Abstracts of papers presented at the
Cold Spring Harbor Meeting
on Cancer Cells

GENETICS AND MOLECULAR BIOLOGY OF BREAST CANCER

September 2–September 6, 1992

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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Arranged by
Mary-Claire King, *University of California, Berkeley*
Marc Lippman, *Georgetown University Medical Center*

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whose untimely loss to breast cancer
strengthens our resolve to
eradicate this tragic disease.

PROGRAM

WEDNESDAY, September 2—7:30 PM

Welcoming Remarks: James D. Watson

SESSION 1 PLENARY SESSION

Chairperson: J.D. Watson, Cold Spring Harbor Laboratory

Fisher, B., University of Pittsburgh, Pennsylvania: The current art of breast cancer prevention and treatment.

Aaronson, S.A., Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Maryland: Aberrant growth factor signaling pathways in human breast cancer. 1

THURSDAY, September 3—9:00 AM

SESSION 2 CURRENT CLINICAL REALITIES, EPIDEMIOLOGY, AND PATHOLOGY

Chairperson: I.C. Henderson, University of California, San Francisco

Henderson, I.C., University of California, San Francisco: Promises, fulfilled and unfulfilled, from adjuvant systemic theory.

Page, D.L., Dept. of Pathology, Vanderbilt University Medical Center, Nashville, Tennesee: Markers of increased breast cancer risk and precursor lesions of breast cancer. 2

Pike, M.C., Spicer, D.V., Rude, R., Pike, L.A., University of Southern California Medical School, Los Angeles: Early experience with a modified hormonal contraceptive aimed at reducing breast-cell proliferation. 3

Norton, L., Memorial-Sloan Kettering Cancer Center, New York, New York: Biokinetic implications of the systemic therapy of human breast cancer.

THURSDAY, September 3—2:00 PM

SESSION 3 GENETICS I

Chairperson: M.-C. King, University of California, Berkeley

Friend, S.H., Barbier, N., Frebourg, T., McIntyre, J., Kassel, J., Massachusetts General Hospital Cancer Center, Charlestown: Lessons to be remembered from the identification of the first breast cancer susceptibility gene, *p53*. 4

Ponder, B.A.J.,[1] Smith, S.A.,[1] Easton, D.F.,[2] [1]CRC Human Cancer Genetics Research Group, Cambridge; [2]Section of Epidemiology, Institute of Cancer Research, Sutton, United Kingdom: Molecular genetics of familial breast-ovarian cancer. 5

Nakamura, Y., Sato, T., Takita, K., Miyagi, M., Sakamoto, T., Cancer Institute, Tokyo, Japan: Accumulation of genetic alterations in breast cancer. 6

Callahan, R.,[1] Cropp, C.,[1] Merlo, G.,[1] Steeg, P.,[1] Liscia, D.,[2] Lidereau, R.,[3] [1]National Cancer Institute, National Institutes of Health, Bethesda, Maryland; [2]Anatomic Pathology, S. Giovanni Hospital, Torino, Italy; [3]Centre Rene Huguenin, St. Cloud, France: Molecular lesions in sporadic human breast carcinomas. 7

THURSDAY, September 3—4:30 PM

Wine and Cheese Party

THURSDAY, September 3—7:30 PM

SESSION 4 POSTERS I (A—F)

FRIDAY, September 4—9:00 AM

SESSION 5 GENETICS II

Chairperson: **M.-C. King,** University of California, Berkeley

FRIDAY, September 4—2:00 PM

SESSION 6 GROWTH FACTORS AND THEIR RECEPTORS I

Chairperson: M. Lippman, Georgetown University Medical Center

FRIDAY, September 4—7:30 PM

SESSION 7 POSTERS II (G—M)

SATURDAY, September 5—9:00 AM

SESSION 8 GROWTH FACTORS AND THEIR RECEPTORS II

Chairperson: **M. Lippman,** Georgetown University Medical Center

SATURDAY, September 5—2:00 PM

SESSION 9 POSTERS III (N—Z)

SATURDAY, September 5

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

SUNDAY, September 6—9:00 AM

SESSION 10 INVASION AND METASTASIS

Chairperson: **S.A. Aaronson,** National Cancer Institute, National Institutes of Health

AUTHOR INDEX

ABERRANT GROWTH FACTOR SIGNALLING PATHWAYS IN HUMAN BREAST CANCER

Stuart A. Aaronson, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland

Accumulating evidence has implicated the aberrant expression of genes that act at rate limiting steps in mitogenic signalling pathways as important determinants of malignancy. Amplification and/or overexpression of genes encoding growth factors or their receptors contributes to a wide variety of human tumors. Other genes that act early in the intracellular transduction of growth factor signals are commonly implicated as oncogenes as well. Efforts to dissect the biochemical cascade initiated by growth factor triggering as well as new approaches aimed at identifying other critical genes in these pathways will be described. Recently, we have isolated novel human growth factors which appear to be major paracrine effectors of normal epithelial cell proliferation. Mechanisms by which these factors may influence breast tumor progression will be discussed. Finally, new approaches for therapeutic intervention based upon our present understanding of the molecular genetic basis of cancer will be described.

MARKERS OF INCREASED BREAST CANCER RISK AND PRECURSOR LESIONS OF BREAST CANCER

David L. Page, Department of Pathology, Vanderbilt University Medical Center, Nashville, Tennessee.

Specific combined histologic and cytologic patterns of epithelial hyperplasia indicate a risk of breast cancer development of four times the general population in only 5-10% of women with otherwise benign biopsies. The length of time at risk is probably best limited to 10-15 years, as longer periods of prediction are not well-supported by currently available experience. The histologic categories which are linked to these moderately increased risk indicators consist of specific combined cytologic and cellular pattern criteria of "atypical hyperplasia." Specific histopathologic lesions indicating varied levels of subsequent risk of the development of invasive breast cancer have gained further acceptance and corroboration from analogous studies in the last five years.

Several studies have indicated an appreciable interaction between atypical hyperplasia and other non-anatomic risk factors, particularly a family history of breast cancer. Also, lower dosage estrogen replacement (specifically conjugated preparation) after menopause does not appear to further affect risk in any histologically defined group.

Probably only non-comedo examples of ductal carcinoma in-situ may be regarded as true precursor lesions in the human breast, and they are not to be regarded as obligate precursors. Currently, most molecular markers are useful only in more advanced lesions such as comedo carcinoma in-situ.

EARLY EXPERIENCE WITH A MODIFIED HORMONAL CONTRACEPTIVE AIMED AT REDUCING BREAST-CELL PROLIFERATION

M.C. Pike, D.V. Spicer, R. Rude, and L.A. Pike
University of Southern California Medical School
Los Angeles, CA 90033-9987

Use of combination-type oral contraceptives is known to significantly reduce a woman's risk of both endometrial and ovarian cancer. The reasons for these effects are essentially understood. Our understanding of normal breast epithelial physiology, and our ability to develop approaches to prevent breast cancer, have trailed significantly behind our understanding of the endometrium and ovary. However, the critical mass of knowledge now exists to permit the design of a hormonal contraceptive that should reduce breast cancer risk.

We will show that the design requirements of a hormonal contraceptive to reduce all three major female cancers are:

Endometrium	Reduce exposure of the endometrium to 'unopposed' estrogen.
Ovary	Reduce the frequency of ovulation.
Breast	Reduce exposure of the breast to 'estrogen plus progestogen'

A prototype contraceptive regimen to achieve these aims is currently in clinical trial at our institution.

The regimen is based on using a GnRHA agonist to prevent ovulation and to give just sufficient add-back steroids to prevent any harmful consequences of the GnRHA-induced hypoestrogenemia.

Preliminary results from this trial will be presented.

LESSONS TO BE REMEMBERED FROM THE IDENTIFICATION OF THE FIRST BREAST CANCER SUSCEPTIBILITY GENE, p53.

S.H. Friend, N. Barbier, T. Frebourg, J. McIntyre, J. Kassel
MGH Cancer Center, Charlestown, MA 02129

It has been estimated that 5% of the over 150,000 women in the US who develop breast cancer each year may carry germline mutations in breast cancer susceptibility genes. The significant heterogeneity in the hereditary forms of breast cancer places potentially significant impediments to many cloning strategies that depend on the analysis of multiple families. We felt that one method to overcome these hurdles was first to focus on families with a unique clustering of breast cancer which might represent inactivations a simple breast cancer susceptibility gene. The Li-Fraumeni Syndrome (LFS) has been defined as the occurrence of cancers in three close relations under the age of 45, one of who has a sarcoma. In families with LFS, breast cancer is the most frequent malignancy. By using a candidate gene approach, we identified germline p53 mutations in affected members of LFS families. Since then we have developed rapid screening techniques which have allowed us to survey several high .populations. It has also allowed us to determine the frequency of de novo germline p53 mutations in women with breast cancer. Because of a concern that some p53 mutations not recognized as common polymorphisms might have variable effects in increasing cancer risk, we have developed several functional assays by which to test whether specific mutations inactivate p53. These studies with the germline p53 mutations should serve as a prototype for working with mutations in other more commonly altered breast cancer susceptibility genes.

MOLECULAR GENETICS OF FAMILIAL BREAST-OVARIAN CANCER

B.A.J. Ponder[1], S.A. Smith[1], D.F. Easton[2], [1]CRC Human Cancer Genetics Research Group, Cambridge; [2]Section of Epidemiology, Institute of Cancer Research, Sutton, UK.

In a study of 31 breast and 14 breast-ovarian cancer families we have obtained clear evidence for linkage to markers on chromosome 17q for the families with ovarian cancer, but only weak evidence in those without ovarian cancer.

To investigate the relationship between allele loss in tumours and the predisposing mutation on chromosome 17q12-21, we have analysed breast and ovarian tumours from 4 breast-ovarian families which show strong evidence of linkage on 17q. 9 of 13 tumours showed allele losses at loci flanking the putative predisposing gene; in each of these 9 tumours the allele losses affected the wild-type chromosome.

These results suggest that the putative breast-ovarian cancer gene is a tumour suppressor gene. The possibility that the predisposing mutation is dominant at the cellular level, and that the allele losses reflect the inactivation of a distinct suppressor gene in the same region, with selection to retain the chromosome carrying the germline mutation, cannot however be excluded.

ACCUMULATION OF GENETIC ALTERATIONS IN BREAST CANCER

Y. Nakamura, T. Sato, K. Takita, M. Miyagi, T. Sakamoto, Cancer Institute, Tokyo, Japan

Nearly 300 breast tumors were examined for loss of heterozygosity at several loci on chromosomes 3p, 11p, 16q, and 17, and amplification of the erbB2 oncogene. These alterations were compared with histopathological and clinical features. A significant association was detected between loss of heterozygosity on chromosomes 17p and 17q and amplification of the erbB2 oncogene (17p, P=0.000721, by Fisher's exact test; 17q, $p<0.001$, $x^2=12.135$). Furthermore, we looked for correlations between metastasis of breast cancer to a regional lymph-node(s) and LOH. However, tumors showing LOH of chromosomes 11p ($\chi^2=10.82$, $p<0.01$) and 17p ($\chi^2=6.78$, $p<0.01$) revealed a significantly higher incidence of metastasis to a regional lymph-node(s) than tumors without LOH on these chromosomal arms. Moreover, only four of 30 (13%) patients with tumors that retained both 11p and 17p had metastasis to a regional lymph-node(s), compared to 24 of 32 (75%) patients with tumors that had lost both 11p and 17p. These results suggested that accumulation of genetic alterations, including loss of function of tumor suppressor genes on chromosomes 3p, 13q, 16q, and 17, and amplification of the erbB2 oncogene, may contribute to tumor development and/or progression in primary breast cancer. In addition, we have isolated the human homologue of the rat prohibitin gene and mapped it to chromosome 17q12-21 where a gene responsible for hereditary breast cancer was localized. DNA sequence analysis in this gene in 50 sporadic breast cancers; one showed a 2-base deletion resulting in truncation of the gene product due to a frame shift; the other had a C to T transition in an intron adjacent to an intron-exon boundary. These results suggest that this gene may be associated with tumor development and/or progression of at least some breast cancers.

MOLECULAR LESIONS IN SPORADIC HUMAN BREAST CARCINOMAS

Robert Callahan[1], Craig Cropp[1], Giorgio Merlo[1], Patricia Steeg[1], Daniel Liscia[2], and Rosette Lidereau[3]. [1]National Cancer Institute, Bethesda, MD 20892, [2]Anatomic Pathology, S. Giovanni Hospital, USL-1, Torino, Italy, [3]Centre Rene Huguenin, St. Cloud, France.

We have undertaken a systematic study of primary human breast tumor DNAs to identify and characterize frequently occurring somatic mutations. Among these mutations, loss of heterozygosity (LOH) on chromosomes 1p (37%), 1q (20%), 3p (30%), 7 (41%), 13q (30%), 17p (49%), 17q (29%), and 18q (34%) in our panel of primary breast tumor DNAs. Specific subsets of tumors could be defined based on the particular collection of mutations they contain. One goal of this study has been to determine whether there is a significant association between specific mutations and clinical parameters of the disease. For instance, LOH on Chromosome 7 in tumor DNA is associated with patients that have a shorter disease-free period ($p=0.00022$) and poor overall survival ($p=0.0036$). In addition, we have recently found that mutations affecting chromosome 17p are associated with breast tumors having a high proliferative index, a parameter closely linked to poor prognosis. We have begun to search for putative tumor suppressor genes within the regions of the genome affected by LOH. There are two apparent target loci on chromosome 17p. One of these is the p53 gene. The other is an as yet undefined locus telomeric to the p53 gene. On chromosome 17q, loss of expression of the NM23 gene in tumors was also found to be linked to patients having a poor prognosis ($p=0.018$). Although a significant trend ($p=0.044$) was found between LOH of the NM23 gene and loss of NM23 expression, no point mutations were found within the coding region of the NM23 gene by SSCP or nucleotide sequence analysis. These and others results suggest to us that there may be potential tumor suppressor genes both centromeric and telomeric of the NM23 locus on chromosome 17q.

RETINOIC ACID RESPONSE IN BREAST CANCER CELL LINES

N. Ahuja, G. Huper, J. Iglehart and J. Marks, Dept of Surgery, Duke University Medical Center, Durham, North Carolina.

Retinoic acid (RA), a metabolite of vitamin A, functions in normal cellular differentiation, vertebrate morphogenesis, and may have a role in oncogenesis. Retinoic acid receptors (RAR) are members of a superfamily of nuclear steroid hormone receptors and respond to RA and its various derivatives. These receptors function by binding to *cis*-acting DNA sequences termed retinoic acid response elements (RARE). We examined RA pathways in breast epithelial cells by transient transfection of chimeric DNA constructs containing RARE modified promoters linked to chloramphenicol acetyl transferase (CAT). CAT activity was used as a measure of RA response. Ten established human breast cancer cell lines were transiently transfected with RARE-CAT constructs and treated with 10^{-6}M RA. Eight out of ten cell lines showed greater than 20 fold induction of CAT activity upon treatment with RA. Short term cultures of normal mammary epithelial cells responded to a similar magnitude. Two cell lines, MDA-MB-231 and MDA-MB-468, showed less than five fold induction of RARE-CAT expression. Co-transfection of an expression plasmid for the RAR-α gene did not increase the response. An independent measure of retinoic acid responsiveness is growth inhibition in the presence of RA. In assays of cell growth, RA inhibited the growth of the two non-responsive cell lines by less than 30%, whereas, RA inhibited the growth of selected responsive lines by greater than 70%. Therefore, a subset of breast cancer cell lines appear to be defective in RA signalling pathways and insensitive to growth inhibitory effects of RA.

ASSOCIATION OF HER-2/neu ONCOGEN AMPLIFICATION WITH LOW NATURAL KILLER CELL ACTIVITY IN BREAST CANCER

P.Aiginger[1], C.Wiltschke[2], E.Tyl[2], A.Steininger[2], J.Spona[2], R.Zeilinger[2], F.Kury[2], K.Czerwenka[2], E. Kubista[2], P.Speiser[2], P.Preis[1] and C.C.Zielinski[1]

[1]1st Dept.Internal Medicine and [2]1st Dept.Obstetr. and Gynecol., Univ.Hosp., Vienna, Austria.

Increased expression of the HER-2/neu oncogen in breast cancer correlates with a decreased level of estrogen receptor and is an important prognostic factor. We have investigated whether there is a connection between HER-2/neu oncogen expression and immunologic parameters directed against tumor defense. Thus, HER-2/neu oncogen expression was investigated by Westernblot analysis and immunochemistry, whereas natural killer (NK) cell activity was measured by a chromium-release assay using the K562 cell-line as targets with three effector:target ratios (100:1, 50:1, 25:1). In patients with breast cancer, NK-activity was significantly higher, as compared to patients with benign tumors or healthy control individuals ($p < 0,001$). Moreover, 25% of patients with breast cancer showed an overexpression of HER-2 protein. Within this group of patients NK-activity was significantly lower ($45,6\pm16,1\%$, $37,2\pm12,4\%$, $16,8\pm4,1\%$), as compared to the group with no HER-2 protein overexpression ($57,3\pm11,0\%$; $p < 0,006$; $46,1\pm10,1\%$; $p < 0,005$; $20,8\pm8,4\%$, $p < 0,02$). NK-activity was never increased with HER-2/neu overexpression. Thus there was a significant correlation of cytolytic effector cell activity with HER-2/neu expression ($p=0,006$), and HER-2/neu overexpression correlated with a negative hormone receptor status ($p=0,004$). In conclusion, these data add further evidence to previous observations from this laboratory that certain tumor characteristics may be associated with reactions of the host in breast cancer.

LINKAGE ANALYSIS OF CHROMOSOME 17q MARKERS AND BREAST-OVARIAN CANCER IN ICELANDIC FAMILIES, AND POSSIBLE RELATIONSHIP TO PROSTATIC CANCER.

A. Arason, R. B. Barkardóttir and V. Egilsson, Laboratory of Cell Biology, Department of Pathology, University Hospital of Iceland, P.O. Box 1465, IS 121 Reykjavik, Iceland

Seven families, selected for breast cancer segregation, have been analysed for chromosome 17q12-q23 linkage to breast and ovarian cancer. In two of them linkage is seen with most markers tested, increasing towards the most proximal region, but without informative recombinations above NM23. A third family shows positive signs of linkage only for the most proximal marker, D17S250, and a key recombinant suggests a predisposing gene mapping proximal to D17S579. In the remaining families, no linkage is observed. Families with 17q linkage are not easily distinguished by clinical characteristics such as early onset (mean age at diagnosis ≤45 years) or organs involved. In fact, the family with the highest lod scores (≥2.3) belongs to the "later onset" (>45 years) category of families while the converse is true for one family without 17q linkage. Linkage heterogeneity in familial breast cancer is thus confirmed, but no correlation is seen with clinical parameters.

