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Abstracts of papers presented
at the 1995 meeting on

PROGRAMMED CELL DEATH

September 20–September 24, 1995

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Arranged by

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Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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Cover: "A Night in Apoptosis City," by James Duffy, Cold Spring Harbor Laboratory.

PROGRAM

WEDNESDAY, September 20—7:30 PM

SESSION 1 INVERTEBRATE DEVELOPMENT

Chairperson: H. Steller, Massachusette Institute of Technology

THURSDAY, September 21—9:00 AM

SESSION 2 IMMUNOLOGY/NEUROLOGY

Chairperson: **S. Nagata,** Osaka Bioscience Institute, Japan

THURSDAY, September 21—2:00 PM

SESSION 3 POSTER SESSION I

THURSDAY, September 21—4:30 PM

Wine and Cheese Party

THURSDAY, September 21—7:30 PM

SESSION 4 *bcl-2* FAMILY

Chairperson: **S. Cory,** Walter and Eliza Hall Institute of Medicine, Australia

FRIDAY, September 22—9:00 AM

SESSION 5 BIOCHEMISTRY

Chairperson: **J. Yuan,** Massachusetts General Hospital East

FRIDAY, September 22—2:00 PM

SESSION 6 POSTER SESSION II

FRIDAY, September 22—7:30 PM

SESSION 7 VIRAL

Chairperson: **G. Evan,** Imperial Cancer Research Fund, London, United Kingdom

SATURDAY, September 23—9:00 AM

SESSION 8 ONCOGENESIS

Chairperson: A. Levine, Princeton University

SATURDAY, September 23 1:30—3:30 PM

SESSION 9 VERTEBRATE DEVELOPMENT

Chairperson: **M. Raff,** University College, London, United Kingdom

SATURDAY, September 23 3:30—5:30 PM

SESSION 10 POSTER SESSION III

SATURDAY, September 23

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

SESSION 11 DISEASE

Chairperson: **A. Wyllie,** University Medical School, Edinburgh, United Kingdom

PROGRAMMED CELL DEATH IN *DROSOPHILA*

Hermann Steller, Julie Agapite, Cerinda Carboy-Newcomb, Alba I. Cid, Florence Davidson, Megan Grether, Anne-Francoise Lamblin, Kim Mejia, Zhiwei Song, Jan Tittel, Chia-Lin Wei, and Kristin White[1], Howard Hughes Medical Institute, Depts. of Brain and Cognitive Sciences and Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, [1]Massachusetts General Hospital, Charlestown, MA 02129.

During *Drosophila* development, large numbers of cells undergo apoptotic cell death. The onset of these deaths is controlled by many different intra- and extracellular signals that may either promote or suppress apoptosis. We have surveyed a large fraction of the *Drosophila* genome for genes that are required for programmed cell death and identified two genes, *reaper* and *head involution defective (hid)*, which play a central control function for the activation of apoptosis in *Drosophila*. A small deletion that includes both genes protects against apoptosis in response to many different death-inducing signals. During embryogenesis, the *reaper* gene is specifically expressed in cells that are doomed to die, and its expression precedes the first morphological signs of apoptosis by 1 to 2 hours. Expression of either *reaper* or *hid* is sufficient to induce apoptosis in cells that would normally survive. These ectopic deaths can be prevented by co-expression of the anti-apoptotic baculovirus p35 protein, indicating that p35 acts downstream from these genes. Our results suggest that multiple signalling pathways for the activation of apoptosis converge onto a common cell death pathway that is controlled by the activity of *reaper* and *hid*.

GENETIC CONTROL OF PROGRAMMED CELL DEATH IN DROSOPHILA. J. Agapite, F. Davidson, K. Mejia, A. I. Cid, A. Newberg and H. Steller. Dept. of Biology and Dept. of Brain and Cognitive Science, MIT, Cambridge, MA

Programmed cell death plays an important role in development and tissue homeostasis in virtually all organisms. At least some components of the programmed cell death pathway(s) are conserved from C. elegans to mammals. We are exploiting Drosophila genetics to gain further insight into the cell death process. Previously two Drosophila genes, *reaper* and *head involution defective* (*hid*), were isolated and both appear to be involved in programmed cell death. Both genes can induce ectopic cell deaths when overexpressed and each can do so in the absence of the other. Targeted expression of either gene in the developing compound eye results in a reduced, rough eye phenotype. This eye phenotype can be suppressed by coexpression of the baculovirus p35 gene which can inhibit cell death in C. elegans, Drosophila, and mammals indicating that the eye phenotype results from an excess of cell death and that this death occurs via a conserved pathway.

In order to identify genes which function downstream of *reaper* and/or *hid*, a deficiency collection representing ~65% of the Drosophila genome was screened for dominant modifiers (suppressors and enhancers) of the reduced, rough eye phenotype caused by overexpressing *reaper* or *hid*. Deficiencies were identified that specifically modify only the *reaper* or only the *hid* dependent eye phenotype. Other deficiencies were identified that modify both phenotypes. These results suggest that *reaper* and *hid* function in parallel pathways which eventually converge. The modifiers identified in the deficiency screen are currently being analyzed further. In addition, a screen for chemically induced mutations that dominantly modify the *reaper* dependent reduced, rough eye phenotype is underway. A lethal P-element collection is also being screened. The results of these screens will be presented.

IDENTIFICATION OF CELL DEATH EFFECTORS IN *DROSOPHILA*

Michal Reichman-Fried, Phani Kurada, Elvan Tahaoglu, and Kristin White CBRC, Massachusetts General Hospital, Harvard Medical School

The active elimination of cells by programmed cell death, or apoptosis, is a fundamental aspect of normal development and homeostasis of many organisms. The long term goal of our research is to understand the molecular mechanisms underlying this process. By using the powerful genetic and molecular techniques available in *Drosophila* we hope to identify and characterize some of the genes involved in apoptosis. In our initial studies we identified a gene, *reaper* (*rpr*), which appears to encode a key control function in the initiation of the cell death program.

Our previous work has shown that the *rpr* gene acts upstream of the effectors of cell death. These effectors are responsible for the stereotyped changes in the cell which are typical of apoptosis. We hope to identify such effectors in a genetic screen, as suppressers of ectopically induced cell death. The ideal genetic tool with which to conduct such a screen would be flies with an easily identified phenotype associated with excess cell death, which is sensitive to changes in levels of activity. Transgenic flies which express *rpr* ectopically in the eye have these characteristics (pGMRrpr). These flies show phenotypes ranging from a normal eye, to a very rough eye, to an almost eyeless fly with increasing gene dosage. Therefore, a slight reduction in cell death due to the decrease in the dose of an effector gene should be easily visible, as a return of the eye to a more normal appearance. A collection of fly strains carrying previously characterized deletions has been tested for dominant interactions with pGMRrpr, and a number of suppressers have been identified. Some of these suppressers can also be shown to suppress the effect of ectopic *rpr* expression in other tissues, and to have effects on the levels of endogenous cell death, indicating that these deletions contain genes which act as general suppressers of *rpr* function. Using this strategy we expect to identify a number of the effector molecules which are involved in apoptosis in this organism, and possibly other organisms as well.

CED-3 IS A CYSTEINE PROTEASE THAT CONTROLS PROGRAMMED CELL DEATH IN *C. ELEGANS*

Ding Xue and H. Robert Horvitz, HHMI, Dept. Biology, MIT, Cambridge, MA 02139, USA

The gene *ced-3* is required cell-autonomously for cells to die by programmed cell death in the nematode *Caenorhabditis elegans. ced-3* encodes a protein similar to mammalian interleukin-1ß converting enzyme (ICE), a cysteine protease that has been implicated in programmed cell death in mammals. Three additional mammalian proteins (NEDD-2/ICH-1, CPP32 and TX) have sequence similarity to CED-3 and can initiate programmed cell death in mammalian cells. These findings indicate that a cysteine protease family may be involved in regulating programmed cell death in many different species.

We found that CED-3 protein expressed in *E. coli* undergoes autocleavage. This activity is abolished when a completely conserved cysteine at the active site is mutated. The activity is also inhibited by iodoacetic acid, a cysteine protease inhibitor, but not by other protease inhibitors. In addition, *ced-3* alleles of different severity affect *ced-3 in vivo* cell death activities and CED-3 *in vitro* protease activities in parallel. These results indicate that CED-3 is a cysteine protease and suggest that its protease activity is responsible for its role in programmed cell death.

To examine how cysteine proteases regulate programmed cell death, we have developed a general method for the purification of this class of proteases from bacteria. Three active proteases (CED-3, ICE and NEDD-2/ICH-1) have been purified to homogeneity. All three proteases autocleave in bacteria and produce similar protease complexes composed of heterodimers: CED-3(p13/p17), ICE(p10/p20) and NEDD-2/ICH-1(p12/p18). Although these proteases all cleave exclusively after Asp residues and before small amino acids (Gly, Ala and Ser), they have different substrate specificities: NEDD-2/ICH-1 cleaves only the NEDD-2/ICH-1 precursor; ICE cleaves the ICE and interleukin-1ß precursors most efficiently; CED-3 cleaves the CED-3 and NEDD-2/ICH-1 precursors best. These differences in substrate specificities may reflect different roles for or mechanisms of these proteases in mediating programmed cell death. These purified active proteases can now be used to seek proteins that modulate or are cleaved by their protease activities. We are now testing whether CED-4 (a novel protein) and CED-9 (a Bcl-2 homologue), both of which are important in controlling programmed cell death in *C. elegans*, can affect the activity of the CED-3 protease *in vitro*. We are also using the yeast two-hybrid system to screen for proteins that interact with CED-3.

PROGRAMMED CELL DEATH IN THE GERMLINE

Tina Gumienny[1], Erika Hartwieg[2], Bob Horvitz[2], and Michael Hengartner[1]

[1]Program in Genetics at SUNY Stony Brook and Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

[2]HHMI, Department of Biology, MIT, Cambridge, MA 02139, USA

Germ cells in *C. elegans* can either remain mitotic stem cells or enter meiosis and undergo spermatogenesis or oogenesis. We are exploring observations of another germ cell fate: programmed cell death (PCD). Our results suggest that PCD occurs extensively in the germ line as a part of or as a by-product of oogenesis.

Most mutations that affect PCD in the soma also affect germ cell deaths. *ced-9*, shown to be necessary to protect cells that should live from dying, is also required in the germ line; many more germ cells undergo PCD in *ced-9(lf)* mutants than in N2 animals, which may explain the partial sterility of *ced-9(lf)* mutants. However, the gain-of-function mutation *ced-9(n1950)*, while blocking somatic cell deaths entirely, has little or no effect on germ cell deaths. *ced-3* and *ced-4*, necessary for somatic cell deaths, are also required for germ cell deaths; we found no corpses in the germline of *ced-3* or *ced-4* mutants. Mutations in the engulfment genes *ced-1, -2, -5, -6, -7,* and *-10*, which prevent efficient engulfment of dying somatic cells by adjacent cells, also obstruct engulfment of dying germ cells by the somatic sheath cells. From the number of accumulated germ cell corpses in these mutants, we estimate that over a third of all possible oocytes undergo PCD.

The germ cells function as nurse cells before becoming oocytes. We suggest that the number of nurse cells generated exceeds the number of oocytes that can be accommodated, and that the superfluent nurse cells are removed through PCD. As evidence, we see corpses in the meiotic part of the gonad soon after oogenesis begins, but identify few to no corpses in larvae and males. In addition, feminized males with oocytes (*fog-1(q253)* and *gld-1(q126)*) show cell corpses and XX animals that do not initiate oogenesis (*mog-1(q223)*, *gld-1(q485)*, and *glp-4(bn2)*) display no germline corpses. In other sex determination mutants, cell death is observed only when oogenesis occurs.

We are currently screening for suppressors of PCD in the gonad using a *ced-1* engulfment mutant. Mutants should suppress cell death and thereby reduce the number of corpses retained in the gonad.

BACULOVIRUS INTERACTION WITH HOST APOPTOTIC PATHWAYS

Lois K. Miller, Somasekar Seshagiri, Domagoj Vucic, Alex Harvey and Jeanne McLachlin. The University of Georgia, Athens, GA, 30602.

Baculoviruses use two different classes of genes to prevent their host cell from undergoing apoptosis during infection: inhibitor(s) of apoptosis (*iaps*) and *p35* (1,2). Both of these viral genes can prevent apoptosis in the absence of other viral gene products (3). In the case of viruses lacking *p35* and *iap*, infected cells can successfully mount an apoptotic response which limits virus replication and results in an abortive infection at both the cellular and organismal levels (4). The *p35* of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is essential for productive AcMNPV infection of most cells of the species *Spodoptera frugiperda* (eg. SF-21 cells) but not *Trichoplusia ni* (eg. TN-368 cells. In collaboration with BASF Bioresearch Corp., we have found that *p35* interacts directly with cysteine proteases of the ICE family and is able to block ICE-induced apoptosis in both SF-21 and TN-368 cell lines (5). Our laboratory is continuing to explore the mechanisms by which *p35* and *iap* interact with the apoptotic pathway of insect cells. We have found that the *Drosophila melanogaster* reaper gene (*rpr*) induces apoptosis in SF-21 cells upon transfection and that both *p35* and *iap* can block *rpr*-induced apoptosis. However, *iaps* are unable to block ICE-induced apoptosis, thus placing the point of *iap* action upstream of cysteine protease activation. Differential display PCR analysis indicates that expression of an anti-apoptotic *iap* in SF-21 cells, either transiently or stably, affects the expression of a host gene. We are also further defining the apoptotic pathway by transfecting various combinations of mammalian activators and inhibitors of apoptosis into SF-21 or TN-368 cells; this system may prove useful in unraveling mammalian as well as invertebrate apoptotic pathways.

1. Clem, R.J., M. Fechheimer, and L.K. Miller, *Prevention of apoptosis by a baculovirus gene during infection of insect cells.* Science, 1991. **254**: 1388-1390.

2. Crook, N.E., R.J. Clem, and L.K. Miller, *An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif.* J. Virol., 1993. **67**: 2168-2174.

3. Clem, R.J. and L.K. Miller, *Control of programmed cell death by the baculovirus genes p35 and iap.* Mol Cell Biol, 1994. **14**: 5212-5222.

4. Clem, R.J. and L.K. Miller, *Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus.* J. Virol., 1993. **67**: 3730-3738.

5. Bump, N.J., M. Hackett, M. Hugunin, et al., *Baculovirus anti-apoptosis protein P35 inhibits the interleukin-1ß converting enzyme and its homolog Ich-2*, 1995, submitted.

A NEW, HIGHLY CONSERVED FAMILY OF APOPTOTIC CELL DEATH REGULATORS. Colin S. Duckett, Jennifer Van Dongen and Craig B. Thompson. Gwen Knapp Center for Lupus and Immunology Research, Howard Hughes Medical Institute, Department of Medicine, The University of Chicago.

Relatively little is known about the mechanisms of regulation of apoptotic cell death in *Drosophila*. Only one gene has been reported, *reaper*, which appears to be involved in the regulation of apotosis in this organism. Reaper exhibits limited homology to the death domains of the Fas and TNF receptor molecules, but its function is not well understood.

We now describe the molecular cloning of a new cellular gene family, designated *ilp*, whose products are involved in the regulation of apoptotic cell death. We have cloned the *Drosophila* and human forms of this gene. They are closely related and appear to be widely expressed in most tissues. Although ILP is unrelated to any cellular genes known to be involved in apoptosis, comparison of the putative peptide sequences with the available sequence databases reveals a strong homology to the baculoviral apoptosis inhibitor protein, IAP. The amino terminus contains three of the previously described baculoviral inhibitory repeats (BIRs). The carboxy terminus contains a RING form of zinc finger, which has been found in several proteins involved in cellular growth and development. Unlike the baculoviral proteins, however, the cellular BIRs are separated from the RING domain by a region of ~160 amino acids which, while similar in predicted structure, are divergent between the *Drosophila* and human forms. Preliminary results suggest that overexpression of ILP in the interleukin-3 dependent cell line FL5.12 can potentiate the rate of apoptotic cell death induced by growth factor withdrawal. However, ILP appears to have no effect on death induced by staurosporine treatment or irradiation. We conclude that a) the high degree of conservation between the *Drosophila* and human genes implies a pivotal role for ILP in the regulation of apoptotic cell death, and b) that the baculoviral IAP genes are probably derived from cellular counterparts, with mutations or deletions which may have led to a *trans*-dominant phenotype.

APOPTOSIS IN A UNICELLULAR EUKARYOTE: REGULATION OF CELL PROLIFERATION AND DIFFERENTIATION AS AN EVOLUTIONARY CONSERVED ROLE OF PROGRAMMED CELL DEATH

JC Ameisen, T Idziorek, O Billaut-Mulot, M Loyens, MA Ouaissi.
INSERM U415, Institut Pasteur, 59019 Lille, France.

The origin of programmed cell death (PCD) is generally linked to the emergence of multicellular organisms. We have explored the hypothesis that PCD may also be operational and fulfill a physiological role in unicellular eukaryotes.

Trypanosoma cruzi is a protozoan unicellular eukaryote parasite whose lifecycle requires 2 different hosts (a haematophage insect vector and a mammalian host, including humans, in which it causes Chagas Disease), and involves 3 distinct differentiation stages (epimastigote, trypomastigote, and amastigote). The strictly extracellular epimastigotes proliferate in the intestine of the insect vector and later differentiate into the G0/G1 arrested trypomastigotes, that are excreted by the insect and penetrate the blood of a mammalian host. The trypomastigotes enter cells, differentiate into amastigotes that proliferate in the cells, come out of the cells as trypomastigotes and either re-enter other cells, or become ingested by the vector in which they differentiate into epimastigotes. Differentiation can be achieved in vitro by culturing epimastigotes at the temperature of the vector intestine in a cell-free defined medium. In these conditions, the epimastigotes proliferate during 3 weeks, and then differentiate into trypomastigotes, that are able to enter mammalian cells if the temperature is switched to 37°C.

After 3 weeks of epimastigote culture, at the time of differentiation into trypomastigote, we observed the onset of massive epimastigote-cell death. Transmission and scanning electron microsopy analysis showed typical cytoplasmic and nuclear morphological features of apoptosis, that were associated with DNA fragmentation in multiples of 200 bp. Epimastigote apoptosis could also be rapidely induced, prior to differentiation into trypomastigote, in particular by culturing parasites at low density and/or in nonconditionned medium, or by raising the temperature to 37°C.

These data indicate that, as in cells from multicellular organisms, the prevention of the induction of PCD is a prerequisite for proliferation and differentiation to proceed. Such a process may have evolved in unicellular organisms in order to achieve optimal adaptation of a given differentiation stage to its particular environment, and to avoid, through a mutual exclusion process, a competition between a differentiation stage endowed with proliferative capacity (the epimastigote), and a G0/G1 arrested stage (the trypomastigote).

FAS-MEDIATED APOPTOSIS

Shigekazu Nagata Osaka Bioscience Institute, Suita, Osaka 565, Japan

Fas is a cell surface protein belonging to the TNF/NGF receptor family, and it mediates apoptosis. A domain (death domain) of about 80 amino acids homologous to the TNF type I receptor in the Fas cytoplasmic region is responsible for the apoptotic signal transduction. The Fas-mediated apoptosis can be inhibited by crmA (serpin family of protease inhibitor) or non-cleavable substrate for ICE (interleukin-1b-converting enzyme), suggesting that this process involves ICE or its related protease. The cytosolic fraction from the Fas-activated cells induces a typical morphological change of apoptosis in nuclei from normal liver, and causes DNA degradation in an apoptotic fashion .

The Fas ligand (FasL) is a member of the TNF family, and exists as a type II membrane protein as well as a soluble cytokine. The soluble FasL is a trimer, and binding of FasL to Fas rapidly induces apoptosis in the Fas-baring cells. FasL is expressed in activated T cells, and works as an effector of cytotoxic T cells.

Mouse lymphoproliferation mutation (*lpr*) and generalized lymphoproliferative disease (*gld*) which causes lymphadenopathy and autoimmune disease are mutations in the Fas and FasL genes, respectively. The Fas/FasL system is involved in the activation-induced suicide of T cells, or peripheral clonal deletion. Since the abnormal T cells accumulated in *lpr* or *gld* mice constitutively express FasL mRNA, it seems that these cells are once activated or chronically activated mature T cells. The Fas mRNA is abundantly expressed in the liver, heart, lung and ovary. Administration of agonistic anti-mouse Fas antibody into mice induced apoptosis in the liver, and quickly killed the mice causing a liver damage. These findings suggest a role of the Fas system in programmed cell death in the liver, and a possible involvement of Fas in pathological tissue damage in CTL-mediated autoimmune disease such as fulminant hepatitis.

THE HODGKIN DISEASE ANTIGEN CD30 IS CRUCIAL FOR ANTIGEN INDUCED DEATH OF DEVELOPING T CELLS

Ryuichi Amakawa[1,2] Josef Penninger[1,2] Tak W. Mak[1,2] Anne Hakem[1,2] Tohifumi Matsuyama[1,2] Drew Wakeham[1,2] Emma Timms[1,2], Hans-Willi Mittruecker[1,2] Arda Shahinian[1,2] Hiroaki Takimoto[1,2] Thomas Kundig[2], and Pamela Ohashi[2]. [1]Amgen Institute, [2]Ontario Cancer Institute, and Departments of Medical Biophysics and Immunology, University of Toronto

The Hodgkin's disease antigen CD30 is normally expressed on activated T and B cells in lymphoid tissues, and in the thymus medula. CD30 is also expressed on T cells implicated in HIV pathogenesis. We have generated CD30 gene deficient mice to investigate its role in vivo. In homozygous mutant mice the thymus contains increased numbers of thymocytes; and activation induced cell death in response to CD3 crosslinking in vitro is defective, while dexamethason and γ-irraditation induced cell death were normal. αβ- and γδ-TCR transgenic mouse strains were separately crossed into the CD30 mutant background. In both models negative selection in the thymus was defective while positive selection remained unaltered. This is the first evidence of a receptor that regulates negative selection in the thymus. These findings suggest that CD30, like TNF-R and FAS are involved in cell death and may play a role in HIV infections, measle virus infections, allergic responses, autoimmune diseases and the pathogenesis of Hodgkin's and non-Hodgkin's lymphomas.

STUDYING THE ROLE OF P53 IN THYMOCYTE APOPTOSIS,
Mariana Nacht, Yvonne Chan, and Tyler Jacks, Howard Hughes Medical Institue, Center for Cancer Research Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

We are investigating the role of p53 in apoptosis using cells derived from mice carrying a germline disruption in the p53 gene. Mouse thymocytes undergo apoptosis in response to ionizing radiation in a p53-dependent manner, while maintaining a p53-independent response to phorpol esters and calcium ionophores. In addition, mice which lack p53 develop thymic tumors at a very high frequency. Several studies have suggested a causal relationship between resistance to apoptosis and oncogenesis. Together these data support a model that in normal mice cells with damaged DNA are eliminated through a p53-dependent apoptotic pathway, whereas thymic lymphomas develop in the genetically altered mice because this activity is lacking.

To test the hypothesis that mice which lack p53 develop thymic tumors because they are unable to eliminate, through apoptosis, cells which have incurred DNA damage, we are combining the p53 mutation with mutations that affect thymic development. By crossing p53-disrupted mice with mice that carry the *scid* mutation or a mutation in either the RAG-1 or RAG-2 locus, we can examine the effects of changes in the quality or frequency of T-cell receptor gene rearrangements on the development of lymphomas. We would predict that decreased rearrangement in the Rag-deficient animals will reduce the tumor incidence since the source for potential mutations has been eliminated. Conversely, we predict that the combination of *scid* and p53 mutations will lead to an increase in tumorigenesis since *scid* mice sustain large genomic deletions and have the potential for harmful recombinations. The results of the *scid*p53-/- double mutant animals are consitent with our model ; they develop thymic tumors 2 to 3 months earlier than the p53-/- animals. However, the Rag-deficient animals are not suppressing the development of thymic lymphomas in mice that also lack the p53 gene. Unexpectedly, preliminary results show that in a p53-deficient background the two Rag mutations are phenotypically different. Specifically, the Rag mutations show differences in early thymoctye development and the age of onset of thymic tumors, suggesting that RAG-1 and RAG-2 may have unique functions.

CD28 COSTIMULATION BLOCKS A FAS-INDEPENDENT MECHANISM OF T CELL DEATH.

Lawrence H. Boise, Patricia J. Noel and Craig B. Thompson. Gwen Knapp Center for Lupus and Immunology Research, Department of Medicine and Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois.

Activation of a T cell through the TCR/CD3 complex in the absence of costimulation leads to anergy or apoptosis. A costimulatory molecule which can prevent these fates for the T cell is CD28. CD28 interacts with members of the B7 family of counter-receptors present on antigen presenting cells. Previously, we have demonstrated that CD28 costimulation can enhance the survival of purified human T cells following treatment with γ-irradiation. The enhanced survival attributed to CD28 costimulation is associated with increased production of the extrinsic survival factor IL-2 and augmented expression of the intrinsic survival factor Bcl-x_L. Through the use of unseparated murine lymph node cells we now present data suggesting that B7 signaling through CD28 is the primary means by which T cells increase their resistance to apoptotic stimuli upon initial activation. Following *in vitro* activation of T cells, present in unseparated lymph node cells, with anti-CD3, survival at 72 hrs is greater than 60% of total cells. This survival can be increased to 80% by the addition of monoclonal antibodies to CD28. Addition of CTLA4Ig, a soluble protein that can block CD28/B7 interactions, leads to a viability of only 20%. When similar studies are performed with cells from CD28-deficient mice, activation with anti-CD3 also results in a 72 hr viability of 20%. In both the CTLA4Ig treated lymphocytes or cells from CD28-deficient mice, expression of Bcl-x_L is significantly lower than in activated cells, while Bcl-2 expression remains unchanged. These data confirm that CD28 costimulation enhances the survival of T cells upon initial activation at least in part through the upregulation of Bcl-x_L.

Overexpression of Bcl-x_L in Jurkat T cells leads to a near complete inhibition of anti-Fas-induced cell death. To determine if the cell death that is blocked by CD28 costimulation is induced by Fas, lymphocytes from *lpr* mice were activated in the presence and absence of CTLA4Ig and viability assessed. Over a 72 hr period of stimulation, no difference in viability was observed between wild type and *lpr* lymphocytes, regardless of stimulation. These data suggest that the cell death seen in T cells activated in the absence of CD28 costimulation occurs through a mechanism other than Fas.

RIP: A NOVEL FAS-INTERACTING PROTEIN WITH KINASE AND DEATH DOMAINS THAT INDUCES CELL DEATH

Ben Z. Stanger[1,2], Emily Kim[2,3], Brian Seed[2,3], and Philip Leder[1,2]. [1]Howard Hughes Medical Institute, [2]Department of Genetics, Harvard Medical School, and [3]Department of Molecular Biology, Massachusetts General Hospital.

Ligation of the extracellular domain of the cell surface receptor Fas/APO-1 (CD95) elicits a characteristic programmed death response in susceptible cells; its signaling mechanism is largely unknown. Using a yeast method for identifying protein-protein interactions, we have identified two gene products that associate with the intracellular domain of Fas: Fas itself, and a novel 74 kDa protein we have named RIP, for receptor interacting protein.

RIP interacts weakly with the p55 tumor necrosis factor receptor (TNFR1) intracellular domain, but not with a mutant version of Fas corresponding to the murine *lpr^{cg}* mutation. The interaction with Fas occurs in a domain corresponding to the region required for transmission of a death signal. RIP contains an N-terminal region with homology to protein kinases and a C-terminal region containing a cytoplasmic motif ("death domain") present in the Fas and TNFR1 intracellular domains.

RIP is expressed at low levels in all mouse tissues, and is slightly upregulated in splenocytes treated with ConA. Its transient overexpression causes transfected cells to undergo the morphologic changes characteristic of apoptosis. A deletion variant lacking the C-terminal half of RIP (including the death domain) is unable to induce cell death, while a variant missing part of the kinase domain retains some killing activity. The ability to associate with Fas/APO-1, and to induce apoptotic changes in transfected cells, suggests that RIP represents a new form of apoptosis-inducing nonreceptor kinase.

