）で内面を覆った内径7㎜，長さ5.7㎝の管を作成し，布製人工血管（有孔性：200cc/㎠，120㎜Hg，H_2O）を作った。対照群には市販のダクロン製人工血管（2.0デニール，20ミクロン，有孔性：200）を採用した。12頭の成犬胸部下行大動脈に作成した人工血管6本および対照群6本を植え込み，植え込み2日目より99日に至るまでの試料を採取した。作成した人工血管はしなやかで軟かく，縫合針の通りも抵抗が少なく，宿主血管壁への縫縮が容易であった。植え込み後の新生内膜形成は対照群に比べ急速かつ安定していた。光顕的観察によって新生血管壁を形成する諸細胞と超極細ポリエステル繊維との親和性の良さが，急速治癒をもたらしていると判明した。すなわち，細胞は超極細ポリエステル繊維間隙に侵入しやすく，かつ増殖しやすいことがわかった。また，フィブリン層の薄さも，超極細繊維の軟かい起毛によると思われた。このような現象が安定した新生内膜を得ることになったが，特にこの人工血管のしなやかさは，従来の低有孔性人工血管にない優れたものでもあった。

Noishiki, Y., Yamane, Y., Mori, Y.: **Characteristics of a now, low porous vascular prosthesis made of a textile of ultra fine fibers of polyester.** Soft and pliable, low porous vascular prosthesis has been developed. In general, low porous vascular prostheses, the fabrics of which are knitted very tightly, are hard and tough to use. Besids, the healing of their neointimae are very poor. In this study, a soft and pliable textile made of ultra fine fibers of polyester were used as a low porous vascular prosthesis. The fibers (0.12 denier, 3.0 micron in diameter) are produced by Toray Co. Ltd. for an artificial leather. Six vascular prostheses made of the textile of 7㎜ in internal diameter and 5.7㎝ in length were implanted in the thoracic descending aortae of 6 dogs. The porosity of them was 200cc/㎠, 120㎜Hg, H_2O). As for the control, a dacron knitted prosthesis of the market (2.0 denier, 20 micron in diameter, porosity:400) was used. The prostheses were removed from the experimental animals from 2 days to 99 days after the implantation. Compared with the control, the prosthesis showed a rapid healing of their neointimae. There were no degenerative changes, nor carcification, in their neointimae. Fibroblasts invaded very rapidly into the intersticis of the fibers of the ultra fine polyester. The ultra fine fibers of the prosthesis showed excellent affinity with the cells which composed the neointimae. From these results, the softness of the prosthesis and the cell affinity of the fibers are the biggest advantages of the new prosthesis. **Keywords**, Low porous graft, Ultra fine polyester fiber, Knitted vascular prosthesis, Soft graft.
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はじめに

内径6㎜以上の太い布製人工血管は完成されたものとして広く臨床に用いられているが，現実には欠点も多い[1]。例えば，それらには抗血栓性がないため，植え込み直後の血栓性閉塞や壁在血栓過形成による狭窄のおそれがある。そのため抗凝固療法を余儀なくされているが，出血傾向が出るほどに強力な療法をしても閉塞するものもある。また，抗凝固療法を行うことによって，人工血管布の各繊維間隙から出血しやすくなる。そこでこの出血を防止するため，一般には編目のつまった，いわゆる低有孔性人工血管を用いている。しかしながら，この低有孔性人工血管にはいくつかの問題点がある。すなわち，布が硬く，吻合操作が難しいのみならず，植え込み後人工血管内面に形成される新生内膜の治癒が著しく悪く，それらが形成されても変性，潰瘍化，脱落等が後を絶たない[2]。このような欠点を解消し，低有孔性であってもしなやかで，そして人工血管布の繊維と，新生血管壁を構成する諸細胞との親和性が良好で，新生内膜が早く形成され，かつ安定して存在し続けられるような新しい型の人工血管を作成したので報告する。

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材料と方法

I 超極細繊維製人工血管の作成

一般に広く使用されているポリエステル繊維（0.2デニール）布の内表面を超極細ポリエステル繊維[3]（0.12デニール，繊維断面直径3.0ミクロン）で覆った特殊な布で内径7㎜，長さ5.7㎝の管を作成し，布製人工血管（有孔性：200cc/㎠，120㎜Hg，H_2O）を作成した。対照として，

入を認めた（図４）。植え込み21日目の例でも，域間隙のフィブリン域に形成質細胞と多数の線維芽細胞の侵入を認めた。この線維芽細胞の侵入は起毛繊維の間隙に含まれたフィブリン層内に限られており，起毛繊維のない部分のフィブリン層内には侵入していなかった。15日目に比べて，21日目では治癒はさらに進み，中央部でも広く内皮細胞の被覆がみられた（図４）。61日目の例では，新生内膜は全面にわたって完成しており，内皮細胞が完全に内面を覆っていた（図５）。85日目の例でも治癒は完了していた。なお，硝子様変性，脱落，潰瘍化，石灰化等は認められなかった。これに対し，対照群では，吻合部の治癒は実験群と同様にすみやかに進行したものの，中央部では遅延しており，99日目の例でも中央部は内皮細胞に覆われておらず，表面に新鮮な赤色血栓の付着を認めた。壁内部でのポリエステル繊維の間隙への細胞侵入は悪く，線維芽細胞の侵入はごく僅かであった（図６）。

考　察

超極細繊維は東レで開発された鹿革感触の人工皮革，エクセーヌ用原糸であり，これで作った布はたとえ緻密に編んでも硬くならないという特徴をもっている。作成した人工血管は低有孔性であるにもかかわらず，しなやかで軟かく，人工血管に鹿革感触を導入したものである。この布は起毛してあり，ビロード様の感触があるが，ビロード布のような厚みはなく均一に薄い。この柔軟性は，従来の低有孔性人工血管を用いた手術時の，人工血管の硬さに由来する不都合，不便さ，扱いにくさ等[4]をことごとく解消しており，有孔性200であっても，従来の1200ないし2000程度の軟かさをもっている。この柔軟な人工血管を用いれば，石灰化を来した病的血管に縫縮しつつ吻合してゆく血管手術で，手術中の苦労を大巾に軽減するのみならず，吻合部からの出血も減少させられよう。