Interestingly, prostatic cancer is the most frequent malignancy after breast cancer in our families (12 cases total, all metastasizing), and is especially prevalent in males presumed to carry the trait. Of 16 obligate male carriers, seven (44%) had developed prostatic cancer. Haplotype analysis in families with 17q linkage reveals two further prostatic cases as potential carriers. We propose that breast cancer genes may predispose to prostatic cancer in male carriers.

ASSOCIATION BETWEEN $p185^{HER2}$ SIGNAL TRANSDUCTION AND CISPLATIN-INDUCED CYTOTOXICITY IN HUMAN BREAST CARCINOMA CELLS

CL Arteaga, SJ Stewart, TL Carty-Dugger, AF Russell-Winnier, LK Shawver. Depts of Medicine and Cell Biology, Vanderbilt University School of Medicine, Dept of Veteran Affairs Medical Center, Nashville, TN, and Berlex Biosciences, Alameda, CA

The c-erbB-2 (HER-2/neu) proto-oncogene encodes a 185-Kda transmembrane glycoprotein with intrinsic tyrosine (tyr) kinase activity. Specific antibodies against p185 enhance the cytotoxic effect of the DNA alkylator cisplatin against c-erbB-2-overexpressing human carcinoma cells (Cancer Res 51:4575, 1991). We have studied the possible relationship between the receptor signal transduction and cisplatin-induced cytotoxicity utilizing the human breast cancer SKBR-3 cell line and the monoclonal IgG1 TAb 250 raised against p185. TAb 250 induced tyr phosphorylation of p185 and of the receptor substrate phospholipase C (PLC)-γ1. Simultaneously, PLC-γ1 activity, measured by [3H]-PIP2 hydrolysis, was increased 61±12% above control, and 20% of the total cell PLC-γ1 protein translocated to the membrane after antibody treatment. Tyr phosphorylated p185 was detectable in Western blots of PLC-γ1 immunoprecipitates as early as 1 min after TAb 250 treatment, indicating association of the receptor substrate with p185. Concordantly, up to 90% of the total cell protein kinase C activity, measured as incorporation of ^{32}P into histone III-S, translocated to the membrane after p185 stimulation. Finally, we tested whether blockade of the p185 tyr kinase with the tyr kinase inhibitor tyrphostin 50864-2 would abrogate the TAb 250-mediated enhancement of cisplatin sensitivity in SKBR-3 cells. Preincubation of SKBR-3 cells with a non-toxic (20-30 μM) concentration of tyrphostin 50864-2 abrogated the TAb 250-induced tyr phosphorylation of p185, the stimulation of PLC-γ1 catalytic activity, and the enhancement of cisplatin-mediated cytotoxicity. Experiments examining cellular uptake of ^{195m}Pt-cisplatin and drug binding to DNA in response to antibody treatment in these cells are currently in progress. Taken together these data support an association between elements of the p185 signal transduction pathway and cisplatin sensitivity. The clinical implications of this association in the treatment of c-erbB-2-overexpressing carcinomas remain to be established.

DNA SEQUENCE POLYMORPHISMS ASSOCIATED WITH ENDOGENOUS MURINE LEUKEMIA VIRUS-RELATED ELEMENTS IN MOUSE MAMMARY CARCINOMAS

B.B. Asch[1], H.L. Asch[1], D.L. Stoler[2], and G.R. Anderson[2], Departments of [1]Expt. Pathology and [2]Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York

About 30-40 copies of murine leukemia virus-related (MuLVr) elements reside in the mouse genome. The full-length elements are 8-9 kb in size and contain *gag*, *pol*, *env*, and long terminal repeat sequences, hallmarks of retroviruses. MuLVr elements are etiological agents of leukemia and lymphoma in certain mouse strains, wherein they induce transformation by transposing and causing insertional mutagenesis of cellular oncogenes. Recent studies in our laboratory have revealed that expression of endogenous MuLVr elements is elevated 10-70 fold in many primary mammary carcinomas of chemical, hormonal, and viral etiologies compared to normal mammary gland of BALB/c mice. As increased expression of endogenous retrotransposons is associated with an increased risk for transposition, mutations due to MuLVr transpositions may have occurred during the development of these tumors. We have therefore begun to search for evidence of such mutations involving MuLVr sequences in the mammary tumors. In our first approach, we compared restriction fragments of DNA isolated from normal mouse mammary gland and the mammary carcinomas. The DNAs were digested with *Eco*R-I, *Hin*d-III, or *Bam*H-I to obtain fragments comprised of sequences from both MuLVr elements and their cellular flanking regions. The fragments were analyzed by Southern blotting and hybridization with a cDNA probe which detects all MuLVr elements. No differences in the set of MuLVr-containing fragments produced by *Eco*R-I and *Hin*d-III were detected between normal and tumor DNAs. After restriction with *Bam*H-I, all samples contained prominent fragments of 9.5, 5.1-5.8, 3.9, 2.8, 2.0, and 1.7 kb in size which hybridized with the MuLVr probe. In addition, 2 extra bands of 2.6 and 2.5 kb were present in DNA from 4 tumors which had elevated levels of MuLVr RNA. The extra bands were not found in samples from normal mammary gland or from tumors which did not have increased MuLVr expression. The basis for these DNA sequence polymorphisms is currently under investigation.

DEREGULATION OF ENDOGENOUS RETROTRANSPOSONS IN MOUSE MAMMARY CARCINOMAS OF DIVERSE ETIOLOGIES

H.L. Asch[1], B.B. Asch[1], D.L. Stoler[2], and G.R. Anderson[2], Departments of [1]Expt. Pathology and [2]Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York

The genome of most mice contains four major families of endogenous retrotransposons: intracisternal A-particles (IAPs), murine leukemia virus-related (MuLVr) elements, VL30 sequences, and murine mammary tumor virus (MMTV). The ability of some of these endogenous retrotransposons to transpose in nuclear DNA and alter expression of genes critical in growth control by insertional mutagenesis is now well-documented from research on several types of cancers, including mammary carcinomas. However, with the exception of endogenous MMTV, very little is known about the expression and activities of these elements in normal and malignant mammary epithelium. We have begun a series of investigations to analyze and compare expression of the three other types of endogenous retrotransposons, IAPs, MuLVr elements, and VL30 sequences, in normal mammary gland and mammary neoplasms of different etiologies in BALB/c mice. In the present study, primary mammary carcinomas induced by chemical, hormonal, viral, and unknown agents were examined by slot and Northern blot analyses. Each of these cancers, despite their various origins, had aberrant expression of one or more of the latter three endogenous retrovirus-like elements. The level of IAP and/or MuLVr transcripts was elevated 5-100-fold in most of the tumors compared to normal mammary tissue, whereas VL30 RNA was markedly decreased from 5-40-fold. Moreover, many of the tumors expressed two IAP transcripts of 5.0 and 1.9 kb not detected in normal samples. Our results thus indicate that there are substantial changes in the expression of as many as three families of endogenous retrotransposons during mouse mammary tumorigenesis, regardless of etiology. Interestingly, these changes are more widespread and consistent than those reported for endogenous mouse mammary tumor virus. IAPs and MuLVr elements have already been shown to contribute directly to neoplastic progression by insertional mutagenesis in cells of hematopoietic lineage. Their activation in mammary carcinomas raises the possibility that they may also play a role in some pathways of mouse mammary carcinogenesis.

CHARACTERISTICS OF P53 PROTEIN IN NORMAL AND HUMAN PAPILLOMA VIRUS IMMORTALIZED HUMAN MAMMARY EPITHELIAL CELLS

Vimla Band, Laurie Delmolino, Sorab Dalal[1] and Elliot Androphy[1,2]
Departments of Radiation Oncology, Molecular and Microbiology[1] and Dermatology[2], New England Medical Center Hospitals, 750 Washington Street, Boston, MA 02111

The long term goal of our studies is to understand the molecular mechanisms underlying immortalization of human mammary epithelial cells. Previously we have demonstrated that human papilloma virus (HPV) 16 E6 gene is essential and sufficient for immortalization of human mammary epithelial cells (HMECs). Consistent with the results obtained by others in rabbit reticulocyte lysate system in vitro, we found a dramatic reduction of p53 tumor suppressor gene product in HPV16 E6-immortalized cells. To elucidate the mechanism of loss of p53 protein in vivo, we characterized the p53 gene and protein in normal and HPV E6-immortalized cells. First, all normal HMEC strains examined show easily detectable levels of p53 protein when analyzed by immunoprecipitation from metabolically labelled cells. To rule out the possibility of missence mutations in p53 gene in these in vitro established cells, we sequenced the entire coding region of the p53 mRNA in one of the normal cell strain called 76N and found it to be wildtype. The p53 protein in HMECs was unusually stable (half life of > 6 hrs.) in contrast to widely examined rodent fibroblasts (half life < 30 min.). This long half life may account for easy detectability of p53 protein in HMECs. In contrast to normal HMECs, the half life of p53 protein in HPV16 E6-immortalized cells was markedly reduced (about 30 min.). This result provides direct evidence for in vivo degradation of p53 protein by E6 gene product. To examine if p53 degradation is essential for E6-induced immortalization, we have transfected E6 genes of HPV6 and BPV1 which have been reported not to degrade p53 protein in in vitro experiments. Surprisingly, each of these E6 genes resulted in immortalization of HMECs, though the efficiency was lower than HPV16 E6. We are currently examining the status of p53 protein in these immortalized cells. These analyses should help determine the importance of p53 loss in E6 immortalization.

A CANCER FAMILY WITH AN ABNORMAL EXPRESSION OF p53 PROTEIN IN MALIGNANT CELLS AND IN A SPECTRUM OF BENIGN TISSUES

D Barnes[1], A Hanby[1], C Gillett[1], S Mohammed[2], S Hodgson[2], I Leigh[3], T Purkis[3], C MacGeoch[4], N Spurr[4], J Bartek[5], B Vojtesek[6], S Picksley[6], D Lane[6].

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As part of our immunohistochemical studies into the expression of p53 protein in patients who are members of families with an inherited susceptibility to cancer, we have encountered a mother and daughter who have a very unusual pattern of p53 protein expression. Both have had mammary carcinoma and we have found an abnormal accumulation of p53 protein in tumours from both patients but, exceptionally, we have also found expression of the protein in many normal endothelial and epithelial cells, in particular in the epidermal keratinocytes. The mother has now died but the daughter is alive and well and we have obtained a skin biopsy from her which enabled us to culture fibroblasts and keratinocytes for further study. Both cell lines also express vastly elevated amounts of the protein. Experiments to date show that the protein has all the features of wild type and none of the features of mutant protein. Furthermore, despite exhaustive analysis of the DNA in 2 laboratories, no mutations have been found in the coding regions of the p53 gene, suggesting that there might be an abnormality in the post translational control mechanism affecting the degradation of p53. It appears that we have identified a new type of inherited cancer syndrome which involves the p53 gene in a way which is different from the mutations found in the Li-Fraumeni syndrome.

AN INTRON MUTATION IDENTIFIED IN A LI-FRAUMENI SYNDROME FAMILY WHICH RESULTS IN A SPLICED FORM OF P53

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The Cancer Surveillance Program of Orange County, a population-based cancer registry, has been used to ascertain early-onset (<30 years at diagnosis) breast cancer cases. 72 cases were identified, and family history was obtained for 24 individuals. One of these families appeared to be a Li-Fraumeni cancer syndrome family, with multiple cases of early breast cancer, brain and bone cancers. Germ-line p53 mutations have been described in Li-Fraumeni syndrome individuals, and may represent the cancer predisposing mutation for this family cluster of cancers. A lymphoblastoid cell line was established from the only surviving obligate carrier, and p53 sequence analysis performed. Following isolation of poly A^+ mRNA, reverse-transcriptase PCR amplification was performed, and the entire coding domain sequenced following asymmetric PCR. Two p53 mRNA species were expressed: a wild-type, and a species in which exon 10 is spliced out. These data suggest that there is a mutation in the splice acceptor sequence for exon 10 such that it is spliced inappropriately. The presence of this mutation is now being examined in paraffin-embedded tumor tissue from other affected individuals in this family.

ALLELIC IMBALANCE IN PRIMARY BREAST CARCINOMAS AND LYMPH NODE METASTASES: RELATIONSHIP WITH TUMOR PLOIDY.

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Recently, we have completed an allelotype study of 86 primary breast cancers in which all non-acrocentric chromosome arms were screened with at least one polymorphic marker per arm for allelic imbalance. Allelic imbalance (AI) includes both allelic loss as well as a gain in copy number of one allele. Highest frequencies of allelic loss (40-60%) were found on chromosomes 16q, 6q, 8q and 17p whereas allelic gain was found predominantly for 1q, 11q and 20q. More than 10 different chromosome arms showed AI in more than 30% of the informative cases. The majority of tumors (73%) showed concurrent AI on 2 or more chromosome arms. Comparison with flow cytometric DNA index determinations showed that AI on 3p and 17p was associated with aneuploidy. Fractional AI was highest in tumors with hypotetraploid DNA indices suggesting an association between tetraploidization followed by chromosome loss and extensive molecular-genetic alterations. We now have extended our allelotype study with 9 primary tumors and their corresponding lymph node metastases. In all 9 tumors AI was found on 4-10 different chromosome arms. Lymph node metastasases showed identical allelotypes as their corresponding primary tumors, irrespective of intra-tumor DNA-ploidy differences. These results indicate that the molecular genetic evolution of breast cancers is largely completed before the formation of metastases. In addition the allelotype can be used as a unique and highly specific clonal marker for individual tumors.

IDENTIFICATION OF GENES INVOLVED IN MAMMARY DEVELOPMENT USING A PROMOTER-TRAP RETROVIRUS.

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In an effort to improve our knowledge of the genetic program of mammary differentiation we are using a recombinant promoter trap retrovirus to identify genes involved in this process. The retrovirus makes use of the well established cooperation of the *myc* and *ras* oncogenes in causing tumors (we therefore have designated the virus COPT for *c*ooperating *o*ncogenes *p*romoter *t*rap retrovirus). Replacing the structural genes of the retrovirus is a c-*myc* gene driven by the ubiquitously expressed phosphoglycerate kinase promoter. In the long terminal repeat of the virus is a promoterless v-Ha-*ras* oncogene. If the proviral form of this virus integrates adjacent to an active cellular promoter, both oncogenes will be expressed and a tumor will form.

Infection of mouse mammary epithelium will be performed at adolescence by direct injection through the nipple into the main duct of the gland. Specific infection of the end buds is currently being tested using retroviruses harboring marker genes. Tumors appearing shortly after infection will likely be due to transcription of *ras* from "housekeeping" type promoters. Mice will then be bred to reinitiate development. Tumors arising during pregnancy will likely be the result of activation of mammary-specific promoters, those of interest. The cellular sequences at this locus can than be recovered using the rapid PCR cloning strategy of Silver and Keerikatte, for further analysis.

PUTATIVE INVOLVEMENT OF CYCLIN D GENES IN HUMAN MAMMARY CARCINOGENESIS

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In breast cancer, some of the complex amplification events encompassing various portions of 11q13 include *CCND1*, a gene encoding a cyclin of the D type, also referred to as *PRAD1*. Although expression of this gene in mammary tumors correlates with its amplification, there is no definite proof that it is the gene under selection in the process of tumor formation.

Two other members of the cyclin D gene family have been cloned from human DNA. They are located in genomic regions where no amplification has ever been shown to take place in breast cancers : *CCND2* is on 12p13 and *CCND3* is on 6p21*.

In order to test the hypothesis that cyclin D genes play an important role in mammary carcinogenesis, we have studied their amplification status in more than 100 primary breast carcinomas. We have found that all three genes were amplified in *ca* 1/5 tumors (18.5%, 24.5%, and 23.5% for *CCND1*, *CCND2* and *CCND3*, respectively). Moreover, *CCND2* and *CCND3* were shown to be co-amplified in a statistically significant number of tumor samples. We have now collected a large series of mammary carcinomas for which both RNA and DNA could be prepared in order to study a possible correlation between RNA expression and cyclin D gene copy numbers. Such a correlation would implicate them as relevant physiologic targets for gene amplification in this disease.

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INDUCTION OF THE METASTATIC PHENOTYPE BY DNA TRANSFECTION IN A RAT MAMMARY MODEL

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We have used the technique of DNA transfection to attempt to isolate and identify DNA sequences which can induce the metastatic phenotype in a benign rat mammary epithelial cell line.

Cotransfection of restriction enzyme-fragmented DNA from malignant human cell lines from a primary breast carcinoma (1) or from pleural effusions (2) and the drug resistance plasmid pSV2*neo* (3) yielded several transfectants which metastasized to lungs and/or lymph nodes in syngeneic rats. No metastasis was obtained when non-transfected recipient cells, or cells transfected with pSV2*neo* plasmid alone, or cells transfected with DNA isolated from benign mammary tumour cells were injected into rats under the same conditions. Cells which metastasized were re-established in culture and DNA isolated from them. Hybridisation with an oligonucleotide specific for the human *Alu* sequence has revealed the presence of human DNA integrated into the genome of those cells. These results demonstrate that the metastatic phenotype can be transferred from malignant human cells in a genetically dominant manner in the model system employed. We now intend to isolate the DNA sequences responsible for induction of the metastatic phenotype by standard cloning and sequencing techniques.

This work has been complemented by transfecting the same recipient cell line with the gene for p9Ka, whose expression has been correlated with metastatic breast disease and a plasmid containing an activated form of the *ras* oncogene, Ha-*ras*-1 (4).

p9Ka and its mRNA are expressed at a high level in a malignant rat mammary epithelial cell line which has a high metastatic potential, whereas non-metastatic mammary epithelial cells fail to express p9Ka mRNA or protein (5). The mRNA of the mouse homologue of p9Ka, called *mts1*, is expressed at a high level in a subline of mouse mammary tumour cells with a high metastatic potential, but it is not expressed detectable in a closely related subline of the same cells that does not metastasized under the same conditions (6).

When our cloned gene for p9Ka is transfected as part of a plasmid containing the *neo* gene into the same benign recipient cell line and injected into syngeneic rats, metastatic tumours in lungs and lymph nodes are formed (7). However, cells transfected with Ha-*ras*-1 reproducibly fail to metastasize in the same cell system.

These results suggest that the presence and/or expression of additional copies of the *p9Ka gene* can induce the metastatic phenotype in those cells, whereas transfection of *ras* does not confer metastatic properties.