BCL-2 AND FAS/APO-1 REGULATE DISTINCT PATHWAYS TO LYMPHOCYTE APOPTOSIS A Strasser*, A W Harris*, D C S Huang*, P H Krammer+ and S Cory*. *The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, Victoria 3050, Australia and +Tumor Immunology Program, Division of Immunogenetics, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

Apoptosis is an evolutionarily conserved physiologic death mechanism responsible for the removal of unwanted cells. Genetic studies on the nematode *C. elegans* have provided insight into its regulation. Cells fated to die during development of this organism do so only if they express two genes, *ced-3* and *ced-4* and cell survival requires expression of *ced-9*. A homologous process operates in vertebrates since *ced-3* encodes a protein homologous to a family of mammalian cytoplasmic cysteine proteases and *ced-9* is homologous to the human proto-oncogene *bcl-2*, which inhibits killing by diverse cytotoxic agents.

In contrast to Ced-9, which blocks all programmed cell deaths in *C. elegans*, Bcl-2 blocks some but not all physiologic forms of cell death in vertebrates, since *bcl-2* transgene expression has only minimal effects on the deletion of autoreactive B and T cells. This provides evidence for a pathway to cell death which can override the action of Bcl-2.

Induction of apoptosis by activation of Fas/APO-1 (CD95) and other members of the TNF receptor family plays an important role in immunoregulation. We have found that Bcl-2 provides little protection against Fas/APO-1 transduced apoptosis in B lymphoid cell lines, thymocytes and activated T cells. Signaling through Fas/APO-1 did not downregulate Bcl-2 or induce its antagonists Bax and Bcl-xS. In Fas/APO-1 deficient *lpr* mice, *Bcl-2* transgenes markedly augmented the survival of antigen-activated T cells and the abnormal accumulation of lymphocytes. In contrast, the cowpox virus protein CrmA, which specifically inhibits ICE but not other cysteine proteases, blocked Fas/APO-1 transduced apoptosis, but did not affect cell death induced by γ-radiation or growth factor deprivation. These data provide evidence that Bcl-2 and Fas/APO-1 are regulators of distinct pathways to lymphocyte apoptosis. Different stimuli may activate distinct cysteine proteases and Bcl-2 only inhibit the activation of some of these cell death effectors.

THE ORPHAN RECEPTOR Nur77/N10 INDUCES APOPTOSIS OF THYMOCYTES IN TRANSGENIC MICE

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We are studying the function of transcription factors encoded by immediate-early genes like Jun, Fos, Rel/NF-κB and zinc finger proteins. One of these growth-factor induced genes, *nur77*/N10, shows significant homology to members of the steroid/thyroid hormone receptor superfamily. The gene products of this family are characterized by a highly conserved DNA-binding domain containing two zinc fingers. The transcriptional activity of these factors is often ligand dependent, although so far none could be identified for Nur77/N10. *nur77*/N10 mRNA is expressed in mouse thymus and recently the *nur77*/N10 gene product has been involved in the apoptosis of T cell hybridomas.

To further characterize the role this gene plays during T cell development and programmed cell death, we established several transgenic mouse lines expressing the wild-type *nur77*/N10 gene under the control of T cell-specific regulatory sequences (*lck* promoter and CD2 locus control region). Thymocytes isolated from these transgenic mice show strongly increased DNA binding activity for Nur77/N10. Lines expressing high levels of Nur77/N10 have thymic atrophy coinciding with a dramatically reduced population of $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymocytes whereas early CD25/IL-2Rα-positive cells are not affected as demonstrated by FACS analysis. As a result, the spleen contains no mature T cells. The reduction in the number of thymocytes is due to an increased and early onset of apoptosis. Interestingly, in thymocytes of the transgenic lines overexpressing Nur77/N10 we find an increase in the expression of FAS ligand (FasL/APO-1-L), a molecule implicated in the apoptosis of peripheral T cells. In conrast, basal level expression of the Fas receptor (APO-1/CD95) is not altered, suggesting that Nur77/N10 induces apoptosis in double-positive thymocytes by upregulating the expression of FasL and thereby increasing signaling through Fas.

NUR77 TRANSCRIPTION FACTOR AS A REGULATOR OF APOPTOSIS IN NEGATIVE SELECTION OF THYMOCYTES AND T CELL HYBRIDOMAS

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Signalling through the T cell antigen receptor (TCR) mediates positive and negative selection of thymocytes. Positive selection promotes differentiation and maturation of thymocytes, whereas negative selection results in apoptosis. T cell hybridomas stimulated through the TCR also result in apoptosis in addition to a G1 cell cycle arrest. The transcription factor Nur77, an orphan member of the steroid receptor family, is highly induced during T cell receptor signalled apoptosis. and is transiently expressed in response to growth and differentiation stimuli. Dominant negative Nur77 blocks apoptosis in T cell hybridomas.

We have generated two sets of transgenic mice, one in which the dominant negative Nur77 is expressed in developing thymocytes and another set in which the wild-type Nur77 protein is constitutively expressed. We report that the dominant-negative Nur77 protein perturbed T cell development and inhibited antigen-induced negative selection of thymocytes. Constitutively expressed wild-type. Nur77 protein induced apoptosis in developing thymocytes, resulting in a decrease in the number of thymocytes that matured. Those T cells that mature also die in an age-dependent manner. These data suggest that Nur77 plays a crucial role in the downstream events in antigen-induced negative selection. A model for Nur77 function during apoptosis versus proliferation will be presented. Its regulation by a novel CsA sensitve protein and the cell cycle machinery through the $p19^{ink4d}$ inhibitor protein will also be presented.

AXOTOMIZED NEURONS IN THE LATERAL GENICULATE NUCLEUS (LGN) OF THE RAT ARE PROTECTED FROM DYING BY BRAIN DERIVED AND CILIARY NEUROTROPHIC FACTORS (BDNF AND CNTF) AND BASIC FIBROBLAST GROWTH FACTOR (BFGF).

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Many *in vitro* studies have shown that neurotrophic factors can be essential in sustaining neurons in the central (CNS) and peripheral nervous system. While relatively few examples have been reported in the mammalian CNS, there is evidence that trophic factors can slow the rate and/or extent of neural degeneration *in vivo*. The present experiments extend this evidence by showing that three neurotrophic factors, BDNF, bFGF and CNTF can reduce the rate and extent of cell death following axotomy of neurons in the LGN.

The axons of LGN neurons were cut by making a unilateral lesion of visual cortex in adult rats. A gelatin sponge soaked in BDNF, bFGF, CNTF or saline (controls) was placed on the surface of the lesion and the animals survived for periods that ranged from 1 to 12 weeks. The animals then were perfused and serial sections through the LGN were stained with cresyl violet. The number of surviving LGN neurons and their mean cross-sectional areas in treated and control rats were compared with the number and size of LGN neurons in normal animals.

BDNF, bFGF and CNTF have little, if any, effect on the sizes of axotomized LGN neurons. In contrast, each of these neurotrophic factors promotes a significant increase in the number of LGN neurons that survive damage to visual cortex. At 1 week postoperatively, treated animals displayed an improvement in neuronal survival that ranged from 65% to more than 100% compared to controls. Remarkably, a marked increase (>100%) in the number of survivng LGN neurons was seen up to 4 weeks postoperatively in rats treated with CNTF. These results indicate that neurotrophic factors drawn from 3 distinctly different gene families can be effective neuroprotectants for axotomized neurons in the LGN when administered *after* the axotomy. Moreover, after a single administration neuroprotective effects are evident as early as 1 week after axotomy and persist for at least 8 weeks.

FIBROBLAST GROWTH FACTOR ACTIVITY RESCUES DIFFERENTIATED TERA-2 CELLS FROM APOPTOSIS IN COLLAGEN GEL

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Tera-2 is a pluripotent human embryonal carcinoma cell line. Retinoic acid (RA) induces the cells to differentiate into quiescent cells that express neuronal markers. According to our previous results differentiating Tera-2 cells become dependent on exogenous fibroblast growth factor (FGF) activity when grown on or inside a collagen (type I) gel. Under these conditions the cells die within a few days after addition of RA. Their survival can be dramatically improved by adding physiological quantities of FGF-2 to the culture medium. In contrast with this, differentiating cells grown on a conventional cell culture plate or cells covered with collagen but in contact with the plate surface survive for several weeks but cease to divide. During differentiation on cell culture dish, FGF-4-mRNA becomes non-detectable while the expression of FGF-2 is only slightly down regulated. However, upon differentiation on a collagen gel, both FGF-2 and FGF-4 become almost non-detectable. Morphological changes characteristic of apoptotic cell death can be detected in differentiating Tera-2 cells in the absence of FGF on collagen gel. DNA staining reveals that the nuclear chromatin becomes dense and in 4 days most of the cells are fragmented into vesicles containing both cytoplasm and parts of the nucleus. Even before this, fragmentation of the genomic DNA into 50 kbp fragments can be detected by pulsed field electrophoresis. This data indicates that in our model a change in the composition of the ECM alters the growth factor expression and requirements of the differentiated cells. In the absence of FGF stimulation, a change in the ECM induces apoptosis in the differentiating cells.

PROGRAMMED CELL DEATH IN SYMPATHETIC NEURONS: A STUDY BY 2D PAGE WITH COMPUTER ANALYSIS.

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During the development of the vertebrate nervous system many neurons die by programmed cell death (PCD). The survival of sympathetic neurons during this process is dependent on the trophic factor nerve growth factor (NGF), which is produced in limiting amounts by the tissues which these neurons innervate. This dependence on NGF for survival is retained by neonatal rat sympathetic neurons cultured *in vitro* and therefore serves as a suitable model for the study of the mechanism of PCD.

The technique of two-dimensional polyacrylamide gel electrophoresis (2D PAGE) coupled with computer image analysis was used in this study to examine changes in protein synthesis occuring during the onset of programmed cell death (PCD) in rat sympathetic neurons from superior cervical ganglia of P_{0-1} Wistar rats, following withdrawal of NGF [1]. Proteins were metabolically labelled with [^{35}S]-L-methionine for 2 h at the onset of PCD and compared to control neurons maintained in the presence of NGF. Analysis of proteins by 2D PAGE was performed using immobilised pH gradient gel strips (IPG strips) in the first dimension and a custom built electrophoresis system capable of running up to 6 SDS PAGE slab gels in a vertical configuration with temperature control, for the second dimension. Resolved proteins were visualised using storage phosphor technology and the digitised images subjected to rigorous analysis using the QUEST II software system (obtained under license from Cold Spring Harbor Laboratories, NY, USA). Proteins were located whose relative synthesis either decreased, or increased, significantly upon withdrawal of NGF.. Further work aims to identify these proteins and also to locate, by Western blotting and ^{32}P labelling of phosphoproteins, components of signalling pathways stimulated by NGF withdrawal and NGF re-addition.

[1] B Amess and A M Tolkovsky, *Electrophoresis* **16** (1995) *in press.*

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STUDIES ON THE ROLE OF ICE/CED3-RELATED PROTEASES IN FAS-INDUCED JURKAT CELL DEATH

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Stimulation of cell death by antibody-mediated ligation of the Fas antigen was studied in the human T-cell line, Jurkat. An anti-Fas monoclonal antibody, CH-11, stimulated rapid, apoptotic cell death that was substantially reduced in cells stably transfected with the bcl-2 proto-oncogene. The possible roles of members of the interleukin-1-beta convertase (ICE)/Ced3 protease family in Fas-induced Jurkat cell death was examined. Jurkat cells were shown by PCR to express ICE, ICH-1 and CPP32 RNA's. In order to inhibit the proteolytic activities of these proteases in Jurkat cells, small peptide inhibitors of aspartate-directed, cysteine proteases were synthesized and characterized with regard to their abilities to inhibit the proteolytic activities of bacterially expressed human ICE, ICH-1 and CPP32. These inhibitors were then tested for inhibition of Fas-mediated Jurkat cell death. Two inhibitors, ZVAD-fluoromethylketone and DEVD-aldehyde substantially inhibited Jurkat cell death induced during a 24 hour incubation with antibody CH-11. Of the two, ZVAD-fluoromethylketone was more potent, exhibiting an IC50 of about 2 μM. Both active inhibitors were potent inhibitors of recombinant ICE proteolytic activity *in vitro*, consistent with a role for ICE in Fas-induced Jurkat cell death. In order to further study ICE/Ced 3-related proteases in Fas-mediated cell death, extracts of cells treated with antibody CH-11 were prepared by Dounce homogenization and centrifugation. Addition of Fas-stimulated extracts to normal rat liver nuclei produced chromatin condensation and margination, as judged by Hoechst dye no. 33342 and DAPI immunofluorescent staining. These "apoptotic" extracts are currently being evaluated for ICE/Ced 3 - related protease activity

TH1 CD4+ LYMPHOCYTES DELETE ACTIVATED MACROPHAGES THROUGH THE FAS/APO-1 PATHWAY. Dalit Ashany, Xin Song, Elizabeth Lacy, Janko Nikolic-Zugic, Steven M. Friedman, Keith B Elkon. SCOR in SLE, Hospital for Special Surgery-CUMC, New York, NY; Immunology and Molecular Biology Programs, Memorial Sloan-Kettering Cancer Center, New York, NY.

The APO-1/Fas cytotoxic pathway plays an important role in the regulation of peripheral immunity. Recent evidence indicates that this regulatory function operates through deletion of activated T and B lymphocytes by CD4+ T cells expressing the Fas ligand. Since macrophages play a key role in peripheral immunity, we asked whether Fas was involved in T cell-macrophage interactions.

Two color flow cytometry revealed that Fas was expressed on resting murine peritoneal macrophages. Fas receptor expression was upregulated following activation of macrophages with cytokines (IFN-γ, TNF-α, IL-4) or LPS although only TNF-α rendered macrophages sensitive to anti-Fas antibody mediated death. To determine the consequence of antigen presentation by macrophages to CD4+ T cells, macrophages were pulsed with antigen and then incubated with either Th1 or Th2 cell lines or clones. Th1, Fas ligand (+), but not Th2, Fas ligand (-), T cells induced lysis of 60-80% of normal macrophages whereas macrophages obtained from MRL/MpJ-*lpr*/*lpr* or CBA/K1Jms/lpr^{cg}/lpr^{cg} mice with mutations in the Fas receptor were totally resistant to Th1 mediated cytotoxicity. Macrophage cytotoxicity was dependant upon specific antigen recognition by T cells and was MHC restricted.

These findings indicate that, in addition to deletion of activated lymphocytes, Fas plays an important role in deletion of activated macrophages following antigen presentation to Th1 CD4+ T cells. Failure to delete macrophages that constitutively present self antigens may contribute to the expression of autoimmunity in Fas receptor (*lpr*) and Fas ligand (*gld*) deficient mice.

GENETIC ANALYSIS OF THYMOCYTE APOPTOSIS: CROSS-RESISTANCE OF APT$^-$ WEHI7.2 CELLS TO DEXAMETHASONE, CERAMIDE, STAUROSPORINE, FAS AND CD3/TCR STIMULATION

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During maturation thymocytes undergo a selection process which allows only cells with functional antigen binding and T-Cell Receptor (TCR) signaling activity to live (positive selection) and eliminates cells with inappropriate antigenicity against self (negative selection). Current models employ two distinct cellular signaling components, the CD3/TCR complex and the Glucocorticoid Receptor (GR), in this selection system. Activation of either of these components alone leads to apoptosis in immature thymocytes. However, moderate activation of both signaling components antagonizes apoptosis induction, resulting in positive selection and survival of maturing thymocytes. The GR target genes and specific events downstream of CD3/TCR activation responsible for apoptosis have not been identified. Additionally, the molecular mechanism of cross-signaling between these two pathways has not been clearly elucidated.

Previously, a set of immature thymocyte cell lines, resistant to glucocorticoids and containing functional GR, were described (Flomerfelt and Miesfeld, 1994, J. Cell Biol. 127:1729.). The Apt$^-$ cell lines were found to be cross-resistant to gamma irradiation and cAMP suggesting that some gene functions are shared between these induction pathways. We are now using five dex-resistant, Apt$^-$ cell lines, representing distinct complementation groups, to examine the induction of apoptosis initiated by ceramide, staurosporine, Fas stimulation, and CD3/TCR activation.

Using propidium iodide staining and flow cytometry, we have found that the WEHI7.2 cell line undergoes apoptosis in the presence of 10 nM Dexamethasone (Dex), 30 μM ceramide (C-2), 30 nM staurosporine, or Fas stimulation. Another cell line, which over expresses the Bcl-2 protein, WHb.12, is sensitive to Fas stimulation but shows no increase of apoptosis following 24 hr Dex, ceramide, or staurosporine treatments. Preliminary data suggests that at least one of the Apt$^-$ lines is ceramide-resistant. Sensitivity to staurosporine, Fas stimulation and CD3/TCR activation is being investigated in all five Apt$^-$ cell lines.

nur77 gene expression is required for apoptosis induction by CD3/TCR stimulation. We show that GR activation antagonizes TPA/ionomycin-stimulated nur77 gene expression in WEHI7.2 cells. The induction of nur77 and this cross-signaling interaction is also being analyzed in the five Apt$^-$ cell lines.

INHIBITION OF P53 INDUCED GROWTH ARREST AND APOPTOSIS BY HUMAN PAPILLOMAVIRUS PROTEINS.

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The Human Papillomavirus (HPV) E6 and E7 oncogenes cooperate to induce immortalisation of primary human cells. We have been interested in the effects of these two oncogenes on p53 function. A mutational analysis of HPV-18 E6 identified three regions of the protein which are important for association with p53. The ability of E6 to inhibit p53 transcriptional activity was also shown to be independent of the ability of E6 to target p53 for ubiquitin mediated degradation. We are now investigating the effects of these activities of E6 upon the biological functions of p53. We have been able to demonstrate that inhibition of p53 growth suppressor activity is performed equally well by both E6 and E7. Abolition of E7 binding to pRb removes its ability to inhibit p53 suppression of cell growth. However, E7 has no effect on p53 inhibition of cell transformation whereas this is strongly suppressed by E6. Mutants of E6 which fail to target p53 for ubiquitin mediated degradation are defective in inhibiting p53 suppression of cell transformation. In addition, the ability of p53 to suppress transformation appears to correlate with its ability to induce apoptosis. Thus, E6's ability to abolish p53 induced apoptosis appears to correlate with its ability to label p53 for ubiquitin mediated degradation.

INHIBITION OF THE GROWTH AND INDUCTION OF APOPTOSIS IN GLIOBASTOMAS BY CGP 41251 Martin Begemann[1], Sharafadeen A. Kashimawo[2], Yu-jeong A. Choi[3], Susan Kim[1], Kim M. Christiansen[1,4], Gregg Duigou[3], Marcel Mueller[5], Ira Schieren[1], Subrata Ghosh[3], Doriano Fabbro[5], Nina M. Lampen[6], Peter B. Schiff[2], Jeffrey N. Bruce[3], I. Bernard Weinstein[1,2]; [1]Columbia-Presbyterian Cancer Center, [2]Department of Radiation Oncology, [3]Department of Neurological Surgery, [4]Department of Genetics and Development, Columbia-Presbyterian Medical Center, New York, NY 10032, [5]Department of Oncology-Virology, Ciba-Geigy, CH-4002 Basel, Switzerland, [6]Department of Electron Microscopy, Memorial Sloan-Kettering Cancer Center, New York, New York 10021.

Protein kinase C (PKC) plays a central role in growth control and might be an appropriate target for the chemotherapy of human brain tumors. The staurosporine derivative CGP 41251, a potent and selective inhibitor of PKC, markedly inhibited cell proliferation in 9 cell lines derived from primary human brain tumors (5 glioblastoma multiforme, one gliosarcoma and glioblastoma and 2 astrocytomas and one choroid plexus carcinoma). BrdU incorporation studies show that CGP 41251 leads to suppression of DNA synthesis. As shown by growth curves with drug reversal and clonogenicity assays, CGP 41251 suppresses growth in a partially reversible/ irreversible fashion, suggesting that CGP 41251 acts through combined cytostatic/ cytocidal mechanisms. Cultures treated with CGP 41251 displayed an increase in the fraction of cells in the G_2/M-phase of the cell cycle, a decrease of cells in the S-phase, and no consistent effect on the G_0/G_1 phase. Immunohistochemical analyses demonstrated that growth inhibition by CGP 41251 was associated with the formation of giant nuclei with extensive fragmentation and apoptotic bodies. These effects of CGP 41251 were abrogated by withdrawal of serum from the medium or by exposure of these cells to aphidicolin, actinomycin D, cycloheximide or TPA. Thus CGP 41215 is a powerful inducer of apoptosis in glioblastoma cell cultures and might be a useful agent for the clinical treatment of gliomas.

IRF-1 AND IRF-2 EXPRESSION IN RAT THYMOCYTES UNDERGOING APOPTOTIC CELL DEATH OR CELL PROLIFERATION

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Previous data from our laboratory suggest that an intriguing relationship exists between cell proliferation and cell death and that similar molecular mechanisms are involved in the early steps of both processes. Indeed, we observed that G0-G1 progression genes (like c-fos, c-jun, c-myc, c-myb, ODC) and transcription factors (AP-1, NF-kB) involved in cellular proliferation are induced also in thymocytes undergoing apoptosis (Grassilli et al, BBRC, 188: 1261, 1992; Sikora et al, BBRC, 192: 386, 1993; Sikora et al, BBRC, 197: 709, 1993; Desiderio et al, CGD, 6: 505, 1995). Interferon Regulatory Factor-1 (IRF-1) and its mutual antagonist IRF-2, originally characterized as regulators of transcription of IFNα and β genes and IFN-inducible genes, has been shown to be involved also in the regulation of ODC expression. Moreover, it has recently been reported that IRF-1 might be involved in apoptosis. Since we observed an early ODC induction either in proliferating or in apoptotic thymocytes, we decided to assess, by Northern blot analysis, IRF-1 and IRF-2 expression in rat thymocytes undergoing apoptosis upon dexamethasone (Dex) addition or stimulated to proliferate by concanavalin A (Con A) treatment. Low levels of IRF-1 mRNA were detectable in untreated thymocytes but underwent a precocious (0.5 h) upregulation in Dex-treated cells and then gradually declined starting from 1 h, to finally disappear after 4 h. Also IRF-2 mRNA was present in untreated thymocytes and rapidly accumulated peaking at 0.5 h after Dex addition. IRF-2 mRNA levels decreased thereafter remaining quite high and constant until 4 h, then disappeared at 8 h. In Con A-stimulated thymocytes both IRF-1 and IRF-2 showed a biphasic kinetic characterized by an early peak, in correspondence to the G0-G1 transition, followed by a second later increase preceding the entry into the S-phase. Thus, the kinetics of IRF-1 and IRF-2 induction preceded ODC early peaks of mRNA accumulation either in apoptotic or proliferating thymocytes while followed the later ODC mRNA increase in proliferating cells, suggesting that IRF-1 might be involved in the regulation of early ODC expression. Moreover, our data suggest for the first time an involvement of these two transcription factors in lymphoid cells apoptosis. It is noteworthy that IRF-1 and IRF-2 showed similar kinetics of precocious expression in Con A- and in Dex-treated cells reinforcing the hypothesis that the early phases of cell proliferation and apoptosis share the same molecular events.

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POLYAMINE CHANGES IN RAT THYMOCYTES UNDERGOING APOPTOSIS BY DIFFERENT STIMULI

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We previously reported that a rapid and reversible expression (mRNA and enzymatic activity) of ornithine decarboxylase (ODC), the rate limiting enzyme of polyamine biosynthesis, occurred in thymocytes undergoing apoptosis upon dexamethasone (dex) treatment. However, polyamine levels did not increase but rapidly decreased starting from 1 h after dex addition, and reached very low levels between 4 and 8 hs. Spermine depletion was observed at 12 hs. The decrease of intracellular polyamines (putrescine, spermidine, spermine) preceded the onset of DNA fragmentation. The addition of exogenous spermine to dex-treated thymocytes rescued them from cell death. Therefore, we hypothesized that polyamine depletion could be a critical signal for the cell to undergo the apoptotic process, at least in this model (Desiderio et al., Cell Growth Diff. 6, 505, 1995). In order to assess whether cellular polyamine decrease might be a general feature of apoptosis, we examined polyamine levels in thymocytes undergoing apoptosis upon heat shock treatment or γ irradiation. In these models too we found that, despite ODC activation, polyamine levels decreased before the appearance of DNA laddering, being strongly diminished 4-8 hs after the application of the apoptotic stimuli. We then decided to investigate the mechanisms responsible for the reduction of polyamines using dex-treated thymocytes as a representative model of apoptosis in which the decrease of polyamines occurs. Since polyamines are known to be substrates for tissue transglutaminase (tTG), an enzyme activated in many models of apoptotic death, we examined tTG mRNA and enzyme activity in dex-treated thymocytes. mRNA accumulation (1-4 hs) preceded the enzyme activation (4-8 hs) that was, however, only 3-4 fold. Thus, the tTG induction and activation we demonstrated can contribute to, but is probably not the only mechanism responsible for the polyamine decrease observed in dex-treated thymocytes. Other possible mechanisms accounting for polyamine depletion, such as polyamine excretion and oxydative catabolism are currently under investigation.

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NEURONAL ACIDOSIS: A POTENTIAL STIMULUS FOR APOPTOSIS IN CEREBRAL ISCHEMIA

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Cerebral ischemia rapidly induces anoxia, hypoglycemia and acidosis that results in both necrotic and apoptotic neuronal loss. A central question is how these early and transient metabolic insults induce apoptosis. We report that intracellular acidification of similar extent as during ischemia induces neuronal loss in cultured hippocampal slices. Analysis of neuronal loss by TUNEL staining and DNA fragmentation suggests that acidosis induces apoptosis. Apoptosis was blocked by infecting slice cultures with recombinant vaccinia virus vectors that inducibly express the bcl-2 gene. Apoptosis is not blocked when bcl-2 is not induced or when cultures are transduced with vectors that express E. Coli β-galactosidase. Apoptosis is blocked by α-tocopherol, N-acetyl cysteine and desferrioxamine suggesting that acidosis generates reactive oxygen species, perhaps by a Fenton-like reaction. RNA synthesis and protein synthesis inhibitors also block apoptosis suggesting that reactive oxygen species may act by altering neuronal gene expression. Similar mechanisms induced by acidosis may contribute to apoptosis following cerebral ischemia.
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CLUSTERING OF APOPTOTIC CELLS IN IMMUNE TISSUES SHOWN BY A HIGH SENSITIVITY *in situ* END-LABELING TECHNIQUE

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Although suspected for many years, it was not until recently that direct evidence for the death of immature thymocytes within the thymus was provided (Nature (1994) **372**, 100-103). This was accomplished by an *in situ* end-labeling technique which allows detection of dying cells by virtue of the fragmented DNA within their nuclei. Apoptotic cells were found to be dispersed throughout the thymic cortex, and to represent a small but significant proportion of mononuclear cells. Here we have used a more sensitive modification of *in situ* end-labeling, termed ISEL+, which reveals a higher prevalence of apoptosis in the thymus than previously reported. In addition, ISEL+ shows that in the thymus as well as the fetal liver (a developing B-cell compartment) apoptotic cells are not randomly distributed, but form well-defined clusters.

ISEL+ was developed by systematically evaluating procedural variables, including tissue preparation, DNA polymerase, labeled nucleotide, and visualization system. ISEL+ uses frozen tissue prepared for RNA *in situ* hybridization, Terminal deoxynucleotidal Transferase (TdT), [digoxigenin]-dUTP, and visualization by an extended antibody incubation before alkaline phosphatase histochemistry. The resulting technique specifically identifies dying cells in known models of programmed cell death, and is approximately 10X more sensitive than previous techniques. In addition, using a tissue-scraping procedure that we developed, DNA end-labeled *in situ* by ISEL+ in sections of dexamethasone-treated thymus was shown to be in the form of apoptotic nucleosomal ladders.