また一方，試作人工血管の大きな特徴として，形成される新生内膜の急速な治癒，安定性が挙げられる。一般に高有孔性においては治癒は早い。初期血栓層内の赤血球は急速に流れ出し，２日目にはフィブリン網となる。低有孔性では赤血球が流れ出しにくく，治癒が遅れる[4]。しかし，試作人工血管においては，高有孔性に似て急速である。

初期血栓層[1]，フィブリン層の薄いのも特徴の一つである。繊細な繊維からなる軟かな起毛がフィブリン層を薄く保持していると思われる。起毛の高さにフィブリン層はとどまっており，それ以上肥厚していない。これは起毛繊維の軟かさが何らかの作用をはたしていると考えられる。従来の硬い起毛ではこのような現象はみられていない。また一方，内皮細胞の被覆にも特徴がある。一般に低有孔性人工血管では内皮細胞の被覆はほとんど吻合部からの連続的伸展に限られており[5]，外膜側から細血管が人工血管繊維間隙を貫通して内膜面へ開口し，そして集落形成という内皮細胞被覆形態は望みがたい。それにもかかわらず，試作人工血管の中央部でも内皮細胞被覆がみられたのは，フィブリン層が内皮細胞の生着に有利な状況をもっていたと思われる。吻合部では若い内皮細胞が分裂，増殖をしており，これが血流に流されてフィブリン層上に付着することもあるので[5]，この生着に有利であれば，内皮細胞は集落も作りやすいと思われる。

15日目，21日目の例にみられるように，起毛された超極細繊維間隙には多数の線維芽細胞侵入があり，起毛繊維のない部分のフィブリン層ではそれが認められないというのは，超極細繊維の存在が細胞侵入，増殖に良好な環境を作っていると思われる。細胞は超極細繊維から何らかの情報をとらえ，急速に繊維間隙に侵入し，増殖してゆく，これを養うべく細血管も侵入するであろうし，この細血管が人工血管内面に開口すれば，細血管由来の内皮細胞集落を作ることも当然考えられる。このように新生内膜内部での急速治癒が内皮細胞の内表面へ生着増殖するのに好影響を与えると考えられる現象は，従来の太い繊維を用いた場合には見られないものである。従来の人工血管では，新生内膜内部での治癒よりも内皮細胞が内表面を被覆する過程が先に完成するし，また治癒も遅延するものと思われていた。本研究での人工血管では繊維の太さを細胞と同じ，もしくはそれより細くすることによって，細胞との親和性を大巾に改善させた結果，治癒を著しく促進させることとなった。

繊維の太さと細胞との親和性の研究は，繊維の形態をも含めて，どの太さで，どの形態が細胞にとって最適かという研究を今後すすめてゆくことで，人工血管に限らず，人工臓器開発研究に貢献してゆけるものと思われる。

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人工臓器 19(3), 1369-1372 (1990)

血管壁細胞を人工血管壁に導入する一方法

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布製人工血管の治癒を促進させるための一方法として，血管小片を細切し，これを人工血管布の網目にからませた後に植え込むという新しい方法を考案した。この方法を用いて動物実験を行なった結果，人工血管は急速な治癒を示し，短期間のうちに内皮細胞によって内面は覆われた。具体的方法としては，末梢静脈小片を剪刀にて細切したのち，約20ccの生理的食塩水に入れて静脈組織細切片浮遊液を作った。次に高有孔性人工血管の一端を結紮し，他端より吸引管を挿入し，これを作成した液に入れ，吸引によって組織片を外側から人工血管壁にからませた。次に新鮮な血液を注ぎ，組織片をさらに固着させた。成犬胸部下行大動脈へこのような処理をしたポリエステル布製人工血管を植え込んだところ，植え込み5日目に新生血管壁内部に無数の内皮細胞の増殖像がみられ，35日目の例では吻合部はもとより，人工血管の中央部ですら内皮細胞による完全な被覆を認めた。

Noishiki, Y., and Yamane, Y. A METHOD TO INTRODUCE CELL COMPONENTS OF BLOOD VESSELS INTO A MATRIX OF VASCULAR PROSTHESIS A simple method to introduce cell components of blood vessels into a fabric vascular prosthesis was developed. This method showed prompt healing of the graft after implantation in an animal study. Tissue fragments suspension was prepared using a piece of peripheral vein. Highly porous fabric vascular prosthesis was immerced into the suspension fluid. The tissue fragments were trapped from the outer surface of the prosthesis by suction from the inside. Preclotting procedure was adopted to immobilize the tissue fragments to the fabric of the prosthesis. The prostheses were implanted into the thoracic descending aortae of 15 dogs. There was no bleeding at the time of the implantation. Numerous endothelial cells proliferation was observed in the specimen for 5 days after implantation. The surface was covered with thin layer of fibrin. Full endothelialization was noticed in the specimens of 35 days. The graft wall was completely healed. This method was very simple and easy to prepare, but the efficacy to promote the healing of the neointima was excellent. KEYWARDS : Endothelialization, Tissue fragment transplantation, Vascular prosthesis, Preclotting, Neointima.
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はじめに

布製人工血管の新生内膜治癒はヒトにおいて遅延するのが常であり[1]，時には長期間経過しても単にフィブリン層が内面を覆っているのみで，内皮細胞による被覆が全くみられないこともある。この原因はまだ明らかにされてはいないが，その対策として，何らかの方法で内皮細胞を人工血管壁に導入すればそれらが分裂増殖して内面を覆ってくれる可能性のあることが最近明らかにされてきた[2,3]。この目的のために現在行われている方法は，人工血管植え込み時に患者自身から内皮細胞を採取し，preclotting時に血液に混ぜて付着させる方法と，前もって患者から内皮細胞を採取しておき，細胞培養技術を用いて増殖させ，手術時に人工血管壁に大量に付着させる，もしくは手術前に人工血管に大量に付着させておき使用する等の方法がある。これらはそれぞれ一長一短があり，まだ一般臨床に用いる所まで至っていない。例えば前者は採取できる細胞数が少なく，効果が不確実である。後者は細胞培養技術および培養期間が必要である。このような欠点を補うべく我々は新しい方法の開発に取り組んできた。この度，非常に単純で簡単であり，設備も準備期間も不必要でありながら大量の細胞を手術時に播くことができ，その効果が確実である方法を考案したので報告する。