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p53 GENE MUTATIONS AND LOSS OF HETEROZYGOSITY (LOH) IN BREAST CANCERS DETECTED BY POLYMERASE CHAIN REACTION (PCR)

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Mutations of the tumor suppressor gene, p53, have been implicated in the pathobiology of breast cancer. Because mutant p53 has a longer half life than wild type, accumulation of mutant protein results in immunopositivity. In a number of studies, p53 immunopositivity has been used as a surrogate for mutations of the p53 gene and we have recently shown a strong correlation between p53 immunopositivity and poor prognosis, estrogen receptor negativity, and high histologic grade (Thor et al., JNCI 84:845-855, 1992).

To further characterize the nature of p53 mutations presents in breast cancers, and to verify the concordance between immunopositivity and mutations, we have sequenced the conserved region of the p53 gene (exons 5 - 8, and their flanking sequences) in selected breast cancer cases. To detect the mutations, DNAs from tumors were amplified by PCR with four pairs of primers covering exons 5 - 8 and their flanking sequences. The mutations were first detected by single stranded conformation polymorphisms (SSCP) analysis, and further proved by double stranded DNA sequencing. Mutations were detected in 16 of 38 cases (42%). Of the 13 immunopositive cases, 12 (92%) had missense mutations distributed among all four exons examined. *The single exceptional case had only 1% immunopositive cells.* Among 25 cases that were negative by immunohistochemistry, 4 additional mutations were found including 1 frameshift and 3 missense mutations.

To determine whether p53 mutations occurred together with deletions of the second allele, LOH of the p53 gene was also measured. Tumor DNAs were amplified by PCR using 3 pairs of primers covering the polymorphic sites in exons 4, 6, and intron 6; the amplified DNA was digested with restriction enzymes and the LOH determined after agarose gel electrophoresis. 13 of 21 informative cases (62%) showed LOH. Surprisingly, there was no correlation between LOH and p53 mutations. Only 3 of 12 (25%) cases with LOH had mutations indicating that many breast cancers with p53 mutations retain a wild type allele. LOH of the p53 gene was also seen in 9 cases with no detectable p53 mutations suggesting the possibility that a gene other than p53 was the target of the LOH for these cases.

The successful use of PCR based technology and SSCP for measuring mutations and LOH enables us to readily screen small, paraffin-embedded samples. Currently, we are evaluating the nature of the p53 mutations and the status of the 2nd allele in a cohort of p53 immunopositive tumors which did and did not recur. Supported by NIH PO1 CA 44768.

MAPPING ALLELE LOSS REGIONS IN BREAST CANCER ON 16q AND 17q USING MICROSATELLITE POLYMORPHISMS

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Somatic loss of heterozygosity (LOH) in breast cancer is a common phenomenon, particularly on chromosomes 3p, 6q, 16q, and 17p. The target genes for these events have for some chromosome arms been identified, for others they remain to be discovered. Detailed mapping of the region undergoing LOH in a series of tumors may aid in this identification. In addition, LOH-mapping may aid in mapping genes that have been localized by means of genetic linkage in families, such as the gene for early-onset breast cancer (BRCA1) on 17q12-q21.

LOH-mapping is intrinsically limited by the number of available markers for a chromosome arm, and their informativeness. A further limitation might be the amount of tumor DNA isolated from each specimen, and a low frequency of informative LOH-breakpoints, necessitating the analysis of large series of patients. A new source of polymorphisms are the recently discovered microsatellites. These loci usually have a high polymorphic information content and are visualized by PCR which significantly reduces the amount of DNA used per typing and makes DNA isolated from paraffin embedded tissues accessible for analysis. However, the effect of asymmetric amplification of alleles as well as the presence of contaminating non-malignant DNA from stromal cells in tumor samples, on the eventual extent of allelic imbalance in lymphocyte versus tumor DNA, has not yet been thoroughly sorted out.

Here we present the results of LOH-mapping studies on 16q and 17q in more than one hundred breast tumors. The use of RFLP, VNTR, and CA-repeat type of polymorphisms, in conjunction with laser densitometry of autoradiographs, enables an evaluation of the applicability of PCR able markers in LOH-studies in both qualitative and quantitative terms.

DIETARY FAT INCREASES THE INCIDENCE OF MAMMARY TUMORS IN MMTV/v-Ha-ras TRANSGENIC MICE ("ONCOMICE")

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The incidence of breast cancer is highest in countries in which fat contributes 40% or more of total calories. Experimental studies indicate that dietary fat promotes mammary tumors, but the mechanism is unclear. Female oncomice were fed diets providing 0-25% of calories from corn oil (CO). Mammary tumor incidence correlated with fat intake, varying from 7% (0% CO) to 52% (25% CO). Ras oncogene copy number and ras mRNA levels were increased in mammary tumors derived from the 25% CO group. The ras oncogene was hypomethylated in all mammary tumors.

The type or amount of fat in the diet influences the transcription of genes encoding lipogenic enzymes and transport proteins. Mammary tumor Stearoyl CoA desaturase (SCD) and apolipoprotein E mRNA levels were altered by fat intake in concert with liver. Fat intake also affected C/EBP isoform mRNA levels. In contrast, p53 gene structure and mRNA levels were unaffected by fat intake.

High fat diets alter gene expression and transcription factor mRNA levels. These changes may alter the metabolism, growth or differentiation of initiated cells.

HAPLOTYPE ANALYSIS OF SISTER PAIRS AFFECTED BY EARLY ONSET BREAST CANCER.

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Following the initial report of linkage to markers on chromosome 17q by Hall et al., 1990, an analysis of over 200 families by an international consortium of groups has estimated that about 45% of breast cancer families in which there is no case of ovarian cancer are linked to BrCa1. However, the study also suggests that BrCa1 may make a lesser contribution to kindreds in which there are only a small number of affecteds.

In an attempt to investigate this issue further, we have typed markers D17S250, D17S579 and D17S589 which flank BrCa1 in forty five sister pairs affected by early onset breast cancer. On the basis of epidemiological studies, over 70% of such families should be the result of genetic susceptibility. The degree of haplotype sharing indicates a significant contribution of BrCa1 in this group of families ($p<0.05$). However, the proportion of sister pairs attributable to BrCa1 still has wide confidence limits, and further studies are required to provide an accurate estimate of the contribution of this gene to familial breast cancer.

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GENETIC LINKAGE ANALYSIS IN FAMILIAL BREAST AND OVARIAN CANCER - RESULTS FROM 214 FAMILIES.

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Breast cancer is known to have an inherited component, consistent in some families with autosomal dominant inheritance; in such families the disease often occurs in association with ovarian cancer. Previous genetic linkage studies have established that in some such families disease occurrence is linked to markers on chromosome 17q. We report the results of a collaborative linkage study involving 214 breast cancer families, including 57 breast-ovarian cancer families; this represents almost all the known families with 17q linkage data. Six markers on 17q, spanning approximately 30cM, were typed. The aims of the study were to define more precisely the localisation of BRCA1, the extent of genetic heterogeneity and the characteristics of linked families and to estimate the penetrance of the 17q gene. Assuming genetic homogeneity, the strongest linkage evidence was obtained with D17S588 (Zmax = 21.68 at θf = 0.13) and D17S579 (Zmax =13.02 at θf = 0.16). Multipoint linkage analysis allowing for genetic heterogeneity provided evidence that the predisposing gene lies between the markers D17S588 and D17S250, an interval whose genetic length is 6.3cM in males and 16.7cM in females. This position was supported with odds of 66:1 over other intervals. The location of the gene with respect to D17S579 could not be determined unequivocally. The best estimate of the proportion of linked breast-ovarian families was 1.0 (lower LOD-1 limit 0.71). In contrast, there was significant evidence of genetic heterogeneity amongst the families without ovarian cancer, with an estimated 45% being linked. These results suggest that BRCA1 accounts for the majority of families in which both early onset breast cancer and ovarian cancer occur, but that other genes predisposing to breast cancer exist. By examining the fit of the linkage data to different penetrance functions, the cumulative risk associated with the 17q gene was estimated to be 59% by age 50 and 82% by age 70. The corresponding estimates for the breast-ovary families were 67% by age 50 and 76% by age 70, and for the families without ovarian cancer 49% by age 50 and 90% by age 70; these penetrance functions did not differ significantly from one another.

EXPRESSION OF ONCOGENES IN MOUSE MAMMARY EPITHELIUM IN VIVO BY TISSUE RECONSTITUTION

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We have developed a method for expressing genes such as oncogenes specifically in mouse mammary epithelium in vivo. Normal epithelial cells are isolated from one mouse, oncogenes are inserted in brief primary culture using helper-free retrovirus, and the cells are implanted into the 'cleared' mammary fat pad of another mouse. A 'cleared' mammary fat pad is one from which the endogenous epithelium has been removed early in development. The transplanted cells reconstitute an epithelial 'tree' in its natural tissue environment in which some of the cells express the introduced gene. This approach has advantages over transgenic mice expressing genes from MMTV or WAP promoters: e.g. clones of cells express the inserted gene against a background of normal cells; and hormone-insensitive promoters can be used, with efficient expression in the virgin gland.

To show expression of introduced genes the enzyme beta-galactosidase was inserted, which can be stained for in whole mount. The cells expressing the gene occured in clusters interspersed with cells not expressing the gene.

The oncogenes myc, int-1/Wnt-1, Ha-ras, hst and neu (erb-B2) have been introduced and gave different abnormal growth patterns of the epithelium. myc caused mild hyperplasia, in which ducts were more closely packed than usual [Edwards et al. (1988) Oncogene 2, 407]. int-1/Wnt-1 caused vigorous hyperplasia, the epithelium showing sidebranching rather like early pregnancy [Edwards et al, Oncogene in press]. neu (erb-B2) produced small to medium foci of closely-packed, hyperplastic epithelium. Hst caused local development of alveoli on major ducts.

When hyperplasias obtained by expressing myc were isolated and Ha-ras introduced to model sequential activation of oncogenes, tumours were obtained [Bradbury et al. (1991) Int J Cancer 48, 908-915].

GROWTH STIMULATION AND GROWTH INHIBITION INDUCED BY AN ErbB-2 STIMULATORY FACTOR SECRETED BY RAT MAMMARY CARCINOMA CELLS.

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Rat mammary carcinoma (RMC) cells isolated from carcinogen-induced mammary tumors secrete a factor into conditioned medium that stimulates tyrosine phosphorylation of the erbB-2 protein. The factor can be partially purified from conditioned medium by ultrafiltration through a 30 kDa cutoff membrane and heparin-agarose chromatography of the 30 kDa retentate. Gel filtration chromatography of the activity obtained from the heparin-agarose column yields a factor with an apparent molecular weight of 105 kDa. The erbB-2 factor secreted by RMC cells has different effects on the proliferative potential of different mammary cell types. First, the RMC cells that secrete the factor proliferate continuously in serum-free medium in the absence of peptide growth factors and, under these conditions, express high levels of tyrosine-phosphorylated erbB-2. Second, this factor stimulates tyrosine phosphorylation of erbB-2 in normal human mammary epithelial cells (MCF-10A cells) which are growth stimulated by this factor when cultured in serum-free medium in the absence of EGF. By contrast, a human breast cancer cell line recently developed in our laboratory (SUM-44PE) is potently growth inhibited by the erbB-2 factor. SUM-44PE cells are cultured in serum-free medium supplemented with insulin and hydrocortisone and under these conditions express moderate levels of erbB-2 protein and undetectable levels of tyrosine-phosphorylated erbB-2. Addition of the erbB-2 factor to the medium of SUM-44PE cells induces rapid tyrosine phosphorylation of erbB-2 and completely inhibits growth of these cells at all concentrations tested.

Both the RMC cells that secrete the erbB-2 factor and the MCF-10A cells that are growth stimulated by the factor express EGF receptors in the range of 100,000 receptors per cell and expression of erbB-2 protein in both cell lines is down regulated by EGF. By contrast, SUM-44PE cells do not express detectable high affinity EGF binding sites. Thus, EGF receptors and erbB-2 receptors appear to interact and the nature of the biological response of cells to the erbB-2 factor may be determined by the presence or absence of EGF receptors.

EXPRESSION IN HUMAN BREAST TISSUE OF DNA SEQUENCES HOMOLOGOUS TO THE MOUSE MAMMARY TUMOR VIRUS

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Mouse Mammary Tumor Virus (MMTV) is the etiological agent of breast cancer in high incidence inbred mouse strains. Although no human breast cancer virus has been detected, the presence of DNA sequences homologous to the MMTV genome in human DNA has lead to the question of whether the expression of these MMTV-like sequences could play a role in the development of human breast cancer. We have recently detected for the first time the expression of these human MMTV-like sequences in hormonally stimulated primary human breast tissue. Using as a probe a cloned human DNA sequence homologous to the pol region of MMTV we have screened a cDNA library made from poly A RNA isolated from non-involved normal breast tissue of a woman with a breast tumor who underwent a mastectomy in her eighth month of pregnancy. From this cDNA library, we have isolated four recombinants which hybridize under stringent conditions to our human MMTV-like pol gene probe. DNA sequencing shows these cDNAs to be greater than 87% homologous to various regions of the entire pol gene of a 9.2 kilobase human endogenous MMTV-related retrovirus genome designated HERV-K10 (1986,J.Virol.60:589-598) which contains open reading frames (ORFs) corresponding to gag, protease (prt), reverse transcriptase (pol), and envelope (env) genes. Each individual clone contains an ORF which when combined overlap and cover the HERV-K10 putative pol gene. However, we have not as yet been successful in isolating a single cDNA capable of expressing the entire pol gene. In addition, three of our four recombinants contain cellular sequences which are non-homologous to the putative pol gene and which may preclude their being transcribed into one complete pol gene transcript.

THE RELATIONSHIP BETWEEN HUMAN BREAST CANCER AND THE INDUCTION OF MAMMARY TUMORS BY MURINE POLYOMAVIRUS.

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We have been studying the patterns of replication and oncogenesis in mice (Balb/c) infected by polyomavirus (PyV). We have found two major groups of organs that differ with respect to their ability to replicate PyV as a function of age. In infection of adult mice (athymic mice were used to bypass the effect of the immune response), viral replication is high in the mammary gland, the skin and the bone (group I organs), but very low in the kidney, the liver and the lung (group II). This contrasts with the pattern in neonatal mice, in which replication is very high in all six organs. We have also shown that the B enhancer domain is crucial for replication in neonatal tissues (in particular in group II organs), but is dispensable for replication in adult group I organs. By deduction, this suggests that the A enhancer domain controls replication in adult group II organs.

The A enhancer domain contains binding sites for transcription factors PEA1 and PEA3, the mouse homologs of AP1 and c-ets. We have demonstrated that middle T antigen, the PyV oncogene, has an essential role in viral DNA replication, mediated by the A enhancer domain, which can be bypassed by serum factors or TPA, i.e. other activators of protein kinase C. Altogether, the results suggest that the middle T pathway, which activates PKC and culminates in the activation of AP1 and c-ets, is organ restricted and cannot function in group II adult organs.

We and others have shown that the major targets of oncogenesis by PyV following infection of neonatal or athymic adult mice consist of group I organs. Our hypothesis is that the activation of AP1 and c-ets provides a major pathway of oncogenesis in group I but not in group II organs. These results may be generalized to oncogenesis by other inducers.

MUTATION ANALYSIS OF THE THRA1 GENE IN BREAST CANCER CELL LINES, SPORADIC BREAST CARCINOMAS, AND FAMILIAL BREAST CANCER PATIENTS.

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We have previously described a region of frequent allelic loss on proximal chromosome 17q in sporadic breast cancer that is likely to contain a tumor suppressor gene. This interval was bordered by the makers D17S250 on the centromeric side and D17S579 on the telomeric side which map to the 17q11-q12 region, and overlaps that of the familial breast cancer susceptibility locus BRCA1. The most frequent marker loss in this region was seen at the candidate tumor suppressor gene, thyroid hormone receptor alpha (THRA1). Southern analysis of both primary tumor tissue and breast carcinoma cell lines for gross rearrangements of THRA1 revealed a breast carcinoma cell line (BT474) with a rearrangement that deleted exons 8-10, a region encompassing the ligand binding domain of the THRA1 gene. Northern analysis revealed an altered THRA1 transcript in BT474 cells. RACE and sequence analysis of the 3' end of the mutant RNA demonstrated a splicing event to a novel sequence, which encodes for an expressed gene. This coding region was designated BTR for "breast tumor rearrangement", and was mapped to 17q by Southern and FISH analysis. Single strand conformation polymorphism analysis of all 9 protein encoding exons of THRA1 and of the expressed transcript revealed no mutations in the corresponding unrearranged THRA1 allele in BT474 nor in a series of sporadic tumors, breast carcinoma cell lines, and normal DNA samples from the youngest affected members of several German breast cancer pedigrees. Additionally, no evidence for altered THRA1 function was found in BT474 upon TRE CAT transfection experiments. We have thus ruled out THRA1 as a candidate sporadic breast cancer tumor suppressor gene and as the BRCA1 gene itself, and have begun analysis of a novel 17q gene that is rearranged in BT474.

FURTHER MAPPING OF THE BREAST/OVARY CANCER SUSCEPTIBILITY GENE ON 17q21. IMPLICATIONS FOR DNA SCREENING IN FAMILIES.

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Nineteen North American causasian families which contain a minimum of four confirmed cases of breast or ovarian cancer have been studied. Four polymorphisms (cLB17.1, D17S579, D17S598 and D17S74), which span a region of approximately 15 cM on chromosome 17q21, were typed. Our data confirm the location of a dominant gene conferring susceptibility to breast and ovarian cancer (maximum lod = 9.78). Two recombinants in one large family suggest that the breast-ovarian cancer locus lies between D17S588 and D17S579. The cross-over that places the cancer gene above D17S588 is strongly supported by its identification in several affected individuals. In contrast, the recombination that places the cancer gene below D17S579 is evident only in a woman who developed breast cancer at an age significantly higher than the mean age of breast cancer onset in her family and therefore might represent a sporadic case. The possibility that chromosome 17 marker data may be used to evaluate individual risks for women from high-risk families will certainly now be raised. Because of the linkage heterogeneity, a generalized marker-based counselling remains problematic at present; nevertheless, for a few very large families where linkage is not in doubt we feel that marker information may now be introduced with caution into the interpretation of individual risk.