In sections of postnatal mouse thymus, ISEL+ shows clustered cells within the cortex; the medulla is sparsely labeled. Circular clusters of 30-50μm in diameter are distributed throughout the cortex, and consist of approximately 10-20 cells each. They are regularly spaced, and separated by regions of unlabeled cells. A similar pattern of apoptotic cells is seen in the fetal liver, although the clusters are bigger, more irregularly shaped, and involve a larger percentage of the tissue. One possible explanation for these observations is that the groups of dying cells identified by ISEL+ are clonal. Apoptosis in both T-cell and B-cell compartments is known to involve the deletion of clones of self-reactive immunocytes, but this process has yet to be directly observed *in situ*. If the clusters of cells observed by ISEL+ are indeed clonally related, patterns of labeling shown here may be visual evidence for clonal selection in the immune system. [supported by the Klingenstein Fund, the UC Tobacco-Related Disease Research Program, and NIH/NIGMS GM07178]

WIDESPREAD PROGRAMMED CELL DEATH IN MITOTIC AND POSTMITOTIC ZONES OF THE EMBRYONIC CEREBRAL CORTEX

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Neurons of the mammalian cerebral cortex are generated and begin their differentiation during embryonic life, arising in mitotic zones surrounding the lateral ventricles, then migrating to more superficial zones to differentiate postmitotically. Details of this development have been revealed in many previous studies which have examined birthdates, migration patterns, and lineage relationships of neuronal cohorts. An important mechanism that functions in other developing systems and that could alter the numbers and types of neurons, as well as other cells, during embryonic cortical development is programmed cell death (PCD). Previous studies, however, have not reported, nor generally considered the existence of PCD during this period. In this study, *in situ* end-labeling (ISEL) was used to determine the presence of PCD in the embryonic cerebral cortex of the mouse, during the period surrounding cortical neurogenesis, embryonic days 10-18 (E10-E18).

Tissue sections of Balb/c embryo cortices were labeled by ISEL, and compared to adult cortex. Few dying cells were seen in the cortex at E10 or in the adult (less than 1%), however from E12-E18, many dying cells (averaging over 50%) were observed throughout the cerebral wall. The peak of PCD was at E14, when over 70% of the cells appeared to be dying. Cells undergoing PCD were observed throughout the cerebral cortex, but a surprising majority were found within mitotic rather than postmitotic regions. Since the young neurons in these mitotic regions do not make synaptic connections, PCD here suggests that non-synaptic mechanisms must regulate cell death in these regions. The overall extent of embryonic PCD indicates that it is an important variable to consider in studies of cortical development. PCD could significantly alter the number and composition of cell cohorts, perhaps allowing the selection of desirable cell phenotypes that survive into postnatal life. [supported by the Klingenstein Fund, the UC Tobacco-Related Disease Research Program and NIH/NIGMS GM07198]

EXTENSIVE APOPTOSIS IN DUCTAL CARCINOMA IN SITU OF THE BREAST

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Greater than 50% of breast ductal carcinoma in situ (DCIS) cases contain histologic areas of "necrosis," the biological significance of which is unclear. Clinical studies have suggested that the presence of prominent intraductal "necrosis" is associated with a higher rate of recurrence and progression to invasive cancer. 25 untreated DCIS cases were examined for apoptosis using the two criteria of morphology and terminal transferase (TUNEL) staining. All 19 cases with intraductal "necrosis" were found to contain massive apoptosis which was present within all "necrotic" zones. The p53 gene has been shown to modulate apoptosis by a number of triggers and may be important in breast cancer. However, immunohistochemistry revealed no correlation between p53 overexpression and apoptosis in DCIS. Thus the extensive intraductal "necrosis" in DCIS appears to represent apoptosis, and this apoptosis is likely mediated by a p53-independent pathway. Analysis of tumors containing both DCIS and adjacent invasive cancer suggest that spontaneous apoptosis is lost concurrently with invasive transformation.

THE INVOLVEMENT OF FAS-FAS LIGAND INTERACTIONS IN MODELS OF APOPTOSIS IN HUMAN T LYMPHOCYTES.

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Apoptosis is a form of programmed cell death that plays an important role in diverse biological systems. An apoptotic cell undergoes a specific program of events that apparently depends on active metabolism and leads to its own destruction. There is strong evidence to suggest that apoptosis plays a vital role in shaping and maintaining the thymic and peripheral T cell repetoire.

This death has been shown to be mediated partly through signalling via Fas- Fas ligand (Fas-L) interactions. Fas-L has been found expressed on some cytotoxic T cells which would implicate Fas-Fas-L interactions in T cell cytotoxicity.

We are interested in exploring the role of Fas-Fas-L interactions in apoptosis of human T lymphocytes. Therefore we have developed two models of apoptosis : 1) using PHA we have induced apoptosis in Jurkats , a human T cell line and 2) using the superantigen Staphylococcal Enterotoxin B(SEB) apoptotic death was induced in SEB responsive T cell blasts. We have shown time and dose dependent death in both these systems.

Apoptosis induced in Jurkats using PHA was not blocked by cyclosporin A and may proceed via a Fas-independent pathway. In contrast SEB - induced death appears to be Ca-dependent and we have also shown that SEB induced cytotoxicity is dependent on Fas-Fas-L interactions by inhibiting this cytotoxicity using an anti-Fas blocking antibody and Fas-Fc, a chimaeric construct combining the extracellular region of human Fas and the Fc portion of a mouse immunoglobulin molecule. We are now investigating the induction of Fas L expression in these systems using RT PCR and have demonstrated induction of Fas-L in human PBMCs and T cell blasts using various stimuli.

Hopefully, these models will provide useful tools for unravelling the importance of apoptosis in the maintenance of the balance between T cell tolerance and autoimmunity.

INDUCTION OF PROGRAMMED CELL DEATH BY C-JUN

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Members of the AP-1 transcriptional complex, such as c-Jun, are selectively up-regulated during several cases of programmed cell death. To establish a functional link between c-Jun induction and programmed cell death, an estrogen-inducible system has been generated by fusion of the mouse *c-jun* gene with the hormone binding domain of the human estrogen receptor (ER). Activation of the c-Jun-ER fusion protein in NIH3T3 fibroblasts lead to the induction of apoptotic cell death, displaying features such as cytoplasmic shrinkage, chromatin condensation, and DNA fragmentation, demonstrated by TUNEL positive cell labeling. Cell death by c-Jun occurred in high serum, however was enhanced in serum deprived cultures. Apoptosis took place in a c-Jun dose-dependent manner. Stable clones expressing high level of the fusion protein died faster than low expressing clones. Cells, which became resistant to the induction of apoptosis, lost the expression of the c-Jun-ER fusion protein. The cell death by c-Jun was not accompanied by an increase in proliferation. Flow cytometry analysis showed that c-Jun induction had no effect on the distribution of cell cycle phases, but resulted in an increase of cells with sub-2n DNA content, a characteristic of apoptotic cells. c-Jun mutants carrying deletions in the transcriptional activation domain or the leucine zipper region were defective in their ability to induce apoptosis. Interestingly, v-Jun, the oncogenic version of c-Jun, showed also reduced cytotoxicity. These observations imply that c-Jun is inducing apoptosis through transcriptional activation of target genes. In fact, the expression of tissue transglutaminase (*tTG*), a gene coding for an enzyme thought to be involved in the assembly of apoptotic bodies, became increased during c-Jun-induced cell death. Transient transfections using CAT reporter plasmids demonstrated that c-Jun is regulating the *tTG* gene promoter. Ectopic expression of *bcl-2* delayed the cell death by c-Jun. The possibility of c-Jun modulating the expression level of Bcl-2 related proteins will be discussed.

IMPLICATIONS FOR A ROLE OF GAS2 IN REGULATING CELL SHAPE CHANGES DURING APOPTOSIS.

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Cell death by apoptosis is characterized by dramatic alterations of cell shape. Activation of proteolytic enzymes plays an important role in regulating apoptosis. We have found that Gas2, a component of the microfilament system highly expressed in growth arrested fibroblasts and in various human and mouse tissues, is cleaved during apoptosis.

By using antibodies specific for the carboxy or amino-terminal ends of Gas2 we demonstrate that during the course of apoptosis Gas2 carboxy-terminal is removed.

When overexpressed in different cell types only the artificially carboxy-terminal truncated forms of Gas2 (Δ236-314 and Δ276-314) are able to trigger dramatic alterations in the microfilament system and in the cell shape. Overexpression of the Gas2wt completely lacks such an effect.

Comparison of the electrophoretic mobility of the apoptotic processed form of Gas2 with those of the different artificially carboxy-terminal deleted derivatives indicates that the apoptotic induced cleavage site within Gas2 should lie very close to aminoacid 276.

The sequence from aa 278 to 280, Val-Asp-Gly, corresponds to a consensus site for the ICE family of proteolytic enzymes. These results suggest a regulated function of Gas2 during apoptosis that is functionally associated with changes in cell architecture.

APOPTOTIC-LIKE PHENOTYPE INDUCED BY GAS3/PMP22 OVEREXPRESSION RELATES TO THE MODE OF INHERITANCE ASSOCIATED WITH CMT1A POINT MUTANTS.

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Gas3, originally isolated from NIH 3T3 for its growth arrest dependent expression, has been subsequently characterized as a major component of the peripheral nerve myelin and named accordingly as Peripheral Myelin Protein. We shown that gas3/PMP22 overexpression in growing NIH3T3 cells leads to an apoptotic phenotype, characterized by membrane blebbing, rounding up and chromatin condensation. Recently several point mutations of human gas3/PMP22 gene have been associated with Charcot-Marie-Tooth 1A, a common hereditary dysmyelinating neuropathy. When Gas3/PMP point mutants (L16P, S79C, T118M and G150D) are similarly overexpressed in NIH3T3 cells the apoptotic phenotype as induced by the wild type is significantly reduced. Both of the point mutants (L16P, S79C) for CMT1A behave as dominant negative with respect to the wild type, while T118M, the only described recessive mutant, behaves as recessive under the same co-expression experiments. These data suggest a role for altered Schwann cells apoptosis in the pathogenesis of CMT1A.

IDENTIFICATION OF GENES REGULATING DRUG-INDUCED DEATH

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Using a clonogenic assay we have shown that after a 24 hour exposure to dexamethasone, the human lymphoid leukaemic line CEM C7A cells undergoes a rapid decline in viability which precedes the appearance of visibly apoptotic cells (acridine orange positive) by approximately 8 to 12 hours. Cells treated for 24-36 hours with dexamethasone therefore represent cells committed to die (as shown by irretrievable loss of clonogenicity) but which have not yet undergone extensive apoptosis (as determined by acridine orange staining).

To identify genes whose regulation is required for commitment to programmed cell death, we have transfected CEM C7A cells with expression cDNA libraries prepared from treated and untreated cells. Resistant clones are currently being screened for the presence of transfected cDNAs which will confer resistance upon a second round of transfection.

In parallel to transfection studies using entire cDNA libraries, we have used PCR-generated cDNA subtraction to enrich for 3' sequences which are either up or down regulated during dexamethasone treatment. Two reciprocal sets of subtractions have now been carried out with cDNA from treated and nontreated cells. Initial hybridisation data indicate successful removal of shared sequences. Currently, 3' material from each subtraction is being used to select full length cDNAs to generate cDNA libraries enriched for genes which are either up or down regulated following dexamethasone treatment. To tackle the problem of transfecting genes whose expression is potentially lethal, we have established a tetracycline-modulatable system in CEM C7A (Gossen, M. and Bujard, H., Proc. Natl. Acad. Sci. USA 89: 5547 1992). In our hands this system allows efficient repression to background levels in the presence of tetracycline and a 100 fold induction following teracycline removal and will be used to assess individual candidate cDNAs.

FUNCTIONAL REGULATION OF *REAPER*-MEDIATED APOPTOSIS

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Programmed cell death (apoptosis) is a highly conserved, gene-directed process that is normally required during animal development and also established in the pathology and treatment of some human diseases. We recently developed a cell culture model system to study the mechanisms that regulates the activity of *Reaper* (*rpr*), an essential cell death gene in *Drosophila*. Within hours of induction of *Rpr* expression, L2 cells bearing a conditionally expressed *rpr* gene show signs of extensive cell death. Our preliminary data indicated that *Rpr*-mediated apoptosis in cultured cells could be blocked by p35 protein, a virally-encoded negative regulator of apoptosis, which functions in worms, insects and mammals. Our data also suggested that *Rpr* might physically interact with p35. Furthermore, we are using this cell culture system to investigate the interaction between *Rpr* and other known cell death regulators. These studies should provide fundamental insights into mechanisms of apoptosis and may provide novel rationales for treatment of various human diseases.

APOPTOSIS AND ALTERED REDOX STATE INDUCED BY CAFFEIC ACID PHENETHYL ESTER (CAPE) IN TRANSFORMED RAT FIBROBLAST CELLS

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Caffeic acid phenethyl ester (CAPE), was previously shown to block tumor promoter- and carcinogen-generated oxidative processes in several assays and to engender differential toxicity to some transformed cells. To study the mechanisms of CAPE-induced differential cytotoxicity, nontumorigenic rat embryo fibroblasts (CREF) and adenovirus (type 5)-transformed CREF cells (Wt3A) were used. As shown by nucleosomal-length DNA degradation, morphological alterations by EM, *in situ* labeling of 3'-OH-ends, and appearance of hypodiploid cell population by bivariant flow cytometry, cell death induced by CAPE in the transformed Wt3A cells was apoptosis. Under the same CAPE-treatment condition, CREF cells transiently growth arrested. Both CREF and Wt3A cells were radioresistant, suggesting deficiencies in the proteins controlling G1 checkpoint. To explore possible mechanisms of CAPE-induced apoptosis, it was determined whether CAPE-induced toxicity was influenced by the redox state of the cells. Depletion of cellular glutathione (GSH) with buthionine sulfoximine (BSO) prior to CAPE treatment, caused CREF sensitive to CAPE-induced cell death. GSH levels were also determined in CAPE-treated CREF and Wt3A cells. The GSH level in the CREF cells was unaffected by CAPE, whereas the Wt3A cells showed a significant reduction. Reducing agent, N-acetyl-cysteine (NAC) and antioxidant, catalase could rescue Wt3A cells from CAPE toxicity. Furthermore, Bcl2, which protects cells from oxidative stress, had a protective effect against CAPE-induced apoptosis in Wt3A cells. These results suggest that CAPE can modulate the redox state of cells. Sensitivity of cells to CAPE-induced cell death may be determined by the loss of normal redox state regulation in transformed cells.

IDENTIFICATION OF DOMAINS WITHIN BAK THAT MEDIATE CELL DEATH AND PROTEIN BINDING FUNCTIONS

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Physical interactions among Bcl-2 family members contribute to the regulation of the cell death program. Bak is a Bcl-2 homolog that promotes apoptosis and can counteract the protection against cell death afforded by Bcl-2 expression. Bak has been shown to form complexes with several anti-apoptotic proteins including Bcl-2, Bcl-x_L, and adenovirus E1B 19 kDa. It is, therefore, possible that Bak promotes apoptosis by binding, and inactivating, cell death suppressors. Alternatively, anti-apoptotic proteins such as Bcl-x_L and E1B 19 kDa may function by binding, and inactivating, Bak and/or other proteins such as Bax, that actively promote cell death.

To explore the relationship between the cell killing and protein binding functions of Bak, we examined the effect of mutations spanning the Bak protein on the capacity to induce cell death and bind to Bcl-x_L. Enforced expression of Bak was previously demonstrated to induce apoptosis in stably transfected Rat-1 cells. In order to rapidly assess the cell death function of a large number of Bak mutants, we used a transient ß-galactosidase co-transfection assay in Rat-1 cells. Bak-induced cell death in this assay was manifested by the elimination of ß-gal expressing cells upon co-transfection with Bak expression plasmids. In parallel, we tested the ability of Bak and various Bak mutants to interact with Bcl-x_L. Binding of Bak to Bcl-x_L was measured in vitro, by the specific interaction of Bak with a GST-Bcl-x_L fusion protein, and in transfected COS cell extracts, by co-immunoprecipitation of epitope-tagged forms of Bak and Bcl-x_L. Thus far, the cell killing and protein binding activities of Bak have not been clearly separated by mutation, suggesting that these functions are closely linked.

A NOVEL BCL-2-RELATED GENE, BFL-1, IS ACTIVATED IN STOMACH CANCER

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Programmed cell death (apoptosis) is an active process which is genetically controlled, and plays an important role in several cellular activities such as embryonic development, deletion of autoreactive T-cells, and tissue homeostasis. Deregulation of apoptosis has been implicated in the development of cancer. Several genes regulating apoptosis have been reported, including p53, a tumor suppressor, c-myc, a proto-oncogene, and various kinds of Bcl-2-related genes which either block or induce apoptosis.

A new cDNA clone which is homologous to Bcl-2, named as Bfl-1, has been isolated from a human fetal liver at the 22nd week of gestation. This clone was identified by computer analysis of random cDNA sequences that were obtained in an effort to expand the expressed sequence tag (EST) databases to be used for human genome analysis. The homology was recognized by 72 % amino acid similarity to the murine A1 gene, a member of the Bcl-2 gene family. The homology to the BH1 and BH2 domains of Bcl-2 was especially significant, suggesting that Bfl-1 is a new member of the Bcl-2-related genes. Bfl-1 is abundantly expressed in the bone marrow and at a low level in some other tissues. In situ hybridization results also indicate that this gene is preferentially expressed in hemopoietic cells. Interestingly, a strong correlation was noted between the expression level of this gene and the development of stomach cancer in eight sets of clinical samples. It is conceivable that Bfl-1 is involved in the promotion of cell survival in stomach cancer development or progression, suggesting the possibility of Bfl-1 to be an oncogene.

MULTIPLE PATHWAYS ORIGINATE AT THE FAS/APO-1 (CD95) RECEPTOR: SEQUENTIAL INVOLVEMENT OF PC-PLC AND ACIDIC SPHINGOMYELINASE IN THE PROPAGATION OF THE APOPTOTIC SIGNAL.

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The early signals generated following crosslinking of Fas/APO-1, a transmembrane receptor whose engagement by ligand results in apoptosis induction, were investigated in human HuT78 lymphoma cells. Fas/APO-1 crosslinking by mAbs resulted in membrane sphingomyelin hydrolysis and ceramide generation by the action of both neutral and acidic sphingomyelinases. Activation of a phosphatidylcholine-specific phospholipase C (PC-PLC) was also detected and it appeared required for subsequent acidic sphingomyelinase (aSMase) activation, since PC-PLC inhibitor D609 blocked Fas/APO-1-induced aSMase activation, but not Fas/APO-1-induced neutral sphingomyelinase (nSMase) activation. Fas/APO-1 crosslinking resulted also in Extracellular Receptor Kinase-2 (ERK-2) activation and in phospholipase A2 (PLA2) induction, independently from the PC-PLC / aSMase pathway. Evidence for the existence of a pathway directly involved in apoptosis was obtained by selecting HuT78 mutant clones spontaneously expressing a newly identified death domain-defective Fas/APO-1 splice isoform which blocks Fas/APO-1 apoptotic signaling in a dominant negative fashion. Fas/APO-1 crosslinking in these clones fails to activate PC-PLC and aSMase, while nSMase, ERK-2 and PLA2 activities are induced. PC-PLC and aSMase were expressed and functional in Fas/APO-1-resistant clones, as they could be activated by TNF. These results strongly suggest that a PC-PLC / aSMase pathway directly contributes to the propagation of Fas/APO-1-generated apoptotic signal in lymphoid cells.

P53 AND MYB PHYSICALLY INTERACT AND MODULATE EACH OTHER,S FUNCTION

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The oncogene c-myb and the tumor suppresser gene p53 encode transcriptional regulatory nuclear proteins expressed during hematopoietic cell differentiation. The Myb protein functions to activate transcription, whereas the p53 protein acts to both stimulate and suppress transcription. C-myb and p53 have opposing effects on cell proliferation. In hematopoietic cells, expression of the oncogene c-myb is essential for entry into S-phase of the cell cycle and DNA synthesis, while expression of p53 results in G1 arrest. Paradoxically, in mitogen stimulated normal cells, growth and differentiation proceeds normally despite the expression of p53 coincident with that of c-myb. In addition to its role in cell cycle transit in normal cells, constitutive expression of c-myb blocks mouse erythroleukemia (MEL) cell differentiation, whereas expression of p53 induces apoptotic cell death during the G1 phase of the cell cycle. However, in normal hematopoietic cells that express p53, enforced expression of c-myb does not block differentiation. We postulated that p53 may target the Myb protein and inhibit it's ability to function as a transactivator of transcription. To determine whether c-myb and p53 interact, we co-expressed both genes in mouse erythroleukemia (MEL) cells during differentiation. We now show that c-myb counteracts p53-induced apoptosis, and that p53 overcomes the c-myb mediated block of MEL cell differentiation. Cotransfection of p53 with c-myb blocked Myb-induced transactivation of the c-myc P2 promoter. Futhermore, we have evidence that p53 and Myc physically associate *in vivo* in both MEL cells and fibroblasts. Anti-Myb antibodies coimmunoprecipitate p53, and anti-p53 antibodies coimmunoprecipitate Myb. These data suggest that in hematopoietic cells the cell cycle regulatory proteins Myb and p53 can interact and modulate each other's function. These data also suggest that a mechanism by which p53 acts as a repressor of transcription includes binding to the Myb oncogene.

SELECTIVE CLEAVAGE OF BCL-X BY THE IL-1ß CONVERTING ENZYME

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The IL-1β converting enzyme (ICE) is a member of a family of cysteine proteases that has been implicated in the induction of programmed cell death. Although the death-inducing function of this family is presumably through proteolysis, the protease substrates critical for cell death are not known. Bcl-2 and some of its family members are potent inhibitors of apoptosis induced by a wide variety of stimuli including ICE-like proteases and virus infection. To investigate the mechanism by which ICE and Bcl-2 family members modulate apoptosis, we tested the ability of purified recombinant ICE to cleave Bcl-2 and related proteins in vitro. We found that ICE specifically cleaves Bcl-x, but not Bcl-2 or Bax. Furthermore, an apoptosis-inducing mitotic cell extract also cleaves Bcl-x at the same site, providing evidence for a direct interaction between effectors of death and survival.

IDENTIFICATION OF hIAP-1, A HUMAN HOMOLOG OF THE BACULOVIRUS ANTI-APOPTOTIC GENE *iap*

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The baculovirus gene *iap* encodes an anti-apoptotic zinc finger protein (IAP) with no previously known cellular homologs, although a novel repeated motif found in IAP (the BIR, or Baculovirus Iap Repeat) is conserved in NAIP, a gene implicated in spinal muscular atrophy. Screening of an EST (Expressed Sequence Tag) database resulted in the identification of a human gene, hIAP-1, with significant overall homology to baculovirus *iap*. hIAP-1 encodes two BIRs and a ring finger, as do the baculovirus IAP proteins, but is larger than baculovirus IAP (predicted M*r* of 49K vs 30K). The level of homology between hIAP-1 and baculovirus *iap* is around 37% at the amino acid level, excluding a single large gap in the baculovirus sequence. hIAP-1 ESTs were identified in cDNA libraries from a number of different tissues, as well as in several tumor cell lines, suggesting that the gene is expressed in a variety of tissues. This was confirmed by Northern blot analysis. Although the hIAP-1 transcript was present in nearly every tissue examined, it appeared to be most prevalent in immune system organs such as thymus, spleen, and small intestine, as well as in pancreas. The potential role of hIAP-1 in blocking cell death is under investigation.

AN APOPTOTIC ENDONUCLEASE ACTIVATED EITHER BY DECREASING pH OR BY INCREASING CALCIUM.

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Fragmentation of chromatin in isolated nuclei from the murine IL-3-dependent cell line BAF3 could be stimulated either by a decrease in pH to 6.5, or by the presence of sub-μM calcium at neutral pH. A 45kD endonuclease, which retained these dual signals for activation, was purified more than 10^5 fold from BAF3 cells. Both fragmentation of chromatin in nuclei and activity of purified endonuclease was inhibited by mM magnesium and potassium at concentrations above 50 mM. These characteristics are distinct from those described for other mammalian endonucleases. Apoptosis could be induced in BAF3 cells by using the ionophores valinomycin or nigericin to lower intracellular pH. Cell death induced by the ionophores could not be inhibited by a combination of IL-3 and over-expressed bcl-2. This is consistent with the ionophores activating a late step in the apoptotic pathway, such as the endonuclease itself.

ANNEXIN V AS A MARKER OF PROGRAMMED CELL DEATH IN B CELLS

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A number of methods have been developed in recent years to identify and study programmed cell death (PCD), including light and electron microscopy, TUNEL, 2-dye staining, etc. However, this phenomenon has been very difficult to analyze in vivo. A particular problem has been the study of development, where there is an additional need to correlate markers of PCD with markers of developmental stages.

There is circumstantial evidence that developing B cells undergo PCD at stages corresponding to positive selection for functional immunoglobulin receptor heavy chains and to negative selection against cells whose immunoglobulin receptors respond to self antigens. However, this phenomenon has never been directly demonstrated. Here we describe the use of fluoresceinated recombinant annexin V (AnxV) as an early marker of PCD in B cell development. Annexin V is a protein that binds phosphatidyl serine in a calcium dependent manner. Phosphatidyl serine appears on the outer leaflet of plasma membranes as a consequence of membrane lipid redistribution, which is part of the "death program" but can also occur physiologically, as seen in the activation of platelets and neutrophils. We have used calcium-dependent cell surface binding of a fluorescein-tagged version of recombinant human annexin V as a marker of PCD for flow cytometric analysis. This method has the advantages of (1) being applicable to live, unfixed cells, (2) being compatible with the use of several other probes as markers of B cell development by flow cytometry, and (3) being easily controlled by staining under the same conditions in the presence of a calcium chelator.

In a model system of cultured cells induced to undergo apoptosis, annexin V (AnxV) staining preceded staining by TUNEL and permeability to propidium iodide (PI), and both of these markers appeared only in $AnxV^+$ cells. In the same system, a sorted population of PI excluding, $AnxV^+$ cells did not survive in culture, whereas the corresponding Anx^- fraction survived and proliferated. Further, EM analysis demonstrated that almost all $AnxV^+PI^-$ cells showed features characteristic of apoptosis (including chromatin condensation and margination, vesiculation of the cytoplasm, and formation of apoptotic bodies), whereas only a minority of $AnxV^-$ cells showed these features. We also used the same experimental scheme to show that in mouse B lineage cells ex vivo, the $AnxV^+PI^-$ population is significantly enriched for cells that demonstrate the above EM markers of apoptosis.

Preliminary analysis of ex vivo mouse bone marrow using AnxV in conjunction with markers of B cell development suggests that PCD is occurring at detectable levels at all stages in the lineage. The highest proportion of $AnxV^+$ cells was seen at the earliest stage ($B220^+CD43^+$), presumably corresponding to cells that have failed to generate a functional, positively selecting immunoglobulin heavy chain, and at the immature B cell stage (IgM^+IgD^-), where negative selection against self-reactive cells is thought to occur. Our results show that staining with AnxV is a reliable and very convenient marker for the study PCD in a complex developmental sequence that occurs in the heterogenous environment of the bone marrow.

APOPTOSIS IN XENOPUS EGG EXTRACTS

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One obstacle to understanding the molecular events of apoptosis or Programmed Cell Death (PCD) has been the difficulty in obtaining sufficient amounts of synchronised cultured cells to study the biochemical processes. Recently, cell-free systems that reproduce nuclear events typical of apoptosis have been developed. We have used concentrated *Xenopus laevis* egg extracts adapted from a system commonly used to study nuclear and cytoplasmic events during the cell cycle. Isolated HeLa nuclei added to such "apoptotic" egg extracts undergo a synchronous process of nuclear destruction involving chromatin condensation and DNA fragmentation initiated after 3 hours of incubation in the extract. This apoptotic process requires ATP and is blocked by $ZnCl_2$ or recombinant human Bcl-2 protein. It does not require protein synthesis or activation of mitotic cyclin-dependent protein kinases. In contrast, isolated HeLa nuclei added to normal interphasic egg extracts remain stable for up to 10 hours and show no DNA fragmentation. This cell-free apoptotic system is ammenable to a biochemical analysis and may be useful to gain an insight into the molecular events controlling the triggering and execution of apoptosis.