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材料と方法

I 組織細切片吸着操作

成犬の頸静脈を長さ約10 cmにわたり切除し，これを剪刀にて細切し，20ccの生理的食塩水中に入れて血管組織片浮遊液を作った。静脈片の重さは約0.2 gであった。次に内径8 mm，長さ7 cm高有孔性のポリエステル製人工血管の一端を閉じ，他端より吸引管を挿入したものを液中に入れ，吸引によって血管組織細切片を人工血管布の網目に硬くからませた。この操作は繰返し行ない，空気も生理的食塩水も吸引できなくなるところまで網目にからませ，目詰まりさせた。次に人工血管のみを取り出し，乾燥したガーゼでくるんで余剰の水分を除き，そ

こに新鮮な血液を注ぎ，preclottingの要領でフィブリンを析出させ，組織片をさらに強固に人工血管網目に固定させた。

Ⅱ　人工血管植え込み

体重8～12 kgの成犬15頭を用い，全身麻酔下に開胸し，胸部下行大動脈を5 cm切除し，ここに作成した人工血管長さ5.7 cmを端々吻合にて植え込んだ。縫合糸は5－0テフデックを用い連続縫合を行なった。術中に創部に抗生物質を感染防止のため使用したが，抗凝固薬は一切使用しなかった。

Ⅲ　人工血管の採取

植え込まれた人工血管は植え込み直後より35日目に到るまで期間をおいて12頭より採取し，肉眼的に観察したあと1％グルタールアルデヒド固定を行ない，光顕的に観察した。残り3頭は長期変化観察のため継続飼育中である。

結　　果

Ⅰ　作成した組織細切片吸着人工血管

用いた人工血管を図1 aに示す。これに組織片を吸着させたのが図1 bであり，外側から組織が薄く，まんべんなく付着し，網目が完全にシールされ，自家組織管の状態となっている。外側はまだ起伏があるが，内腔面は光沢があり平滑であった。この状態に血液を注いで組織片をフィブリンで固めたものが図1 cであり，一般に行われるPreclottingした血管のようであるが，その中には組織片が散在している。血液による組織片の固着化は一度新鮮な血液をかけるだけで十分であり，一般に行われているPreclottingのように，何度も血液を注ぐ必要はなかった。

Ⅱ　人工血管の植え込み

胸部下行大動脈への植え込みに当っては，人工血管は柔軟性に富み，扱いやすく，切断端はほつれることなく，糸の通りも良好であった。吻合操作は容易で，宿主血管壁への縫縮も容易であり，あたかも自家組織管の植え込みのようであった。吻合操作中に付着しておいた組織片がはがれるようなことはなかった。血流再開時には人工血管壁面からの出血はなく，また植え込み後の線維素溶解現象によると思われる後出血はまったく認められなかった。

Ⅲ　植え込み結果

15頭のうち1頭は手術翌日に衰弱死した。残り14頭は健康そうに見えたが，4頭に胸腔内局所的小膿瘍を認めた。残りの動物には異常はなく，長期観察用の3頭の健康状態は良好であった。試料を採取した動物にあって，末梢動脈の塞栓症に基因すると思われる合併症は認められなかった。また腎の割断面には塞栓症を思わせる肉眼的所見はみられなかった。

切除標本の内面肉眼的観察では，植え込み1日目で血管壁全体に赤味があるものの，内面は光沢があり平滑で，ポリエステル繊維が透見できた。内面への血栓の付着はみられなかった。植え込み3日目ではさらに光沢が増し，5，7，14，28日と日数を重ねるごとに壁の赤味は減少し，次第に白色となり，35日目の試料ではまったく白色の新生血管壁を形成していた。この間肉眼的には全く血栓の付着は認められなかった（図2）。

Ⅳ　切除標本光顕観察結果

植え込み直前の人工血管断面の光顕標本観察では，ポリエステル束外側に血管組織の細切片が点在していた。組織片は繊維束の間隙に入り込んでいるものもあったが，これを越えて人工血管内腔側に入っているものはなかった（図3）。植え込み1日目では人工血管内面は薄いフィブリン層に覆われており，血管壁内部には多数の赤血球，白血球が認められた。内面のフィブリン層は薄く，約10～100ミクロンであった。植え込み5日目には人工血管の外側にある血管組織片から多数の内皮細胞および線維芽細胞，平滑筋細胞様細胞等が増殖していた。内皮細胞の増殖している部分のフィブリンは消失していた。またいくつかの内皮細胞が集まって，管腔を形成しているのも認められた。植え込み7，14日と日がたつにつれ，この傾向は強まり，28日目では無数の細胞が新生内膜側にみられた。このような変化は人工血管のすべての部分，すなわち，吻合部，中央部分等の区別なく，等しく観察することができた。植え込み35日目の例においては，人工血管全面にわたって内皮細胞が覆い，内皮細胞下には線維芽細胞が層をなして，これらが細胞線維性の新生血管壁を形成していた。人工血管の外膜側では多数の線維芽細胞と細血管がみられ，付着させた組織片と，新しく侵入してきた，もしくは分裂増殖した細胞や組織等を区別することはできなかった（図4，5，6，7）。

考　　察

血管壁を構成する諸細胞のうち，外科的な目で注目を集めているのは内皮細胞である。この取り扱い，培養等は熟練すれば容易であるが，一般の外科医には無理な事が多い。従って，前述した様な手術時に採取するとか，前もって採取し，培養で増殖させることは一般臨床の場で広く実施するには難しい。また現在行われている方法を完璧に行ったにしても，その効果が必ずしも予期した通り発揮されるかどうか不明であるというのが現状である。このため米国をはじめとして，世界中の研究施設で巨額の費用と人員を注ぎ込んでいるにもかかわらず，まだ臨床への道が開けておらず，次の段階へ進んでいない。