GENETIC AND PHYSICAL MAPPING OF THE BRCA1 REGION ON 17Q

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We have made a detailed somatic cell hybrid map of human chromosome 17q11.2-23, containing the familial breast and ovarian cancer locus (BRCA1) at 17q12 and highly informative closely linked CA repeat markers. We are using these markers to build up a genetic map of this region and to examine LOH in 200 premenopausal breast tumors. An X-irradiation panel of 38 hamster/human and mouse/human hybrids with fragments of 17q was used to localise 28 genes in linear order in this region. More detailed mapping of over sixty probes onto chromosome 17q was done using a panel of hybrids with well defined breakpoints and ten CMGIs. Our localisation of RARA, TOP2 and 17HSD between D17S181 and THRA, the two closest known flanking markers for BRCA1, suggests that any of these is a potential candidate for the BRCA1 locus. We are building up a PFGE map and a YAC contig covering the BRCA1 region, in the hope of detecting PFGE alterations in either breast tumors or in individuals from linked families.

Regulation of Estrogen Receptor Function in Breast Cancer by Naturally Occurring Variants Suzanne A.W. Fuqua, The University of Texas Health Science Center, San Antonio, TX. 78284

The loss of hormone dependence of certain breast tumors may be due to the presence of mutated or truncated estrogen receptors (ERs) that activate transcription even in the absence of hormone, or alternatively, inactivate wild-type receptor function. We have identified a 40,000 dalton variant ER that was truncated within the hormone binding domain from supposed ER-negative human breast tumor specimens. This variant (termed an exon 5 deletion variant ER) was unable to bind hormone, however it was capable of stimulating the expression of estrogen-responsive genes in vitro in a hormone-independent manner at approximately 10-15% of the stimulatory activity of wild-type ER, hence we have termed this variant a dominant-positive receptor. Transfection of this dominant-positive receptor into MCF-7 breast cancer cells rendered these cells unresponsive to endocrine therapies such as tamoxifen and high dose progestins. However, these cells remained sensitive to ICI 164,384. These results suggest that tumors containing elevated levels of the exon 5 deletion variant may be resistant to the effects of tamoxifen, but sensitive to pure steroidal antiestrogens. We have also identified another truncated ER variant (deleted of exon 7) which was present at elevated levels in ER-positive, progesterone receptor (PgR)-negative breast tumor specimens. This 52,000 dalton exon 7 deletion variant was unable to function as a transcription inducer of estrogen-responsive genes in vitro. But when it was cointroduced into cells with wild-type ER, then the activity of wild-type receptor was reduced by approximately half. These dominant-negative effects upon wild-type receptor function could have wide ranging effects upon cells. Growth arrest was observed when MCF-7 cells were transfected with the exon 7 variant.
Conclusion: Both dominant-positive and dominant-negative ER variants may regulate normal ER function in human breast cancer cells.

BIOLOGY OF EPIDERMAL GROWTH FACTOR (EGF)-RELATED PEPTIDES IN BREAST CANCER. David S. Salomon[1], Nicola Normanno[1], Toshiaki Saeki[1], Nicholas Kenney[1], Nancy Kim[1], Fortunato Ciardiello[1], Gibbes Johnson[2], William J. Gullick[3], Gregory Plowman[4], Mohammed Shoyab[4] and George Todaro[5]. [1]National Cancer Institute and [2]Food and Drug Administration, Bethesda, MD. [3]Imperial Cancer Research Fund, London, England, [4]Bristol-Myers Squibb Pharmaceutical Research Institute and [5]Fred Hutchinson Cancer Research Center, Seattle, WA. Transforming growth factor α (TGFα), amphiregulin (AR) and cripto (CR) are members of the EGF family of proteins. TGFα and AR bind to and activate the EGF receptor. TGFα, AR and CR mRNA transcripts and/or proteins are found in approximately 50 to 70% of primary human breast tumors and in a number of human breast cancer cell lines. In breast cancer cell lines that are estrogen-responsive, physiological concentrations of 17β-estradiol (E2) can induce an increase in TGFα mRNA expression and protein production suggesting that the growth-promoting effects of E2 may be mediated in part through the intermediary action of this growth factor via an autocrine mechanism. This may be the case since infection of estrogen-responsive MCF-7 or ZR-75-1 cells with a amphotropic recombinant retrovirus containing a fragment of the human TGFα cDNA oriented in the 3' to 5' orientation to generate an antisense mRNA can significantly inhibit the induction of TGFα mRNA and protein in response to E2 and can partially block both E2 stimulated anchorage-dependent and anchorage-independent growth. The expression of TGFα and AR is also modulated by specific oncogenes that have been identified as potential growth effectors in the pathogenesis of primary human breast tumors. For example, immortalized, nontransformed MCF-10A human mammary epithelial cells are transformed by an activated c-Ha-*ras* protooncogene or by overexpression of the c-*erb* B-2 protooncogene. MCF-10A cells express approximately 3 x 10^5 EGF receptor sites/cell and require either exogenous EGF or AR for growth. Transformation of these cells by c-Ha-*ras* results in a 4-8-fold increase in the expression TGFα mRNA and a 30-40-fold increase in AR mRNA expression which is accompanied by a concomitant loss in the mitogenic responsiveness of these cells to exogenous EGF or AR. Soft agar growth of the *ras* transformed cells is partially blocked by treatment of these cells with either a TGFα neutralizing monoclonal antibody (50% growth inhibition) or with an EGF receptor blocking monoclonal antibody (85% growth inhibition) demonstrating that TGFα and possibly AR are able to function as potential autocrine intermediaries in the transformation pathway which is utilized by an activated *ras* gene. In contrast, *erb* B-2 transformed MCF-10A cells exhibit an increase in AR expression but not in TGFα expression demonstrating these two EGF-related peptides may be differentially regulated by various oncogenes. Although endogenous AR protein was immunocytochemically detected in the cytoplasm of MCF-10A cells, *ras* or *erb* B-2 transformed MCF-10A cells exhibit a higher level of immunoreactivity both in the cytoplasm and in the nucleus. In some cases nuclear staining was also be observed. AR phosphorothioate 20-mer antisense oligodeoxynucleotides are able to inhibit by 70-80% the growth of *ras* and *erb* B-2 transformed MCF-10A cells suggesting that AR may also function as an autocrine growth factor for both *ras* and *erb* B-2 transformed cells. TGFα appears to function as a dominantly acting oncogene in mammary epithelial cells that express a sufficient complement of functional EGF receptors. Overexpression of TGFα in MCF-10A cells following infection with an amphotropic retroviral expression vector containing the human TGFα cDNA is able to transform these cells *in vitro*. However, neither TGFα, *ras* nor *erb* B-2 is able to elicit a fully malignant phenotype *in vivo*. Overexpression of these genes either alone or in combination was insufficient to affect tumor formation in γ irradiated or NK deficient nude mice inoculated with MCF-10A cells suggesting that the expression of an additional oncogene(s) or the loss of expression of a tumor suppressor gene is probably required to complete the malignant process.

EGF/TGFα AND TGFβ CONTROL OF NORMAL AND IMMORTALIZED HUMAN MAMMARY EPITHELIAL CELL GROWTH IN CULTURE.

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The effects of EGF/TGFα and TGFβ on growth control and specialized functions have been examined and compared in finite lifespan vs. immortally transformed human mammary epithelial cells (HMEC). Our HMEC culture system permits active long term growth (45-80 pd) of finite lifespan cells derived from individual reduction mammoplasty tissues. Two established cell lines, 184B5 and 184A1, were derived from normal HMEC specimen 184 following in vitro exposure to benzo(a)pyrene. Normal HMEC have a stringent requirement for EGF for clonal growth, but grow in mass culture in the absence of exogenously added EGF due to the endogenous production of TGFα. 184B5 and 184A1, although synthesizing TGFα mRNA, do not secrete sufficient TGFα protein to support growth in the absence of added EGF. A rapid, efficient, and reversible Go-like growth arrest can be achieved in both cell types by effective blockage of the EGF signal. In normal HMEC this requires addition of a blocking antibody to the EGF receptor while removal of EGF is sufficient in the cell lines. Re-exposure to EGF leads to a synchronous entry into the cell cycle with high levels of synthesis of mRNA for the early response genes c-myc, c-fos, c-jun, and MGSA observed within 1hr. The cell lines differ from normal HMEC in that c-myc and c-fos mRNA levels are not reduced during the Go arrest. TGFβ causes a late G1 growth arrest in normal HMEC. Protein synthesis, particularly components associated with the extracellular matrix (e.g., fibronectin, laminin, collagen IV, PAI-1), is stimulated. Although 184B5 and some variants of 184A1 can maintain active growth in the presence of TGFβ, they remain TGFβ responsive. They show similar induction of secreted protein synthesis and have a similar profile of TGFβ1 receptors. Thus, the effects of TGFβ on cell growth can be dissociated from its effects on specialized responses, suggesting at least two independent pathways for TGFβ activity, one which leads to cessation of proliferation and one which induces a specific set of differentiated cellular responses. We are currently comparing specific mRNA expression and phosphorylation patterns of cell cycle related proteins in synchronized populations of these growth inhibited vs. non-growth inhibited HMEC. These studies may help define the mechanisms of TGFβ induced growth inhibition in this cell system. Since tumorigenic progression is characterized by derangements in signal transduction, growth control and differentiation processes, analysis of the effects of EGF/TGFα and TGFβ in cultured normal and transformed HMEC may provide information relevant to the carcinogenic processes occurring in the breast in vivo.

CELLULAR AND MOLECULAR HETEROGENEITY OF ESTROGEN AND PROGESTERONE RECEPTORS AND HORMONE RESISTANT BREAST CANCER

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The tumors of patients undergoing hormone treatment for metastatic breast cancer invariably become resistant. Hormone antagonists, which are expected to suppress tumor growth, often have paradoxical agonist-like effects. The antiproliferative effects of tamoxifen are mediated by estrogen receptors (ER). We will discuss two mechanisms by which resistance to this drug may develop. First, the increasing evidence from breast tumor cell lines and solid human tumors for the existence of mutant forms of ER that modify cellular responses to estrogens. Second, the cellular heterogeneity of breast cancers in which subpopulations of cells may emerge during hormone treatment that are growth-stimulated rather than growth-inhibited. There is also growing interest in the use of progesterone antagonists to treat breast cancer. These drugs, which act through progesterone receptors (PR), appear to target different antiproliferative pathways than do the antiestrogens. We will discuss two mechanisms by which progesterone antagonists may have agonist-like effects, focusing on the heterogeneity of the PR A- and B-isoforms in regulating antagonist-mediated transcription, and on the role of cAMP in modulating antagonist to agonist reversals of transcription.

NOVEL MODULATORS OF SYNTHESIS AND DEGRADATION OF EXTRACELLULAR MATRIX IN BREAST CANCER

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Invasive breast cancer is characterized by degradation of basement membrane and extracellular matrix and by the frequent appearance of a stromal fibrotic reaction. We have identified two novel activities which may be involved in these processes. The first is a major matrix-degrading protease secreted by all hormone dependent breast cancer cell lines examined to date. The enzyme is also found in hormone dependent breast cancer cell lines grown as tumors in the nude mouse. This 80 kDa glycoprotein activity has an alkaline pH optimum, a dependence upon Mg^{++}, Ca^{++}, or Mn^{++} for activity, and a broad substrate specificity including gelatin, Type IV collagen, laminin, and fibronectin (but not casein). It is inhibited by EDTA and leupeptin but not by TIMP-2 and it has been purified 2000-fold from breast cancer cell conditioned medium. The second novel activity (termed MDGF-1) is an acidic, 62 kDa growth factor which selectively stimulates synthesis of collagen by fibroblasts. It appears to be the major growth factor with this characteristic in human milk and in extracts of primary human breast cancer. This factor, in addition to effects on stromal collagen metabolism, is capable of stimulating the proliferation of receptor containing breast cancer cell lines in vitro. Amino terminal sequencing of MDGF-1 has revealed no homology with other growth factors and it binds to a 130 kDa binding site on responsive cells in culture. Binding triggers rapid tyrosine phosphorylation on a 185-200 kDa cellular substrate. Full characterization of the structure and function of the protease and growth factor are underway.

AN APPROACH TO THE STUDY OF DIFFERENTIAL GENE EXPRESSION DURING MAMMARY TUMORIGENESIS IN TRANSGENIC MICE

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Our laboratory has been studying mammary tumorigenesis in Wnt-1 transgenic mice (Tsukamoto, A. *et al. Cell* **55**, 619-625 (1988); Kwan, H. *et al. Mol. Cell Biol.* **12**, 147-154 (1992)). The transgene in these animals mimics a mouse mammary tumor virus (MMTV) integration at the *Wnt-1* locus, with the MMTV LTR enhancer directing Wnt-1 expression to the mammary and salivary glands and male reproductive tract. Tumorigenesis in this model is assumed to be a stochastic process with predisposition to mammary tumors caused by overexpression of the Cys-rich secreted growth factor Wnt-1. All animals exhibit preneoplastic mammary gland hyperplasia with progression to mammary adenocarcinoma in 50% of virgin females by six months of age and in 15% of males by one year of age. Metastases to the lung are also occasionally observed.

We are using several strategies to identify secondary events that mediate carcinogenesis in this model: genetic crosses with other transgenic animals, viral superinfection of Wnt-1 transgenics, analysis of DNA amplifications in tumors and metastases, and determination of differential gene expression via subtractive hybridization. To analyze differential gene expression, cDNA libraries have been constructed from several tissues of Wnt-1 transgenic females: hyperplastic mammary gland, primary adenocarcinoma, and pulmonary metastatic tumor. The libraries were constructed in a vector λEXLX (Palazzolo, M.J. *et al. Gene* **88**, 25-36 (1990)) which allows simple conversion into a plasmid library via site-specific recombination at two lox sites in λEXLX. T7 and SP6 transcription allow generation of RNA in either orientation and an f1 origin allows generation of cDNA.

Subtractive probes will be used to retrieve cDNA's from the appropriate libraries. These cDNA's will then be analyzed as candidates for genes whose over- or under- expression correlates with the development of tumors or metastases. Combining this approach with our other studies, we hope to elucidate what events collaborate with Wnt-1 overexpression to promote murine mammary tumorigenesis.

CELL CYCLE DEPENDENT EXPRESSION OF P53 mRNA AND PROTEIN IN NORMAL HUMAN MAMMARY EPITHELIAL CELLS

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One of the most common abnormalities detected in breast and other epithelial cancers is mutation of the the p53 tumor suppressor gene. To better understand why this gene becomes mutated in such a high proportion of breast tumors, we have undertaken studies to determine the temporal expression and function of wild type p53 protein in normal human mammary epithelial cells (HMEC) derived from reduction mammoplasties. Normal HMEC were synchronized by two different methods and the expression of p53 mRNA and protein was examined in the arrested and restimulated cells. Our results indicate that the p53 protein is expressed at high levels in cells arrested by treatment with lovastatin or by growth factor deprivation. The amount of p53 protein decreased dramatically as the cells were restimulated to proliferate and traversed the early and mid G1 phases. p53 protein accumulated again to high levels concomitant with the entry of cells into S phase. In these normal HMEC p53 protein could only be detected in the nucleus. Because p53 mRNA showed very minimal changes during the cell cycle, the large differences in p53 protein levels are likely due to posttranscriptional controls.

A POPULATION BASED STUDY OF EARLY ONSET BREAST CANCER IN VICTORIAN FAMILIES

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A family study based on women diagnosed with breast cancer before aged 40 years has commenced. Cases are sampled via the Victorian Cancer Registry, a population based registry to which cancer registration is a statutory requirement. Ascertainment is considered to be complete. The aim is to quantify: the extent of familial clustering of breast cancer in Victoria, the increased risk to relatives of different relationships, the extent to which increased risk might be explained by familial aggregation in risk factors, or by measured genetic markers, and in the longer term, the extent to which family history influences prognosis. Statistical pedigree analyses will also be carried out to determine if familial aggregation is consistent with a putative autosomal dominant genetic locus with either partial or complete penetrance, taking into consideration measured epidemiological risk factors. A questionnaire addressing major risk factors is administered face-to-face or by telephone to the proband and to her female and male relatives. All first degree relatives and grandparents on both the paternal and maternal side, for both the proband and, as a control, for her spouse or partner (if applicable) are in the study. A sequential sampling scheme is used to study first degree relatives of any previously studied relative with breast cancer. Proxy information is collected for deceased subjects. Reported cancer cases in relatives will be verified. Early experience of the study is reported. Procedures have been developed to obtain the cooperation of treating surgeons, and to produce a proband response rate of over 80% and good cooperation from relatives. Blood samples are to be collected for testing hypotheses relating to specific markers and candidate DNA probes for breast cancer in this population-based sample of families.

GENETIC ALTERATIONS OF THE TUMOR SUPPRESSOR GENE REGION 3p IN HUMAN BREAST CARCINOMA AND RENAL CELL CARCINOMA

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The short arm of chromosome 3 is frequently affected by cytogenetic changes associated with human malignancies. We have found loss of heterozygosity (LOH) in sporadic renal cell carcinoma (RCC) and breast carcinoma, in 84% and 29% of cases, respectively. It remains unknown whether the disorders reside in lesions of the same gene. Thus, the data strongly support the presence of at least one powerful tumor suppressor gene in the 3p region. The 3p loss in breast carcinoma seems to correlate with higher degree of malignancy.

Since these tumor types are seen both in mouse and rat it appeared important to localize the mouse and rat homologues on the chromosome map. Our findings showed that both are localized in regions previously shown to be syntenic with human chromosome 3, chromosome 9 in mouse and chromosome 8 in rat.

We are doing linkage studies on families in Iceland showing high rate of breast cancer. Some of the family members develop also other tumor types, known to carry a 3p abnormality, suggesting 3p as possible site for breast cancer susceptibility locus. Blood samples from 105 persons (affected and relatives) from 8 breast cancer families have been collected for an initial screening concerning 5 loci on chromosome 3p. Linkage to 3p has been excluded in 4 breast cancer families and multipoint analysis is under way for the remaining families.

REGULATION OF WILD-TYPE AND MUTANT *p53* IN MURINE MAMMARY NEOPLASIAS: MULTIPLE PATHWAYS FOR OVEREXPRESSION.

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Treatment of breast cancer relies on early detection of primary lesions. The earliest recognizable intermediate in the development of breast cancer are regions of focal hyperplasia. However, the genetic changes associated with transition of mammary epithelial cells to hyperplasia remain undefined. To study early genetic changes, mammary epithelial cell lines that form hyperplastic outgrowths when transplanted to cleared mammary fat pads have been established from BALB/c mice. Tumors form from the hyperplastic outgrowths at a frequency that is predictable for each outgrowth line, but differs among the lines. Because alteration in *p53* expression is the most commonly identified defect in breast cancer, the status of *p53* was determined in the cell lines, the in vivo outgrowths and tumors derived from the outgrowths.