ABUNDANT APOPTOSIS IN HUMAN ATHEROSCLEROTIC PLAQUES WITH A RECENT HISTORY OF RUPTURE

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Abstract

Rupture of atherosclerotic plaques occurs preferentially at sites of degradation and weakening of the connective tissue in the fibrous cap. Morphologically these sites are characterized by a loss of smooth muscle cells and an accumulation of inflammatory cells. The aim of the present study was to analyse the frequency and mechanism of cell death in atherosclerotic plaques with a recent history (<6 months) of rupture.

Methods: Atherosclerotic plaques were obtained from patients with symptomatic ipsilateral carotid stenosis undergoing vascular surgery. Biotin end-labelling of fragmented DNA by Klenow polymerase, agarose gel electrophoresis of plaque DNA, propidium iodide staining of chromatin and electron microscopy were used to identify cell death by apoptosis (programmed cell death) and necrosis.

Results: The mean number of cells containing fragmented DNA in the plaques was 12.7±3.5% (n=15). Focal accumulations of cells with DNA fragmentation appeared in the fibrous cap, at sites of ruptures and close to lipid deposits and necrosis.Electrophoretic separation of DNA isolated from plaques resulted in ladders of 180-200 multiple fragments characteristic for apoptosis. The presence of apoptotic cell death was further confirmed by electron microscopy demonstrating nuclear condensation and fragmentation as well as formation of organelle-containing apoptotic bodies. Immunohistochemical and electron microscopic analysis suggested that most of the apoptotic cells were smooth muscle cells. Apoptotic smooth muscle cells were generally surrounded by T lymphocytes and macrophages.

Conclusion: The present observations suggest that apoptosis of smooth muscle cells in the fibrous cap is involved in plaque destabilzation and rupture.

LIGHT-INDUCED RETINAL DEGENERATIONS AND THE ACTIVATION OF APOPTOSIS IN *DROSOPHILA* Florence F. Davidson, Anne-Francoise Lamblin and Hermann Steller. Howard Hughes Medical Institute at M.I.T., E25-441, 45 Carleton St., Cambridge, MA 02139

Visual perception in vertebrates and invertebrates relies on a shared signal transduction pathway that is activated by the photoreceptor rhodopsin, a member of the G protein-coupled seven helix receptor superfamily. Subsequent second messenger and effector events leading to synaptic transmission and vision have been well studied. However, little is known about the relationship between light reception and homeostasis of the photoreceptor cell. Nevertheless, mutations in a number of the proteins of the light signal transduction cascade cause degeneration of the retina leading to blindness. Human, mouse and *Drosophila* retinal degenerations have been identified and it has been shown in some cases that the same genetic lesions are responsible in all three organisms. We have now found that in two light dependent forms of retinal degeneration in the fly, rdgC and ninaE^{RH27}, rescue from the degeneration phenotype is achieved by crossing in one to two copies of the baculoviral cell survival factor p35 expressed under the control of the GMR promoter. rdgC flies exhibit a rhodopsin (ninaE) dependent degeneration caused by defects in the structural gene of a 2A-type protein phosphatase, whereas ninaE^{RH27} mutant flies have a point mutation, C200Y, in their main form of rhodopsin. TdT-labeling of cryostat sections of the rdgC degenerating retina, electron microscopic examination of the degenerating photoreceptor cells, and the rescue by p35 indicate that cell death in rdgC occurs by apoptosis. TdT and EM analyses are currently underway for ninaE^{RH27}. Northern analyses, tests of activation of a lacZ reporter construct under the control of the rpr upstream regulatory elements (rpr-lacZ), and mosaic studies are being pursued, in an investigation of the role of the cell death gene rpr in these rdgC and ninaE^{RH27} degenerations. The rpr-lacZ transgenic reporter line was made as part of ongoing studies in our laboratory focussing on the transcriptional regulation of rpr. Using this and several other constructs, the minimal promoter of rpr is in the process of being identified, by comparison of the expression pattern of the reporter gene to endogenous rpr mRNA *in situ* patterns in wild type and mutant embryos and third instar larvae.

NUCLEAR OVEREXPRESSION OF PROENKEPHALIN INDUCES APOPTOTIC DEATH.

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The opioid precursor proenkephalin is well recognised to exist as a secretory protein in a range of non-neural as well as neural cell types. We have recently shown that it exists in the nucleus of embryonic fibroblasts and undifferentiated myoblasts where it is responsive to growth arrest and differentiation signals. We have commenced a series of mutagenesis experiments to begin to address the mechanism of nuclear targeting. Our results show that a nuclear fate is conferred to proenkephalin transiently transfected into COS cells in two ways: 1) removal of the signal peptide sequence by PCR deletion (PE ΔSS); 2) site-directed mutagenesis of the normal ATG codon to force translation initiation from a dowstream codon (PE ATC) (Boettger and Spruce, 1995, J. Cell Biol. In Press).

We have recently shown that cytoplasmic proenkephalin confers a survival function (Dewar et al., in preparation). We have also observed that, following DNA damage, there is reorganisation of nuclear proenkephalin which precedes the onset of apoptosis. To explore the relationship of nuclear proenkephalin to cell death and survival, we have begun to analyse the behaviour of proenkephalin selectively targeted to different subcellular compartments. In COS cells transiently transfected with PEΔSS, the transfected gene product is localised in both nucleus and cytoplasm but it does not enter the secretory pathway; PE +SS is localised exclusively in the cytoplasm in a secretory pathway-like distribution. Cell viability is unchanged in both. However, in 3T3 cells transiently transfected with PE ΔSS or PE ATC, death with apoptotic features is seen; shrunken cells are visible within 24 hours of transfection and by 48 hours virtually no survivors are seen. In contrast, cells transfected with PE +SS survive and apart from some transient post-transfection alteration in morphology, transfectants survive and flatten out by 48 hours. In 3T3 cells, nuclear localisation of proenkephalin is seen at early time points with PE ΔSS and PE ATC but not with PE +SS where it is localised exclusively in the cytoplasm. Identification of transfectants and the cellular localisation of proenkephalin has been confirmed in all experiments by the use of proenkephalin tagged with a p53 epitope in parallel with non-tagged constructs.

In view of the differential behaviour of nuclear-targeted proenkephalin in COS and 3T3 cells, we have begun to explore the relationship of the induction of apoptosis to the p53 status of the cell. We have transfected mutated and non-mutated PE into C6 rodent fibroblasts which express a temperature sensitive mutant form of p53, which adopts a wild-type conformation at 32°C but is mutant at 37°C. At 37°C PE ΔSS is localised in nucleus and cytoplasm, and the cells appear largely viable and have processes; however, at 32 °C there are very few survivors after 24 hours, and these look typically apoptotic in having a shrunken, blebbing appearance with condensed nuclei. Cells transfected with PE +SS express PE in the cytoplasm and are viable at both temperatures. From these data we tentatively conclude that nuclear proenkephalin mediates apoptosis through a p53-dependent pathway.

CD40 LIGATION, A SURVIVAL SIGNAL IN B CELLS, POTENTIATES EPITHELIAL CELL APOPTOSIS

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CD40, a member of the tumour necrosis factor receptor family, is expressed on the surface of B lymphocytes where its ligation provides a potent survival signal. CD40 is also expressed in basal epithelial cells and in a number of different carcinomas where its function remains unknown. We observed that contrary to the studies in normal B cells, cross-linking of CD40 by the G28.5 mAb or soluble CD40 ligand resulted in inhibition of carcinoma cell growth and enhanced susceptibility to apoptosis induced by TNFα, Fas, ceramide and anti-neoplastic drugs such as *cis*-platin and adriamycin. This effect was also observed in CD40-transfected Rat-1 fibroblasts. In addition, CD40 ligation partially reversed the resistance of certain carcinoma cell lines to chemotherapeutic agents.

Whilst Bcl-2 did not affect the growth inhibition induced by CD40 ligation in epithelial cells, expression of the Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) efficiently blocked the effect. These results suggest that CD40 regulates cell growth and survival in a cell lineage-specific manner and that this pathway is important in regulating the growth of epithelial cells.

SUPPRESSION OF LYMPHOCYTE APOPTOSIS IN TRANSIENT HETEROKARYONS

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Suppression of apoptosis has been implicated in the development of cancer and resistance of tumor cells to chemotherapy and radiation. Several genes encoding suppressors of apoptosis have been identified, including *bcl*-2 and related genes. Since these suppressor genes are not expressed in all tumors which possess resistance to the induction of apoptosis, it is reasonable to expect that novel suppressors of apoptosis exist. We are using a genetic approach to search for additional suppressors of programmed cell death in somatic cells. In these studies, HTC rat hepatoma cells (which are resistant to apoptosis induced by glucocorticoids and calcium ionophores) are fused to BW5147 murine thymoma cells (which undergo apoptosis with either activator) to form transient heterokaryons. This experimental design allows determination of whether suppressors of apoptosis are diffusable and whether their activity is dominant. Prior to fusion, HTC cells are loaded with a green fluorescing cytosolic dye, and BW5147 cells are loaded with an orange fluorescing cytosolic dye, which allows subsequent differentiation of heterokaryons. These heterokaryons were exposed to two apoptotic stimuli (dexamethasone and a calcium ionophore) and apoptosis measured by nuclear condensation and the formation of membrane-bound vesicles containing DNA. These data demonstrate that both HTC cells and HTC/BW5147 heterokaryons are completely resistant to apoptosis under conditions in which BW5147 cells undergo programmed cell death. In addition, this suggests that HTC cells possess a dominant cytosolic suppressor which is able to protect BW5147 cells present in the same heterokaryon from apoptosis. To biochemically examine potential cytosolic suppressors of apoptosis, we have developed an *in vitro* system in which HTC cytosol is mixed with BW5147 nuclei. Preliminary data suggest that HTC cytosol contains an activity which can protect BW5147 nuclei from autodigestion in this assay.

SELECTIVE INDUCTION OF APOPTOSIS IN CULTURED DERMAL PAPILLA FIBROBLASTS BY DIFFERENT PKC INHIBITORS.

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The dermal papilla (DP) plays an inductive role in the control of hair follicle morphogenesis and cycling. In contrast to the cells of the hair matrix which undergo apoptosis during hair cycling, the dermal papilla is a permanent cell population. This specialized and long-lived cell population of fibroblasts appears to be protected from programed cell death (PCD) by gene products such as Bcl-2, and Nerve Growth Factor Receptor, that are strongly expressed in dermal papilla in vivo.

The genetic pathway controlling apoptosis is modulated by different factors, some of which stimulate or inhibit PKC-mediated signal transduction, an important pathway involved in cell survival. The importance of this pathway in PCD of dermal papilla fibroblasts was investigated by inhibition of PKC activity using staurosporine, a potent but non-specific inhibitor of PKC, and bisindolylmaleimides (Bis), a selective inhibitor of PKC α, β, and γ isoforms.

Dermal papilla microdissected from human fetal scalp were characterized in culture by their specific aggregative behavior, a fundamental property related to their function. Homogeneity was determined by a strong expression of a specific marker of cultured dermal papilla, a-actin . Early passaged dermal papilla fibroblasts were treated with staurosporine (10^{-6}-10^{-9}M) and Bis (10^{-6}-10^{-9}M) for 24 hours. Apoptotic cells were identified by TUNEL staining and flow cytometry. DNA content analysis showed selective loss of DNA, indicative of apoptosis, from staurosporine treated cells but not from Bis treated cells. TUNEL staining demonstrated that the dermal papilla cells within the clumps were more affected than those forming the monolayer, suggesting that the sensitivity to induction of cell death may be related to the physiologic state of the dermal papilla cell with typical aggregative behavior. Staurosporine-induced cell death was partially suppressed by TPA, indicating that PKC activity is required for induction of apoptosis in human fetal dermal papilla. Surprisingly dermal papilla cells treated with Bis did not show the extent of cell death compared to the control with carrier alone.

In conclusion, we determined that PKC-mediated signalling is important in controlling the apoptotic pathway in dermal papilla and that this pathway can be selectively activated by different PKC inhibitors. PKC α, β, and γ may not be the major isoforms that function in dermal papilla fibroblasts, or their inhibition is not sufficient to induce apoptosis in these cells. Identification of the specific PKC isoforms expressed in dermal papilla fibroblasts as well as the use of other inhibitors may determine which isoform is involved in dermal papilla apoptosis.

FUNCTION AND EXPRESSION OF THE BCL-X GENE IN THE NERVOUS SYSTEM

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In order to understand the mechanisms controlling neuronal cell death we have studied expression and function of the Bcl-x gene. Bcl-x is a member of the Bcl-2 family. Bcl-xl and Bcl-xs are two splice variants of these gene. We have shown that, like Bcl-2, Bcl-xl rescues sympathetic neurons from cell death induced by nerve growth factor deprivation. Expression of Bcl-x mRNA was studied by Northern blot analysis and in situ hybridisation. By these two approaches, we identified Bcl-x mRNA in neurons of the central and peripheral nervous system. We observed that Bcl-x mRNA expression is much more abundant in adulthood than during embryonic stages. This is in contrast to expression of Bcl-2 protein whose expression is higher during embryogenesis. Polymerase chain reaction analysis allowed us to identify Bcl-xl as the more abundant variant of Bcl-x in the nervous system. These results strongly suggest a role for Bcl-x in survival of neurons not only during embryogenesis but also during adulthood.

FUNCTIONAL ROLE OF IL-1ß IN APOPTOSIS MEDIATED BY THE ICE FAMILY.

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The interleukin-1ß converting enzyme (ICE) family plays an important role in regulating vertebrate cell death. Previous work has shown that ICE overexpression induces apoptosis, that its inhibition by CrmA prevents cell death, and that during TNF-α mediated apoptosis ICE is activated as indicated by the release of mature IL-1ß (mIL-1ß). To date no ICE substrate relevant to apoptosis has been identified. We investigated the role of IL-1ß in cell death mediated by the ICE family. Our results indicate that processing of pro-IL-1ß, followed by mIL-1ß secretion and extracellular receptor binding are integral events modulating apoptosis following ICE activation. We demonstrate that apoptosis mediated by TNF-α in L929 and HeLa cells, and by trophic factor deprivation in primary DRG neurons, is inhibited by the IL-1 receptor antagonist. In addition, in G_1/S phase arrested HeLa cells, mIL-1ß itself induces apoptosis through an ICE independent pathway. COS cells which are normally resistant to *Ice* and *Ich-1L* induced apoptosis, undergo cell death by expressing *Ice* and *Ich-1L* with *pro-IL-1ß* or in the precence of mIL-1ß or TNF-α. These findings identify pro-IL-1ß as the first substrate of ICE, whose cleavage product (mIL-1ß), is a downstream mediator of the apoptotic cascade. Modulation of IL-1ß activity *in-vivo* could provide a novel pathway for the therapy of diseases with aberrant apoptosis.

DISRUPTION OF CELL CYCLE CONTROL BY A VIRAL ONCORPOTEIN DURING DEVELOPMENT OF THE MAMMARY GLAND ALTERS CELL FATE AND FUNCTION: INDUCTION OF p53 INDEPENDENT APOPTOSIS IS FOLLOWED BY IMPAIRMENT OF MILK PROTEIN SYNTHESIS IN SURVIVING CELLS

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Disruption of cell cycle regulation is associated with developmental abnormalities and tumorigenesis. The simian virus 40 large T antigen (Tag) interferes with cell cycle control by interacting with pRb family members and p53. Mice carrying a transgene composed of the whey acidic protein (WAP) gene promoter linked to the Tag coding sequence express Tag during pregnancy and are unable to nurse their young. We examined the role of cell cycle control in mammary gland development of these mice. The primary reason for nursing failure is a lack of milk protein synthesis. Cells which expressed Tag differentiated throughout pregnancy as measured by the sequential activation of milk protein genes, but could not produce milk proteins. Notably, Tag also induced apoptosis in 5% of the mammary epithelial cells during late pregnancy. In contrast, less than 0.2% of mammary epithelial cells in non-transgenic littermates were undergoing apoptosis. Apoptosis in Tag mice was associated with increased steady state RNA levels of bax and bcl-x_{L+S}, with a relative increase in bcl-x_S expression. Since p53 was sequestered by Tag, it is likely that p53 independent mechanisms precipitated apoptosis and caused the observed changes in bax and bcl-x expression. In conclusion, we propose that the inactivation of p53 and pRb in differentiating mammary alveoli alters cell fate and function. Induction of p53 independent apoptosis in mammary epithelial cells is followed by failure of milk protein synthesis in surviving cells.

Bcl-2 INHIBITS APOPTOSIS IN HAEMATOPOIETIC CELLS INDUCED WITHOUT CHANGES IN ENERGY METABOLISM OR FREE RADICAL PRODUCTION. EVIDENCE THAT bcl-2 PROMOTES A STATE OF METABOLIC HIBERNATION.

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We have investigated whether bcl-2 might protect murine IL3-dependent Bo cells from apoptosis following IL3 withdrawal by increasing glycolytic ATP production, activating ATP-dependent repair mechanisms or inhibiting other ATP consuming reactions. Parental cells and cells over-expressing human bcl-2a (B15) were metabolically indistinguishable. Both maintained similar mitochondrial membrane potentials measured by Rhodamine 123 fluorescence, although ATP was derived entirely from glycolysis. On IL3 removal both Bo and B15 cells virtually ceased glycolysis but maintained intracellular (ATP); only Bo cells underwent apoptosis. Uncoupling of mitochondria with 2,4-dinitrophenol (DNP) stimulated lactic acid production and reduced intracellular (ATP) in both Bo and B15 cells, inducing apoptosis in the Bo cells even in the presence of IL3, but not in the B15 cells. Using either the sensitive chemiluminescent probe Pholasin(R) or the fluorescent probe dichlorofluorescin diacetate we found no evidence for the involvement of reactive oxygen species (ROS) in the induction of apoptosis by IL3 withdrawal or DNP addition. However, both Bo and B15 cells could be induced to produce ROS by maleic acid diethyl ester. Surprisingly, the antioxidant N-acetyl-cysteine accelerated apoptosis in IL3-deprived Bo cells and induced it in the presence of IL3. We conclude that bcl-2 stabilises a state of metabolic arrest induced by IL3 withdrawal in parental cells rather than activating alternative metabolic pathways or ATP-dependent housekeeping or repair mechanisms.

OXIDATIVE STRESS PROMOTES MAINTENANCE OF CELL SHAPE, CELL CYCLE PROGRESSION AND INHIBITS APOPTOSIS IN MURINE HAEMATOPOIETIC CELLS

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Murine IL3-dependent haematopoietic cells rapidly apoptose on withdrawal of IL3. This has been ascribed to involvement of free radicals in the apoptotic process and it has been postulated that the proto-oncogene bcl-2 acts on an anti-oxidant pathway. However, this has been challenged by other data showing that apoptosis and its protection by bcl-2 still occurs under conditions where free radicals are not generated. We have studied the response of a variety of IL3-dependent cells to oxidative stress induced by agents which modulate the glutathione redox cycle. We have found:

1. Oxidative stress induced by maleic acid diethyl ether (DEM) promotes maintenance of cell shape in the absence of IL3.
2. IL3 induces free radicals, withdrawal of IL3 reduces free radicals.
3. Oxidative stress induces progression of cells from G1 to S-phase over a period of six hours.
4. Intracellular reducing agents increase apoptosis and cell contraction.
5. Cell lines express different physiologies in respect of production of free radicals, effects of inhibitors on glutathione levels and changes in DNA synthesis during glutathione inhibition. However, all maintain shape and cycle progression with DEM.

We suggest that pro-oxidants and IL3 suppress apoptosis, promote cell shape and cell cycle progression, and hypothesise that these effects are related to redox control of the cytoskeleton.

SPHINGOID AND ALIPHATIC AMINES ARE SPECIFIC INDUCERS OF APOPTOSIS IN HL-60 CELLS.

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Apoptosis or programmed cell death is a naturally occurring, genetically determined program of autonomous cellular suicide required for the maintenance of normal tissue homeostasis. Inhibition of apoptosis by the deregulation of certain oncogenes or loss of tumor suppressor genes results in clonal expansion leading to carcinogenesis. Induction of apoptosis in an expanding population of tumor cells therefore has therapeutic potential. The promyelocytic leukemic cell line, HL-60, is known to undergo apoptosis upon treatment with a variety of agents including DNA topoisomerase I and II inhibitors, pyrimidine antimetabolites, tyrosine kinase inhibitors, etc. In the present study, we found that sphingoid and aliphatic amines are potent inducers of apoptosis in HL-60 cells. The sphingoid amine, sphingosine, induced apoptosis at a concentration of 15μM as early as 4 hours after treatment of HL-60 cells in the presence of 10% fetal calf serum. The effect of sphingosine was highly stereospecific. Only D-erythro and D,L-erythrosphingosine caused apoptosis; other stereoisomers or modified sphingosines were ineffective. The double bond in the sphingoid base was expendable; thus dihydrosphingosine was effective in inducing apoptosis. The mechanism of sphingosine-induced apoptosis appears to exclude involvement of either ceramide or protein kinase C since the effects of sphingosine were not reversed upon prior treatment of cells with either fumonisin (45μM) or phorbol ester, TPA (50nM). Besides sphingosine, a series of primary aliphatic amines (tested at 15μM in serum-containing media) were found to be potent inducers of apoptosis in HL-60 cells. A minimum of 12-carbon chain was required - thus hexylamine, octylamine or decylamine did not induce apoptosis, whereas dodecylamine, tetradecylamine, hexadecylamine and octadecylamine (sterarylamine) were all effective. The efficacy of these compounds was also dependent on the primary amine-functionality; thus stearic acid (sodium salt), stearyl methyl ester, and stearyl alcohol were all ineffective. Similarly, N,N,N-trimethylsphingosine or N-acetyl sphingosine were ineffective in inducing apoptosis. The biochemical and genetic mechanisms underlying the apoptotic effects of sphingoid and aliphatic amines are currently being investigated.

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THE TUMOR-PHYSIOLOGICAL STRESS OF HYPOXIA SELECTS FOR ONCOGENICALLY TRANSFORMED CELLS THAT HAVE LOST THEIR ABILITY TO UNDERGO APOPTOSIS

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Regions of low oxygen (hypoxia) are a common characteristic of solid tumors from diverse histological origins, and are correlated with a poor prognosis independent of therapy. Cellular and genetic analysis of differences between tumors of successive pathological stages suggests that a physiological stress(es) within a tumor selects for cells with reduced apoptotic ability and loss of p53 function, an integral part of stress inducible apoptotic pathways. As of yet no candidate selective stress has been identified in solid tumors. We report here that hypoxia can induce apoptosis in oncogenically transformed cells, and that further genetic alterations such as loss of p53 function or overexpression of the apoptosis inhibitor protein Bcl-2 can significantly reduce hypoxia induced cell death. We found that small numbers of transformed cells devoid of p53 activity will overtake wild-type p53 cells when mixed together and treated with hypoxia *in vitro*. When these same transformed cells are transplanted in immune deficient mice, developing tumors derived from $p53^{+/+}$ cells, but not $p53^{-/-}$ cells contain significantly more apoptotic cells in regions of the tumor which stain positive for hypoxia. Thus, we propose that hypoxia, resulting from tumor growth exceeding angiogenesis, may be the common physiological stress in tumors which selects for cells with reduced apoptotic potential, and in particular for p53 mutations.

K252a, A HIGH-AFFINITY NGF-RECEPTOR INHIBITOR, INDUCES APOPTOSIS IN CULTURED HUMAN KERATINOCYTES.

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Keratinocytes synthesize and release nerve growth factor (NGF). NGF has been demonstrated to stimulate the proliferation of human keratinocytes via phosphorylation of the high affinity NGF-R (trk). Accordingly, K252a, which specifically inhibits trk phosphorylation, is able to blocks NGF-induced keratinocyte proliferation. Since in other cell systems NGF has been shown to act by suppressing cell death, we investigated whether NGF blockade by K252a could lead to apoptosis rather than inhibition of proliferation in human keratinocytes. Keratinocytes, taken from neonatal foreskin, were cultured in serum-free medium w/wo the addition of NGF, K252a or NGF+K252a and collected after 24, 48, 72 and 96 hs. NGF+K252a-treated cells underwent apoptosis starting from 72 hs, as assessed by morphological observation of cells using either light or electron microscopy and by electrophoretical analysis of DNA laddering. Surprisingly, also keratinocytes treated with K252a alone underwent apoptosis within 72 hs. K252a-induced apoptosis in absence of exogenous NGF could be due in part to inhibition of NGF released by keratinocytes themselves. Alternatively, K252a could cause apoptosis by inhibiting tyrosine kinases other than trk. The latter possibility was however in part ruled out by the demonstration that fibroblasts and lymphocytes, which do not express trk, were resistant to K252a apoptotic effect. To elucidate the mechanisms of K252a-induced apoptosis in keratinocytes, we measured IL-1a release by ELISA and bcl-2 levels by Western blot. As reported in other cell types, we showed that apoptotic keratinocytes secreted significantly increased amounts of IL-1a ($p<0.001$). Furthermore, bcl-2 levels were lower in apoptotic keratinocytes than in control or in NGF-treated cells. Therefore, we suggest that endogenous NGF could act as a survival factor for keratinocytes and that its action could be mediated through the up-regulation of bcl-2.

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PROTEASOME INHIBITION IN THYMOCYTE APOPTOSIS

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The selective elimination of proteins is an important tool in regulating cell state. The ATP-dependent 26S multisubunit protease is critical in achieving such regulation. The multisubunit complex is composed of a 20S protease catalytic core called the proteasome and regulatory subunits necessary for control and specificity. The 26S protease targets polyubiquitinated proteins for degradation and recycles ubiquitin monomers by freeing them from polyubiquitin chains.

The importance of the ubiquitin-proteasome pathway becomes evident when the substrates for this proteolysis are identified. Many short-lived proteins that are critical for abbreviated periods are regulated by the ubiquitin -proteasome pathway (eg. cyclins, p53, c-fos). In addition, proteasomes target the bulk of abnormal and long-lived proteins.

The use of peptide inhibitors to the 20S proteasome have identified an additional role for the proteasome as a processor. Inhibition of the proteasome blocks the generation of peptides presented on MHC class I molecules, and inhibitor studies have also determined a requirement for the proteasome in the processing of the p105 precursor into the p50 subunit of the NF-κB complex.

Because the proteasome is an important regulator of cell activities with an impressive range of substrates, work was undertaken to investigate the involvement of the proteasome in apoptosis. The identification of the inhibitors LLM (calpain inhibitor II) and LLnL (calpain inhibitor I, MG132) as effective blockers of the chymotrypsin-like activity of the 20S proteasome provided an easy way to assess proteasome involvement in apoptosis. Mouse thymocytes were preincubated with 50 uM LLM and LLnL for 1 hour prior to treatment with dexamethasone, γ-irradiation, PMA , or PMA + A23187. At 10 hours post-treatment with these agents, death was delayed under all conditions except treatment with PMA + A23187. Because these inhibitors also inhibit calpains, papain, and the lysosomal proteases cathepsins B and L, the control protease inhibitors E64 and leupeptin were also tested. These control proteases did not significantly delay death in any of the pathways. These data support the idea of proteasome involvement in many apoptotic pathways in thymocytes and introduce another role for this very important protease.

CLEAVAGE OF POLY(ADP-RIBOSE) POLYMERASE BY INTERLEUKIN-1β CONVERTING ENZYME AND ITS HOMOLOGS TX AND NEDD-2

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The proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) is an early biochemical event which occurs during apoptosis. A recent study suggested that PARP cleavage can be mediated by a novel cytosolic protease (prICE) that resembles interleukin-1β converting enzyme (ICE), but can not be mediated by ICE itself [Lazebnik *et al.*, (1994) *Nature 37*, 346-347]. We have used a COS cell co-transfection assay to investigate if ICE or any known ICE-like protease is active in PARP cleavage within the cell. Here we report that co-expression of human PARP with human ICE, or the ICE homologs TX and Nedd-2, resulted in a cleavage of PARP identical to that observed in apoptotic cells. Experiments with purified recombinant human ICE indicated that PARP polypeptide can be specifically cleaved *in vitro* by ICE in a time- and enzyme concentration-dependent manner. PARP cleavage, however, requires a 50-100 fold higher ICE concentration than does processing of the interleukin-1β precursor at equivalent substrate concentration. The abilities of ICE, TX and Nedd-2, when expressed at high intracellular concentrations, to cleave PARP are consistent with their induction of apoptosis in transfected cells.