我々のこの度行った研究の第1の特徴はだれにでも容易に行うことのできる点にある。末梢静脈片を少し集めれば人工血管布の網目をシールするのに十分である。また，吸引操作を用いているため，シールにむらが生じていても，数回繰り返すことによって均質なシールが得られる。これに要する静脈片は形態が整っていなくても，少量ずつ集めれば良いことが判った。

本方法の第2の特徴は血管組織を形成するすべての細胞および線維を一度に同時に扱っている点にある。これ

まで世界中で行われてきた方法はいづれも内皮細胞を重視して，内皮細胞のみを採取し培養していた。これは線維芽細胞や平滑筋細胞が混在すると内皮細胞が負けてしまい，後者のみが増殖することに基因していた。これはin vitroにおいて確かにその通りである。しかし我々は細胞培養の場をin vivoに求めている。in vivoでは同時に異なった種類の細胞を培養させてもそれぞれの特性に従って増殖してゆく，例えば，内皮細胞は増殖しつつ，フィブリンを溶かし，血流に面する所に出てゆこうとする。また互いに連がり，管腔を形成しようとする働きもある。線維芽細胞や平滑筋細胞では増殖の速度は速く，そしてそれぞれは血流から離れ，下層に入ってゆこうとする。これらは自然に住み分けが進み，互いを押さえ合うようなことはない。むしろ，線維芽細胞や平滑筋細胞等が下層に存在することでフィーダーセルとなり，その表層には内皮細胞の被覆が有利となる現象もおこりうると思われる。5日目の所見ではこのような活発な所見が人工血管の全面にわたって観察することができ，我々の意図した，全細胞成分を同時に使用する方法に無理のないことを支持してくれたと考えている。

第3番目の特徴は組織片を外側から吸着させることにある。組織片自体はたとえ静脈片であっても切断端を多くもち，コラーゲンが露出しているため血栓性が高い。そのため，一度血液を注いだだけでPreclotting様の操作が完了するほどである。このような組織片が血管内腔に入ることはきわめて危険である。本研究では外側にあれほど組織片が吸着されていても，内側には侵入しておらず，内面は光沢ある平滑な面を保っていた。また，組織片が流れて塞栓症を引き起した様子もない。このような事から，安全に大量に組織片を人工血管壁に導入するには外側からが優れている。もし少量を有効的に導入するのであれば内側からの方が良いと思われるが，現在の所，だれにでもできるという点から考えると，外側の方が簡便である。

第4番目の特徴としては，人工血管布の網目の目詰まりにある。一般に高有孔性人工血管では植え込み前にPreclotting操作を行って，網目を目詰まりさせる。これには新鮮な血液と，フィブリンを析出させる時間とが必要である。そのようにして完全に目詰まりさせても，手術後数時間たって線維素溶解現象によってフィブリンが溶解し，はずれて大出血を生じることもある。我々の方法では組織片で目詰まりしているため，基本的にはPreclottingは不必要である。また線維素溶解現象の影響を受けなくてすむ，従って，思いきった術後の抗凝固療法を行うことができる。この度行った新鮮な血液をふりかけて凝固させる操作は組織片を人工血管布に固着させておくためのものであった。組織片の一端が吸着されていても，他端がフラフラとしていれば，吻合操作時，組織片が縫合糸にからまって，不便を来すことがあるので，この予防のために行ったものである。従って，一般に言われているPreclotting操作を行ったものではない。

さて，本方法の最も大きな特徴，これが第5の特徴となるが，人工血管壁の新生血管壁形成促進作用にある。一般に，新生血管壁形成は長時間必要で，我々の成犬における実験でも20週間を必要とした[4]。しかし本研究では5週間でそれが完成している。しかも，治癒が吻合部とか，中央部といった区別なく，平行してすべての部位において進行している。これは組織片を播いた効果が著明に出ていることを示している。我々はその理由として，組織片における破断面の広さ，多さを考えている。皮膚の移植でも，ただ単に皮膚を置くより，小片にして播いたり，短冊状に切って拡げたりする方が，組織の破断面から無数の細胞が出てきて，急速な治癒を生じることがすでに知られている。このような現象が人工血管壁でも生じているものと我々はみている。また，細胞は単一な細胞をバラバラで扱うのではなく，複数の種類の細胞をグループで扱う方が，個々の細胞が本来の機能を発揮しやすいことも最近明らかにされているが，このような現象も本人工血管の治癒過程において発揮され，急速な治癒がおきたのかもしれないと推測している。

本研究はまだ長期例を観察していないので結論じみたことは言えないが，現在得ている情報をもとに判断する限りにおいては，これまで世界中で行われてきたいかなる方法よりも単純で，その効果は最も優れていると思われる。

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ヘパリン徐放性癒着防止膜

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癒着現象が，フィブリンの折出→フィブリンのポリマー化→細胞浸潤，線維芽細胞出現→コラーゲン線維網形成→細胞線維性癒着組織の完成，という過程を経て進行することに注目し，ヘパリン徐放で癒着の初期段階であるフィブリン折出を完全に阻止することによって，われわれは癒着防止に成功した。ヘパリンを含有し，徐放する担体は，ヘパリン徐放後生体内に埋没され，あるいは吸収されることが望ましいことからコラーゲン膜をそれに利用した。動物実験として，成犬の大腸漿膜に5 cm平方の欠損を作り，この部をヘパリン化したヒト羊膜で覆い，100%の抗癒着性を得た。60日以上の経過例では，ヘパリン化羊膜はすでに正常な漿膜細胞に覆われていた。従って，作成した癒着防止膜を用いることによって，初期はヘパリンの徐放により，その後は正常な漿膜細胞による天然の抗癒着力によって，永久に癒着を阻止しつづけることが明らかとなった。

Noishiki, Y., and Miyata, T. **Heparin releasing antiadhesive membrane**
A hewly developed antiadhesive membrane with slow release of heparin was demonstrated. In the animal experiments, an excellent antiadhesive effect using heparinized amnion was observed on the large intestine surface of which serosa (5x5 cm area) was pealed off previously. The amnion membrane can be ionically heparinized along the procedure described as follows. 1. The membrane was dipped into 1% solution of protamine sulfate. 2. The protamine impregnated membrane was treated with 0.25% glutaraldehyde solution (pH 4.0) to crosslink protamine to collagen and wash with distilled water. 3. The membrane was soaked into 1% heparin solution for 24 hours at 45℃ and repeatedly washed with distilled water. Heparin prevents the fibrin deposition, which is ahead of the adhesion. After the heparin releasing, natural serosa covered on the membrane. Thus, this antiadhesive membrane can induce the natural antiadhesive ability permanently. **Keywords.** Heparinized collagen, Antiadhesive membrane, Heparinization, Fibrin deposition.