The relative expression of *p53* protein was determined by immunohistochemical staining and immunoprecipitation with monoclonal antibodies that discriminate between wild-type and mutant conformations. The nucleotide sequence of the *p53* mRNA coding region was directly sequenced allowing the amino acid sequence of the *p53*s to be deduced. Both alleles of *p53* were mutated in TM4 cells and tumors (Δ123-129/Trp^{138}) and overexpression of *p53* protein was observed by immunohistochemical staining. Although both alleles were mutated and all cells overexpressed *p53* in vitro, less than 50% of the cells in the hyperplastic outgrowths stained positively for *p53*. These data demonstrate that the milieu of the in vivo outgrowth serves to regulate overexpression of mutant *p53* in a large proportion of the cells. During progression to tumors, this regulation is lost and all cells overexpress *p53*. TM9 cells provide a contrasting example. Both TM9 cells and tumors expressed only wild-type *p53* transcripts. However, these cells and tumors also overexpressed *p53* protein. These data suggest that there are multiple pathways that result in overexpression of *p53* in mammary epithelial cells. We believe that these include mutation of the coding sequence of *p53* as well as alteration of genes that regulate accumulation of *p53*. Elucidation of these regulatory pathways may suggest potential forms of therapy for breast cancer.

MULTIPLE CHROMOSOMAL REGIONS MAY UNDERGO COPY NUMBER ALTERATIONS IN BREAST CANCER

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Cytogenetic analysis of breast tumors is sometimes difficult due to the sparsity or poor quality of representative metaphase spreads, and to the common occurrence of highly abnormal chromosomes. The nature of these aberrant chromosomes as well as the origin of sequences amplified in HSRs or DMs often cannot be established by karyotypic analysis. We have developed a new technique, comparative genomic hybridization (CGH), for detection and chromosomal localization of DNA sequence copy number changes anywhere in the genome in a single hybridization. In CGH, differentially labeled total genomic test DNA and normal reference DNA are allowed to compete with each other during hybridization to normal metaphase spreads. Following immunofluorescent detection of the labeled DNAs with two different fluorochromes, relative copy numbers of all regions in the test genome are quantitatively established by measuring the ratio of the fluorescence intensities from the two fluorochromes along the length of each normal chromosome. Increased relative copy number (amplifications) and reduced relative copy number (deletions) of the tumor DNA is readily mapped on the normal metaphase chromosomes.

We have applied CGH with DNA from the near-diploid 600PE breast cancer cell line and from a normal female control to show that the color ratios accurately revealed all the cytogenetically known chromosomal aberrations (tetrasomy for 1q, and deletions of 9p, 11q, 16q and 17p) of this cell line. In another experiment, CGH using DNA from five fibroblast cell lines with 1 to 5 copies of the X chromosome allowed accurate determination of this range of X chromosome copy number.

Amplified DNA sequences could be detected and mapped by CGH in tumor cell lines with DMs or HSRs and occasionally also in those with no previous cytogenetic information on gene amplification. Besides known oncogenes such as MYC and ERBB2, the chromosomal origins of other amplified sequences that previously were not known to contain amplified genes were discovered. Regions of increased copy number in breast tumor cell lines and/or primaries were mapped to 1q, 8q (proximal to myc), 13q, 17q (terminal to erbb2), and 20q. Sometimes two or three separate regions on the same chromosome were amplified in one cell line. Supported by NIH CA 44768, CA 45919 and DOE AC-03-76SF0098.

ESTROGEN RECEPTOR MUTATIONS IN TAMOXIFEN RESISTANT HUMAN BREAST CANCER

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The problem of hormone resistance is a major challenge in the treatment of breast cancer. Although endocrine therapy with antiestrogens (eg. Tamoxifen) often results in a remarkable improvement in breast cancer patients, tumor resistance to these drugs develops over time. The molecular mechanisms by which receptor positive tumors develop resistance to antiestrogens, however, remain unknown. The estrogen receptor plays a central role in mediating the effects of estrogens and antiestrogens. Therefore, any alteration in the receptor could theoretically render the cell endocrine resistant. We hypothesize that endocrine resistance in ER^+, PR^+ human breast cancer is due to altered or mutated estrogen receptors that are unable to mediate the actions of estrogens and antiestrogens. To search efficiently for ER mutations, our strategy is to isolate RNA from a large number of ER^+, PR^+ tumors (Tamoxifen resistant and sensitive), amplify the ER cDNA by RNA-PCR and then assay the amplified products for sequence variation by single-strand conformation polymorphism (SSCP). Frozen human breast tumor samples clinically diagnosed as Tamoxifen resistant or sensitive were obtained from the tumor bank at CCF. Detailed clinical information and response of patients to Tamoxifen over several years is readily available. We first amplified exon 2 (codes for first zinc finger) from wild type ER cDNA and from 17 tumor samples. A 246 bp fragment (including primers) was amplified, denatured and analyzed by SSCP. A variant exon-2 band was detected in a Tamoxifen resistant tumor from a patient with recurrent disease. The patient responded to Tamoxifen for about two years before developing metastatic carcinoma of breast origin of the right supraclavicular lymph node. The exon 2 variant was seen in both the primary and metastatic tumors from this patient. Since exon 2 of ER codes for the first zinc finger, it is interesting to speculate that a faulty or defective first zinc finger is responsible for Tamoxifen resistance seen in this patient. Our efforts are now directed toward sequencing the ER variants and analyzing the functional significance of this mutation.

ALLELIC IMBALANCE STUDIES AND ANALYSIS OF CANDIDATE GENES IN SOMATIC AND TUMOUR TISSUE IN A FAMILY WITH BREAST/OVARIAN CANCER LINKED TO CHROMOSOME 17

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In 1990 Hall et al. (Science 1990, 250, 1684-1689) showed the first strong evidence for a breast cancer gene localised to the long arm of human chromosome 17 by genetic linkage. They obtained a LOD score of 2.35 at a θ of 0.20 with the marker D17S74. The LOD score was significantly increased when the families were stratified by age and a LOD score of 5.98 was obtained in those with a mean age at diagnosis of less than 46. This initial finding was confirmed by Narod et al. (The Lancet 1991, 338, 82-83) who obtained a LOD score of 2.20 with the same marker in five breast ovarian cancer families. Following these results a collaborative study was established to help locate the gene on 17q. A total of 214 families were studied with six markers and a region spanning approximately 10CM was defined flanked by the markers D17S250 and D17S588. One of the critical findings from the linkage analysis of these families was that all families with a mixture of breast and ovarian cancers were linked to this gene. In our panel of families we have one large breast/ovarian family with a total of 12 breast and three ovarian tumours. A LOD score of 2.35 at θ 0.003 was obtained with the marker NME in this family. We have obtained blood samples and fixed blocks of tumours from a number of these individuals and carried out PCR studies looking at loss of heterozygosity in tumours. Initial results indicate that in one tumour from this family all the chromosome 17 below the marker THRA1 is lost thus indicating that the region in which the BRCA1 gene lies can be narrowed between THRA1 and D17S588. Also sequence analysis using somatic and tumour tissue looking for mutations in the candidate genes for example prohibitin and the two estradiol 17-beta dehydrogenase genes EDH17B1 and EDH17B2 has been carried out.

THE TRANSFORMING POTENTIAL OF WNT GENE FAMILY MEMBERS

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The *Wnt*-1 gene was originally identified as a target for insertional activation in MMTV-induced mouse mammary tumors. The gene encodes cysteine-rich secreted glycoproteins that associate tightly with the extracellular matrix or cell surface. The protein encoded by the *Wnt*-1 gene is believed to function normally as a secreted growth factor or morphogen involved in the development of the central nervous system. Ten members of the murine *Wnt* gene family have been cloned and the expression of several of these genes in the developing mammary gland suggests they may play a role in normal mammary gland development.

We have been assesing the ability of *Wnt*-1, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, and *Wnt*-5B to transform the cultured mammary epithelial cell line C57MG. Toward this goal, we have generated cDNAs encoding *Wnt* gene family members fused to an influenza hemagluttinin (HA) epitope to allow detection of the gene products with an anti-HA monoclonal antibody. Epitope tagged *Wnt*-1 retains the ability to transform cultured mammary epithelial cells. The transforming potential of the epitope tagged *Wnt* gene products are being tested by using retroviral vectors to express these genes in C57MG mammary epithelial cells. Paracrine transforming capability of *Wnt* genes is being assessed by co-cultivating C57MG cells with *Wnt*-expressing Rat fibroblasts (which themselves show no response to *Wnt*-1 and act as donors of *Wnt* proteins to adjacent C57MG cells). Transient transfection of cDNAs encoding the epitope tagged *Wnt* family members into quail QT6 cells results in the production of secreted glycoproteins that associate tightly to either the cell surface or extracellular matrix, as is seen with *Wnt*-1 proteins.

In addition, we have constructed cDNAs encoding *Wnt*-1 fused to the transmembrane and cytoplasmic region of the CD4 protein or encoding *Wnt*-1 with the ER retention sequence KDEL added to the C-terminus. To date, we have shown that *Wnt*-1-CD4 expression results in autocrine but not paracrine transformation suggesting that these cell membrane-associated-*Wnt*-1 proteins do not trasmit signals from one cell to another.

A NOVEL MECHANISM OF TRANSCRIPTIONAL STIMULATION BY THE ANTIESTROGENS TAMOXIFEN AND ICI 164,384.

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Antiestrogens, such as tamoxifen or ICI 164,384, bind to the estrogen receptor and inhibit transcription at classical estrogen response elements. This ability is thought to underlie their therapeutic effect. Although antiestrogens usually block estrogen action, they are agonists on diverse and unpredictable targets, and this complicates their use in treating or preventing breast cancer. Tamoxifen, for example, has potent estrogenic activity on the growth and gene expression of endometrial cells and stimulates transcription of some estrogen responsive genes in breast cancer cells.

Here we show that sites for the AP-1 family of transcription factors (jun/fos and relatives) act as non-classical estrogen response elements with a variety of promoters, including the human collagenase and herpes virus tk promoters, and in several cell types. The collagenase promoter shows up to a 20 fold induction with estrogen that is not shown by derivatives deleted for the AP1 site. Activation at the AP-1 site requires AP-1 protein, because it does not occur in F9 cells, which lack AP1 proteins, unless they are transfected with expression vectors for jun and fos. The amino terminus of the estrogen receptor is needed for this response, but the DNA binding domain is not required. Remarkably, antiestrogens are potent activators of transcription at AP1 sites in the presence of estrogen receptor. Tamoxifen induction is often as strong as estrogen. ICI can also be a potent agonist, but only when receptor levels are very high. These results may explain some of the agonist effects of antiestrogens on gene transcription as due to action at AP1 sites rather than classical response elements. These results also suggest new ways to screen antiestrogenic drugs.

CYTOCHROME P450 ACTIVITY IN BREAST CANCER

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Cytochromes P450 are a superfamily of enzymes that is mainly present in hepatocytes and converts 17 ß-estradiol to 2-hydroxy-estradiol. Recent reports showed that some human breast cancer tissues could be stained by specific P450 antibodies. Induced expression of P450 has been shown to lead to increased metabolism of estrogen resulting in antiestrogenic effects in several human breast cancer cell lines. We attempted to evaluate the prognostic value of cytochrome P450 for overall survival of breast cancer patients by measuring the P450 activity in biopsies. Using 7-ethoxy-4-trifluoromethyl-coumarin (EFC) as a substrate, P450 activity was measured by determining its product 7-ethoxycoumarin fluorometrically. We measured the activity of this enzyme in various breast cancer cell lines and in 100 biopsy samples from primary breast cancer patients. The results demonstrate that breast cancer tissue contains much lower P450 activity (10'000 times less) per mg protein than rat liver tissue . 2 out of 3 estrogen receptor (ER) positive cell lines, MCF-7 and ZR-75-1 showed detectable P450 activity. ZR-75-1 cells contained 41 % of the activity of MCF-7 cells. Another ER positive cell line BT-47D and the ER negative cell lines BT-474, MDA-MB-231, BT-20, HBL-100 and SKBR-2-III showed no P450 activity in our assay. The P450 activity could be increased by estrogen pretreatment of MCF-7 and ZR-75-1 cells but not of BT-47D and all ER negative cell lines tested. In 9% of all biopsy samples, ER positive (6/9) and ER negative (3/9), P450 activity was detected. The P450 positive tissue samples showed ten times less activity than MCF-7 cells . No correlation was found between P450 activity and estrogen receptor, progesterone receptor and Cathepsin D content. However 8 out of 9 P450 positive samples were epidermal growth factor receptor positive. The independent prognostic value of this enzyme in breast cancer is under investigation in a long term study.

STUDIES OF ALLELIC IMBALANCE IN HUMAN BREAST CANCER

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A number of groups have reported varying levels of allelic imbalance of polymorphic markers on human chromosome 1 in primary breast tumour samples. Loss of heterozygosity (LOH) has been reported at two regions, at 1q21-23 (Chen *et al.*, 1989, Proc. Natl. Acad. Sci. USA, **86**, 7204) and at distal 1p (Genuardi *et al.*, 1988, Am. J. Hum. Genet., **45**, 73). Cytogenetic studies have implicated additional regions on chromosome 1, most notably 1p13 (Mitchell and Santibanez-Koref, 1990, Genes, Chromosomes and Cancer, **2**, 278). We have used a number of informative probes on chromosome 1 to determine the frequency of LOH and the smallest region(s) of common overlap. Three regions show levels of allelic imbalance above background: 1p13-p35 (D1S73 and D1S7), 1q21-q23 (MUC1 and D1S61) and 1q32-qter (D1S81 and D1S8). Perturbations of sequences at these three regions appear to represent independent events, and indicate that a number of sequences on chromosome 1 are involved in breast cancer. The detailed discussion of the patterns of allelic imbalance will be presented, together with data correlating the alterations to various clinical parameters.

Additional regions of the genome are also being studied for LOH in the same panel of breast tumours, including known tumour suppressor loci (p53, RB1 and DCC). Loss or mutation of these sequences will be compared to loss of regions of chromosome 1 to determine whether subsets of breast tumours can be identified having specific patterns of LOH. Other regions of the genome also show loss at relatively high frequency, and at least one of these regions has not previously been shown to be involved in breast cancer. A detailed description of these latter studies will be presented.

TRANSCRIPTION FACTOR AP-1 AND THE TERMINAL DIFFERENTIATION AND ONCOGENIC TRANSFORMATION OF MAMMARY EPITHELIAL CELLS

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The lactogenic hormones, prolactin, glucocorticoids and insulin induce terminal differentiation and milk protein synthesis (e.g. β-casein) in the mouse mammary gland and in mammary epithelial cells in culture. In mammary epithelial cells in culture we measured the levels of β-casein promoter activity, as a correlate of hormone induced differentiation, and of the transcription factors AP-1 and Oct-1. The activity of the β-casein promoter was very low in growing and confluent cells but it was high after lactogenic hormone treatment. Measurements of the DNA-binding activity of transcription factor AP-1 by gel retardation analysis revealed high activity in growing cells, and significantly reduced activity in cells stimulated to terminally differentiate with lactogenic hormones. Transformation of the cells with oncogenes encoding Mos, Ras or Src (but not Myc) led to maintained high levels of AP-1 activity (and of fos and jun mRNA) and was accompanied by a failure of the lactogenic hormones to trigger activation of the β-casein promoter. Over-expression of Fos had a similar effect. Glucocorticoid receptor function was impaired in the transformed cells. Interestingly, at no stage in the life cycle of the mouse mammary gland was AP-1 activity high. In a sample of malignant human mammary tumours AP-1 activity levels varied from undetectable to very high.

CHROMOSOME 17q LINKAGE STUDIES OF 19 FRENCH BREAST CANCER AND BREAST-OVARIAN CANCER SYNDROMES FAMILIES.

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A gene for early onset familial breast cancer (BRCA1) has recently been mapped to the chromosome 17q12-q21 region. A same locus was assigned for the breast and ovarian cancer syndrome. In order to confirm the gene location and to test for possible genetic heterogeneity, we have conducted linkage analysis in 19 French breast and breast-ovarian carcinoma syndrome families (14 and 5 respectively) with five chromosome 17q markers (from centromere to telomere: D17S250, D17S579, 42D6, NM23 and D17S74). The five breast-ovarian cancer syndrome families as a group give positive evidence for linkage (Lod score=2.29 at O=0.00 with D17S579, the most closely linked marker to breast cancer) whereas the breast cancer families as a group do not (Lod score = 0.05 at O - 0.01 with D17S579). Heterogeneity of linkage of breast cancer is significant in France and support the existence of more than one susceptibility gene. From the study of two extensive families in which 6 members with other cancers carry the haplotype linked to susceptibility, it is suggested that the BRCA1 gene could also be related to the development of other cancers.

SEQUENCE ANALYSIS OF THE *int-2/fgf-3* GENE IN AGGRESSIVE HUMAN BREAST CARCINOMAS

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A number of primary human breast carcinomas have amplification of the chromosome 11 region containing the *int-2/fgf-3* proto-oncogene, and progression of breast cancers has been correlated with *int-2* amplification or with certain restriction fragment length polymorphisms (RFLPs) of the *int-2* gene. Using the polymerase chain reaction (PCR), we have obtained the *int-2* coding sequences from six primary tumors, four of which had amplification of the *int-2* gene and one contained amplification of the *neu* gene. The majority of these tumors (5/6) were aggressive as judged by their early recurrence and/or metastasis. Nucleotide sequencing of PCR products revealed that previously described BamHI and PstI RFLPs of the *int-2* gene, as well as a previously undescribed polymorphism at position 9154, were located within the intron between the second and third exons. A seventh tumor was used to localize one of the PstI RFLPs 5 bp from the splice acceptor site of the third exon. However, none of the tumors analyzed showed differences in the *int-2* protein coding regions compared to normal placenta DNA. These results imply that aggressive human breast cancers encode an unaltered form of the *int-2* protein.

p53 mutations and abnormal pattern of p53 protein expression in human breast cancer.

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Every cancer type looked at so far has been shown to habour inactivating mutations in the p53 tumor suppressing gene, however, differences have been encountered from one tumor type to the other, in the incidence and the pattern of nucleotide substitution.