FAS INDUCES ACTIVATION OF PI-3-KINASE VIA SRC-TYROSINE KINASES

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Programmed cell death is a highly conserved cellular mechanism induced by a variety of stimuli, e.g. corticosteroids, UV-light, oxygen radicals or by triggering T-cells via the Fas-receptor. The Fas receptor seems to have an important function in the homeostasis of the immune system since mutations of the receptor or its ligand result in lymphadenopathy, lymphoaccumulation and autoimmune symptoms of lpr or gld mice. We and others have demonstrated an activation of sphingomyelinases and p21Ras upon Fas receptor triggering. To identify further signaling events important for Fas induced apoptosis, we stimulated Jurkat cells via the Fas receptor using the monoclonal antibody CH11 (2µg/ml) and detected a tyrosine phosphorylation of several intracellular proteins. Since a 110kDa protein was highly phosphorylated, we determined tyrosine phosphorylation and activity of phosphoinositide-3-kinase (PI-3-K). Fas induced a 5-fold stimulation of PI-3-K activity correlating with an increase of tyrosine phosphorylation of the p110kDa subunit of PI-3-K. Preincubation of cells with the kinase inhibitors Herbimycin A or Staurosporine prevented PI-3-K phosphorylation and activation. Likewise, PI-3-K-activation and tyrosine phosphorylation was abolished in two different mutant T-cell clones lacking the tyrosine kinase p56lck and transfection of these mutants with p56lck reconstituted phosphorylation/activation of PI-3-K upon Fas receptor triggering.

To demonstrate the significance of PI-3-K stimulation for programmed cell death, we measured DNA-fragmentation after Fas in the presence or absence of the PI-3-K inhibitor Wortmannin (100nM). Wortmannin almost completely inhibited Fas induced programmed cell death pointing to an important function of src-tyrosine kinase regulated PI-3-K activity in apoptosis.

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REGULATION OF LYMPHOCYTE SURVIVAL BY THE *BCL*-2 GENE FAMILY

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The oncogenic role of mutations that promote cellular proliferation is well accepted. Equally important, however, are mutations that curb apoptosis, the process of physiologic cell death. The link between enhanced cell survival and cancer emerged with the discovery that *bcl*-2, the gene translocated in follicular lymphoma, enhances cell survival. Transgenic mouse models have directly demonstrated the lymphomagenic potential of *bcl-2*. The cytoplasmic protein encoded by *bcl-2* has been shown to be located on the nuclear membrane, endoplasmic reticulum and outer mitochondrial membrane. The biochemical basis for its survival function remains unclear, although genetic studies in *C. elegans* suggest that it interferes with the activity of cysteine proteases that play an essential role in cell death. By identifying proteins that can complex with Bcl-2, we hope to gain insight into its mode of action. Several *bcl-2*-related genes have been described; one close homologue, *bcl-x*, also inhibits cell death, but other relatives such as *bax* and *bak* appear to facilitate it. Using a PCR-based strategy, we have recently identified a novel gene that enhances cell survival and are assessing its ability to counter diverse death signals in a range of cell types. Although *bcl-2* protects cells from diverse cytotoxic stimuli, including cytokine deprivation and radiation, it provides little protection against apoptosis transduced via the cell surface receptor Fas/Apo-1. Our data raise the possibility that there is more than one effector mechanism for cell death.

BAX DEFICIENT MICE DEMONSTRATE LYMPHOID HYPERPLASIA BUT MALE GERM CELL DEATH.

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Bax, is a heterodimeric partner of bcl-2, which in gain of function experiments countered bcl-2 and promoted apoptosis. Site directed mutagenesis of bcl-2 suggests that bcl-2 may need to interact with bax in order to block cell death. Moreover, bax can heterodimerize with multiple bcl-2 family members. In order to address the role of bax in cell death and identify its critical roles in development, we generated bax deficient animals using homologous recombination in ES cells. Deficiency of bax does not appear to affect embryonic viability as -/- mice appear healthy and are generated at the expected Mendelian frequency. However, the mice demonstrate selective hyperplasia of lymphoid tissues. The organ weights of both thymus and spleen are increased. Total thymocytes are increased 1.6 fold without a change in the distribution of each maturational subset. In vitro survival of thymocytes with or without dexamethasone or gamma irradiation treatment was not significantly different than controls. Thus, bax does not appear to be necessary for p53 dependent apoptosis within thymocytes. In spleen the -/- mice display a greater increase in B cells (1.8 fold) than T-cells. Purified B cells demonstrate decreased apoptosis whether resting or activated with anti-IgM or LPS. Bax deficient males are infertile with a complete cessation of mature sperm cell production. Seminiferous tubules are markedly disorganized with disruption of the normal spermatogenic cycle. Many tubules contain an expansion of premeiotic germ cells representing atypical spermatogonia to preleptotene spermatocytes. DNA content analysis reveals these increased 2N cells but a complete absence of condensed 1N spermatozoa. Multinucleated giant cells and abnormal mitotic or meiotic figures accompany massive cell death with clustered apoptotic germ cells. In contrast, *bax* deficient females are fertile but the ovaries do contain unusual atretic follicles with numerous atrophic granulosa cells which presumably failed to undergo apoptosis. Thus, the loss of bax can result in hyperplasia or hypoplasia depending on the cellular context. Bax deficient mice signify a prominent role for the apoptotic pathway in monitoring gametogenesis, and provide evidence for an interrelationship of proliferation, differentiation and cell death.

Bip1, a distantly related member of the Bcl-2 family of proteins with a killer instinct, interacts with cellular and viral survival promoting proteins. Janice M. Boyd[1], Gregory J. Gallo[2], B. Elangovan[1], Erik Uhlmann[1], Thomas Chittenden[2], Robert J. Lutz[2] and G. Chinnadurai[1]. Institute for Molecular Virology, St. Louis University Medical Center, 3681 Park Avenue, St. Louis, MO 63110[1] & Apoptosis Technology Inc., 148 Sidney Street, Cambridge, MA 02139[2].

Bip1 is a novel cellular protein and was isolated by two-hybrid interaction cloning in yeast using Bcl-2 as the bait. Bip1 interacts strongly with the survival-promoting proteins Bcl-2, Bcl-x_L, adenovirus E1B 19 kD and EBV BHRF1 in *in vitro* and *in vivo* protein binding studies. It is a 160-amino acid protein with a C-terminal hydrophobic membrane spanning domain. Unlike other known members of the Bcl-2 family of proteins, Bip1 does not contain sequences homologous to the conserved BH1 and BH2 domains. However, it contains a nine amino acid region (termed BH3) conserved among several members of the Bcl-2 family of proteins. In transient transfection studies, Bip1 promotes cell death which can be suppressed to varying extents by coexpression of survival-promoting proteins. In growth factor-dependent hematopoietic cell lines that stably express Bip1, it antagonizes the survival promoting activity of Bcl-2. Mutational analysis of Bip1 indicates that a minimal 53-amino acid region encompassing the BH3 domain interacts with a bipartite sequence motif in Bcl-2 and Bcl-x_L. This sequence motif is located within the N-terminal half (that excludes the BH1 and BH2 domains) of these proteins. Mutational analysis of the adenovirus E1B 19 kD protein has also identified a similar bipartite domain. In both Bcl-2 and 19 kD proteins, these bipartite sequence motifs are essential for survival-promoting activity. Implications of Bip1 interactions with these sequence motifs in modulating death *vs* survival-promoting activities will be discussed.

CROSSTALK BETWEEN CELL DEATH AND CELL CYCLE PROGRESSION: BCL-2 REGULATES NFAT-MEDIATED T CELL ACTIVATION

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Genetic models of bcl-2 -/-, bcl-2 +/-, bcl-2 +/+ (wt), and lck^{pr}-bcl-2 transgenic (gf) mice provided a gradient of bcl-2 levels within normal thymocytes and T cells. Bcl-2 levels progressively decreased the number of thymocytes in S phase (bcl-2 -/-, 9.5%; bcl-2 gf, 1.5%). Bcl-2 even affected the degree of quiescence in G0 phase, resting T cells in which cell volume was inversely related to bcl-2 protein levels. Following activation with anti-CD3 or co-stimulation together with anti-CD28, the presence of bcl-2 markedly delayed cell cycle progression. For example, at 24 hours post-activation, 32% of bcl-2 -/-, 22% bcl-2 +/-, 9% bcl-2 +/+, but only 3% bcl-2 gf were in S+G2/M phase. The cdk inhibitor p27kip1, which is normally regulated by IL-2 signaling, was altered by bcl-2 with a marked retention of p27 in bcl-2 gf T cells following activation. However, specificity was noted in that p21 levels were not altered by bcl-2. Bcl-2 also affected activation induced cell death. At 24 hrs. post-activation, 27% of bcl-2 -/-, but only 7% of bcl-2 gf T cells were apoptotic. Proximal signal events such as calcium mobilization, tyrosine phosphorylation, and immediate early gene transcription (myc, jun, Fos, NGFI-B) were unaffected by bcl-2. However, transcription and synthesis of IL-2 was markedly attenuated by bcl-2. Other delayed early cytokine responses which follow T cell activation including IL-3 and GM-CSF were also inhibited by bcl-2. EMSA analysis demonstrated decreased NFAT binding activity in activated bcl-2 gf T cells while other transcription factors, NFκB, AP-1, Oct-1 required for IL-2 transcription were normal. This selectivity was confirmed when expression of Bcl-2 impaired the response of an NFAT-LacZ reporter in Jurkat T cells. These data indicate an inter-relationship of cell death and cell activation in which bcl-2 exerts a prominent effect upon the NFAT pathway.

BCL-2 AND MYC REGULATE P53 CONFORMATION AND NUCLEAR TRAFFICKING

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Both the mechanism by which members of the *bcl-2* gene family modulate apoptosis and the functional interaction of tumor suppressor genes with oncogenes are poorly understood. In MEL cells, we have recently shown that the Myc and Bcl-2 proteins can cooperate to overcome p53 tumor suppression. Alone, Bcl-2 delays p53-induced apoptosis, but does not affect p53 nuclear localization or growth arrest. Together, Myc and Bcl-2 overcome p53 cell cycle arrest and p53-induced apoptosis and inhibit p53 trafficking into the nucleus during G1, the critical period during which p53 induces apoptosis. Bcl-2 and Myc appear to modulate p53 function by affecting the conformation (and by inference, the subcellular localization) of the p53 protein. In MEL cells that express p53, c-*myc*, and *bcl-2*, the p53 is folded in a mutant conformation. Expression of Myc or Bcl-2 alone with p53 does not affect the latter protein's conformation. To determine whether *bcl-2* plays a role in the subcellular localization of p53 in *de novo* cancers, a recombinant adenovirus that expresses *bcl-x$_S$*, a transdominant repressor of members of the Bcl-2 protein family, was made. This virus was used to infect MCF-7 breast cancer cells, which express large amounts of Bcl-2, Myc and cytoplasmic wild type p53 protein. Consistent with a role for Bcl-2 in the regulation of p53 nuclear trafficking, infection of these cells with the *bcl-x$_S$* adenovirus, but not a control adenovirus, results in the translocation of the p53 protein from the cytoplasm into the nucleus with subsequent induction of apoptosis. In some cells, p53 has been shown to mediate some forms of radiation-induced cytotoxicity while the Bcl-2 protein can protect cells from apoptosis induced by such agents. In MCF-7 cells, although radiation induces the expression of wild type sequence p53, the protein remains in the cytoplasm and is not recognized by an antibody specific for p53 wild-type conformation. Also, radiation-induced cell death is independent of p53 in these cells. In contrast, in mouse fibroblasts transformed with E1A and *ras*, p53 enhances radiation-induced cell death. In such cells, the radiation induces the expression of nuclear p53 folded in a "wild type" conformation. These observations are consistent with the concept that Bcl-2 overexpression plays a role in functionally inactivating wt p53 in these cancers. Taken together, these data suggest that a critical function of Bcl-2 is the regulation of the conformation and subcellular distribution of wt p53.

THE MCL-1 GENE EXPRESSED IN TRANSGENIC MICE EXHIBITS ENHANCING EFFECTS ON CELL SURVIVAL

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The mcl-1 gene was isolated from ML-1, a human myeloid leukemia cell line that undergoes differentiation upon induction with the phorbol ester, TPA. The sequence of the mcl-1 gene exhibits significant homology to bcl-2, which is known to inhibit apoptosis in many cell types. By using gene transfer techniques, we have found that mcl-1 is also capable of enhancing cell survival. For example, FDCP-1 cells transfected with mcl-1 show a survival advantage over vector controls under several conditions that cause apoptosis.

We have now applied a transgenic mouse approach to gain an understanding of the role of mcl-1 in the process of cell death in vivo. The transgene construct used consisted of a cloned 17kb human mcl-1 genomic fragment, which contains all of the exons and introns of mcl-1, as well as 10 kb of 5'-flanking sequence and 4 kb of 3'-flanking sequence. This places mcl-1 under the control of its normal cis-acting regulatory sequences. The ability of this construct to direct mcl-1 expression was confirmed by transient expression in NIH 3T3 cells, with detection by Western blotting. Injection of this construct into pronuclei resulted in the production of two founder mice and, from one of these, four transgenic sublines were derived, each exhibiting slight differences in their mcl-1 genomic banding patterns on Southern blots.

We have initiated studies on heterozygotes from one of these transgenic sublines. In a tissue survey assaying for transgene expression by Western blot, We found expression of the mcl-1 trangene to be tissue specific, spleen, thymus and liver having the highest levels of expression. Kidney, lung and small intestine exhibited weak expression and transgene expression was not detectable in heart, brain and muscle. Both T and B lymphocytes from the spleen exhibited high levels of mcl-1 expression when they were assayed separately. We also observed that some transgenic mice had enlargement of the spleen, with histochemical studies showing an expansion of white pulp. In vitro suspension cell culture of splenocytes from three 20 week old transgenic mice demonstrated a survival advantage over cells from control littermates. These findings suggest that mcl-1 expression in vivo can enhance the survival of selected cell types. The effects of mcl-1 in the transgenic mouse model in vivo therefore appear to be similar to those seen upon transfection of the gene into cell lines in vitro.

CHARACTERIZATION OF CRITICAL AMINO ACID RESIDUES OF BCL-X_L TO REPRESS SINDBIS VIRUS-INDUCED APOPTOSIS AND TO HETERODIMERIZE WITH OTHER BCL-2 FAMILY MEMBERS

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Sindbis virus, a prototype alphavirus, induces classic morphologic apoptosis in many cultured cell lines. Using Sindbis virus genome as a vector to express death regulatory genes, we devised a new system to test the ability of candidate genes to repress or accelerate viral-induced apoptosis. We have demonsatrated that Bcl-2, Bcl-x_L, and BHRF1 can delay viral-induced apoptosis . In contrast, Bax and Bak can accelerate viral-induced apoptosis.

Yin et al. have mapped the death repressor domain of Bcl-2 to the BH1 and BH2 regions by site-specific mutagenesis (Nature 369, 321-323). A model in which Bcl-2 must bind Bax in order to exert its activity was proposed because mutations that affected Bcl-2 function also disrupted its heterodimerization with Bax. We have identified critical amino acid residues required for Bcl-x_L to repress apoptosis and to interact with Bax by site-specific mutagenesis, which suggests that Bcl-x_L probably acts differently from Bcl-2. Correlation of the protective function of Bcl-x_L with its ability of heterodimerization with Bak is undertaken.

THE GENES THAT CONTROL PROGRAMMED CELL DEATH: FROM WORM TO MAMMAL. Robert Friedlander, Yong-Keun Jung, Masayuki Miura, Suyue Wang, Valeria Gagliardini, Louise Bergeron and Junying Yuan*
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The mammalian interleukin-1β converting enzyme is a homolog of *C. elegans* cell death gene *ced-3* product. Expression of the murine *Ice* induced programmed cell death. Expression of *crmA*, a cowpox virus gene encoding a specific inhibitor of ICE, in DRG neurons effectively blocks the cell death induced by trophic factor removal. We identified a homolog of ICE, named *Ich-1*. We found that *Ich-1* mRNA is alternatively spliced into two different forms. One mRNA species encodes a protein product of 435 amino acids, named ICH-1_L, which contains amino acid sequence homologous to both the P20 and P10 subunits of ICE as well as the entire CED-3 protein. The other mRNA encodes a 312 amino-acid truncated version of ICH-1_L protein, named ICH-1_S, that terminates 21 amino acid residues after the pentapeptide QACRG of ICH-1_L. We found that expression of *Ich-1*$_L$ and *Ich-1*$_S$ has opposite effects on cell death. Overexpression of *Ich-1*$_L$ induces programmed cell death, while overexpression of the *Ich-1*$_S$ suppresses Rat-1 cell death induced by serum deprivation.

Because inhibition of apoptosis may be an essential step in tumorigenesis, we investigated the interaction of various oncogene products with the ICE family. In an *in vitro* system, we found that adenovirus E1A-induced cell death can be suppressed by expression of *bcl-2* and *crmA*, suggesting that activation of the ICE family may be critical for E1A-induced cell death. Expression of simian virus 40 large T antigen (T ag) prevents both E1A- and the ICE family-induced apoptosis. The mutant T ag (K1), which is defective in pRb binding, has equal anti-apoptotic activity in rat fibroblasts as wild type T ag. p53, but not pRb or p107, antagonizes the effects of T ag suppressing ICE-induced cell death, but not ICH-1_L-mediated cell death. Thus, wild type p53 may potentiate ICE-induced apoptosis. Expression of a temperature sensitive mutant p53Val^{135} sensitizes COS cells to death induced by ICE at permissive temperature but not at non-permissive temperature. Reciprocal regulation of Bcl-2 and Bax proteins are evident in the cells expressing T ag. COS cells do not die when transfected with *Ice* or *Ich-1*$_L$. However, cotransfection of *bax* with *Ice* or *Ich-1*$_L$ into COS cells induces apoptosis effectively. Thus, the anti-apoptotic effects of T ag on ICE- and ICH-1_L-induced cell death pathway may result from modulation of the cell's susceptibility to death by regulating the relative ratio of effectors including Bcl-2 and Bax by p53.

APOPTOSIS IN IL-1ß CONVERTING ENZYME (ICE)-DEFICIENT MICE

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IL-1ß converting enzyme (ICE) cleaves pro-IL-1ß to generate mature IL-1ß. ICE is homologous to other proteins that have been implicated in apoptosis, including the products of C.elegans cell death gene *Ced3* , *Nedd-2/Ich1* , *Ich2* and *CPP32*. We generated ICE-deficient mice to elucidate the role of ICE in apoptosis. ICE-deficient mice developed normally, appeared healthy and were fertile. Post-lactation involution of mammary glands was normal in the female ICE-/- mice. There were no significant differences in peripheral blood counts and the percentages of various T cell subsets and B cells in freshly isolated thymuses, spleens and lymph nodes from nine week-old ICE-deficient mice compared to their age-matched wild-type littermates. The oldest mice (15 months) are phenotypically normal with no evidence of tumors.

Peritoneal macrophages from ICE-/- mice underwent apoptosis to a similar extent as cells from ICE+/+ mice in response to 5 mM ATP in vitro. Induction of apoptosis in vitro by 10^{-6} M dexamethasone, 5 Gy gamma irradiation or 18 h culture with medium alone (aging) in thymocytes from ICE-/- and ICE+/+ mice was similar. However, thymocytes from ICE-/- mice were resistant to apoptosis induced by anti-Fas antibody treatment in vitro as also reported by Kuida et.al [Science, *267* , 2000-2002 (1995)]. Our studies indicate that ICE is not critically involved in apoptosis during development. In the adult animal, however, ICE may be required for specific pathways of apoptosis induced by various stimuli. We will report on the role of ICE in Fas and granzyme B mediated apoptosis and its physiological relevance in lymphocyte function.

DEATH SUBSTRATE POLY(ADP-RIBOSE) POLYMERASE (PARP): ITS BIOCHEMICAL FUNCTIONS AND FATE DURING DNA REPAIR AND APOPTOSIS

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One of the early reactions occurring in the cell after DNA damage is the activation of poly(ADP-ribose) polymerase (PARP), which is a nuclear enzyme present abundantly among the higher eukaryotes. Activated PARP catalyzes the formation of a polymer of ADP-ribose on nuclear acceptor proteins, including PARP itself (automodification), topoisomerases and histones, resulting in altered functions of these proteins (1). PARP is known to participate in two major cellular responses to DNA-damage, i.e., recovery or death. It is implicated in DNA base excision repair (2,3). During the active phase of apoptosis, PARP is cleaved by some proteases of the ICE family and this cleavage is a biochemical marker of apoptosis (4,5).

We show here that transient overexpression of the partial cDNA of PARP representing its DNA-binding domain (DBD) causes not only the *trans*-dominant inhibition of PARP, but it also leads to apoptosis in COS-1 cells (where p53 is sequestered), and not in CV-1 cells (with wild type p53). The DBD which has two Zn^{2+} fingers, helix-turn-helix region and a nuclear localization signal, prevents activation of native PARP by competing for binding to DNA strand breaks. This overexpression was shown by others to reduce DNA base excision repair following damage by alkylating agent but not the nucleotide excision repair following damage by UVC. We also show that cellular recovery after oxidative DNA damage requires activity of PARP, and that cellular survival is completely blocked in presence of a potent chemical inhibitor of PARP.

We will also compare the cleavage pattern of PARP in the apoptotic cells and in vitro by the apoptotic proteases such as CPP 32.

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FADD, A NOVEL DEATH DOMAIN-CONTAINING PROTEIN, INTERACTS WITH THE DEATH DOMAIN OF FAS AND INITIATES APOPTOSIS

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The proximal signal transduction of Fas and the receptor for Tumor Necrosis Factor (TNF) has been enigmatic. The activation of these receptors is caused by aggregation mediated by the respective ligands or agonist antibodies. The signal is thought to be transduced by clustering of the intracellular domain, which encompasses a region which is significantly conserved in the Fas antigen as well as in TNFR-1. This shared "death domain" suggests that both receptors interact with a related set of signal transduction molecules that, thus far, remain unidentified.

Using the cytoplasmic domain of Fas in the yeast two-hybrid system, we have identified a novel interacting protein, FADD, which binds Fas and Fas-FD5, a mutant of Fas possessing enhanced killing activity, but not the functionally inactive mutants, Fas-LPR and Fas-FD8. FADD contains a death domain homologous to the death domains of Fas and TNFR-1. A point mutation in FADD, analogous to the *lpr* mutation of Fas, abolishes its ability to bind Fas, suggesting a death domain to death domain interaction. Overexpression of FADD in MCF7 and BJAB cells induces apoptosis, which, like Fas-induced apoptosis, is blocked by CrmA, a specific inhibitor of the interleukin-1ß converting enzyme (ICE). These findings, along with subsequent studies in our laboratory, implicate FADD in the proximal signal transduction of Fas.

THE APO-1(FAS/CD95)-MEDIATED DEATH-SIGNAL

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APO-1 (Fas/CD95), a member of the tumor necrosis factor (TNF) receptor superfamily induces apoptosis upon receptor oligomerization. The receptor and its ligand are important for apoptosis of peripheral T cells, for downregulation of an immune response and most likely, at least in part, also for peripheral T cell tolerance. In Aids, apoptosis mediated by this system might contribute to the depletion of T helper lymphocytes.

In a search to identify intracellular signalling molecules coupling to oligomerized APO-1 several cytotoxicity-dependent APO-1-associated proteins (CAP) were immunoprecipitated from the apoptosis-sensitive human leukemic T cell line HUT 78 and the lymphoblastoid B cell line SKW6.4. CAP1-3 and CAP4 instantly detectable after crosslinking of APO-1 were only associated with aggregated and not with monomeric APO-1. CAP1 and CAP2 were identified as phosphorylated MORT1 (FADD) [Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H. and Wallach, D. (1995) J. Biol Chem **270**, 7795-7798; Chinnaiyan, A.M., O'Rourke, K., Tewari, M. and Dixit, V.M. (1995) Cell **81**, 505-5122.] . Association of CAP1-4 with APO-1 was not observed with C-terminally truncated non-signalling APO-1. CAP1 and 2 did also not associate with an APO-1 cytoplasmic tail carrying the lpr^{cg} amino acid replacement. Moreover, no APO-1/CAP association was found in the APO-1^{+}, anti-APO-1 resistant pre B cell line Boe. CAP1-4 form a death-inducing signalling complex (DISC) with the APO-1 receptor and are, thus, the first APO-1 associating proteins of a signalling cascade mediating apoptosis.

A novel protein, FAF1, potentiates Fas-mediated apoptosis

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Fas, a member of tumor necrosis factor (TNF) receptor family, can induce apoptosis when activated by Fas ligand binding or anti-Fas antibody crosslinking. Genetic studies have shown that a defect in Fas-mediated apoptosis resulted in abnormal development and function of the immune system in mice. A point mutation in the cytoplasmic domain of Fas (a single base pair change from T to A at base number 786), replacing isoleucine with asparagine, abolishes the signal transducing property of Fas. Mice homozygous for this mutant allele (lpr^{cg}/lpr^{cg} mice) develop lymphadenopathy and a lupus-like autoimmune disease. Little is known about the mechanism of signal transduction in Fas-mediated apoptosis. In this study, we used the two-hybrid screen in yeast to isolate a Fas-associated protein factor, FAF1, which specifically interacts with the cytoplasmic domain of wild type Fas but not the lpr^{cg} mutated Fas protein. This interaction occurs not only in yeast but also in mammalian cells. When transiently expressed in L cells, FAF1 potentiated Fas-induced apoptosis. A search of available DNA and protein sequence data banks did not reveal significant homology between FAF1 and known proteins. Therefore, FAF1 is a novel protein that binds to the wild type but not the inactive point mutant of Fas. FAF1 potentiates Fas-induced cell killing, and is a candidate signal transducing molecule in the regulation of apoptosis. FAF1 is the first molecule cloned which can interact with the death domain of Fas, can potentiate Fas apoptotic signal and does not contain a death domain in the protein sequence. FAF1 may be a signalling molecule downstream of the death domain containing proteins during apoptotic signal transduction. Further study on the mechanism of FAF1 function during apoptotic signal transduction will give us more insight about this process.

THE RESCUE OF NOVEL DEATH GENES THAT MEDIATE RECEPTOR-INDUCED PROGRAMMED CELL DEATH.

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A functional approach of gene cloning, that is based on transfections with anti-sense cDNA libraries, was developed for the rescue of genes that function as positive mediators of cell death. The system was applied in epithelial cells that in response to interferon-γ undergo a type of cell death with cytological characteristics of programmed cell death. Three novel anti-sense cDNA fragments that reduced the susceptibility of the cells to the interferon-γ -induced cell death were isolated. The anti-sense RNA expression also protected the cells from APO-1/Fas-induced cell death. The full length sense cDNAs corresponding to the novel sequences were cloned, the deduced amino acid structure of the three proteins was determined, antibodies were raised and initial structural / functional analysis of the recombinant proteins was made. One of these genes, designated Death Associated Protein-1(DAP-1) codes for a basic proline-rich 15kDa protein that carries a potential SH3-binding motif. The second codes for a unique calcium/calmodulin-dependent serine/threonine kinase that was found localized to the cytoskeleton. This novel 160kDa kinase, (named DAP-kinase) carries 8 ankyrin repeats, 2 P-loop motifs and a region responsible for the cytoskeletal binding. In addition, this kinase possesses at the C-terminal end of the protein the "death domain"of the type present in Fas, p55 TNF receptor, and other recently cloned death proteins. The cytoskeletal localization may be functionally important since we found that specific cytoskeletal disruptions preceded the nuclear alterations during the interferon-γ - and the Fas/APO-1-induced apoptosis in epithelial cells. DAP-3 codes for a 1.7kb mRNA and the resulting 46kDa protein is an ATP/GTP-binding protein. The three DAP genes were found to be widely expressed in all the examined adult and embryonic tissues. We propose that the three novel DAP genes are novel positive mediators of programmed cell death.