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はじめに

腹腔内，胸腔内，心嚢内，頭蓋内，腱鞘内，神経周囲等における手術後の予期せぬ癒着は重大な合併症を惹起させることがある。従来は癒着防止の方法として，テフロンやシリコーン等の生体になじみにくい材料を癒着防止膜として使用するか，あるいはヒアルロン酸，プロトポルフィリン，コンドロイチン硫酸等の薬剤による癒着防止が試みられてきた[1,2]がいずれの方法でも有効な結果は得られていない。われわれは癒着がフィブリン折出→フィブリンのポリマー化→細胞浸潤，線維芽細胞出現→コラーゲン線維網形成→細胞線維性癒着組織の完成という過程を経て進行する[3,4]ことに注目し，その初期段階であるフィブリン折出をヘパリンで阻止することによって，次々と進行する癒着過程を完全に防止することに成功した。その方法として，ヘパリンを癒着の進行する場に徐放させ，その担体としてはヘパリン徐放後，組織修復に良好な場を与え，そして可能ならば生体内で吸収されてしまうことが望ましいことを考慮して，その両方の条件を満たす物質としてコラーゲン膜を採用した。動物実験として，ヒト羊膜を酵素処理でコラーゲン膜とし，それにヘパリンをイオン結合させて，成犬大腸漿膜剥離面に応用し，良好な成果を得たので報告する。

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材料と方法

1　癒着防止膜の作成

ヒト羊膜を無菌的に採取し，生理的食塩水中に浮遊させながら，羊膜を破損しないよう注意して付着している余分な結合組織を剥離した。次いで0.01%フィシン溶液（pH 7.4，0.2Mリン酸バッファー）中で室温，24時間処理し，コラーゲン線維以外の細胞，蛋白成分を除去し，その後リン酸バッファーで洗滌した。次に1%硫酸プロタミン水溶液に浸漬し，充分にプロタミンをコラーゲン網にからませた後に0.25%グルタールアルデヒド水溶液中でプロタミンをコラーゲンに架橋した。次いでこれを充分に水洗し，余剰のグルタールアルデヒドを洗い流した。次にこれを1%ヘパリン液中に浸漬し，45℃，24時間かけてコラーゲンに架橋されたプロタミンにヘパリンをイオン結合した。その後ヘパリン化された羊膜を流水中にて水洗し，結合していない余剰のヘパリンを流し

止膜の出現は待望されていた感がある。本研究では癒着に先行するフィブリン折出をヘパリンが完全に阻止することによって癒着過程への進行を阻止し，しかもヘパリン徐放後は天然の抗癒着性をもつ漿膜細胞へと癒着防止機能を引き継いでいくことから，たとえコラーゲン膜がヘパリン放出後に新生した細胞によって埋もれてしまっても，永久に完全な，そして自然の抗癒着性を維持できるところに特徴がある。

ヘパリンを徐放するための担体となる膜は，これまでの基礎実験ではポリウレタンなどの合成高分子膜でも良い結果を得てはいるが，合成高分子膜の多くは生体内では非吸収性で，異物としていつまでも残存することとなる。コラーゲン膜は生体内で吸収されるようにすることが可能であり，しかもコラーゲンの網目は細胞の侵入，増殖に良好な立場を提供することから，漿膜細胞の被覆をも促進させることが可能である。以上のことより，コラーゲン膜を用いてヘパリンを徐放させるという癒着防止膜のデザインは優れていると考えている。

ヘパリンの徐放速度についてのin vitro実験結果では，3か月間に最初含有されていた76%のヘパリンが徐放されることが判明しており，徐放がきわめて緩やかであることがわかる。ヘパリンの結合量については，使用するプロタミン量でコントロール可能であり，癒着を非常にしやすい場に用いる癒着防止膜ではさらに高濃度のヘパリンを結合させることも可能である[6]。

漿膜剥離部分は完全に癒着防止膜に覆わせておく必要があるだろうか。この疑問に関しては図　で明らかなように，癒着防止膜がはがれている部分でも癒着が発生していない。すなわち，フィブリンは折出を阻止するに充分な量のヘパリンが近くで徐放されていれば，必ずしも完全に漿膜剥離部分が覆われていなくても良いと考えられる。従って，癒着防止膜は必ずしも膜状でなくても，例えば，メッシュ状やヒモ状でも良いことを示唆しており，膜の形状については，使用部位，場所によって，種種に変化させることが可能となるだろう。また，腹壁のような張力のかかる場所においては，ポリエステルメッシュを張り合わせて力学的な補強をしてやることもできるであろう。このようにして，この癒着防止膜を用いることによって，多くの場所での術後の癒着にもとづく合併症を阻止することが可能となってきた。

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穿刺可能な細口径人工血管の開発

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動物の頸動脈を化学的に処理することによって、抗血栓性を賦与し、同時に、生体特有の柔軟性を維持させておくことで、注射針による易穿刺性、針の抜去後の易止血性をもたせた細口径人工血管を開発した。生体材料を人工臓器用素材として利用する場合、生体内での吸収性、異物反応、抗原性をおさえるとともに強度をもたせるため、一般にはグルタールアルデヒド処理を行う。しかし、グルタールアルデヒド処理をすると、生体材料特有の柔軟性、親水性は失われる。我々は新しく親水性エポキシ系架橋剤をそれに導入した。作成した人工血管は生体血管に似た柔軟性と強度があり、内径 3.0～3.5 ㎜、長さ 6 *cm*の graft でも、植え込み后18G注射針による刺入されてすら、開存率 87.5％であった。注射針抜去後の止血時間は、平均 10 分 12 秒であった。対照群のグルタールアルデヒド処理血管の開存率は25％であり、止血時間は 22 分 25 秒と長かった。作成した人工血管は以上の実験結果より、人工腎用Ａ－Ｖ内シャント用に使用可能と思われる。