Using a PCR-SSCP approach we analyzed 96 human primary breast tumors for the presence of mutations in the p53 coding sequence. 18/96 (18.6%) tumors presented such an anomaly and Chi^2 analysis revealed an inverse correlation with steroid receptor content, thus indicating an association with aggressive breast tumors. Most of the mutations (16/18) were single nucleotide substitutions (the remaining 2 being internal deletions of 11 and 23 bp respectively) and although we found 6 G:C to A:T transitions it is difficult to conclude on a dominant pattern of mutation in our tumor series. Interestingly only 3 tumors out of the 18 mutated at the p53 gene clearly showed the concommitant loss of the wild type allele. Taking the cellular heterogeneity of breast tumors into account, the presence of wild type sequences in some SSCP assays could be contributed by normal or non mutated cancer cells present in the analyzed tissue. To address this we performed PCR-SSCP on DNA extracted from 20μm thick tissue sections cut into parafine embedded blocks from tumors showing both the mutated and the wild type conformers. The four sections selected were composed of 80 to 90% tumor cells and all four displayed concommitantly the mutated and the wild type allele, thus possibly suggesting that only a fraction of the tumor cells composing the tumor may harbour the p53 mutation. Moreover this cellular heterogeneity may also lead to an underestimation by molecular techniques of the incidence of p53 mutations in breast cancer because of their dilution by the wild type signal. Such limitations may be obviated using *in situ* methods such as immunocytochemistry whith which isolated foci of positive cells may be detected. Taking advantage of the potential relationship between the presence of a mutation at the genetic level and positive immunohistostochemical staining with anti-p53 antibodies we searched for mutations by PCR-SSCPin tumor sections showing variable levels of anti-p53 staining (from 30 to 80% of stained cells). Sections with 60 to 80% of stained cells displayed a mutation upon PCR-SSCP, whereas some sections with 30 to 40% proved more elusive. This lead us to test further for the relationship between immunotaining and mutation of the gene. To do this we analyzed a subset of tumors which we knew the SSCP results of by immunostaining using 3 anti-p53 antibodies (CM1, DO7 and 1801). A detailed comparison of the molecular and immunocytochemical data, as well as results obtained with different antibodies, will be presented.

Two distinct mechanisms alter p53 in human breast cancer : Mutation and nuclear exclusion

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Twenty-seven cases of inflammatory breast cancer were screened for level and location of the p53 protein using immunocytochemical methods and monoclonal antibody directed against the p53 protein. Three groups were detected : eight cases (30%) contained high levels of p53 in the nucleus of the cancer cells; nine cases (33%) showed complete lack of detectable staining; ten cases (37) showed a pattern of cytoplasmic staining with nuclear sparing. Nucleotide sequence analysis of p53 cDNAs derived from the samples with cytoplasmic staining revealed only wild type p53 alleles in 6/7 cases. The seventh case showed an unusual in-frame deletion of serine 241. An eighth case was determined to be wild-type by single strand conformation polymorphism. In contrast, the samples containing nuclear p53 showed a variety of missense mutations and one nonsense mutation. Three of the tumors analyzed which lacked detectable p53 staining were all wild type in their nucleotide sequence suggesting normal p53 expression in these cases. Interestingly, a case of normal lactating breast tissue also showed intense cytoplasmic staining for p53 with nuclear sparing.

These data suggest that some breast cancers that contain the wild type form of p53 may inactivate its tumor suppressor activity by sequestering this protein into the cytoplasm, thus excluding it from its site of action in the nucleus. This leads us to propose a novel, mutation - independent mechanism of p53 inactivation. The detection of cytoplasmic p53 in normal lactating breast tissue suggests that intracellular translocation and stabilization of p53 might be a normal event used for specific physiological situations which require transient cell proliferation.

HYDROCARBONS - THE URBAN FACTOR IN BREAST CANCER

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The urban factor in breast cancer can be understood by examining human exposure to the "urban toxic soup" which is composed primarily of hydrocarbon combustion by-products. These hydrocarbons are found either as volatile organic compounds or as compounds bound to particulate matter and contain a complex mixture of polycyclic aromatic hydrocarbons, polar substituted polycyclic aromatic hydrocarbons, simple aromatic and aliphatic hydrocarbons.

Many of these environmental pollutants are lipophilic substances which are stored, concentrated and metabolized in the breast to carcinogenic metabolites. Some produce electrophiles which adduct to DNA and initiate carcinogenesis. Other aromatic and aliphatic metabolites also produce oxygen free radicals, consume glutathione and induce lipid peroxidation of cellular membranes. The consequence of long term hydrocarbon exposure is an increased intracellular pro-oxidant state which destabilizes DNA, is clastogenic and allows for initiation and promotion of breast cancer.

In urban communities there is an increased exposure to hydrocarbons caused by the consumption of fossil fuels for energy production as well as increased personal contact with hydrocarbon sources. Low dose long term human exposure to many mammary specific hydrocarbon carcinogens and to the promotional effects of perhaps hundreds of other carcinogenic and non-carcinogenic hydrocarbon metabolites accounts for the urban factor in breast cancer.

Recognizing the relationship of human breast cancer to hydrocarbon exposure allows for control of this disease by reducing environmental hydrocarbon pollution and by the personal use of anti-oxidants and binding site blocking agents.

SIGNAL TRANSDUCTION IN NEU MEDIATED MAMMARY TUMORIGENESIS AND METASTASIS.

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Overexpression and amplification of the neu proto-oncogene has been implicated in the development of aggressive human breast cancer. To directly assess the effect of mammary gland-specific expression of the neu proto-oncogene, transgenic mice carrying unactivated neu under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer were established. By contrast to the rapid tumor progression observed in several transgenic lines carrying the activated neu transgene, expression of unactivated neu in the mammary epithelium resulted in the development of focal mammary tumors after long latency. Interestingly, many of the tumor bearing transgenic mice developed secondary metastatic tumors in the lung.

The neu induced tumors expressed elevated levels of neu mRNA and protein. Overexpression of neu in the mammary tumors was also associated with elevated neu intrinsic tyrosine kinase activity, de-novo tyrosine phosphorylation of several cellular proteins and elevated expression of the ets related transcription factor PEA3. Furthermore expression of activated neu in established cell lines transcriptionaly activates reporter constructs bearing both PEA3 and AP1 responsive elements. We have initiated studies designed to understand the role played by these transcription factors in neu induced tumorigenesis and metastasis.

GROWTH FACTOR AND CELL CYCLE GENES IMPLICATED IN MAMMARY TUMORIGENESIS.

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Breast cancer in the mouse is frequently associated with the inappropriate expression of normally silent proto-oncogenes instigated by the nearby integration of mouse mammary tumor virus (MMTV). Two such genes, int-2/FGF3 and hst-1/FGF4, are members of the FGF family, and were shown to be physically linked on mouse chromosome 7 and human chromosome 11 band q13. When tested in vitro, FGF3 is a poor mitogen and weak transforming gene, as compared to FGF4, yet it is a more frequent target for MMTV. To further investigate the role of FGF3 in mammary tumorigenesis we assessed its properties in a transgenic mouse model in which FGF3 expression was regulated by MMTV. The transgenic mice developed pregnancy-dependent hyperplasia as a result of FGF3 expression in the mammary gland. However, in situ hybridization revealed that FGF3 transcription in these lesions was non-uniform, in striking contrast to the clonal tumors that arise from MMTV infection. These findings confirm the role of FGF3 in tumorigenesis but point to mechanistic differences between the virally induced and transgenic tumors.

In human breast cancers, both FGF3 and FGF4 are modestly amplified, in approximately 15% of cases, particularly in estrogen receptor positive tumors. However, neither gene is consistently expressed in the tumors, suggesting either an early cryptic role in t development or that these genes are merely passeng on the 11q13 amplicon in which another closely linked gene is more directly involved. A candidate gene that meets most of the expected criteria is cyclin D1 which is about 120kb centromeric of FGF4. Cyclin D1 is always co-amplified with FGF3/FGF4 and shows elevated expression in these tumors. Its association with parathyoid adenomas and chromosomal translocation in centrocytic lymphomas provides additional credentials for a role as an oncogene. We are presently investigating the properties of this cyclin to assess its contribution to breast cancer.

EXPRESSION OF THE ERBB3 PROTO-ONCOGENE PRODUCT IN BREAST CANCER

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Abnormalities of the EGF receptor and/or the related ERBB2 receptor occur in a significant proportion of cases of human breast cancer and are important influences in the behaviour of this tumour type. We now demonstrate by nucleic acid analysis and immunohistochemistry that the recently recognised third member of this receptor gene family, ERBB3, is overexpressed in 43 out of 199 cases (22%) of primary breast cancer. Overexpression of ERBB3 appears to result from increased levels of gene transcription since none of the cell lines or primary cancers analysed showed evidence of gene amplification. Overexpression of ERBB3 is positively associated with the presence of lymph node metastases. Our results suggest that the ERBB3 oncogene, like its relatives EGFR and ERBB2, may also be involved in the pathogenesis of breast cancer.

ROLE OF THE HER-2/neu GENE IN HUMAN BREAST AND OVARIAN CANCER

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The HER-2/neu proto-oncogene encodes a growth factor receptor which is overexpressed in 20-30% of human breast cancers. This overexpression is associated with a decreased relapse free as well as overall survival in those patients whose tumors contain the alteration. The overexpression is most often due to amplification in a significant number of cases. This association between HER-2/neu amplification/overexpression and outcome suggests that the alteration may play some causal role in the pathogenesis. To test the potential role of HER-2/neu overexpression in altering the biologic activity of human breast normal and malignant epithelial cells, a number of in vitro studies were conducted in which single-copy, low expressing cell lines were converted to multiple copy, high expressing cell lines. The biologic effects of HER-2/neu overexpression were then measured including effects on DNA synthesis, cell growth, anchorage independent growth, and tumorigenicity. Overexpression of HER-2/neu resulted in an increase in those parameters in the malignant cell lines as well as the non-transformed immortalized breast cell lines. In the normal primary breast cells there was no evidence of these effects with HER-2/neu overexpression alone.

Monoclonal antibodies directed against the extracellular domain of the receptor can suppress all of the biologic effects induced by HER-2/neu overexpression both in vitro and in vivo. Preclinical studies indicate that these antibodies can be effective in completely suppressing growth of human tumor cells as well as malignant breast tissue xenografts when either are growing in vivo. The suppression is specific to cells and tissues overexpressing the HER-2/neu gene. Strategies using these antibodies in combination with other therapeutic modalities indicates that this cytostatic effect can be converted into a cytocidal effect. These observations have led to the development of new treatment strategies directed at this molecular alteration and these strategies are now in clinical testing. In addition, the recent identification, cloning and sequencing of a ligand for the HER-2/neu receptor has allowed for its recombinant expression. The availability of this ligand has led to further insights into the role of the HER-2/neu protein in the pathogenesis of human breast cancer.

HORMONAL CONTROL OF TRANSACTIVATION/REPRESSION OF THE HUMAN PROGESTERONE RECEPTOR GENE IN BREAST CANCER CELLS

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Female sex steroids control the growth and progression of hormone responsive cancers through the coordinate regulation of specific gene networks. A useful model system for studying the molecular basis of this activity is the human progesterone receptor (hPR) gene, which is transcriptionally regulated by estrogens, progestins, and antagonists via their cognate receptors in cultured human breast cancer cells. To better understand some of the underlying mechanisms, genomic DNA and T47D cDNA clones encompassing the entire translated portion of hPR mRNA and 6.3 kb of upstream hPR DNA were isolated and sequenced. The promoter region contains a CAAT box and a consensus binding site for the Sp1 transcription factor, but no classical TATA box. A long GC-rich untranslated region (744 bp) precedes the initiation codon (ATG_B) for the larger B form of hPR. Three low-to-moderate affinity recognition sites for hER and two for hPR have been located by DNA competition experiments and/or gel shift studies in the untranslated region preceding the ATG_B codon as well as in the intragenic region that includes the ATG_B codon. To determine whether these sequences comprise functional EREs or PREs, transfection experiments were performed with reporter plasmids that contained appropriate segments of the hPR gene linked to CAT cDNA through the heterologous thymidine kinase (tk) promoter. Initial studies with several hPR-tk-CAT reporters indicate that an estrogen responsive element exists within the +731/+761 region of the hPR gene, which includes the ATG_B codon at +744 bp. A 31-bp oligonucleotide containing this sequence bound hER with about 50-fold reduced affinity when compared to the frog vitellogenin A2 gene ERE. Interestingly, progestins inhibit the estrogenic induction of the "ERE" located at +744, presumably via the progesterone receptor present in MCF-7 cells. However, an ERE-tk-CAT reporter containing the perfect palindromic vitellogenin A2 ERE is unaffected by progestins in these cells. We are currently trying to determine the role of this region in regulating hPR gene expression in MCF-7 cells. At present, it appears that both transcriptional activation and repression can occur through this downstream *cis*-acting element within the transcribed region of the hPR gene, similar to the regulation of the rabbit PR gene. Repression may involve a direct or indirect interaction between the estrogen and progesterone receptors at this site. We are also trying to determine whether one or more of the other cis-acting sites in the hPR gene may involve interactions between ER and other transcription factors such as fos and jun, thereby providing an additional level of regulation of hPR gene expression.

These studies were supported by NCI grant CA02897.

THE INCIDENCE OF BREAST CANCER IS INCREASING IN A LARGE FAMILY WITH HEREDITARY CANCER. Steven Narod(1), Henry Lynch(2), Theresa Conway(2), Patrice Watson (2), Jean Feunteun(3) , Jillian Parboosingh(1), Jane Lynch(2), Gilbert Lenoir(4). (1) McGill Centre for Human Genetics, Montreal, Canada; (2) Creighton University School of Medicine;(3)Institut Gustave Roussy, Villejuif, France;(4) International Agency for Research on Cancer, Lyon, France

A large family containing 23 women with breast or ovarian cancer is linked to a susceptibility gene on chromosome 17; the maximum two-point LOD score seen was 4.20 at 5 centiMorgans distance from the D17S250 locus. Because linkage in this family is not in doubt, DNA probes can be used to indentify probable gene carriers from birth. Using five linked DNA markers, and by incorporating clinical data, 34 probable gene carriers have been identified. By age 70 the cumulative risk for breast cancer among carriers is estimated to be 73% and the risk for ovarian cancer is 83% - but the risk is not evenly distributed among generations of women. The incidence rate of breast cancer among women born after 1930 was five times greater than that for those born before 1930 ($p = 0.044$). The increase could not be explained by improved methods of detection or by changing patterns of fertility and is more likely to be the effect of a changing environment.

OVER-EXPRESSED HUMAN PLACENTAL 17ß-HYDROXY-STEROID DEHYDROGENASE IN COS-m6 CELLS AND T-47D HUMAN BREAST CANCER CELLS HAVE PREDOMINANT REDUCTIVE ACTIVITY IN CULTURE

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17ß-hydroxysteroid dehydrogenase (17-HSD) is an enzyme catalyzing the interconversion between estradiol and a less active estrogen, estrone. Thus the enzyme may have a major role in the pathophysiology of estradiol-responsive growth in breast cancer. As some reports have suggested the existence of multiple forms of 17-HSD, we studied estradiol metabolism catalyzed by placental-type 17HSD in cultured cells.

The 17HSD cDNA was cloned under the SV-40 early promoter of the PSG5 eucaryotic expression vector and the enzyme was transiently and stably over-expressed in COS-m6 cells and T-47D human breast cancer cells, respectively. The resulting recombinant 17HSD was immunologically reactive and had an expected molecular weight of 35 kDa, as judged by Western blot analysis. Both the transiently and the stably over-expressed enzyme had predominantly reductive activity in the intact cells in culture when the substrate was introduced into the culture medium. In addition, a high endogenous oxidative activity catalyzing the conversion of estradiol to estrone was detected in the COS-m6 cells, while the endogenous enzyme had no reductive activity, in contrast to the recombinant placental 17HSD. The data suggest that over-expressed placental 17HSD has predominantly reductive activity and that there seems to be another enzyme responsible for the oxidative activity in the COS-m6 cells which could not be detected with the antibodies to human placental 17HSD.

HUMAN BREAST CANCER AUTOANTIGEN DETECTION IN EXPRESSION cDNA LIBRARIES.

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In an effort to determine whether breast cancer patients can mount an autologous humoral immune response directed against tumor associated antigens, we applied the approach that has been successfully used to identify cDNAs of proteins which elicit an autoimmune response in various autoimmune disease states: the screening of expression libraries with autologous serum.

cDNA libraries in expression vector λgt11 were constructed from mRNA extracted from different primary ductal breast carcinomas, and were screened in their entirety with autologous patient serum. Four immunoreactive clones have been identified and isolated to date. Sequence analysis of the isolates showed that the four isolates actually represented two species which could not be matched to any sequences present in the current Genbank database release. The closest match for one of the two sequences is a 62% homology over a 120 base pair stretch with the sequence for a human angiogenic vascular endothelial growth factor.

In an initial experiment to test the reactivity of these clones against additional patient and control sera, we observed that five out of ten breast cancer patient sera reacted with one clone and four out of the ten with the second clone. A few control sera were found to react, but much more weakly. By northern blot analysis the mRNA of one isolate is 2.2kb and the other hybridizes to two bands (1.6kb and 2.0kb). The messages for both isolates are detectable in RNA isolated from primary breast tumors as well as breast tumor cell line MCF-7, indicating that they are expressed in the transformed epithelial cells of the tumor mass.

ALLELIC LOSS ON CHROMOSOME 17 IN DUCTAL CARCINOMA IN-SITU

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Loss of heterozygosity (LOH) for a number of chromosomal loci has been reported in invasive breast cancer, suggesting a role for tumor suppressor genes in the oncogenesis of this disease. LOH of 17p13, which contains the p53 gene, has been observed in up to 70% of cases of invasive breast carcinoma. No studies to-date have investigated LOH of this region in preinvasive cancer, such as ductal carcinoma *in situ* (DCIS).

Because of the diffuse nature of DCIS usually found within the breast, a microdissection technique was developed to separate malignant cells from adjacent normal stroma. Tumor DNA was extracted from microdissected archival, paraffin embedded sections, and compared with normal control DNA from either adjacent normal breast, uninvolved lymph node or white cells.

PCR was performed using the CA repeat polymorphism at D17S513, which localizes to 17p13. A total of 9 tumor normal pairs have been analyzed thus far. LOH was seen in 3 tumors, 2/6 of the comedo high nuclear grade variety and 1/3 of the mixed/cribriform low nuclear grade subtype.

This preliminary finding of LOH at 17p13 in DCIS suggests a role for a tumor suppressor gene early in the development of breast cancer.