CYCLOPHILINS: CATABOLIC COMPONENTS OF THE APOPTOTIC SYSTEM

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Nucleic acid degradation is a key component of apoptosis in all cells. Additionally, our laboratory has recently shown that RNA is degraded during apoptosis. Although the enzymes responsible for the nucleic acid fragmentation during apoptosis have yet to be conclusively identified, we have purified an 18 kD nuclease (NUC18) from apoptotic rat thymocytes whose activity correlates with the DNA and RNA degradation in these cells. Interestingly, NUC18 displays a remarkable sequence and structural similarity to the cyclophilin family of proteins. Cyclophilins were first identified as the intracellular receptors for the immunosuppressant drug Cyclosporin A. Cyclophilins and related peptides have since been observed in a wide variety of species and exhibit varied cellular localization. Following this surprising connection between NUC18 and cyclophilins, we examined cyclophilins for nuclease activity. Three different forms of purified cyclophilin (cyclophilin A, B, and C) demonstrate the ability to degrade a variety of nucleic acid substrates, including single- and double-stranded DNA, supercoiled DNA and RNA. Cyclophilin digestion of DNA results in an increase of 3' OH ends, corresponding to the types of DNA ends generated during apoptosis. Furthermore, a banding pattern is observed after electrophoresis of digested plasmid, implying that cyclophilins might have preferred sequences for binding and cutting the nucleic acid substrates. This notion is supported by electron microscopic analysis of cyclophilin B-DNA complexes. The images reveal clusters of cyclophilin B binding to specific sites along the strand of linearized plasmid. In addition, we have also shown that cyclophilin B can selectively degrade tRNA, suggesting that cyclophilin acts in the cytosol as well as the nucleus. Taken together, these data suggest that cyclophilin acts at multiple sites and stages during the apoptotic process.

CHARACTERIZATION OF APOPTOSIS INDUCED BY SUSPENSION CULTURE IN THE HUMAN PAPILLOMAVIRUS 16 POSITIVE CASKI CERVICAL CANCER CELL LINE

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Suspension culture of epithelial cells has been reported to be sufficient to induce apoptosis. The p53 and Rb genes play an important role in apoptosis, and because human papillomavirus (HPV) E6 and E7 proteins contribute to immortalization by interfering with these proteins, we have studied suspension culture-induced apoptosis in a variety of HPV-containing epithelial cell lines (J Cell Biochem, Supp19B; pp 312). Despite being positive for HPV 16, the Caski cervical cancer cell line undergoes apoptosis in cell suspension, and we therefore sought to characterize the mechanism of this process in Caski cells.

In each experiment, the cells were studied in suspension culture using tissue culture plates coated with poly-HEMA as well as in attached culture as a control. DNA was extracted from the cells by Triton X treatment and subjected to agarose gel electrophoresis. DNA fragmentation was detected with a terminal transferase reaction (ApopTag™). HPV E6 and E7 transcripts in the RNAs from both suspended and attached cells were detected by RT-PCR. Caski cells in suspension culture formed multicellular spheroids (MCS). ApopTag staining showed positive cells throughout the MCS. Light and transmission electron microscopic observation revealed typical apoptotic morphology such as nuclear condensation and chromosomal margination throughout the MCS in accordance with the ApopTag results. Time course experiments showed that DNA laddering of suspended Caski cells correlated with MCS formation. Cycloheximide treatment, treatment with antibodies to E-cadherin, and culture in calcium free medium each suppressed DNA laddering as well as MCS formation of Caski cells. In the attached culture control, a low level of DNA degradation was observed as background due to spontaneous apoptosis.

These results suggest that Caski cells undergo apoptosis by suspension culture despite active transcription of the HPV 16 E6 and E7 genes. Apoptosis in suspension culture is closely linked to MCS formation, which may be mediated in part by E-cadherin. E-cadherin is mutated or downregulated in some malignant tumors, and our data suggest that its possible role as a tumor suppressor may be through mediation of signals for apoptosis of abnormal cells. Further characterization of apoptosis in Caski cells, as well as in cell line resistant to apoptosis in suspension culture is currently underway.

CHARACTERIZATION OF THE FUNCTIONAL INTERACTION BETWEEN E1B 19K PROTEIN AND BAX.

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The E1B 19K protein is the adenovirus functional equivalent of Bcl-2. Multiple sequence alignment between E1B 19K and Bcl-2 family members indicates regions of homology in conserved amino acid residues and many of these sites are required to maintain 19K and Bcl-2 function. Both E1B 19K and Bcl-2 are functionally indistinguishable and cooperate with E1A to transform cells and block p53-dependent apoptosis, but not growth arrest. Since the anti-apoptotic function of E1B 19K protein works in the absence of other adenovirus proteins, it suggests that E1B 19K protein functions through interaction with cellular proteins.

The yeast two hybrid system was used to find E1B 19K binding proteins by screening a Hela cDNA library. One of the four clones which showed a strong interaction is Bax, a member of Bcl-2 family and a previously identified functional antagonist of Bcl-2 by direct interaction. Regions of Bax and 19K required for protein-protein interaction have been mapped and coincide with regions required for regulating cell survival. Bax and E1B 19K protein interacted in an *in vitro* coimmunoprecipitation assay. Immunoflourescence studies showed an overlapping *in vivo* staining pattern between the Bax and the E1B 19K protein. Overexpression of the Bax relative to E1B 19K suppressed the anti-apoptotic function of E1B 19K protein. Thus Bax antagonizes 19K function similar to Bcl-2, suggesting that 19K and Bcl-2 may function by interacting with the same cellular proteins to regulate apoptosis.

P53 STATUS AFFECTS THE RATE OF ONSET, BUT NOT THE OVERALL EXTENT OF CELL DEATH INDUCED BY DNA DAMAGE IN RAT-1 FIBROBLASTS WITH DEREGULATED C-MYC EXPRESSION

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Mutation of the p53 gene has been associated with tumorogenesis both in clinical and in experimental studies. Moreover, it has been recently proposed that p53 inactivation in tumor cells may cause their resistance to radiation and chemotherapy. Deregulated expression of proto-oncogene c-myc has been found in many tumors and is believed to play a major role in tumorogenesis. It has been recently demonstrated that c-myc can sensitize rat-1 fibroblasts (Rat-1 cells) to apoptosis upon serum withdrawal through a p53-dependent pathway. Futhermore, we found that constitutive c-myc expression in Rat-1 cells accelerates apoptosis induced by DNA damage (as measured by FITC dye exclusion test and DNA fragmentation). We examined, therefore, if this myc-mediated apoptosis by DNA-damage is dependent on the p53 status of the cells. Endogenous wild-type p53 activity has been suppressed by indroducing a full-length dominant negative p53 mutant gene into Rat-1 cells which constitutively overexpress c-myc (Rat-1^{myc} cells). The onset of cell death was significantly delayed in Rat-1^{myc} $p53^{-}$ cells as compared to Rat-1^{myc} cells. However, abrogation of wild-type p53 activity did not enhance long-term survival and clonogenic growth of Rat-1^{myc} cells after pre-treatment with a DNA damaging agent for 24 hours. These results show that abrogation of wild-type p53 activity by overexpression of a dominant negative p53 mutant in Rat-1 fibroblasts delays, but does not affect overall extent of c-myc-mediated apoptosis by DNA damage.

EVIDENCE FOR A G2-CHECKPOINT IN *p53*-INDEPENDENT APOPTOSIS INDUCTION BY X-IRRADIATION AND ITS REGULATION BY KU:DNA-PK. Zhiyong Han[1], Devasis Chatterjee[1], Dong Ming He[1], Janet Early[2], Panayotis Pantazis[2], James H. Wyche[1] and Eric A. Hendrickson[1]. [1]Department of Molecular Biology, Cell Biology and Biochemistry, Divison of Biology and Medicine, Brown University, Providence, RI 02912, [2]The Stehlin Foundation for Cancer Research at St. Joseph Hospital, Houston, TX 77003.

The tumor suppressor gene *p53* is thought to be required for the induction of programmed cell death (apoptosis) initiated by DNA damage. We show here, however, that the human promyelocytic leukemia cell line, HL-60, which is known to be deficient in *p53* due to large deletions in the gene, can be induced to undergo apoptosis following x-irradiation. We demonstrate that the decision to undergo apoptosis in this cell line appears to be made at a G2 checkpoint. In addition, we characterize an HL-60 mutant, HCW-2, which is radioresistant. HCW-2 cells display identical DNA damage induction and repair capabilities as the parental HL-60 cell line. Thus, the difference between the two cell lines appears to be that x-irradiation induces apoptosis in HL-60, but not in HCW-2, cells. Molecular analysis demonstrates that HCW-2 cells paradoxically no longer express the apoptotic suppressor gene, *bcl-2* and overexpress the apoptotic effector gene, *bax*. However, HCW-2 cells also overexpress *bcl-x* and this expression was transiently enhanced following x-ray irradiation. Therefore, the apoptosis induction in HL-60 cells and the radio- and apoptotic resistance shown by HCW-2 cells is due to the presence of a *p53*- and *bcl-2*-independent mechanism possibly mediated by *bcl-x* at a G2 checkpoint.

Since the Ku:DNA-PK complex is known to be critically involved in the cellular response to x-irradiation and in possibly mediating a G2 checkpoint we have characterized Ku and DNA-PK activities in HL-60 and HCW-2 cells. We demonstrate here that the HL-60 Ku DNA end-binding activity is altered such that it results in different salt and DNase sensitivities as well as an altered EMSA profile. Consequently, DNA-PK activity, which is dependent upon functional Ku, is greatly reduced in HL-60 cells. Finally, we show that the reduction in DNA-PK activity is responsible for the defective G2 checkpoint in HL-60 cells. Thus, we have demonstrated a role for Ku and DNA-PK in a G2-checkpoint and suggest that this repair activity may be important in regulating a cell's decision to undergo apoptosis following DNA damage.

BAX AND BCL-X_S ARE INDUCED AT THE ONSET OF APOPTOSIS IN INVOLUTING MAMMARY GLANDS

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Involution of the mammary gland is characterized by apoptotic death of epithelial cells and the collapse of lobuloalveolar structures. Few apoptotic figures were observed in mammary epithelium of lactating mice. After weaning the number of apoptotic cells increased sharply and reached a maximum on day 3. Active cell death continued until day 5 after weaning. Only little parenchyma remained on day 8, when remodelling of the gland was completed.

bcl-x and bax are members of the bcl-2 family and known as regulators of apoptosis. Low levels of bcl-x and bax mRNAs were expressed during lactation. They increased rapidly during the first 2 days of involution, suggesting a role in the regulation of cell death in mammary epithelium. Different mRNAs of bcl-x have been reported due to differential splicing. A short form, bcl-x_S, can induce cell death, and a long form, bcl-x_L, has a protective function. Using RT PCR, we distinguished these 2 mRNAs. The ratio of bcl-x_S *vs.* bcl-x_L remained unchanged in the virgin, pregnant and lactating gland. However, it changed during the first 2 days of involution, when bcl-x_S expression increased 6-fold more than bcl-x_L. These findings point to a role of bax and bcl-x_S in predisposing mammary alveolar cells to PCD imediately after completion of lactation.

APOPTOTIC CELL DEATH IN HUMAN ATHEROSCLEROSIS

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Comparative studies of human atherosclerosis between underdeveloped and industrialised societies show that the pattern of lesions differs between the two. The former develops early lesions (fatty streaks) just as much as developed populations but have a much diminished incidence of potentially fatal complications. Most characteristic of the advanced lesion is the core, which contains lipids, oxidised lipids along with cell debris which is, at least partly, macrophage-derived.

The cause of cell death in human atherogenesis is still not known. Oxidised low density lipoprotein (LDL) is a potential candidate and it is found to be toxic for macrophages *in vitro* resulting in apoptosis. Certain oxidation products of cholesterol which are known to be toxic are found in lesions during atherogenesis.

We began to investigate the mode of cell death *in vivo* using surgically excised samples of human atherosclerotic lesions. Several methods were used including terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) method, confocal laser microscopy and transmission electron microscopy (TEM).

Apoptotic cells were found in human atherosclerotic lesions in addition to necrosis, identified by TEM and by TUNEL method at the level of light and electron microscopy. Cell death was most prevalent at the boundaries of the largely acellular core of the lesion suggesting that the potential damage to the cells was greatest in this area. Confocal laser microscopy showed that some TUNEL positive cells also expressed proliferating cell nuclear antigen (PCNA).

We propose that oxidised LDL-induced apoptosis may have a role in atherogenesis. It is well known that the severity of insult determines the route of cell death. A mild insult may cause apoptosis and a severe insult can lead to necrosis. Apoptosis may also be involved in the regulation of cellularity of the lesion. The exact mechanisms involved in human atherogenesis merit further investigation.

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SEGMENT-SPECIFIC, STEROID-MEDIATED DEATH OF IDENTIFIED INSECT MOTONEURONS IN CELL CULTURE. K.L. Hoffman, L.C. Streichert, J.T. Pierce and J.C. Weeks. Institute of Neuroscience, University of Oregon, Eugene OR 97403-1254.

During metamorphosis of the hawkmoth, *Manduca sexta*, a class of steroid hormones, the ecdysteroids, regulates the developmental death of motoneurons at stage transitions. At the larval-pupal transformation, a stereotyped subset of motoneurons that innervate larval proleg motoneurons die. In larvae, each accessory planta retractor muscle, APRM, is innervated by a pair of motoneurons, the APRs. At pupation, the APRs die in a segment-specific pattern: APRs in abdominal segments 5 and 6 [APR(5)s and APR(6)s] die while APR(3)s and APR(4)s survive and are respecified for new pupal/adult roles. Experimental manipulations of the APRs' interactions with pre- and postsynaptic cells *in vivo* fail to perturb the segmental pattern of death, suggesting that ecdysteroids act directly on the APRs.

We tested this hypothesis by isolating APRs from the nervous system and examining their fates in culture. APR(4)s and APR(6)s were retrogradely labeled *in vivo* with the fluorescent dye, DiI, dissociated from ganglia and placed in low-density cell culture in serum-free medium. In one study, APRs were placed in culture on the day prior to the onset of the prepupal peak of ecdysteroids, which normally triggers their death *in vivo*. The cells were photographed every 2-3 days and scored as dead if they fragmented. Addition of 20-hydroxyecdysone (20-HE) to the medium caused APR(6)s to die in a dose-dependent manner whereas APR(4)s survived. Thus, APRs respond to 20-HE with the correct segment-specific pattern of death *in vitro*, consistent with their being direct targets of ecdysteroid action. In another study, APR(4)s and APR(6)s were cultured on the day of pupal ecdysis, which is after the prepupal peak and one day before the APR(6)s normally die *in vivo*. 20-HE was not added to the medium. APR viability was assessed at 0, 24 and 48 hr by a number of staining methods. Preliminary data indicate that the APR(6)s carried out the death program *in vitro* while the APR(4)s survived. These findings suggest that, by pupal ecdysis, the APR(6)s are committed to die and that the death program occurs *in vitro* over the same time course as *in vivo*. Experiments are underway to investigate the biochemical and molecular events underlying APR death, and its segmental specificity.

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THE ROLE OF P53 IN TUMOR GENOMIC INSTABILITY, APOPTOSIS AND RESPONSE TO CHEMOTHERAPY. Jeff E. Hundley[1], Dean A. Troyer[2], Steve K. Koester[4], Susan G. Hilsenbeck[3], and Jolene J. Windle[1,4]. [1]Dept of Cellular and Structural Biology; [2]Dept of Pathology; [3]Dept of Medicine/Oncology, University of Texas Health Science Center at San Antonio; and [4]Cancer Therapy and Research Center, San Antonio, TX.

We have established an in vivo model in which to evaluate the role of the p53 tumor suppressor gene in various aspects of tumor progression. This model involves interbreeding MMTV-ras transgenic mice[1] (which reproducibly develop mammary and salivary tumors) and p53 "knock-out" mice[2], in order to generate animals which develop tumors that differ in their p53 functional status. The tumors arising in the p53-deficient background differ from those arising in a p53+/+ background in a number of ways, including tumor type distribution, age of onset, and chromosomal content. While somewhat greater than half of the tumors arising in ras\p53+/+ mice are mammary in origin, nearly all of the tumors in the ras\p53-/- mice are salivary, resulting from an acceleration of salivary tumor onset (mean age = 4.6 mth for ras\p53+/+, 2.6 mth for ras\p53-/-). Preliminary results suggest that tumors arising in p53-/- mice have a higher S-phase fraction, consistent with a role for p53 in mediating growth arrest at the G1/S boundary. We also expected that tumors arising in the p53-/- background would exhibit lower levels of spontaneous apoptosis, since p53 has been shown to mediate apoptosis in response to oncogene-induced cell cycle perturbations. Surprisingly, however, tumors in both the p53+/+ and -/- backgrounds have low levels of apoptosis (<1%), with slightly higher levels in the p53-/- tumors. This result is consistent with the finding that activated ras expression renders cells less susceptible to apoptosis in certain experimental situations. We are therefore investigating the possibility that tumors arising in MMTV-myc transgenic mice will exhibit higher levels of spontaneous apoptosis, since myc expression has been shown to sensitize cells to apoptosis.

We have also compared the DNA content of tumors arising in the p53+/+ and -/- backgrounds by flow cytometric analysis, and find that the p53-/- tumors are more frequently aneuploid and generally exhibit a higher degree of intratumor heterogeneity, consistent with the hypothesis that p53 plays a role in maintaining genomic integrity.

Finally, we are investigating the role of p53 as a determinant of tumor response to chemotherapy. While ras\p53+/+ tumors respond immediately and efficiently to adriamycin treatment, ras\p53-/- tumors are consistently unresponsive until about the fifth or sixth day of treatment, at which time their growth begins to slow considerably. This delayed response appears to be associated with an impaired ability to undergo adriamycin-induced apoptosis. Thus, there are p53-independent mechanisms of tumor response to adriamycin, but they are less efficient or sensitive than those mediated by p53. In contrast, taxol appears to act by p53-independent mechanisms, since p53-/- tumors respond efficiently to this agent. These results have important implications with regard to p53 function during tumorigenesis and progression, and provide evidence that p53 status represents an important determinant of tumor response to certain forms of anticancer treatments.

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Control of Fas Ligand expression and activation-induced apoptosis of T cell hybridomas by NF-κB p65-p50. Vladimir N. Ivanov*, Richard R. Lee, Eckhard R. Podack and Thomas R. Malek. Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL 33101, USA.

TCR-mediated activation of mouse T cell hybridomas induces programmed cell death (activation-induced apoptosis) by induction of Fas Ligand (Fas L) and Fas expression with subsequent Fas-dependent cell death signaling. To test the hypothesis that the NF-κB p65-p50 transactivator is directly involved in the activation-induced apoptosis of the 2B4 T cell hybridoma, 2B4 was treated with anti-CD3ε mAb alone or in the presence of forskolin, an activator of cAMP-dependent signaling. cAMP signaling is a negative regulator for the release of NF-κB p65-p50 from the cytoplasmic complex with inhibitor IκB *in vivo*. Activation-induced apoptosis was significantly suppressed by forskolin. A profound decrease in nuclear levels of NF-κB p65-p50 was detected after costimulation with forskolin prior to apoptotic commitment. Furthermore, cAMP-dependent signaling inhibited Fas L and Fas expression in 2B4, as shown by Northern hybridization and flow cytometry. To further investigate the involvement of NF-κB p65-p50 in control of Fas L expression and regulation of apoptosis, NF-κB translation was suppressed by use of a specific antisense oligonucleotide that resulted in inhibition of Fas L expression and decreased levels of cell death. These observations indicate a crucial role of NF-κB transactivators in the regulation of activation-induced apoptosis.

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ISOLATION AND IDENTIFICATION OF PROTEINS INTERACTING WITH *NUR77* USING THE YEAST-TWO-HYBRID SYSTEM

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nur77 belongs to the family of immediate-early genes and shows high sequence homologies to members of the steroid/thyroid hormone receptor superfamily, which are characterized by a highly conserved zinc-finger DNA-binding domain and a carboxy-terminal ligand-binding domain. Since there is no specific ligand identified yet, *nur77* belongs to the class of orphan receptors. Previous studies performed in our laboratory showed that *nur77* is required in the process of activation-induced apoptosis of immature thymic T-cells (Liu et al., 1994). In order to elucidate the signal transduction pathways involving *nur77* which result in programmed cell death of immature T-cells, we choose the strategy of cloning and isolating proteins interacting with *nur77* in the yeast-two-hybrid system. This system allows the detection of protein-protein-interactions by the functional activation of the prokaryotic transcription factor LexA. Only yeast transformants containing interacting protein components are capable in activating the expression of a lacZ reporter gene and a blue/white screening procedure is possible. In our approach we constructed a transcriptional inactive, deleted version of the transcription factor *nur77* and fused it to amino acids 1 to 202 of LexA, which contain the DNA-binding domain and dimerization domains (Gyuris et al., 1993). This "bait" was used to screen a mouse T-cell lymphoma cDNA library, in which cDNA fragments are fused to the activation domain of the yeast transcription factor GAL4. After screening of at least $6x10^6$ transformants 61 positive, blue colonies were obtained. Grouping these positive clones into related families, isolation and sequencing of the cDNA fragments and the identification of these potential interactors of the immediate-early gene *nur77* are underway and will lead to a more detailed characterization of these proteins.

FOCAL APOPTOSIS IN OLEIC ACID LUNG INJURY. Jeffrey A. Kazzaz[1], Jing Xu[1], Alan M. Fein[1], Poonam Khullar[2], Allan L. Schuss[2], Stephen Hall[3], Bruno Piedboeuf,[4] Stuart Horowitz[1]. [1]CardioPulmonary Research Institute and [2]Department of Pathology Winthrop-University Hospital SUNY Stony Brook School of Medicine Mineola, NY, [2]HHMI-University of Washington Seattle, WA, [3]Pulmonary and Critical Care Medicine OHSU, Portland, OR. [4]Laval University CHUL, Ste. Foy Quebec, Canada.

Intravenous administration of oleic acid is used in animals as a model of acute lung injury (ALI), which in rabbits is manifested by an influx of polymorphonuclear leukocytes, proteinaceous edema, surfactant inactivation, and impaired pulmonary function. This acute injury is rapid and occurs within 1 hour. Histologic examination of the lungs shows generalized edema, and a few focal patches of severe injury found in peripheral regions, which are characterized by thickened interstitium, hemorrhage, and microthrombi. We were interested in determining whether apoptosis, or programmed cell death (PCD) was occurring in oleic acid-injured lungs. We employed the TUNEL assay (terminal transferase dUTP nick end labeling), to identify apoptotic nuclei *in situ*, in tissue sections. Apoptotic nuclei were rare in untreated and saline-control lungs, and in the relatively undamaged regions of oleic acid-injured lungs. However, apoptosis was evident in all the patches with histologic evidence of injury, with a 1:1 correlation between the two. To ensure that the TUNEL positive cells were still alive, we performed *in situ* hybridizations for tissue inhibitor of metalloproteinases (TIMP) mRNA on serial section. The same TUNEL-postive patches also had abundant TIMP-1 transcripts. These data indicate that apoptosis is a feature of ALI, and its location and severity may influence eventual outcome.

APOPTOSIS IN MODELS OF RESOLVING AND NON-RESOLVING PNEUMONIA. Jeffrey A. Kazzaz[1], Jing Xu[1], Poonam Khullar[2], Allan L. Schuss[2], Alan M. Fein[3], Gregory C. Rhodes[4], Angelina Enno[5], Stuart Horowitz[1]. [1]CardioPulmonary Research Institute, [2]Department of Pathology, [3]Division of Pulmonary and Critical Care Medicine Winthrop Univ. Hosp., Mineola, NY 11501; [4]Smith and Barrett Pathology Labs, Orange County, Australia; [5]Department of Pathology, University of New South Wales, New South Wales, Australia.

Recent evidence suggests that apoptosis or programmed cell death (PCD) plays a role in acute respiratory distress syndrome and recovery from lung injury during removal of granulomatous tissue. We were interested in learning if PCD is a feature that distinguishes the resolution of pneumonia from the progression to fibrosis. We utilized rat models of streptococcal pneumonia. Instillation of *Strep. sanguis* or *Strep. pneumonia* type 25 induces acute pneumonitis in rats. However, rats infected with *S. sanguis* recover after 4 days and at 8 days the lungs appear normal ultrastructurally, with the exception of an abscess that forms in the extreme base of the lung. In contrast, *S. pneumonia* causes the rats to remain sick and after 8 days fibrosis is clearly evident. We utilized the TUNEL (terminal transferase dUTP nick end-label) technique to determine the extent of PCD in sections of lungs taken at various time points postinfection ranging from 8 hours to 8 days. We found little evidence of PCD at the 8 hour or 1 day time points when rats were infecyed to either bacterial strain. By day 2 however, the models diverged in their patterns. In the resolving model, the apoptotic nuclei were localized to the abcess area and the transition area between the abcess and resolved areas. In the fibrotic model apoptotic nuclei were found in a variety of cell types at day 2 and PCD was widespread by day 8. The cell types affected include epithelial cells, type 2 pneumocytes of alveoli, presumptive fibroblasts, and macrophages. From these data, we conclude that PCD is correlated with the failure to resolve and progession to fibrosis following streptococcal pneumonia.

FUNCTIONAL ASSAY SYSTEM FOR THE IDENTIFICATION OF TUMOR SUPPRESSOR GENE(S) ON HUMAN CHROMOSOME 3.

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Cytogenetic analysis and loss of heterozygosity (LOH) studies have indicated that genes within the short arm of human chromosome 3 (chr3), essentially in the regions 3p14, 3p21-p22 and 3p25, are involved in the development and/or progression of many types of solid tumors. Recently, homozygous deletions have been localized at the 3p21 region (in the D3S2 locus) for one malignant breast tumor, at the region 3p22-p21.3 in 5 small cell lung carcinoma (SCLC) cell lines, and at the region 3p21.3-p21.2 in one SCLC cell line. The introduction of human chr3 into renal cell carcinoma (RCC), lung adenocarcinoma, ovarian carcinoma, the mouse fibrosarcoma A9 (MF A9) cell lines as well as the introduction of the short arm of chr3 and the 3p14-p12 band into RCC cell lines, and the presence of 2Mb of DNA from the 3p22-p21 region into MF A9 cell line resulted in the suppression of tumorigenicity.

Our previous results indicated that loss /or absence of the 3p24-p21.3 region of human chr3 from mouse human microcell hybrids (MCH) facilitated tumor outgrowth in SCID mice and therefore suggested the presence at least of one tumor suppressor gene within this region. Fluorescent *in situ* hybridization (FISH) of these tumors have revealed that intact versions of chr3 were regularly eliminated from 2 MCH lines. Only translocated fragments were maintained, as were chromosomes with deleted segments: 3p24-p14; 3q21-q26, 3p22-p14 and 3pter-p21.3. Control MCH lines carrying human chr8 and human chr1, maintained these chromosomes in intact form in all tumors examined so far.

Subsequently, we have tested the mouse-human hybrid containing complete human chr3 in intact form and translocated fragments of chr3 within mouse chromosomes for tumorigenicity in SCID mice. In order to characterize the effect upon tumorigenicity of the loss of various chromosomal segments, 36 human chr3 specific PCR primers were used. The 3q-arm was found in 5 explanted tumors whereas 20 markers distributed along short arm of chr3 were not present. A small fragment from the region 3p21.3-p21.2 (including GNAI2 and D3F15S2 loci), localized between markers D3S1076 and D3S966 was maintained in 2 out of 5 tumors tested.

Therefore, we have identified a small fragment that overlaps the homozygous deletions reported in the literature. Taken together these results indicate that tumor suppressor gene(s) resides telomeric to the D3F15S2 locus within a region between the D3F15S2 and D3S1611, GLB1 markers.