Noishiki, Y., Kodaira, K., Furuse, M., Miyata, T., Yamane, Y. **DEVELOPMENT OF A PUNCTURABLE SMALL CALIBER VASCULAR GRAFT.** A small caliber vascular graft was developed. The graft, which was made of modified carotid artery from animals, has enough compliant property like a native artery. The soft graft wall showed easy puncturability with a 18 gauge needle and it took short bleeding time after the needle was removed. In general, biological materials for implantable artificial organs were crosslinked with glutaraldehyde (GA). The crosslinking with GA makes the materials insoluble, less biodegradable, and less antigenic, but its disadvantage is to make the materials more stubborn. We introduced a new hydrophilic crosslinking reagent, Polyepoxy Compounds (PC), instead of GA. After the crosslinking with PC, biological materials become white in color. Softness and elasticity of the original material could be preserved even after the crosslinking. The vascular graft developed was crosslinked with PC. The graft got high hydrophilic and elastic properties. Thirty-two grafts of 3.0 to 3.5mm in internal diameter and 6 cm in length were implanted in both carotid arteries of 18 dogs. Each graft was punctured 5 times with a 18 gauge syringe needle after the implantation. The grafts were removed from 1 to 30 days after the surgery. The patency rate was 87.5%. The bleeding time after the needle was removed was 10 min. 12 sec. in average. Eight carotid artery grafts crosslinked with GA were adopted as control experiments. It showed 25% patency and 22 min. 25 sec. of the bleeding time. These results showed that the graft developed has an antithrombogenicity and puncturable property. Thus, the graft is very promissing as a A-V bypass graft for artificial kidney. **KEYWORD** Small caliber vascular graft, Puncturable vascular graft, Polyepoxy compounds, crosslink, Biological materials.

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はじめに

人工腎用Ａ－Ｖ内シャントに用いられる人工血管の需要は高まる一方であるが、理想的な人工血管は開発されておらず、EPTFE graftやグルタールアルデヒド(GA)処理された牛頸動脈 graft、臍帯静脈 graft等を、不便ながらも使用しているのが現状である。これらは内径 4～6 ㎜と太いため、小児や身体の小さい患者に使用するのに無理がある。このようなことから、穿刺可能な細口径人工血管の開発によせる期待は非常に大きい。しかし、現実には、まず第一に細口径人工血管の開発が基本的であり、さらに、それが穿刺に耐えることが次の条件となるため、いづれも現在の人工血管開発技術では未解決なものであった。

我々は人工血管に抗血栓性を賦与した細口径人工血管の開発を進めてきたが、この過程において、材料の架橋方法や、抗血栓性賦与方法において新しい方法を開発し

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考　察

Ⅰ　作成した人工血管の抗血栓性

細口径人工血管の基本的条件に抗血栓性がある。我々は、これまで細口径人工血管の開発にあたって、抗血栓性賦与方法として人工血管内面にヘパリンをイオン結合させる方法を採用し良好な成果を得た[1,2,3]。しかし、ヘパリン化人工血管は、注射針による刺入の際止血に関しての不安があった。すなわち、ヘパリンが残存している間は、注射針抜去後の止血時間が長びくことが考えられる。ヘパリンが放出し終った時には安心であろうが、それには術後1ケ月以上待つ必要がある。このことからヘパリンを必要とせずして、抗血栓性を得る方法を検討した結果、高親水性材料の有用性を、森ら[4]の研究で認め、生体材料を高親水性にする方法の開発へと、研究方向が進んだ。森らの研究では、従来、高親水性材料が抗血栓性を有することが指摘されていたなかで、軟質塩化ビニールに長鎖ポリエチレンオキサイドをグラフトし、含水率90%以上のポリマーを作成した。このポリマーは末梢静脈内糸留置法によるin vivo評価法でも、長期間にわたる良好な抗血栓性を示した[5]。そこで、我々はこの成果を参考にして、生体材料を高親水性とし、含水率を高めるため、親水性エポキシ系の新しい架橋剤を、従来のグルタールアルデヒド架橋の代りに導入した。その結果、材料は柔軟性を維持し、しかも高親水性となり、森らが合成高分子材料で得た成果と同様の抗血栓性をコラーゲンでも得た。本研究での作成した人工血管は、このような成果にもとづいた高親水性による抗血栓性である。従って、ヘパリンほどの積極的な抗血栓性はないため、実験結果にもみられるような、手術手技いかんによっては、吻合部で血栓が形成され閉塞するおそれがある。しかし、注意して吻合を行えば、内径3㎜程度の人工血管では充分な抗血栓性を得ることが判明した。

Ⅱ　作成した人工血管の穿刺性

注射針による人工血管の穿刺は、易穿刺性と、注射針抜去後の止血性が重要となる。現在臨床で広く用いられているEPTFE graftは、素材がテフロンであることから、多孔性にしてあるとはいえ注射針刺入には硬く、注射針によって、EPTFE graft壁に孔を開け、壁を破壊しつつ刺入される。従って、針の抜去後も破壊された壁や、その孔は、同じ大きさで残るため止血に時間を要する。本研究で対照に用いたGA処理人工血管も同じ傾向がみられ、針抜去後の孔が大きく開いており、止血時間は長かった。これに比べ、新しい架橋剤で作成した人工血管は、壁の柔軟性にすぐれ、そのコンプライアンスも生体血管に近いものであった[6]。そのため、針の刺入に際して、注射針によって血管壁は線状の傷あとを残すとも壁が柔らかいので、刺入孔が引きのばされて拡がり、針を通す。そして抜去後の孔は、引きのばされていた部分が縮むため縮少する。最終的には図6にみられるように線状の切られた傷あとを残すのみとなる。この現象が止血時間を短縮させた最大の要因であろう。注射針による4種類の血管の易穿刺度では、作成した人工血管が最も抵抗が少なかった。これは、高親水性によって血管壁が多量の水を含むため、新鮮血管壁よりも、さらに刺入抵抗が少なくなったものと思われる。一方、GA処理血管では刺入抵抗は比較的高いことが判明した。