EXPRESSION OF THE CHROMATIN REGULATORY PROTEIN HMG-I IS AN ACCURATE DIAGNOSTIC MARKER FOR NEOPLASTIC PROGRESSION OF MAMMARY EPITHELIAL CELLS

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The DNA binding protein HMG-I has been suggested as a marker for progression of several kinds of cancer, most convincingly for prostate cancer. We have now assessed the accumulation of HMG-I mRNA as a marker for breast cancer development. We utilized a series of mouse mammary epithelial cell lines, all derived from the same parent cell population, that express a range of growth characteristics from preneoplastic to non-invasive to highly aggressive. We utilized Northern blotting procedures to measure the amounts of HMG-I mRNA in these cells under several different growth conditions. At low cell density in culture, the amount of HMG-I mRNA directly correlated with the relative degree of malignancy of the cell lines. At confluent cell density, HMG-I mRNA production ceased in all but the most highly malignant cells, in which it continued unabated. Several different methods have been utilized to transfect the preneoplastic cell line with the HMG-I gene in appropriate expression vectors; the gene provides these cells with the ability to grow in suspension in soft agarose, which they previously lacked. We conclude that HMG-I can serve as a useful marker of the progression status of neoplastic mammary cells, and it may play a significant role in the process of progression itself. Similar studies are underway with human cells in culture and human breast pathology specimens. (Supported by NIH grant CA-46885.)

ASSOCIATION OF CHROMOSOME 17 TO BREAST CANCER AND THE SEARCH FOR A TUMOUR SUPPRESSOR GENE.

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Analysis of early onset breast cancer families has indicated linkage between a breast cancer susceptibility gene and markers on chromosome 17(q12-23) . This locus is believed to lie between the markers for nm23 and GIP (gastric inhibitory peptide).

Loss of heterozygosity studies have indicated that other chromosomes 3p, 13q, 16q, 17p as well as 17q21 were lost in breast tumours . These results suggested that there was an accumulation of genetic alterations, which may include loss of function of tumour suppressor genes. Further studies using the candidate gene prohibitin, indicated mutations in several sporadic breast tumours and we await the familial studies using prohibitin.

We have isolated YACs covering approximately 2Mb of chomosome 17q21-23 including YACs from many known loci, including; erb2b,nerve growth factor receptor, growth hormone1 and 2, nm23, protein kinase c, oestradiol dehydrogenase β , retinoic acid receptor α, hoxII , prohibitin, GIP and glycoprotein IIIa. Additional YACs were isolated from cosmids known by in situ hybridisation to come from 17q21-23..

We have developed vectors to allow the modification of the YACs and to allow introduction of the YACs into breast cancer cell lines to see the effects of any putative tumour suppressor genes.

DNA INDEX, PROLIFERATIVE ACTIVITY AND HISTOLOGIC GRADE AS PROGNOSTIC FACTORS OF DUCTAL BREAST CANCER

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Flow cytometry (FC) analysis and image analysis (IA) including DNA index (ploidy status) and proliferative activity (%S and %S + G2/M) were done on paraffin-enbedded tumors of 119 patients with breast cancer. No significant relationship was found between ploidy and age, estrogen receptor status, tumor size, nodal status, clinical stage and menopausal status; whereas ploidy was significantly related to histological type and grade (p less than 0.001). In addition, the DNA index was significantly related to time of recurrence (p=0.0006). Aneuploid tumors had significantly higher proliferative activity (p less than 0.001). Patients with %S or %S + G2/M activity less than or equal to the median value were significantly different from those with %S or %S + G2/M above the median: patients with %S + G2/M greater than the median value showed shorter time to recurrence (p=0.024) and shorter survival (p=0.046). Multivariate analysis using the Cox model suggested that ploidy and proliferative activity (%S + G2/M) gave prognostic information, independent of clinical parameters. Nevertheless, this independent significance was lost when histological type and grade was included in the analysis.

IDENTIFICATION OF TWO NOVEL PROTO-ONCOGENES ON CHROMOSOME 11Q13, INVOLVED IN HUMAN BREAST CANCER AND SQUAMOUS CELL CARCINOMA.

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Amplification of the human chromosome 11q13 region is frequently observed in breast cancer and squamous cell carcinomas of the head and neck, and is indicative of poor prognosis. The previously identified proto-oncogenes within this region, INT-2 and HSTF-1, are only rarely expressed in these tumors. By differential cDNA cloning, we identified two novel genes within this region, that are amplified and overexpressed in tumors with an 11q13 amplification. One of them, U21B31, is identical to the recently described PRAD-1 (or cyclin D1) gene that is involved in cell cycle regulation. The other putative oncogene, called EMS-1, encodes a protein that is normally associated with the cytoskeleton. It contains a SH3 domain and an internal tandem repeat. However, in tumor cells with an 11q13 amplification, this protein is mainly found in cell-substratum contact sites. Overexpression and relocation of the EMS-1 gene product might contribute to the invasive potential of tumor cells with an 11q13 amplification.

p53 MUTATIONAL SPECTRA IN PATIENTS WITH HISTORY OF ATYPICAL HYPERPLASIA (FEMALES) AND BREAST CANCER (FEMALES AND MALES). A. Seth[1], J. Mariano[1], R. Metcalf[2], D. Palli[3], S. Kottaridis[4], A. Panayiotakis[4], H. Li[1], S. Bianchi[3] and I. S. Papas[1], [1]Laboratory of Molecular Oncology, National Cancer Institute, Frederick, MD 21702-1202; [2]Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, MD 20892; [3]Centro per to Studio e la Prevenzione Oncologica, Florence, Italy; [4]Hellenic Anticancer Institute, Athens, Greece.

Mutations of the p53 tumor suppressor gene are the most common genetic lesion in human cancers and appear to be relatively common (30%) as somatic cell mutations in breast cancer. p53 mutations have also been frequently reported in breast cancers as part of the Li-Fraumini syndrome. In the present study, we tested the hypothesis whether women with both breast cancer and a history of atypical hyperplastic lesions have an increased frequency of germline p53 mutation, as well as whether these atypical hyperplastic lesions exhibit somatic p53 mutations. The study included 32 female patients and the p53 mutations were examined by both SSCP and direct DNA sequencing of the PCR-amplified DNA fragments. In the five samples so far analyzed, two tumor samples showed mutations at amino acid 248 (CGG→CAG, Arg→Gln) and one showed mutation at amino acid 243 (ATG→ATC, Met→Ile). Analysis of the DNA from normal and atypical hyperplastic lesions of the cancer patient samples showed no germline or somatic cell mutations, suggesting that the p53 mutations occurred during advanced stages in the pathogenesis of cancer.

Males with breast cancer are far rarer than females. We were interested in finding out whether the mutational spectra of the p53 gene reflects the different exposure of breast tissue to estrogens in female and male patients. Of 10 samples investigated for p53 mutations in exons 7 and 8, only one sample showed a point mutation in the DNA sequence corresponding to amino acid 290 (CGC→CGT, Arg→Arg); however, this point mutation turned out to be a silent change, thus representing only DNA polymorphism. Although the number of male breast cancer samples so far examined by us are small, unlike females, the male breast cancer does not appear to have frequent p53 mutations. Previously, such low frequency of p53 mutations have been reported in patients with medulloblastoma (10%) and mesotheliomas (20%).

A GERMLINE MUTATION IN THE DNA-BINDING DOMAIN OF THE ANDROGEN RECEPTOR IN FAMILIAL MALE BREAST CANCER.

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Breast cancer in men is approximately one hundred-fold less common than in women. Among the established risk factors is a family history of male or female breast cancer. Although germline mutations in the p53 gene and in the BrCa1 gene on the long arm of chromosome 17 are known to confer a high risk of female breast cancer, there is little evidence for high rates of male breast cancer in kindreds attributable to this gene.

In our set of over 200 breast cancer families we have encountered only one in which there were two males affected. Further investigation revealed that both affected individuals suffered hypospadias. Sequence analysis of the androgen receptor gene revealed a mutation in the second zinc finger of the DNA binding domain at codon 607 resulting in a substitution of glutamine for arginine.

The likelihood of this association being due to chance is extremely small and we suggest that the mutation in the androgen receptor is related to the development of breast cancer in this family. This is therefore the first report of a gene which may predispose to breast cancer in men and of germline mutations in a steroid hormone receptor resulting in cancer predisposition.

COMPLEXITY OF THE 11q13-BASED AMPLIFICATION EVENTS IN BREAST CANCER

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DNA amplification observed in human cancer is thought to activate oncogenic functions which participate directly in the tumoral process. This simple idea is based on the "one amplicon-one gene" model derived from *in vitro* studies on drug resistance. However, selective forces involved *in vivo* in tumor growth and metastasis are such that amplification of several genes -oncogenic or not- may contribute to tumor progression, even by confering only a slight selective advantage. The concept of amplifiable genomic region could thus be more complex than primarily suspected. Moreover, being selected either alone or in various combinations, amplicons could overlap for functional and/or structural reasons. These issues are particularly critical in breast cancer where DNA amplification is frequently encountered.

We have started to dissect amplification events taking place at 11q13 in mammary carcinomas, where they witness a rather negative outcome of the disease. We have shown that (at least) three discrete sub-regions of 11q13 were amplifiable separately. The central one corresponds to the area encompassing *FGF3* (*INT2*), *FGF4* (*HST*) and BCL1 which was the first one demonstrated to be amplified in *ca* 20% mammary tumors. It is tightly associated to the cyclin D1 gene (*CCND1*). On its telomeric side, the newly discovered *GARP* gene defines additional amplicons for which the gene under selection has to be identified.

We have focused our interest particularly onto the region of 11q13 centromeric from BCL1 where D11S97 has been used to define apparently independent amplification events. The use of more centromeric probes confirms the existence of this amplicon and precise the boundaries of the area where the responsible gene(s) should be looked for.

Chromosome 17 allele loss and *c-erb*B2 amplification in breast tumours

Mahvash Tavassoli, Ali Ziaee & Nigel Kirkham

Abstract;

Chromosomal deletions, associated with the loss of normal function in tumour suppressor genes, have been identified in a variety of both familial and sporadic human cancers. Although the molecular pathology of breast cancer is not understood, several studies have detected chromosomal deletions which have been identified by the loss of heterozygosity (LOH). Prominent amongst these are chromosome 17 deletions, suggesting the involvement of chromosome 17 located tumour suppressor gene/s in the development of breast cancer. We have used a number of polymorphic markers including VNTR, restriction site polymorphisms and microsatellite markers to examine chromosome 17 deletions in a panel of 51 malignant tumours, Chromosome 17 LOH and c-erbB2 gene amplification was analysed in these tumours, plus their lymph node metastasis. The majority of chromosome 17 deletions appear to be interstitial and affect chromosome 17p13.3, near the TP53 locus. Amplification of c-*erb*B2 oncogene was detected in 22% of breast tumours analysed and correlated with LOH on 17p. Loss of 17p heterozygosity seemed an early event in the development of breast cancer with no significant correlation to lymph node metastasis and/or histological type of tumours. Our data suggests the involvement of loci, distal to the P53 tumour suppressor gene on short arm of chromosome 17 in the molecular pathogenesis of breast cancer.

FLG/FGFRI and PLAT as markers of amplification at chromosome 8p12 in breast and ovarian cancers.

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Two FGF receptor (*FGFR*) genes, *FLG/FGFRI* and *BEK/FGFR2* located at 8p12 and 10q26 respectively, are amplified in approximately 12% of primary breast tumors (Adnane et al., Oncogene, 1991). We focused our efforts on the 8p12 region and extended our analysis to another marker of this region, *PLAT*.

In our series of 173 breast cancers, *FLG/FGFR1* and *PLAT* were respectively amplified in 14.5% and 15.6% on the cases and both events were frequently concommitant, but also occurred independently. In 101 ovarian cancers, amplification of *PLAT* (12.7%) was observed more frequently than that of *FLGF/FGFR1* (4.9%). The amplification of PLAT in tumors where *FLG/FGFR1* showed a normal copy number casted some doubt on the identity of the gene selected in the amplicon. Therefore, we undertook an analysis of the RNA expression of both *FLG/FGFR1* and *PLAT* in a panel of 68 breast tumors showing normal copy number or amplification at the concerned loci. The expression pattern of *PLAT* excluded this gene since its levels of expression were, in most tumors, indistinguishable from those observed in normal mammary tissue. Contrastingly, *FLG/FGFR1* showed variable levels of expression, with overexpression in amplified and non amplified samples. We also noticed amplified tumors which did not overexpress this gene. These results suggest that neither *FLG/FGFR1* nor *PLAT* behave as targets of the amplification observed at chromosome 8p12.

A SURVEY OF GENETIC ABNORMALITIES IN BREAST CANCER

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Oncogenes, tumor suppressor genes, growth factors and hormonal influences may all play an important role in breast cancer. This study examined a series of untreated primary breast cancers for specific genetic abnormalities.

Eighty invasive breast cancers were snap-frozen in liquid nitrogen at the time of surgery. DNA extracted from tumor tissue and venous blood lymphocyte DNA was examined for loss of heterozygosity at 5 tumor suppressor loci - p53, YNZ22, THH59 (all on chromosome 17); DCC and apc - by Southern blotting. The DNA was also screened for p53 mutations following PCR of exons 5-9, by the HOT technique and mutations confirmed by direct DNA sequencing. Northern blots of RNA from tumor tissue were compared with normal breast for expression of the oncogenes c-myc c-erbB-2 and H-ras, the growth factors TGFβ1 and pS2 and the tumour suppressor genes p53 and DCC. Full clinical details and pathological parameters including tumor estrogen receptor protein content were documented. Loss of heterozygosity (LOH) at YNZ (in 60% informative patients) was the most common feature. p53 mutation, THH59 LOH, LOH at the colorectal loci (DCC/apc) oncogene or growth factor over expression each occurred in upto a third of patients. Genetic profiles for individual tumors suggested that some combination of tumor suppressor loss and oncogene overexpression was present but indicated the diversity of genetic abnormalities involved. This survey points to future approaches in understanding the molecular and genetic abnormalities in breast cancer.

P53 MUTATIONS AND PROTEIN EXPRESSION IN ICELANDIC BREAST CANCER PATIENTS: CORRELATION WITH PROGNOSIS.

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Loss of a functional p53 allele has been found in a large proportion of breast carcinomas, suggesting that it plays an important role in the tumour formation. We have studied tumour and blood samples from 109 breast cancer patients for mutations in the p53 gene, allelic loss, protein expression in tumours and serum, using constant denaturant gel electrophoresis (CDGE), direct DNA sequencing, Southern blotting, immunohistochemistry and ELISA. Mutations in exons 5, 7 and 8 were found in 17 of the 109 samples (16%). Loss of heterozygosity was detected with p53 probes in 71% of the mutated cases. All cases were checked for germline mutations but none were found. P53 protein expression was detected in all mutated samples except those with nucleotide deletions. These data are being analysed for correlations between p53 mutations, the degree of p53 protein expression in the tumour, levels of p53 protein in serum as well as clinical parameters including stage of disease, recurrence of malignancy and survival. Preliminary analysis suggests a strong correlation between p53 mutations and short-term mortality.

RAT MAMMARY TUMOR CELLS AS A MODEL TO STUDY FATTY ACID EFFECTS ON p53 EXPRESSION

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Rat mammary tumors induced by N-nitrosomethylurea (NMU) or 7,12-dimethylbenz(a)anthracene (DMBA) are commonly used as model systems to investigate the modulating effects of dietary factors on breast cancer. It has been shown that 86% of NMU-induced tumors and 23% of DMBA-induced tumors carry a carcinogen-induced mutation in the *Ha-ras* gene (codon 12 for NMU and codon 61 for DMBA). Dietary fats were not found to affect the frequency of *ras* mutations in the NMU model, yet did affect final incidence of tumors when administered during the promotion phase in both models. Since p53 alterations appear to be late events during carcinogenesis in some tissue types, we have investigated whether these fatty acids might affect expression and, in turn, activity of the p53 tumor suppressor gene in cells from these tumor models. *In vitro* analysis of p53 expression was carried out with 2 established cell lines, NMU (ATCC CRL 1743) and RBA (ATCC CRL 1747), derived from rat mammary tumors initiated by NMU and DMBA, respectively.

When cultured in RPMI medium containing 10% FBS, both cell lines over-express p53 as determined by immunocytochemistry using the monoclonal antibody clone Pab 240. Using metabolic pulse chase labeling followed by immunoprecipitation and SDS-PAGE analysis, both cell lines were found to express p53 protein with a prolonged half-life of 1 1/2 to 2 hr. Accumulation of p53 to high levels commonly indicates the presence of a mutant form of the gene, suggesting that both RBA and NMU may carry mutations in this tumor suppressor gene.

To study the effects of serum concentrations in general, and fatty acids in particular, on p53 protein expression, we exposed NMU cells to medium containing high serum (10% fetal bovine serum, FBS), low serum (1% FBS), or low serum plus 1 μg/ml linoleic acid (LA) in the form of LA-BSA complexes. Growth rates for NMU cells are reduced by 30-60% in 1% FBS compared with 10% FBS, while addition of LA-BSA complexes results in an intermediate growth rate. After 3 days in the experimental medium, cells were metabolically labelled with ^{35}S-methionine and the level of p53 protein expression was determined by immunoprecipitation and autoradiography. We found p53 expression to be higher in NMU cells maintained in low serum (1%) than in those grown in high serum (10%), or those exposed to low serum in the presence of LA-BSA. Interestingly, the increased p53 expression in cells grown in low serum was found to correlate with their slower growth rate, and the ability of LA-BSA to increase growth rate is consistent with its activity in reducing p53 expression.

These studies suggest not only that both NMU and DMBA-induced rat mammary tumor cells may contain p53 gene mutations, but also that levels of p53 protein synthesis may be influenced by fatty acid levels in a relationship that is the inverse of the growth rate.

MISSENSE POLYMORPHISM (C/T224) IN THE HUMAN CATHEPSIN D PRO-FRAGMENT DETERMINED BY PCR-SSCP ANALYSIS AND POSSIBLE CONSEQUENCES ON PRO-ENZYME ROUTING IN CANCER CELLS

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Overexpression, in human breast cancers, of the lysosomal protease cathepsin D is associated with a higher risk of relapse and metastasis (1) and pro-enzyme routing is altered in several tumoral mammary cell lines, leading to its hypersecretion (2). The MCF7 cell line carries a C --> T mutation at position 224 which converts Ala n°7 to Val within the cathepsin D pro-fragment, as compared to the normal cathepsin D sequence (3). We used a PCR-SSCP protocol to discriminate the genotypes according to the type of base (C or T) at position 224. A quick purification step of PCR products and a high polyacrylamide concentration in the SSCP gels were required to discriminate C/T224 heterozygotes from C/C224 homozygotes. The variant T allele was found to be carried in 25% of genomes and equally distributed in 22 cancer cell lines, 30 breast cancer tumors and 31 normal tissues. Moreover, genotypes were identical in matched sets of tumoral mammary cells and normal white blood cells from the same patients. The C/T224 transition is thus not caused by a somatic event but polymorphism. However, this missense polymorphism might facilitate increased secretion and delayed maturation of pro-cathepsin D in breast cancer cells when the gene is overexpressed by increasing hydrophobicity of the pro-fragment.