HUMAN RIP REVEALS STRUCTURAL AND FUNCTIONAL HOMOLOGIES TO THE MURINE FAS-RECEPTOR INTERACTING PROTEIN (RIP)

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Recently, we reported the isolation of a novel protein, RIP, that associates with the intracellular domain of FAS/APO-1 (CD95) and more weakly with the p55 tumor necrosis factor receptor (TNFR1). The murine form when overexpressed causes transfected cells to undergo morphological changes characteristic of apoptosis. Here we report the coding sequence of a human RIP cDNA. A specifically primed Daudi library was screened and two overlapping cDNAs of a combined length of 680 bp were isolated. Two additional fragments of 280bp and 190 bp were obtained by PCR of the enriched Daudi library using primers based on cDNA sequences derived from dBEST, the NCBI Expressed Sequence Tag Database, and an N-terminal sequence derived from murine RIP. Together the cDNAs constitute a single open reading frame of 2 kB that shows 71% identity to the murine sequence with the highest degree of homology in the carboxy terminus, the region which interacts with FAS in yeast. Human RIP interaction with the cytoplasmic tail of FAS using the yeast two hybrid system is abrogated by a nucleotide substitution based on the murine lpr^{cg} mutation. Human RIP contains an N-terminal region with extended homology to protein kinases and a C-terminal region containing a cytoplasmic motif (death-domain) present in the FAS and TNFR1 intracellular domains. Although we have yet to identify a substrate on which RIP exerts substantial phosphotransferase activity, the high degree of conservation between species in the kinase domain suggests it may be a functionally important unit.

IDENTIFICATION AND CHARACTERIZATION OF SIGNAL TRANSDUCERS ASSOCIATED WITH THE CYTOPLASMIC DOMAIN OF FAS: J. Kim[1], B.-Q. Li[2], M. Subleski[2], H.-F. Kung[1] and T. Kamata[2], [1]LBP, NCI-FCRDC, Frederick, MD 21702-1201. [2]Biological Carcinogenesis and Development Program, S.A.I.C./Frederick, NCI-FCRDC, Frederick, MD 21702-1201

The Fas antigen is expressed on the surface of various normal and neoplastic cells. The Fas-mediated cell death pathway has been implicated in the T-cell antigen receptor-activation-induced cell death. Mice carrying a point mutation in the cytoplasmic domain of Fas develops a lupus-like lymphoproliferative autoimmune disorder (lpr). These observations suggest an important role for Fas in immune system homeostasis. The molecular mechanism by which Fas transduces cell death signals to a downstream effector(s) reamins unknown.

In order to study the molecular basis for Fas-mediated cell death signal transduction, we attempted to identify and characterize a Fas cytoplasmic domain-binding protein (FBP). GST or GST fusion proteins containing the cytoplasmic domains of wild type mouse Fas and Fas mutants were expressed in *E. coli*, and the fusion proteins immobilized on glutathione-sepharose beads were incubated with detergent-solubilized lysates from [^{35}S]methionine-labeled T-cell hybridoma 2B·4 and HBC cells. In both cell lines, a 35 kDa protein was found predominantly associated with GST-wild type Fas (GST-Fas) but not GST alone. A similar pattern of protein binding was detected with lysates from Jurkat T-cells. Activation of 2B·4 cells with PMA plus inonomycine or anti-CD3 antibody, which induce the expression of Fas ligand and subsequent cell death, had no effect on the binding of 35 kDa FBP to GST-Fas, suggesting their constitutive interactions. When cell lysates were incubated with the GST-fusion protein containing the cytoplasmic domain of functionally inactive FD7, a Fas mutant lacking the COOH-terminal 30 amino acids (GST-FAS FD7), no interaction was detected. Interestingly, 35 kDa FBP strongly bound to GST-Fas FD5, which is a 15 amino acid C-terminal deletion mutant of Fas with enhanced cell killing activity. These results suggest that 35 kDa FBP may be involved in Fas signaling. At least, the FBP was distinct in molecular mass from RIP (74 kDa), FADD (23 kDa) and MORT1 (27 and 28 kDa) Fas death domain-interacting proteins that have recently been identified by yeast two hybrid systems. Molecular cloning of the gene encoding the FBP is in progress.

APOPTOSIS INDUCED IN HUMAN AND RAT GLIOMA CELLS BY INTRONLESS MYC GENE EXPRESSION DOES NOT REQUIRE WILD-TYPE P53 GENE EXPRESSION

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Mammalian cells appear to contain at least one copy of the intronless *myc* gene such as the s-*myc* gene in rodents and the *myc* L2 gene in primates. The s-*myc* and *myc* L2 genes are expressed in rat embryo chondrocytes and human adult testis, respectively, where physiological apoptotic cell death is considered to take place. Recent reports have indicated that expression of wild-type p53 is required for susceptibility to c-Myc-mediated apoptosis. In contrast, s-Myc or MycL2 could effectively induce apoptotic cell death when overexpressed in human and rat glioma cells which express only mutated *p53* gene and has deletion of the *p16* gene. In addition, both intronless *myc* gene expression arrested the glioma cells in the G1 phase of the cell cycle in a manner independent of wild type *p53* gene expression. Both of s-Myc and MycL2 could interact with Max and influence the transcriptional level through the CACGTG core sequence. s-Myc activated transcription through this motif and its DNA binding was required for apoptosis induction in glioma cells, while MycL2 could repress transcription activated by c-Myc. These results suggest that expression of the intronless *myc* genes may induce apoptosis through distinct signal transduction pathways from those of c-Myc-mediated apoptosis.

COMPARATIVE STUDIES ON DELAYED LETHAL MUTATION IN SYNGENEIC HUMAN LYMPHBLASTOID CELL LINES DIFFERING IN p53 STATUS AND SUSCEPTIBILITY TO APOPTOSIS

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We have utilized human lymphoblastoid cell lines derived from the same donor that differ in p53 status and initial sensitivity to the cytotoxic effects of ionizing radiation to explore the interaction of the pathways for programmed cell death with the expression of delayed lethal mutation. The two cells lines compared are each heterozygotes at the tk locus and hemizygous at the hprt locus. The TK6 cell line is wild-type for p53 expression, while the WTK1 cell line has mutations in both p53 alleles. Our preliminary studies demonstrate a differential sensitivity to X-ray-induced cell killing for the two cell lines that varies dramatically with time post-irradiation. TK6 cells recover more rapidly from the cytotoxic effects of ionizing radiation, while the WTK1 cells show marked cytotoxic effects over extended periods of time (at least 12 generations post-irradiation). Susceptibility to X-ray-induced specific locus mutation also differs for these two cell lines. The p53- WTK1 cells are more mutable than are the p53+ TK6 cells. The more marked difference is observed for the autosomal tk locus. As it is known that several of the bcl-2 family members are known to be p53 responsive, we are presently pursuing studies to examine the time course for induction of bcl-2, bcl-X_L, bcl-X_S, and bad as a function of time post-irradiation in both cell lines. In order to independently evaluate the role of apoptosis control proteins in the time course and severity of X-ray-induced delayed lethal or specific locus mutations, we have constructed a series of transformants of TK6 and WTK1 that constituitively overexpress bcl-2 or bcl-X_L. Clones that express high levels of bcl-2 or bcl-X_L mRNA and protein are now being tested for susceptibility to X-ray-induced cytotoxicity and mutagenesis.
These studies are supported by NIH grant CA-62364 to A. Kronenberg.

Bcl-2 EXPRESSION REGULATES SODIUM BUTYRATE-INDUCED APOPTOSIS IN HUMAN BREAST CANCER CELLS.
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Sodium butyrate (butyrate) is a potent growth inhibitor and differentiating agent for many cell-types including breast cancer cells. Programmed cell death, or apoptosis is a physiological mechanism of cell death that is dependent on both pre-exiting proteins and *de novo* protein synthesis. In the studies presented here we explored the role of apoptosis in growth regulation of human breast cancer cells by butyrate. We report that butyrate treatment of breast cancer MCF-7 cells causes non-reversible growth inhibition by inducing apoptosis in a time- and dose-dependent manner, as measured by quantitative DNA damage ELISA. As little as 12 h treatment with butyrate can cause 5.6 fold induction in apoptotic cell death which continued to increases up to 27 fold by 48 h post-treatment. The observed butyrate-induced apoptosis in MCF-7 cells was closely associated with the down-regulation of expression of Bcl-2 mRNA and Bcl-2 protein, a gene product known to be involved in regulation of apoptosis in mammalian cells. The observed relationship between down-regulation of Bcl-2 and induction of apoptosis was not causal as stable overexpression of Bcl-2 in MCF-7 cells resulted in protection of MCF-7 cells from the cytotoxic morphological changes and growth inhibitory effects of butyrate (15% growth inhibition as opposed to 60% growth inhibition in the parental cells). In addition, Bcl-2 overexpressing MCF-7 cells showed significant suppression of butyrate-induced stimulation of apoptosis (5 fold compared to 27 fold in parental MCF-7 cells by 48 h post-treatment). In addition, butyrate also potentiated chemosensitivity of MCF-7 cells to the adriamycin. These findings demonstrate that butyrate-induced apoptosis in breast cancer cells is mediated by down-regulation of Bcl-2, and that butyrate may be useful in potentiating the chemotherapy-induced apoptosis in breast cancer cells.(Supported by the AICR Grant # 94B93).

TRANSCRIPTION OF THE BRAIN CREATINE KINASE GENE (CKB) IN GLIAL CELLS IS INCREASED BY cAMP-DEPENDENT PROTEIN KINASE, CHOLERA TOXIN OR PROSTAGLANDIN E_1 and E_2.

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Creatine kinases (CK) catalyze the reversible transfer of a high-energy phosphate group between creatine phosphate and ADP to regenerate ATP in cell types which rapidly expend ATP. Previous reports suggest that in *some cell types* CKB may play a role in the cell cycle since (i) the CKB protein is associated with mitotic spindles; (ii) CKB expression is high in tumors from different cell types many of which contain mutations in p53 alleles; (iii) transcription of CKB is repressed in some (but not all) cell types when cotransfected with a plasmid expressing wt p53 (Mol. Cell. Biol. **14**:8483-8492 [1994]).

We have previously shown in *primary* cell cultures from rat brain that CKB mRNA levels are high in oligodendrocytes and astrocytes and low in neurons (J. Neurochemistry **59**: 1925-1932 [1992]) and that a forskolin-mediated increase in cAMP significantly activates CKB transcription by a pathway involving protein kinase A (PKA) (J. Neuroscience Res. **39**: 70-82 [1994]). It has been suggested that CKB regenerates ATP needed by oligodendrocytes during myelinogenesis and by astrocytes for ion transport.

We were interested in identifying possible physiological ligands which might activate CKB transcription in vivo during brain development and also to determine if G-proteins were involved in this activation. Therefore, we show that either PGE1 or PGE2 or cholera toxin (an activator of Gαs proteins) activate CKB transcription in human U87 glioblastoma cells. This activation occurs rapidly (within 6 hrs) and requires the activity of PKA but not protein kinase C (PKC). These results suggest that in glial cells CKB mRNA can be regulated by extracellular signals acting through G-protein-coupled receptors. The results are also discussed with regard to (i) the possible mechanism by which the expression of CKB is increased during postnatal rat brain development to meet changes in energy requirements and (ii) the increased production of prostaglandins and (possibly) CKB in cells under the stress of hypoxia and ischemia.

Also, we show in U87 cells that the forskolin-mediated increase in CKB transcription is mediated through sequences located within 188 bp of the CKB promoter and *does not* involve the two consensus CRE elements located in intron 1 and intron 2 of CKB.

A TRAF RELATED GENE IS A CANDIDATE GENE FOR THE *AMNIONLESS* MUTATION, A GASTRULATION-STAGE MOUSE MUTATION, ASSOCIATED WITH EXCESS, REGIONALLY LOCALIZED CELL DEATH. E. Lacy, S.X. Wang, E. Bornslaeger, C. Tomihara-Newberger, O. Haub, H.G. Lee. Molecular Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, NY 10021.

Amnionless (amn) is a recessive prenatal lethal insertional mutation that was generated by the integration of an injected human CD8a transgene. Mutant embryos become arrested in their development during gastrulation, between 7-9 days of gestation. The most obvious morphological defects in the mutant embryos are the absence of an amnion, lack of node and distal streak functions, and growth retardation. Proximal streak functions and differentiation of extraembryonic ectoderm derived structures, however, appear largely intact. An initial investigation into the contribution of cell death to the observed growth defects in the *amn* mutant found excess apoptotic cell death localized specifically to the anterior distal region of the embryonic ectoderm. This region is predicted by the early gastrula fate maps to give rise to neuroectoderm and amnion ectoderm, two tissues lacking in the *amn* mutant. This observation raises the possibility that the *amn* mutant embryo is lacking in the delivery or reception of a signal needed to coordinate the survival, proliferation, and differentiation of these cells. In the absence of this signal, the neuroectoderm and amnion ectoderm precursor cells undergo programmed cell death.

One intriguing candidate for the *amn* gene has already been identified at a junction between the transgene and flanking host DNA. This gene encodes a 567 amino acid protein, which shares homology with Tumor Necrosis Factor (TNF) Receptor Associated Factors 1 and 2 (TRAF1, TRAF2). TRAF1 and TRAF2 define a new family of putative signal transducing proteins that associate with the cytoplasmic domains of members of the TNF receptor superfamily. Thus we have named this gene TRAF*amn*. More recent data have shown that TRAF*amn* is identical to a protein (CD40bp, LAP1, CRAF) that binds the cytoplasmic domain of CD40, a member of the TNFR superfamily that controls the survival, proliferation and differentiation of B cells in germinal centers. Northern analysis showed that TRAF*amn* is transcribed in ES cells and throughout gastrulation, and RNase protection analysis of total RNA from E7.5 mutant and wildtype embryos demonstrated that TRAF*amn* gene expression is completely disrupted in the mutant embryos. Our observations that TRAF*amn* is expressed in wildtype but not in mutant embryos during gastrulation and that the *amn* mutant is defective in the survival, proliferation and differentiation of embryonic ectoderm, make TRAF*amn* an intriguing candidate for the *amn* gene.

BCL-2 INHIBITS OXYGEN RADICAL-MEDIATED ER CALCIUM POOL DEPLETION. Minh Lam, Thomas S. McCormick, Clark W. Distelhorst. Departments of Medicine and Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106.

The endoplasmic reticulum (ER) is the major intracellular reservoir of calcium ions (Ca^{2+}) in non-muscle cells. ER Ca^{2+} sequestration is essential for intracellular signaling, translation, protein processing and cell growth. Recent findings in this laboratory indicate that ER Ca^{2+} pool depletion induces Bcl-2-regulated apoptosis in WEHI7.2 mouse lymphoma cells (PNAS 91:6569, '94). Bcl-2, which has been proposed to inhibit apoptosis by an antioxidant mechanism, is localized to intracellular membranes including the ER. Because the Ca^{2+} sequestering activity of the ER is a prime target of oxidative damage, we investigated the proposed antioxidant mechanism of Bcl-2 action by assessing the effect of hydrogen peroxide (H_2O_2) treatment on ER Ca^{2+} sequestration in WEHI7.2 cells, which do not express Bcl-2, and in two stable WEHI7.2 transfectants, W.Hb13 and W.Hb12, which express low and high levels of Bcl-2 respectively. ER Ca^{2+} pool filling was determined by measuring the amount of Ca^{2+} released from the ER by thapsigargin (TG), a selective ER Ca^{2+}-ATPase inhibitor. In WEHI7.2 and W.Hb13 cells, H_2O_2 treatment produced a significant decrease in the TG-mobilizable ER Ca^{2+} pool, indicating that oxidative damage impaired the ability of the ER to sequester Ca^{2+}. By comparison, H_2O_2 treatment had less of an effect on the ER Ca^{2+} pool of W.Hb12 cells, indicating that Bcl-2 overexpression inhibits oxidative damage and thereby preserves ER Ca^{2+} pool integrity. Concentrations of H_2O_2 associated with significant ER Ca^{2+} pool depletion in WEHI7.2 and W.Hb13 cells induced apoptosis, whereas W.Hb12 cells were resistant to H_2O_2-induced apoptosis.

Our findings suggest that Bcl-2 overexpression is associated with increased cellular antioxidant defenses that preserve the integrity of an ER Ca^{2+} pool essential for cell growth and survival.

ANALYSIS OF APOPTOTIC AND SURVIVAL SIGNALS DURING TUMOUR PROGRESSION IN TRANSGENIC MICE

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It is becoming increasingly apparent that the balance between cell proliferation and programmed cell death (PCD) is a vital regulatory point in the development of malignant tumours. In the Rip1-Tag2 transgenic mouse model of tumourigenesis the rat insulin promoter (RIP1) targets the expression of SV40 large T-antigen (Tag) to the ß cells of the pancreatic islets. This results in the development of ß cell tumours (insulinomas). The importance for the close coupling of cell proliferation and PCD during tumour development is exemplified by recent studies of Rip1-Tag2 mice carrying a homozygous disruption of the IGF-II gene. In the absence of the survival factor IGF-II tumour cells show dramatically increased incidence of PCD and a reduced tumour mass (Christofori et al., 1994). The Rip1-Tag2 transgenic mice thus provide an ideal system for studying apoptotic and survival stimuli during tumour progression.

For an initial analysis we have focused on Ice (Interleukin-1ß converting enzyme) and Ich (Ice and ced3 homologue) family members. RT-PCR data on Rip1-Tag2 tumours and tumour cell lines indicate that Ich but not Ice are expressed during tumour development. Potential changes in the expression of Ich isoforms during multiple stages of tumour development are being analysed by RNAse protection experiments. The function(s) of Ich isoforms during tumour progression is presently being addressed. To this end we are creating cell lines derived from ß insulinomas which are expressing a particular Ich isoform under an inducible promoter. Additionally, we are utilising epitope-tagged Ich variants to study whether the sub-cellular localisation of Ich isoforms in various cell lines is altered upon apoptotic or survival stimuli.

References:

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MECHANISM OF ONCOSUPPRESSION BY AAV: SITE SPECIFIC INTEGRATION IN RODENT CELLS

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To investigate the mechanism of oncosuppression by AAV we have generated stable SV40 transformed Chinese hamster cell lines carrying an integrated AAV/Neo genome by infection of CO60 cells with an AAV/Neo virus and selection for G418 resistant cells. In all the cell lines no expression of the viral genes was detected.

Phenotypic analysis of the cells demonstrated that several of their transformed characteristics were altered. A large fraction of the cells displayed an apoptotic phenotype. The cells lost their capability to grow in soft agar, their plating efficiency was reduced and the colonies were smaller. The cells were highly sensitive to DNA damaging agents and lost their capacity to amplify SV40 DNA upon treatment with carcinogens.

We have cloned and characterized the integrated AAV and the flanking cellular sequences from one of the AAV/Neo cell lines (clone 9-3). In this clone the integration was followed by a massive rearrangement of the viral sequences which led to the loss of the expression of the viral Rep genes. The 5' regulatory region of a cellular gene located in the vicinity of the AAV integration site was affected by the viral integration. The transcription and the activity of this gene were reduced upon treatment with DNA damaging agents while in the parental cells its expression was induced following the treatment. The intact cDNA coding for this gene was cloned from a cDNA library and the clone was stably introduced into 9-3 cells to study its capability to rescue the transformed phenotype.

Analysis of several AAV/Neo Chinese hamster and mouse cell lines by Southern blot hybridization and by FISH demonstrated that in most of them AAV integrated into the same chromosomal locus.

We propose that the site specific integration of the viral gene into this chromosomal locus plays a key role in AAV oncosuppression by induction of apoptosis. The role of the cloned gene in normal and transformed cells and its possible involvement in the regulation of programmed cell death will be discussed.

STRESS-INDUCED LENS EPITHELIAL CELL APOPTOSIS AS A FUNDAMENTAL CELLULAR MECHANISM FOR NON-CONGENITAL CATARACT FORMATION IN HUMANS AND ANIMALS.

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Cataract is a major ocular disease which causes blindness in many developing countries of the world. Studies of the past half century have established that various factors such as oxidative stress, ultraviolet irradiation and other toxic agents can induce both *in vivo* and *in vitro* cataract formation. However, a common cellular basis for this induction has not been previously recognized. The present study of lens epithelial cell viability suggests such a general mechanism. When lens epithelial cells from a group of 20 cataract patients 12 to 94 years of ages were analyzed by terminal deoxynucleotidyl transferase (TdT) labeling and DNA fragmentation assays, it was found that all of these patients had apoptotic epithelial cells ranging from 4.4 to 41.8%. By contrast, in 8 normal human lenses of comparable age, very few apoptotic epithelial cells were observed. We suggest that cataract patients may have deficient defense systems against factors such as oxidative stress and UV at the onset of the disease. Such stress can trigger lens epithelial cell apoptosis which then may initiate cataract development. To test this hypothesis, it is also demonstrated that hydrogen peroxide insult at concentrations previously found in some cataract patients, ultraviolet irradiation and calcium ionophore treatment all induces lens epithelial cell apoptosis of the cultured rat lenses as determined by TdT labeling, electron microscopic examination and DNA fragmentation assays. Moreover, the temporal and spatial distribution of induced apoptotic lens epithelial cells precedes development of lens opacification. These results suggest that stress-induced lens epithelial cell apoptosis seems to be the common cellular mechanism by which various agents initiate non-congenital cataract formation in humans and animals (Supported by NIH grants).

THE DEATH OF SERUM-DEPRIVED AND DRUG-TREATED PC12 CELLS AS A MODEL SYSTEM FOR STUDYING THE MECHANISM OF APOPTOSIS

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When PC12 cells are deprived of serum, they die and exhibit the characteristic features of the cell death termed 'apoptosis'. We examined the relationship of this death process to the cell cycle. Examination of the cell cycle profiles of PC12 cell populations synchonized at different and specific phases of the cell cycle in relation to the appearance of apoptotic cells following serum-deprivation showed that these cells can die from each of the G_0/G_1, S and G_2/M cell cycle phases. Flow cytometry methodology which determines the RNA content of cells in relationship to their position in the cell cycle was used to address these questions in non-synchronized cells. These experiments revealed that apoptosis in serum-deprived exponentially growing cells may also occur at each phase of the cell cycle and is not preceded by growth arrest. Others have shown that nerve growth factor (NGF), fibroblast growth factor (FGF), dibutyrl cAMP (Bt_2cAMP), aurintricarboxylic acid (ATA) and exogenenous expression of bcl-2 resuce PC12 cells from apoptosis induced by serum-deprivation. We found that cycloheximide, actinomycin D, colchicine and EGTA also induce apoptosis in PC12 cells; therefore, the ability of these survival factors to inhibit the cytotoxic effect of these drugs was tested. Our results show that NGF, Bt_2cAMP and bcl-2 inhibit apoptosis induced by cycloheximide, actinomycin D, colchicine and EGTA. FGF inhibits apoptosis induced by EGTA and colchicine. ATA inhibits apoptosis induced by EGTA. These results suggest that apoptosis induced by various treatments is mediated by different pathways which all converge into a final, common pathway. NGF, Bt_2cAMP and bcl-2 affect the final common pathway whereas FGF and ATA are more specific and only affect some of the pathways.

ROLE OF TRANSCRIPTIONAL FACTOR USF IN PROGRAMMED CELL DEATH

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Transcriptional factor USF is a family of basic helix-loop-helix leucine zipper proteins. Two different USF proteins, of apparent molecular weight 43 kDa and 44 kDa, are ubiquitously expressed in human, mouse, and other higher eukaryotic organisms. The USF genes have been cloned and the structure of protein of 43 kDa has been studied by X-ray crystallography. Recent studies clearly show that USF can function as a transcriptional factor in vivo and in vitro. The biological function of USF is no clearly defined. However, USF binding sites exist in the promoter regions of many important genes (e.g., p53 and cyclin B1) and USF can compete with protooncogene Myc family for the same DNA-binding sites, suggesting that USF could play an important role in cell proliferation.

Recent studies in our laboratory revealed that overexpression of USF in cells such as Hela can be strongly growth inhibitory. Using indirect immunofluorescence staining, we also found that ectopic of USF in Hela causes nuclear fragmentation. Most of these events happen two to three days after transfection. Nuclear fragmentation was not observed with a truncated version of USF lacking the N-terminal transcriptional activation domain. Since nuclear fragmentation is characteristic of apoptosis or programmed cell death, these observations suggest that USF could play a role in programmed cell death. Further studies are now being carried out to verify this hypothesis; the importance of putative downstream targets of USF such as p53 is being investigated.

INACTIVATION OF WILD-TYPE P53 FUNCTION IN TESTICULAR TUMORS:

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Given the central role of p53 gene mutations in the progression from hyperplasia to invasive cancer, it is perhaps surprising that some cancers, such as testicula teratocarcinomas, never contain p53 gene mutations. Human teratocarcinomas also appear to express high levels of wild-type p53 as they have strong nuclear staining by immunofluorescence. We have characterized a panel of murine teratocarcinomas which resemble their human counterparts in that they express approximately 40-fold more nuclear p53 than murine fibroblasts. The high levels of wild-type p53 present in these teratocarcinoma cells does not result in enhanced expression of two p53 regulated genes, mdm-2 and p21. The mdm-2 protein is not overexpressed is these teratocarcinoma cells relative to fibroblasts, suggestin that a novel mechanism for p53 gene inactivation exists in these tumors. Differentiation of teratocarcinoma cells with retinoic acid results in markedly decreased levels of p53 which displays normal transcriptional activity, supporting the hypothesis that this mechanism for p53 transcriptional inactivation is developmentally restricted. Finally, treatment of teratocarcinoma cells with DNA damaging drugs results in a further increase i p53 levels and apoptosis, which may explain the extrem sensitivity in vivo of human teratocarcinomas to chemotherapy.

MYOBLAST APOPTOSIS IS ASSOCIATED WITH EARLY TRANSIENT INDUCTION OF SERUM-INDUCIBLE GENES AND DELAYED OVEREXPRESSION OF UBIQUITIN

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Apoptosis is a distinctive type of cell death which requires specific biochemical events activated by the dying cell. Toward investigating the mechanisms of apoptosis in the muscle cell lineage, we established a tissue culture model based on serum deprivation-induced cell death in a cultured line of murine myoblasts. We showed that *de novo* RNA/protein synthesis and extracellular calcium influx are critical for triggering early cell death, which, together with the internucleosomal DNA fragmentation and morphological changes, suggests a classic apoptotic death mechanism. Myoblast apoptosis was also modulated by calpain and protein kinases which are regulated by Ca^{2+}/calmodulin and cAMP. The death of myoblasts appeared to be coupled to cell cycle progression in that quiescent myoblasts and those arrested at G1/S but not G2/M lost viability rapidly upon serum deprivation. Massive cell death was preceded by an early transient induction of several serum-inducible genes such as *c-myc*, *c-fos*, and SRF (serum response factor) peaking at 15 - 20 hr after serum removal. The late phase of myoblast apoptosis (48 - 72 hr) was marked by the accumulation of ubiquitin to high levels and a concurrent increase in the ubiquitin-conjugating activity. This model of myoblast apoptosis thus exhibits multiple diverse molecular events with proximal and distal regulatory points.

THE DECISION TO DIE: REPAIR VERSUS DEATH.

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Death programmed or otherwise, is a process characterised by its irreversibility. In apoptosis the origin of the irreversibility is unknown and very important. An irreversibility threshold, somewhere before nuclear dissolution should exist as otherwise, inappropriate recovery would produce mutated cells with a risk to the whole organism. This points to the irreversibility being near the chromatin collapse phase.

Mammalian nuclei and the much simpler avian erythrocyte nuclei have been compared to examine what processes around the final collapse of chromatin are candidates for the irreversible stage. A simple model; avian erythrocyte nuclei were presented with either energy stress by exhaustion of cellular energy supplies and nucleolytic stress as isolated nuclei. Energy stress from gramicidin-toxicity caused pronounced structural changes of erythrocyte nuclei but these were not quite like normal apoptosis. However, exogenous nucleases did to induce the characteristic structural collapse and cavitation events of apoptosis in the same way, in model avian erythrocyte chromatin, that normal apoptosis does in mammalian chromatin cells although caution was required because one standard EM embedding technique causes a completely artifactual apoptosis-like marginalisation of chromatin. Overall, the results are most compatible with the primary process in apoptopic chromatin collapse being DNA breaks. This is the oldest hypothesis but it has recently been seriously disputed. For it to be credible however, there has to be some mechanism whereby the relatively small numbers of breaks that may initiate apoptopic collapse are not repaired in early apoptosis.