おわりに

新しく開発した人工血管は、細口径であるにもかかわらず、抗血栓性と注射針による刺入性、抜去後の止血性にすぐれており、人工腎用A－V内シャント用人工血管としての可能性が高いと思われる。今後は臨床応用できるところまで改良を重ねてゆきたい。

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●原著　　人工臓器 25(2) 443-447 (1996)

皮下に植え込まれた人工血管の治癒過程におけるbFGFの動向
-皮下結合組織片及び骨髄組織の播種による影響-

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Basic fibroblast growth factor (bFGF)の人工血管における血管新生の状態を血流の影響の無い状態で観察するために実験を行った。①実験動物の皮下組織を細切し布製人工血管壁に圧力注入(TF血管)、②EPTFE人工血管壁に骨髄組織を播種(BM血管)の2種を作成し、試料を採取した同じ動物の皮下に植え込み、1日から14日目まで検討した。肉眼的には2日目よりTF血管の周囲は赤色で周囲の組織と一塊になっていた。光顕所見では細小血管は2日目にはTF血管の周囲に集まり、5日目には繊維間に侵入していた。7日目には細小血管及び繊維間の線維芽細胞が強くbFGF陽性に染色された。対照では7日目でも人工血管周囲の組織のみbFGF陽性に染色された。7日目にはBM血管では外側組織から壁内へ細小血管が侵入していた。TF血管およびBF血管は皮下への植え込み後に、産出されるbFGFにより活発に細胞増殖および血管新生していた。人工血管の早期治癒にbFGFが重要な意味を持つことが示唆された。

Tomizawa Y, Noishiki Y, Okoshi T, Nishida H, Endo M and Koyanagi H. BASIC FIBROBLAST GROWTH FACTOR IN NON-FUNCTIONING VASCULAR GRAFTS: EXPERIMENTAL STUDY. Recently, many different growth factors have been identified and characterized. Basic fibroblast growth factor (bFGF) is an angiogenic agent and affects all cell types involved in wound healing. Autologous connective tissue was minced and impregnated into a fabric vascular graft wall (TF-graft). Autologous bone marrow was obtained and impregnated into an ePTFE graft (BM-ePTFE graft). Untreated grafts were used as control. Pieces (1 x 3 cm^2) of the grafts were implanted subcutaneously in a canine study. These samples were removed from one day to two weeks after implantation and were evaluated. At second day, around the TF-graft, many capillaries were microscopically observed, while no host tissue was adhering to the untreated graft. From day three, cell proliferation was observed in the TF-graft and it was intensely immunoreactive for bFGF in the tissue fragments in the graft wall, whereas the control had no cell infiltration. At day seven, cell proliferation and migration was remarkable in the TF-graft. At day 14, capillaries were infiltrating into the BM-ePTFE graft while no cell migration was observed in the control. bFGF immunoreactivity in the TF-graft and BM ePTFE-graft suggests that wound healing already started early after implantation subcutaneously. KEYWORD: bFGF, vascular graft, fabric prosthesis, EPTFE

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はじめに

Basic fibroblast growth factor (bFGF)は血管新生をおこすサイトカインの中では作用が最も強いものの1つと考えられている。動物実験ではあるが　bFGFは血流に接している布製人工血管壁においては血管新生を介して内皮細胞被覆を促進させ、わずか植え込み後5日目には内皮細胞が人工血管中央の内腔面に島状に出現し、吻合部でさえもプレクロッティングされた対照の人工血管に比べて治癒が促進していた[1]。細胞培養の培地において線維芽細胞及び平滑筋細胞は血清濃度の影響を強く受け、濃度が濃いと細胞の活動が抑制される。皮下結合組織及び骨髄組織を播種した人工血管を人工血管として移植した場合、高い血清濃度が影響して治癒が遅延する可能性も否定できない。人工血管壁に播種された細胞の挙動を血流の影響の無い、皮下組織内で純粋に観察するために布製及びEPTFE人工血管を用いて検討した。

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材料及び方法

1. 組織片を播種した人工血管 (Tissue fragmented graft; TF血管)の作成

全身麻酔下に雑種成犬の皮下から脂肪組織を採取し、ハサミを用いて十分に細切した。布製人工血管(Micron, porosity:1200 Intervascular社、米国)壁に、細切した組織片に10mlの生理食塩水を加え浮遊液としたものを圧注入した。

った目的を持って細胞を導くのでは難しさが異なるように思われる。また今回我々が試みたように自己の組織内に含まれるbFGFと市販のrecombinant human bFGFでは、前者は自然に制御されるが、後者は制御せねばならないため、使用に際して良いデザインが不可欠である。

結語

血流の影響の無い状態においても人工血管の治癒に、皮下結合組織及び骨髄組織等の自己組織由来のbFGFが重要であることが示唆された。人工臓器も生体機能の発現を制御するサイトカインの効果を最大限に利用した”ハイブリッド人工臓器”の時代になりつつあり、生体との調和を保った人工臓器の開発を目指したい。

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ミクロ多孔質構造により内面に自然に血清蛋白層を形成させて内皮化を促進させるA-Cバイパス用人工血管

我々は小口径人工血管にミクロ多孔質と，ある一定以上の漏水性を賦与することにより，A-Cバイパス用人工血管開発を行ってきた．そして，これまでの動物実験では，漏水性をもつミクロ多孔質ポリウレタン製人工血管（内径1.5 mm，長さ10 cm，漏水率39 ml／min／cm²）をループ状に植え込み，3ケ月後の開存性と内腔面の約50%の内皮化を確認している．本研究では，このループ状の人工血管植え込みモデルを用いて，さらに長期の開存性および内皮化の状態を評価することが目的である．ミクロ多孔質ポリウレタン製人工血管(内径1.5 mm，長さ10 cm，漏水率84 ml／min／cm²）を作製した．そして，このグラフトをループ状にしてラットの腎動脈下，腹部大動脈に端々吻合で植え込んだ．現時点で91日目（n=1）と93日目(n=2）の生存ラットを得ている．このうち1匹のラットを76日目に全身麻酔下に清潔手術操作で開腹し，拍動を触知することによりグラフトの開存を確認した．そして再び閉腹した．現在は，さらに長期の経過を観察中である．