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Breast cancer formation in transgenic animals induced by the WAP-SV-T hybrid gene

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Abstract:

After injection of the WAP-SV-T hybrid gene into fertilised mouse eggs, eight independent transgenic mouse lines were obtained. Females from three lines developed mammary carcinomas with high frequency, coinciding mostly with lactation. In contrast to the endogenous WAP gene, expression of the hybrid gene continued after lactation. The tumor cells had a very invasive growth characteristic. Tumor regression in vivo was not observed. However, after transfer into tissue culture 25% of the cells ceased to express the hybrid gene and acquired the growth characteristic of normal cells. It was possible to retransform these cells by injection of wt SV40 DNA, but not after transfer of the hybrid WAP-SV-T gene.

RETINOIC ACID RESISTANCE OF ESTROGEN-INDEPENDENT BREAST CANCER CELLS COINCIDES WITH DIMINISHED EXPRESSION OF FUNCTIONAL RETINOIC ACID RECEPTORS

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Breast tumors often are estradiol (E2)-dependent initially, but they tend to loose this dependency upon progression. While retinoic acid (RA) strongly inhibited proliferation of the E2-dependent breast cancer cell lines MCF7, T47D, and ZR75-1, the E2-independent and E2 receptor (ER) negative lines MDA-MB231, MDA-MB468, BT20 and Hs578T were refractory to the inhibitory effects of RA. The specific sensitivity of the E2-dependent cell lines seems not to be caused by an inhibitory effect of RA on the E2-induced signal transduction pathway. In the first place, RA inhibited the proliferative response of these lines not only to E2 but also background proliferation and that induced by insulin. Furthermore, although RA receptors (RARs) can inhibit ER activity when overexpressed, endogenous RARs hardly impaired transcriptional activity of the ER in transient transfection assays.

RARα mRNA was highly expressed in the RA responsive lines, but not in the unresponsive lines, except BT20. With the exception of Hs578T, also RARβ mRNA expression was low in the unresponsive lines. While in the dependent lines and Hs578T RA activated RA responsive element-dependent transcriptional activity, this response was very low in MDA-MB231, MDA-MB468, and BT20, suggesting that the RA resistance of these latter three ER-negative lines is due to underexpression of functional RARs. Our results suggest that the loss of functional RARs may be a frequent event, leading to RA-unresponsiveness of ER negative breast cancer cells. This implies that both the steroid- and retinoid receptor status of breast tumors may be used to predict a successful treatment with retinoids.

AMPLIFICATION, EXPRESSION AND REGULATION OF CYCLIN GENES IN HUMAN BREAST CANCER.

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Cell cycle progression is coordinated by the action of cell division kinases in association with their regulatory subunits, the cyclins. However, data on the expression of cyclin genes in breast cancer are limited. These genes potentially mediate the effects of breast cancer cell mitogens and growth inhibitors and are also candidate oncogenes. To investigate these possibilities we have analysed the mRNA expression of the G1-phase C, D1 and E cyclin genes in T-47D breast cancer cells stimulated by insulin to progress semi-synchronously through the cell cycle under serum-free conditions. Secondly, we have studied cyclin genes in 20 human breast cancer cell lines and 148 breast tumor samples by Southern and Northern blot analysis.

In T-47D cells, cyclin D1 mRNA was induced within 2 h of insulin treatment (10 μg/ml), to a maximum of 2- to 3- fold. This increase was maintained until 12 h but declined to control levels by 24 h. The increase in cyclin E mRNA was of similar magnitude but occurred later, at 12-15 h, and was accompanied by an increase in histone H4 mRNA as cells entered S phase. Cyclin C mRNA levels were constant through the cell cycle. IGF-1 and fetal calf serum, both potent mitogens for these cells, also stimulated expression of cyclins D1 (6h) and E (18h).

Northern and Southern blot analysis produced the following results- cyclin A: mRNA apparently highly expressed without gene amplification in the MDA-MB-157 and BT-549 cell lines; cyclin B: highly expressed without amplification in the BT-549 cell line; cyclin C: neither highly expressed nor amplified; cyclin D1: amplified and highly expressed in the MDA-MB-134, -330, and -453 cell lines, amplified but not highly expressed in the MDA-MB-175, -361 and ZR-75-1 cell lines; and highly expressed but not amplified in one of three MCF-7 sublines; cyclin E: amplified and highly expressed in the MDA-MB-157 cell line. No gross structural rearrangements of any cyclin gene were detected by Southern analysis. Thirty of 148 tumor samples (20%) also highly expressed cyclin D1 mRNA.

These data are consistent with a role for the G1-phase cyclins D1 and E in mediating mitogenic responses in breast cancer cells and show that cyclin D1 is commonly dysregulated in human breast cancer cell lines and tumors.

CHARACTERIZATION OF CALMODULIN-LIKE PROTEIN (NB-1) WITH RESTRICTED EXPRESSION IN EPITHELIAL TISSUES AND REDUCED ABUNDANCE IN TRANSFORMED HUMAN MAMMARY EPITHELIAL CELLS

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Because of the central role that calcium regulation plays in both growth and differentiation of epithelial cells, proteins involved in calcium mediated pathways are potential targets of carcinogenic changes. The NB-1 gene encodes a 16 kD protein which is identical in size and shares 85% amino acid identity with human calmodulin, the well characterized primary mediator of intracellular responses to calcium fluxes. In contrast with calmodulin, which is expressed ubiquitously in human tissues, expression of NB-1 mRNA and protein is limited to certain cells of pseudostratified and stratified epithelial tissues. Using specific polyclonal antibodies developed in our laboratory, we have localized the expression of NB-1 protein in fixed tissue sections from histologically normal human breast, prostate, skin and cervix. In each of these tissues, NB-1 showed a unique pattern of expression. In the normal breast, NB-1 staining was most abundant in basal cells surrounding small ducts, but was also found in luminal cells surrounding small ducts and in basal cells around large ducts. In several tissue sections from infiltrating breast ductal carcinomas, NB-1 protein expression was reduced or absent in comparison with normal tissue. This finding correlated well with in vitro studies, where reduced NB-1 expression was found in immortally and tumorigenically transformed derivatives of normal human mammary epithelial cells. Expanded immunohistochemical studies are now being conducted to determine at what stage(s) of carcinogenic progression and in what type of lesions NB-1 expression is likely to be downregulated or lost.

Given its restricted distribution, the NB-1 protein may be involved in the initiation or maintenance of certain differentiated functions. In the presence of calcium, calmodulin has been shown to bind to and regulate a variety of cellular proteins, including many protein kinases. Using recombinant NB-1 protein, we have shown that, like calmodulin, the NB-1 protein is capable of displaying calcium dependent binding interactions with a number of human mammary epithelial cell proteins. However, in vitro studies of enzyme activation (Yaswen, P., George, S., Cruzalegui, F. and Means, A., unpublished results) have shown that NB-1 protein can be functionally distinguished from calmodulin. Experiments are now under way to isolate and identify human mammary epithelial cell proteins which are preferentially bound and regulated by NB-1 protein.

SINGLE DOSE RADIOLIGAND BINDING ASSAY FOR THE MEASUREMENT OF THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR): OPTIMIZATION OF THE METHOD AND ANALYSIS OF HUMAN PRIMARY BREAST CARCINOMAS

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Besides nodal status, tumor size, nuclear grading and steroid receptor status, the epidermal growth factor receptor (EGFR) status may be used to predict disease-free and overall survival of breast cancer patients. Furthermore, EGFR has been reported to be an even better parameter for the response to the anti-estrogenic drug tamoxifen. Immunohistochemical procedures and some immuno assays are available, but most authors report the use of radioligand binding assays. However, most of these binding assays are not comparable, since they differ greatly in the preparation of membranes, quality of iodinated EGF, separation of bound from free ligand, and cut-off points used. Therefore, we have designed a sensitive and reproducible single dose radioligand binding assay for the routine analysis of tumor samples. Briefly, the most important component of the assay is HPLC purified EGF, enzymatically radiolabeld using immobilized lactoperoxidase/glucose oxidase and purified by reversed phase chromatography. The specific activity is determined by binding- and displacement analysis. The affinity of iodinated EGF is equal to that of unlabeled EGF. This tracer is stable for at least 4 months without any increase in unspecific binding. For the measurement of unspecific binding a 200fold excess of unlabeld EGF is used, EGF being supplied in a lyophilized form in "ready to use" reaction tubes. The separation of bound from unbound ligand is accomplished by coprecipitation of carrier proteins with PEG. In general, unspecific binding is less than 5% of the total activity, intra assay c.v. about 3.5%, and inter assay c.v. less that 10%. An excellent correlation between data from this single dose assay and data obtained by multipoint scatchard analysis has been noted. For the determination of the membrane protein content a modified BCA method (PIERCE) preceded by the solubilisation of membranes is used. The assay is linear for protein concentrations between 0.15 mg/ml and 0.75 mg/ml. EGFR was determined in membrane preparations of more than 500 primary breast carcinomas. Measurable receptor concentrations ranged from 1 fmol/mg to 768 fmol/mg membrane protein. Arbitrarily a cut-off value of 5 fmol/mg was choosen. These data will be discussed in respect to estrogen and progesterone receptor status, histo-pathological and clinical data.

LOSS OF HETEROZYGOSITY ON CHROMOSOME 17 IN HUMAN OVARIAN CARCINOMA.

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Twenty-seven ovarian carcinomas (Cas) and 3 ovarian tumors of low malignant potential (tLMPs), a closely related malignant epithelial tumor that presents at younger ages, has slower progression, and better stage-for-stage prognosis than Ca, were examined for loss of heterozygosity (LOH) on chromosome 17. We studied 15 loci, 6 on the p arm (144D6(S34), YNZ22(S30), YNH37.3(S28), php53B(TP53), MCT35.1(S31), EW301(S58)) and 9 on the q arm (EW207(S73), EW102(S41), CMM86(S74), EW101(S40), HG7Bg4Apa3(prohibitin), p63 (RARA), pNM23-H1(NM23), THH59(S4), and RMU3(S24). 90% of the tumors had some LOH. Thirteen tumors, all high-stage (III, IV) Cas, had LOH at all informative (inf.) loci, possibly representing monosomy. Fourteen tumors had LOH at some but not all inf. loci; 13 were Cas, 6 low-stage (I,II) and 7 high-stage(III,IV), and the other was a tLMP. Three tumors were heterozygous at all inf. loci, the other two tLMPs and an extremely well-differentiated Ca. The 3 tLMPs had 1 LOH/15 total inf. loci versus 109 LOH/159 loci in the 27 Cas. YNZ22 had the highest LOH rate of all loci, 94%, and this locus constituted the single p arm common region of deletion bracketed by 144D6 (LOH 56%) and by YNH37.3 and TP53 (LOH 67%). Three tumors had LOH only at YNZ22; one, a tLMP, was also informative and heterozygous at TP53. There may be a second locus on p13 distal to TP53 involved in ovarian carcinogenesis, as has been suggested for breast Ca (Sato et al Ca Res 51:5794, 1991). High LOH rates were also noted at EW301(p11) (82%), a q21 cluster including EW102, CMM86, EW101, and prohibitin with LOH of 63-75%, and THH59 (69%). CMM86 has been linked to early-onset familial breast Ca and breast ovarian Ca (Hall et al. Science 250:1684, 1990; Narod et al Lancet 338:82, 1991). One carcinoma has its only LOH at the prohibitin locus. On the p arm, there is a single common region of deletion on p13.3 separated from a second high LOH locus at p11.2 by a low LOH (38%) locus. The q arm has diffusely high LOH at loci distal to q12 but does not have a clear common region of deletion.

DOWN REGULATION OF GAP JUNCTIONAL COMMUNICATION IN BREAST CANCER

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Gene expression may be lost either by a change in DNA, referred to as Class I, or by a change in regulation (Class II). We stress the therapeutic importance of Class II changes because they are potentially reversible by treatment, e.g. drugs. By subtractive hybridization we select candidate tumor suppressor genes expressed in normal human mammary epithelial cells but not in breast tumors. Connexin encoding genes Cx26 and Cx43 were found by this method. Connexins form gap junctions, which function as channels of direct communication for adjacent cells in solid tissues, transferring small molecules such as ions and second messengers up to about 1 kDa.

The hypothesis that gap junctional intercellular communication (GJIC) contributes to normal growth regulation is supported by its absence in most solid tumors and by the correlation between transformation and loss of GJIC in experiments using rodent fibroblast cell lines.

We have shown that the loss of GJIC in breast cancer is regulated transcriptionally, leading to the absence of mRNA, protein, and GJIC in tumor cells grown in culture. Furthermore, *in vivo*, immunocytochemical staining has revealed the presence of connexin protein in normal lumenal mammary epithelium but not in preliminary studies of 8 tumors.

Transfection of either Cx26 or Cx43 (under control of an exogenous promoter) into non-expressing tumor cells has led to re-expression of mRNA, and protein, and restoration of GJIC. Of most significance for potential therapy, we have found that Cx26 can be induced to re-express in breast cancer cell lines by treatment of cells with phorbol ester. Thus, tumor cells have lost GJIC as a consequence of down-regulation of Cx26 and Cx43 genes, but the wild-type gene is still present and can be induced to re-express.

STROMELYSIN-3: A MARKER OF BREAST CANCER PROGRESSION

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Matrix metalloproteinases (MMP) are believed to play an important role in tumor invasion and metastasis. The MMP family comprises type I collagenases, type IV collagenases and stromelysins. In the later group, stromelysin-3 (ST3) has unique characteristics. The ST3 substrate(s) is presently unknown, but the sequence differs from previously described MMPs, in ways suggesting that the new MMP may be distinct in its activation properties and its substrate specificity. ST3 RNA has been found in all the invasive breast carcinomas so far tested, and in some in situ carcinomas, which are often of the comedo-type. ST3 transcripts are exclusively observed in fibroblastic cells immediately surrounding cancer cells. ST3 gene expression can be detected in breast cancer metastases observed in lymph nodes, skin, bone, liver and pleura. Although the expression pattern of ST3 gene is characteristic of the new MMP, several lines of evidence suggest that ST3 may cooperate in tumor invasion with other proteinases and extracellular matrix components, also produced by tumoral fibroblasts of breast carcinomas.

MOLECULAR BIOLOGY OF CATHEPSIN D TRANSCRIPTION AND SECRETION IN BREAST CANCER CELLS

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High cathepsin D concentration in breast cancer cytosol is associated with a higher risk of distant metastasis and breast tumor cells overexpress cathepsin D gene in both estrogen receptor positive and negative breast cancer cells. This enzyme has the potential to degrade components of extracellular matrix at an acidic pH and to activate or inactivate several proteins involved in the control of tumor cell proliferation and invasion. In this talk, we will mostly consider the mechanisms by which estrogens and antiestrogens regulate cathepsin D gene transcription and pro-cathepsin D secretion in ER positive breast cancer cells. The cathepsin D promoter of the MCF7 cells has been cloned and studied for functional estrogen responsive elements (EREs). Two non-canonical EREs located at -261 and -354 and acting in synergy, have been demonstrated to bind *in vitro* estrogen receptor and to mediate activation of transcription of a TK-CAT reporter gene. Moreover, this promoter has mixed property with at least 3 transcription start sites located upstream a TATAA box, and an estrogen regulated start site located downstream the TATAA box. The mRNA length therefore varies according to the presence or absence of estrogens. The secretion of pro-cathepsin D is increased in breast cancer cells lines (both ER+ and -), and its mechanism involving saturation of the Mannose-6-P/IGFII receptor and another membrane bound transport system will be discussed in addition to the significance of a T $\longrightarrow$C, Ala $\longrightarrow$Val missense mutation of the pro-fragment which has been identified by PCR-SSCP analysis as a polymorphism.

Further studies will aim to determine whether there is an alteration of these regulations in cancer cells, compared to normal estrogen responsive cells, and its possible consequence on metastasis.

TRANSFECTION AND BIOCHEMICAL ANALYSIS OF NM23 FUNCTION.
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The human MDA-MB-435 breast carcinoma cell line has been reported to metastasize upon injection into the mammary fat pad of nude mice (Price et al. Cancer Res. 50: 717, 1990). MDA-MB-435 cells were transfected with the full length *nm23-H1* cDNA linked to a constitutive CMV promoter, and two stable high expressing clones identified (H1-170, H1-177). As controls, MDA-MB-435 cells were transfected with the CMV expression construct without a cDNA insert, and two clones randomly selected (C-100, C-103). The *nm23-H1* transfectants exhibited differences in properties related to both tumorigenicity and metastasis, as compared to the control transfectants. This included a reduced in vitro growth rate at low passage number, reduced colonization potential in soft agar, altered responsiveness to TGF-β, and reduced migration in response to serum, PDGF, IGF or AMF. Upon injection into the mammary fat pad of nude mice, only the highest expressing *nm23-H1* clone, H1-177, produced primary tumors that were smaller than control clones, and only from low passage number cultures. These in vivo data stand in contrast to the in vitro growth and colonization data, and stress the importance of orthotopic injections where epithelial-stromal interactions and local production of growth factors may be important. Both the H1-170 and H1-177 clones produced draining lymph node and lung metastases in significantly fewer animals than the control clones. The data indicate that *nm23-H1* can exert a negative regulatory effect on breast cancer progression.

Three biochemical properties of nm23 proteins are under characterization. Both the nm23-H1 and nm23-H2 proteins have nucleoside diphosphate kinase (NDPK) activity, which transfers a terminal phosphate among nucleoside di- and triphosphates and involves a high energy phosphohistidine intermediate. A leucine zipper like motif is also present on nm23 proteins, and was the subject of mutation in a stage IV human neuroblastoma tumor. In addition, we have observed that incubation of nm23 with ^{32}P-γATP or -orthophosphate resulted in both phosphohistidine and phosphoserine labeling in vitro and in vivo, respectively. In vitro, nm23-H1 protein labeled on a serine residue to a greater extent than did nm23-H2. The phosphoserine bond energy is insufficient for subsequent transfer of the phosphate to a nucleoside diphosphate, establishing the nm23-phosphoserine form as being distinct from its NDPK activity.

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