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緒　言

我々はA-C バイパス用人工血管開発へのアプローチのひとつとして人工血管をミクロ多孔質とし，さらにある一定以上の漏水性を賦与することを提唱してきた(1, 2, 3, 4, 5, 6)．この方式により植え込み後，急性期の開存性を向上させ，さらに内腔面の内皮化を促進させて，中期から慢性期にかけて完全な内皮化とその維持を達成することによって，長期間の人工血管開存性を獲得するというコンセプトのもとに研究を行ってきた．そして，これまでの動物実験では，漏水性をもつミクロ多孔質ポリウレタン製人工血管(内径1.5 mm，長さ20 mm，漏水率39 ml／min／cm²）において，植え込み3ケ月後に満足すべき開存性とほぼ完全な内皮化が得られることを示した(1)．また内径1.5 mm，長さ10 cmの同材質の人工血管をループ状に植え込み，3ケ月後の開存性と内腔面の約50%の内皮化を確認した(3)．本研究では，このループ状の人工血管植え込みモデルを用いて，さらに長期の開存性および内皮化の状態を評価することが目的である．

材料と方法

ミクロ多孔質ポリウレタン製人工血管（内径1.5 mm，長さ10 cm，漏水率84 ml／min／cm²）を作製した．このグラフトをループ状にしてラットの腎動脈下，腹部大動脈に端々吻合で植え込んだ．そして長期にわたり開存性および内皮化の状態を評価する計画である．

結　果

現時点で91日目（n=1）と93日目（n=2）の生存ラットを得ている．このうち1匹のラットを76日目に全身麻酔下に清潔手術操作で開腹し，グラフトおよびグラフト吻合部の大動脈の拍動を触知することによりグラフトの開存を確認した（図1）．そして再び閉腹した．さらに長期間飼育して再度評価する予定とした．

考　察

A-C バイパス用小口径人工血管の開発が急務であり，世界中で抗血栓性高分子材料を用い，内面を完全に平滑にした人工血管の研究が精力的に進められてきたが（7, 8)，この研究は行き詰まっている．我々は前述の方法が血栓の付着を阻止すると同時に内皮細胞の付着をも阻止

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人工臓器 16(3), 1527-1529 (1987)

成長できる人工血管の開発の基礎研究

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幼小児期に人工血管の植え込み術をうけると、その人工血管は患児の成長に合せて、成長してゆき、大人になった時に成長は止まり、以後は安定した形態を維持するという「成長できる人工血管」を作るための基礎的実験を行い、その成長のメカニズムを理論的に解説した。その基礎となる安定期、つまり成長しきった時期での人工血管の枠組みに、ポリエステルメッシュチューブを採用し、この状態での安全性を動物実験により評価したところ、1.3㎜×1.3㎜という網目の荒いメッシュでも、血管壁を維持できることが判明した。この結果をもとに考察すると、もとのポリエステルメッシュチューブを、生体内で劣化吸収可能な糸でからめて、網目の数や、幅を減少させておくことで、植え込み時の人工血管のサイズを小さくおさえておくことにより、植え込み後、次第に糸が吸収されて、もとのサイズのメッシュチューブにもどってゆき、これにつれて、新生血管壁も大きくなり、人工血管は成長してゆくことが期待できる。

Noishiki, Y., Kodaira, K., Miyata, T., Furuse, M., Yamane, Y. **FUNDAMENTAL STUDY OF A GROWABLE GRAFT** Vascular grafts being available in the market cannot grow up along with the children patents growth. We designed a new growable vascular graft and the basic experiment was performed using a special vascular graft, which was composed of polyester mesh tube and connective tissue surrounded. They were implanted into the thoracic descending aortae of experimental animals. There was no wall bleeding nor aneurysmal dilatation in the grafts. From these results, a new growable graft was designed as follows. At first, polyester mesh tube was stitched with a biodegradable suture in advance, and was coated with collagen. After the implantation, the biodegradable suture will be absorbed, then the mesh tube will be inflated with inner blood pressure. The neoarterial wall will be synthesized along with the expanding of the flamework of the graft. Therefore, the growable vascular graft will be realized. **KEYWORD** Growable vascular graft, biodegradable suture, Collagen, Polyester mesh tube, Vascular graft.
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はじめに

現在使用されている人工血管は、植え込み後の太さ、長さ等の形状変化がない。この形態維持性は人工血管には必要であるが、時には都合の悪いこともある。例えば乳小児期の人工血管置換術は、患者の成長に伴ってその部で相対的な狭窄症を引きおこす[1,2]。このような場合、成長に伴って2回、3回と、より大きな径の人工血管を植え込む必要があるが、再手術、再々手術は危険性も高く、患児にとって大きな負担である。このようなことから、身体の成長に伴って成長してくれる人工血管の必要性が高い。我々はこの問題を解決するための基礎実験を行った結果、人工血管を安全に、かつ確実に成長させうるという結論を得たので、その理論と、実験の成果を紹介したい。

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材料と方法

3種類のポリエステル布(網目のサイズ、1.3×1.3㎜、0.8×0.8㎜、0.2×0.2㎜)で、内径8㎜、長さ5.7㎝の管を作り、その内腔に合うシリコーンチューブを挿入した。次にこれを成犬皮下組織内に挿入し、20日後にメッシュチューブに付着した組織とともに取り出し、70%エタノール液内に浸漬保存した。人工血管として使用する際には、内腔のシリコーンチューブを抜去後、生理的含塩水中につけて、エタノールと生理的含塩水とを置換した。このようにして、ポリエステルメッシュを枠組みとし、結合組織で肉付けをした人工血管を作成した(図1)[3,4]。

動物実験としては、55頭の成犬胸部下行大動脈を5.5㎝切除し、ここに作成した管を人工血管として植え込み、植え込み直後より850日に至るまで経過観察し、その間に順次試料を採取し、肉眼的、光顕的に観察した。