Study of the DNA termini obtained from somatic mammalian nuclei autolysing in vitro suggests non-ligatable nucleolysis sites are a strong candidate for the irreversibility mechanism. The repair processes may have been paralysed by making the nucleolysis termini non-ligatable. Non-ligatability may involve poly-ADP ribose synthetase because the presence of NAD reduces the non-ligatibility of DNA ends produced in nuclei in-vitro.

EXPRESSION OF THE FAS LIGAND IN SLE. Jeremy McNally, Joern Drappa, Steven M. Friedman, and Keith B. Elkon SCOR in SLE, The Hospital for Special Surgery-Cornell University Medical Center, New York, NY.

Mutations in the genes encoding the Fas receptor (FasR) or its ligand (FasL) are responsible for the expression of lupus-like diseases in three different strains of mice. Although the precise mechanisms are not fully characterized, most evidence suggests that certain T cell subsets expressing FasL are cytotoxic toward activated peripheral T and B cells that express FasR and that deletion of these cells is necessary for the maintenance of tolerance.

To determine whether the FasL was expressed appropriately in SLE, peripheral blood T cells from SLE patients or normal controls (n = 5 in each group) were activated by anti-CD3 and IL-2 in vitro. 5-7 days later, T cells were examined for cytotoxic effector function using transfected Fas sensitive (Fas+) and Fas antisense resistant (Fas-) lymphocyte targets in a ^{51}Cr release assay. Specific^{51} Cr release was compared between SLE patients and controls. SLE T cell cultures had an average 30% lower cytotoxicity of Fas(+) targets compared to the normal controls tested on the same day. Although 3/5 SLE T cell cultures also had lower cytotoxicity on Fas(-) targets, the average reduction was less than Fas(+) targets. To evaluate Fas ligand expression at the molecular level, RT-PCR was performed on SLE lymphocytes with three FasL specific primer pairs. No obvious mutations (insertions or deletions) in FasL cDNA were detected in these samples.

These findings indicate that, unlike *gld* mice, FasL is present and functional in the majority of SLE patients' T cells. However, the lower % cytotoxicity of Fas(+) targets by SLE versus normal T cells is compatible with some impairment of FasL expression and could be important in allowing persistence of activated cells in the periphery.

HYPOXIA PROLONGS NEUTROPHIL SURVIVAL IN VITRO.

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Neutrophil apoptosis is thought to represent a major mechanism involved in the resolution of inflammation. The rate at which these cells undergo apoptosis in vitro can be modified by a number of inflammatory cytokines and bacterial products (eg. lipopolysaccharide, GM-CSF, TNFα) implying that microenvironmental factors can have a profound influence on this process. The potential for hypoxia to affect neutrophil survival may also have considerable relevance in vivo and is of particular interest as these cells do not express Bcl-2 and do not undergo apoptosis following activation of respiratory burst activity by, for example, fMLP or PAF.

Human peripheral blood neutrophils were purified by dextran sedimentation and plasma/Percoll gradients. 6.75×10^5 cells were cultured in 150 µl Iscove's DMEM containing 10% autologous serum in 21%, 2.5% and 0% oxygen environments and apoptosis assessed morphologically. Contrary to expectation, hypoxia caused a major inhibition of apoptosis (% apoptosis 20 hr: 78.7 ± 2.2% in 21% O_2, 61.4 ± 6.5% in 2.5% O_2 and 23.1 ± 3.2% in 0% O_2, n=5). No effect of hypoxia was observed 6 hr into culture where the basal rate of apoptosis was very low (<5%) and hypoxia did not influence cell recovery or viability (trypan blue exclusion). Time-course studies demonstrated <25% apoptosis after culturing neutrophils for 44 hrs at 0% O_2 compared to virtual total apoptosis/necrosis by 30 hrs under standard (21% O_2) culture conditions. The effect was additive to that of GM-CSF (50 U/ml), not associated with the induction of Bcl-2 expression and not mimicked by the soluble antioxidant vitamin E analogue Trolox (10 mM, 8 hrs).

These data demonstrate that extreme hypoxia causes a dramatic inhibition of neutrophil apoptosis mediated via a Bcl-2 and reactive oxygen species-independent mechanism.

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FAS-FAS LIGAND SYSTEM IS INVOLVED IN THE APOPTOTIC CELL DEATH DURING THE NEURONAL DIFFERENTIATION OF P19 EC CELLS.

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P19 embryonal carcinoma (P19EC) cells differentiate into neuronal cells by culturing with retinoic acid (RA) in the aggregate form. The cell aggregation of the RA-treated P19 EC cells induced apoptotic cell death; cytoplasm contraction and DNA ladder formation were observed. Fas ligand and TNF induce the apoptotic cell death of many type of cells, but little is known about the involvement of these factors in the neuronal cell death. ICE-like protease, a homologue of Ced-3, induces the neuronal cells death of vertebrate and apoptotic cell death mediated by Fas is suppressed by inhibitor of ICE, suggesting that Fas is involved in the neuronal cell death. In the present study, we demonstrated that P19 EC cells expressed ICH (ICE/Ced-3 homologue) during the neuronal differentiation of P19 EC cells, and that the expression of Fas and Fas ligand were induced in P19 EC cells by RA treatment and in their neuronal differentiation process followed by the cell aggregation, respectively. The expression of TNF receptors were also induced by RA, but TNF α and β did not express during the neuronal differentiation. The cells overexpressing Fas ligand induced apoptotic cell death of RA-treated P19EC cells. Fas-positive cells were observed in the neuronal differentiated P19 EC cells and in the neuronal cells in the central nervous system of mouse embryos. These observations suggest that Fas/Fas ligand system is involved in the neuronal cell death of P19 EC cells and mouse embryos.

INDUCTION OF APOPTOTIC PROGRAMMED CELL DEATH IN NEURONS BY CAMPTOTHECIN, AN INHIBITOR OF DNA TOPOISOMERASE I

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DNA topoisomerase I (topo-I) is an enzyme used in the relaxation of DNA supercoiling during replication, transcription, and DNA repair. Camptothecin, and its analogs, are anticancer agents which inhibit the activity of topo-I and induce apoptotic cell death in proliferating cells. Although, these agents are thought to be toxic only to cells in the S-phase of the cell cycle, camptothecin induces a dose-dependent cytotoxicity to postmitotic rat cerebral cortical neurons and NGF-treated, growth-arrested PC12 cells (*Soc. Neurosci. Abs.* (1994) 20:246). We now report that camptothecin treatment of cultured neurons results in an apoptotic programmed cell death that is mediated by topo-I.

The ability of camptothecin to induce morphological changes characteristic of apoptosis was measured by nuclear chromatin staining and continuous time-lapse videomicroscopy in mixed cultures of neurons and astrocytes. Nuclear chromatin condensation was determined by labelling cells with the nuclear dye, DAPI. Camptothecin-induced cell death was accompanied by rapid chromatin condensation which occurred only in neurons within 24 hours of treatment. Time-lapse analysis of camptothecin-treated cultures demonstrated neuronal zeiosis (plasma membrane blebbing and contraction), cytoplasmic shrinkage, and neurite fragmentation that lasted approximately 4-8 hours. These morphological changes were characteristic of apoptosis and could be distinguished from rotenone-induced neuronal necrosis. Camptothecin-induced neuronal death could be inhibited by RNA and protein synthesis inhibitors suggesting that this death depends upon ongoing transcription and translation. The S (active) and R (inactive) stereoisomers of the camptothecin analog, 10,11-methylenedioxycamptothecin (MDCPT), were utilized to determine if neuronal death was specific to pharmacological agents which inhibit topo-I. Treatment of neurons with the S isomer resulted in a significant, dose-dependent cell death while treatment with the R isomer had no effect compared to control conditions. The potency of S-MDCPT- and camptothecin-induced neuronal death was consistent with the ability of these agents to inhibit topo-I *in vitro*. These data highly suggest that camptothecin-induced neuronal death is mediated by inhibition of topo-I.

These data demonstrate that the S-phase specific toxin, camptothecin, can induce apoptotic programmed cell death in postmitotic neurons via topo-I. These results are consistent with reports of neuronal apoptosis in response to other cell cycle-dependent antitumor agents. Identification of the biochemical mediators of camptothecin-induced neuronal death may identify important regulators of neuronal programmed cell death via the apoptotic pathway.

PROGRAMMED CELL DEATH IN T CELLS BY SOLUBLE MHC-PEPTIDE COMPLEXES: TREATMENT OF AUTOIMMUNE DISEASES.

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In the absence of second costimulatory signal, the recognition of T cell receptors (TCRs) by purified MHC class II-peptide complexes lead to the induction of T cell non-responsiveness. The present study describes that in addition to the T cell non-responsiveness, the engagement of TCRs by purified MHC II-peptide complexes also induces antigen-specific apoptosis or programmed cell death (PCD) in T cells in vitro. The induction of apoptosis was demonstrated in both mouse and human T cell clones. A dose and time dependent T cell death was observed by exposing cloned T cells to purified complexes containing syngeneic MHC class II and immunodominant antigenic peptides. The quantitative detection of DNA strand breaks was analyzed by the 3' end labeling of fragmented DNA with biotinylated-dUTP in the presence of terminal deoxynucleotide transferase (TdT) using double fluorescence flow cytometry. In this analysis, the T cell apoptosis was predominantly observed in the synthetic (S) phase of cell cycles. Similarly, the fragmentation of DNA was also observed in an enzyme immunoassay using BrdU-pulsed T cells. The specificity of T cell apoptosis was demonstrated by exposing T cells with MHC II alone and MHC II bound to another high affinity epitope from the same antigen. Specific apoptosis in T cells was further characterized by electron microscopy and expression of *bcl-2* and *bax* proteins. These results suggest that the antigen-specific apoptosis can be induced in mature $CD4^+$T cells by purified MHC class II-peptide complexes. Since CD4+ autoreactive T helper cells are known to involve in pathogenesis of various autoimmune diseases, specific elimination of autoreactive T cells may have significant clinical relevance in antigen-specific treatment of various autoimmune diseases.

DEMONSTRATION OF ANTI-APOPTOTIC FUNCTION OF THE BACULOVIRUS p35 AND OpIAP GENES IN MAMMALIAN CELLS USING A VIRUS VECTOR SYSTEM.

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Sindbis virus (SV) induces classical apoptosis in various mammalian cell lines (BHK, N18, AT3 neo and VERO). Infectious recombinant SV vectors (SVV) can be generated by in vitro transcription and transfection of full length viral RNA, providing a convenient tool to study programmed cell death. We have constructed a SVV system to test the anti-apoptotic function of heterologous genes. The baculovirus anti-apoptotic genes p35, from the *A. californica* nuclear polyhedrosis virus (AcMNPV), and OpIAP the inhibitor of apoptosis gene from *O. pseudosugata* nuclear polyhedrosis virus (OpMNPV), were cloned into a double subgenomic SVV (pdsTE12Q), and recombinant viruses were generated to infect BHK (baby hamster kidney) and N18 (mouse neuroblastoma) cell lines. Cell viability was assayed by trypan blue exclusion at different time points using a multiplicity of infection of five. Both p35 and OpIAP inhibited (Confidence Limit = 95%) cell death induced by SVV in BHK and N18 plated at low density (4×10^3 cells x cm^2). In contrast to p35 the protection conferred by OpIAP was not sustained for cells at a four-fold higher density. Protection was abolished by insertion of a premature stop codon into the p35 and the OpIAP genes contained in the SVV. This confirms the evolutionary conservation of programmed cell death, showing the ability of p35 and OpIAP to protect mammalian cells. Additionally, these results suggest the involvement of ICE-like proteases in SV induced apoptosis considering that p35 has been recently proposed as a possible inhibitor of ICE-like enzymes (N. Bump et al. 1995. Journal of Cellular Biochemistry. Supplement 19B: 294). Finally, this data underscores the applicability of the Sindbis virus vector system to study apoptosis.

CELLULAR GENETIC STUDY OF FAS- AND TNF-RESISTANT MUTANT FROM U937 CELLS

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Human monocytic leukemia U937 cells readily undergo apoptosis when cells were treated with various stimuli including antitumor agents, TNF-α , and anti-Fas antibody. To study the mechanism of apoptosis, we isolated and characterized a mutant, UK110, from U937 cells, which was resistant to TNF-α- and anti-Fas antibody-induced apoptosis but was less resistant to etoposide-induced apoptosis. TNF-α -induced signals are mediated by two types of TNF receptors (TNFR), p55- and p75-TNFR, and p55-TNFR is homologous to the Fas antigen. Interestingly, UK110 cells showed resistance to apoptosis by agonistic anti-p55-TNFR antibody, indicating that UK110 cells were resistant to Fas- and p55-TNFR-mediated apoptosis. Because expression of apoptosis-associated molecules, such as c-Myc, Bcl-2 and Bax, or activation of NF-kB by TNF-α were similar between U937 and UK110 cells, an undetermined pathway for apoptosis through Fas and p55-TNFR could be mutated in UK110 cells.

To examine whether the resistant phenotype in UK110 cells is recessive or dominant, somatic cell hybridization was employed between mutant UK110 and parental U937 cells. The obtained hybrid clones, No. 2, 3, 4, 5, and 6, were sensitive to anti-Fas antibody- and TNF-induced apoptosis, which suggested that UK110 cell has recessive mutation. Furthermore, we isolated a revertant, BR20, showing resistance to anti-Fas antibody- and TNF-induced apoptosis from apoptosis-sensitive hybrid No. 5 cells without mutagenesis. Q-band karyotyping study and chromosome painting analysis showed that a particular chromosome decreased in revertant BR20 cells. These observations suggested that the decreased chromosome in BR20 cells may participate in the resistant phenotype against anti-Fas antibody- and TNF-induced apoptosis.

DEATH AND LIFE OF SPHINGOMYELIN SIGNALLING-STIMULATED CELLS

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Fas antigen-mediated apoptosis of T lymphocytes plays an important role in the immune system. A defect in Fas-mediated cell death results in lympho-proliferative disease and autoimmune disease. We have found that both CD28, a costimulatory membrane molecule of T lymphocyte, and Fas stimulate sphingomyelin-ceramide turnover-mediated signalling (SM signalling) in mouse and human cells. Ceramide generation is a key event in SM signalling and since ceramide is a multifunctional molecule that can cause either programmed cell death (PCD) or costimulation in treated cells, regulatory signals must exist which control these pathways. Accordingly, Fas and CD28 must generate different regulatory signals; Fas-regulatory signal will promote death processes while CD28-regulatory signal will prevent PCD. We observed that Fas stimulation increases the stability of mRNA specific for *c-Myc* , therefore the apoptosis of Fas-stimulated cells may be caused by unbalanced expression of cell death- and/or survival-associated genes. Importantly, either CD28 stimulation or ceramide alone stabilizes mRNAs in treated cells. The regulatory signal may limit the specificities of the stabalized mRNAs in SM signalling-stimulated cells. We will discuss the molecular mechanisms of regulatory signals which determine the fate of SM signalling-stimulated T cells.

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SELECTION AGAINST EXPRESSION OF *myb* ONCOGENES IN NIH 3T3 FIBROBLASTS -- POSSIBLE INVOLVEMENT OF PROGRAMMED CELL DEATH

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The v-*myb* oncogene, which was found in avian myeloblastosis virus (AMV) and E26 retroviruses, is known to cause leukemia in chickens and to transform hematopoietic cells *in vitro*. Unlike other oncogenes, v-*myb* has not been reported to transform fibroblasts. Furthermore, expression of the c-*myb* proto-oncogene, the cellular counterpart of v-*myb*, is largely restricted to immature hematopoietic cells. This tissue specificity may have an important implication to the transforming capacity of the oncogene. In order to address this issue, we tried to establish murine fibroblasts overexpressing various Myb oncoproteins.

NIH 3T3 cells were transfected with plasmid expression vectors containing various *myb* genes: E26 v-*myb* (*gag-myb-ets*) and its mutants, AMV v-*myb*, and chicken c-*myb*. After selection with G418, surviving cells were cloned for further analysis. Western blotting with polyclonal anti-Myb antiserum detected no proteins of anticipated sizes in any of the clones. Northern blotting with double-stranded DNA *myb* probe revealed that majority of clones did not show detectable mRNA. On the other hand, hybridization to *ets* probe was readily detected in most of the clones transfected with *ets*-containing constructs. Southern blotting with the same probes showed variable results, indicating the absence or rearranged incorporation of the genes in some clones.

Our failure to establish NIH 3T3 clones that overexpress functional *myb* genes, together with the low frequency of G418-resistant cells after transfection, suggests the existence of negative selection. Overexpression of *myb* oncogenes may be toxic to the proliferation of fibroblasts, possibly through induction of programmed cell death. This hypothesis may explain why *myb* oncogenes, which transform hematopoietic cells, cannot transform fibroblasts.

REGULATION OF THE BCL-2/BAX HETERODIMER BY AN APOPTOTIC STIMULUS

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Bcl-2 exerts its action through heterodimerization with Bax. p53 and TGF-β type apoptotic stimuli are known to alter the ratio of Bcl-2/ Bax hetero- vs. Bax homodimers by modifying the ratio of Bcl-2 and Bax protein levels. Recent reports indicate that the activity of Bcl-2 is also regulated by its phosphorylation status.

We wished to examine how two other distinct apoptotic stimuli effect the Bcl-2/Bax heterodimers. While no direct change in the amount of Bcl-2 and Bax proteins were seen, progressive dissociation of the Bcl-2/Bax heterodimer has occured upon IL-3 deprivation. Bad and Bcl-Xs, known competitive inhibitors of the Bcl-2/Bax heterodimer, were not upregulated, arguing for a different mechanism of heterodimer dissociation. In the presence of IL-3, endogenous Bcl-2, which forms dimer with Bax very inefficiently, was not phosphorylated. However, overexpressed Bcl-2, that is primarily in heterodimer with Bax, was in a phosphorylated form. However, upon IL-3 deprivation Bcl-2 was rapidly dephosphorylated. Bax was not phosphorylated under either conditions. Yet, in a different cell line, overexpressed Bcl-2 was not phosphorylated, either with or without the induction of cell death by glucocorticoid treatment.

Overall our data suggest, that Bcl-2/ Bax dimers may be inactive in proliferating cells and selective phosphorylation of Bcl-2 may represent one such inactivating mechanism. Upon an apoptotic stimulus Bcl-2 may regain its activity, in some cases by its dephosphorylation, but the Bcl-2/ Bax heterodimer is progressively dissociated leading to a secondary inactivation. The activity of Bcl-2/ Bax heterodimers and the rate of heterodimer dissociation appears to define a time window during which Bcl-2 is able to counter an apoptotic stimulus.

GENETIC REGULATION OF APOPTOSIS IN MOUSE T CELLS

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We have isolated several genes that are regulated during the induction of apoptosis in the mouse thymus. We have shown that one of these genes, *nur77*, is required for cell death in T cell lines. Recent data from our laboratory indicates that a selected mutant of this T cell line, mutant 51, does not die when induced by signals through the T cell receptor. Although this mutant does not undergo apoptosis, *nur77* induction is observed. Interestingly, whereas Nur77 protein is made following induction of apoptotic signals, the protein is not phosphorylated. Additionally, changes in intracellular Ca^{++} profiles are observed in this line following inductive signals when compared to wild type cells. The significance of these data with respect to induction of apoptosis in T cells by signals through the T cell receptor will be addressed.

Another gene that is induced during cell death in mouse T cells is *apt-4*. This gene is induced several hours after signaling through the T cell receptor and unlike *nur77*, it is inhibited by cyclosporin A. Additionally, *apt-4* appears to be downstream of Bcl-2 since expression of Bcl-2 can abrogate the induction of *apt-4*. *Apt-4* also is required for cell death in T cell lines as evidenced by antisense experiments where expression of antisense *apt-4* completely inhibits the induction of cell death in a T cell line.

ROLE OF MDR-1, BCL-2 AND P53 GENE PRODUCTS IN REGULATION OF APOPTOTIC RESPONSE TO DAUNORUBICIN IN ACUTE MYELOID LEUKEMIA (AML) BLASTS.

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The aim of our study was to investigate a role of and possible interactions between the mdr-1, bcl-2 and p53 gene products in the *in vitro* susceptibility of AML blasts to Daunorubicin (DNR)-induced apoptosis.

Leukemic blasts from ten AML patients were incubated for one hour with 1.0 μM DNR conjugated with Rhodamine [Fluorescence 2 (FL2), red light] and subsequently sorted on the basis of FL2 intensity, related to DNR uptake which is dependent on the level of the mdr-1 gene product-P-glycoprotein. Four cell populations were studied: I. unsorted blasts incubated with DNR, II. sorted blasts with high levels of DNR (DNR^{high}), III. sorted blasts with low levels of DNR (DNR^{low}) and IV. control blasts incubated without DNR. The expression of p53 and bcl-2 proteins and the presence of apoptosis were evaluated by flow cytometry, using monoclonal antibodies (MAb) and indirect or direct immunofluorescence, and terminal deoxytransferase (TdT)-dependent tailing of DNA breaks using digoxigenin (DIG)-labelled dUTP/dATP together with DNA staining by propidium iodide (PI), respectively. The mdr-1 mRNA was evaluated by solution hybridization assay and quantitative PCR.

In individual cases, the expression of mdr-1 mRNA (in numbers of copies per cell) correlated to the DNR uptake in the total blast population, but not to the distribution of drug between DNR^{high} and DNR^{low} fractions nor to the level of apoptotic response. After DNR treatment, in five out of 10 cases a distinct apoptotic fraction was present (5-21% of cells), confined to DNR^{high} blast populations.

The bcl-2 protein was present in all tested AML cases. In four cases the fluorescence profiles obtained with anti-bcl-2 MAb suggested higher levels of bcl-2 protein in DNR^{high} by comparison to DNR^{low} subpopulation (Kolmogorov-Smirnov statistics, D/S >15). In all tested cases the expression of the p53 protein in a growth promoter orientation (MAb PAb1620-/PAb240+), which is also the phenotype of the mutant p53, was found. However, the fluorescence intensity of p53 did not differ between DNR^{high} and DNR^{low} subpopulations. In DNR^{high} blast population of two samples p53 in a growth suppressor orientation was present and related to the induction of apoptosis. No correlation between the *in vitro* sensitivity of AML blasts to apoptosis and patients survival was found.

We conclude that in some, but not all, AML cases the sensitivity to DNR induced apoptosis is related to the intra-cellular drug concentration, and that even high levels of bcl-2 protein and/or presence of p53 in a growth promoter orientation are not sufficient to protect the blasts from cytostatic-drug induced apoptotic death.

THE FATE OF NUCLEAR LAMINS AND THE NUCLEAR ENVELOPE IN APOPTOSING NEURONS

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Mitotic cells display a number of characteristic features. These include chromatin condensation, detachment from the substrate and the disassembly of the nuclear lamina and the nuclear envelope. Apoptotic cells are known to show the first of these two morphological markers. We wished to ascertain whether additional events were shared between these two processes which might implicate common pathways.

We used primary sympathetic neurons and differentiated PC12 cells as our experimental systems. Both cell types are dependent on NGF for survival *in vitro* and die with a typical apoptotic phenotype when
NGF is removed from the medium. We compared the level and distribution of nuclear lamin proteins in apoptotic cells with those in mitotic cells using both biochemical analysis and immunocytochemical staining.

Reduced lamin immunostaining was seen in apoptosing cells when compared to cells maintained in NGF. However this reduction was due to the degradation of lamin proteins as opposed to lamin solubilisation which occurs in mitosis. This suggests that different enzymes are being utilised in the two processes. To assess the integrity of the nuclear envelope, which fragments into small vesicles and disperses throughout the cytoplasm during mitosis, we microinjected FITC conjugated dextrans into the cytoplasm of SCG neurons. A high molecular weight tracer was excluded from the nucleus of cells undergoing apoptosis while a low molecular weight tracer entered the nucleus rapidly after injection. This demonstrated the existence of a functional nuclear barrier in apoptosing cells and that normal diffusion was not affected by the process of microinjection. The presence of a continuous nuclear envelope in dying neurons was additionally demonstrated by electron microscopy of apoptosing PC12 cells.

These data suggest that there are some clear distinctions between the pathways activated during mitosis and apoptosis and that nuclear envelope breakdown is not a prerequisite for or a consequence of apoptotic cell death.

REGULATION OF APOPTOSIS BY P53 AND THE BCL-2 GENE FAMILY IN MOUSE MODELS OF RETINAL DEGENERATION. Rachel M. Phipps[1,2], Nancy Ranson[2], Kimberly A. Howes[1], David S. Papermaster[2], Gabriel Nunez[3], Jolene J. Windle[1,2]. [1]The Cancer Therapy & Research Center, San Antonio, TX 78229; [2]The University of Texas Health Science Center at San Antonio, TX 78284; [3]The University of Michigan Medical School, Ann Arbor, MI 48109.

It has been demonstrated that a variety of forms of retinal degeneration result from apoptotic death of photoreceptor cells. For example, mutation of the gene encoding the β-subunit of the rod photoreceptor cGMP phosphodiesterase in *rd* (retinal degeneration) mice results in total loss of photoreceptors between postnatal days 14-16. In addition, we have generated a line of transgenic mice expressing the human papillomavirus E7 oncoprotein which binds pRb specifically in photoreceptor precursor cells (under the control of the interstitial retinol-binding protein, IRBP, promoter). The photoreptors of IRBP-E7 mice exhibit apoptosis at precisely the developmental stage during which the cells would normally terminally differentiate. Thus, apoptotic retinal degeneration can be induced either by perturbing cell cycle regulation during differentiation or function of mature photoreceptors.

To dissect the molecular pathways regulating cell death in photoreceptors, we are creating lines of mice coexpressing the genes predisposing to retinal degeneration and various genes which potentially regulate this process. By interbreeding IRBP-E7 mice with p53-/- ("knock-out") mice, we demonstrate that p53 is not absolutely required for E7-mediated apoptosis of photoreceptors, but modulates the onset and rate of apoptosis in this system. Furthermore, absence of p53 allows some cells to escape apoptosis, resulting in tumor formation. We also demonstrate that p53 is not required for retinal degeneration in *rd* /p53-/- mice by assaying for apoptosis at postnatal days 12, 14 and 16 using the TUNEL (TdT-mediated dUTP-biotin nick end labelling) assay.

In addition, we have generated lines of transgenic mice expressing members of the bcl family of apoptosis regulating genes under the control of the IRBP promoter. IRBP-bclxl and IRBP-bclxs mice show normal photoreceptor morphology and number despite evidence of high RNA and protein expression of these genes. IRBP-bcl-2 mice have also been generated and are being characterized. We are currently investigating whether bcl-xl overexpression can prevent retinal degeneration in either the *rd* or IRBP-E7 mice; these results will be presented. Supported by NIH EY09213 (JJW), EY06891(DSP) and EY10992 (JJW and DSP).

TRANSLATIONAL FACTOR eIF-4E SUBSTITUTES FOR GROWTH FACTORS IN SUPPRESSION OF MYC-DEPENDENT APOPTOSIS

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Translation initiation factor eIF-4E is a 24-kDa phosphoprotein which is rate limiting for protein synthesis. It can be activated by peptide growth factors through signaling pathways involving Myc. Overexpression of eIF-4E stimulates cell proliferation and induces malignant transformation which is dependent on Ras. Here we report that overexpression of eIF-4E substitutes for serum or individual growth factors in the suppression of apoptosis in fibroblasts expressing physiologically regulated or constitutively upregulated Myc. Cells overexpressing eIF-4E do not have altered expression of Bcl-2 and Bcl-X_L and are able to undergo apoptosis in response to camptothecin or an anti-CD44 antibody. These data suggest that inhibition of apoptosis by eIF-4E is mediated by translational activation of death suppressor proteins distinct from Bcl-2 and Bcl-X_L which operate upstream of the cell death machinery. The effect of eIF-4E is not reproduced by Ras overexpression or acquisition of a transformed phenotype. Thus, growth factor induced activation of eIF-4E may represent a physiological safeguard mechanism interdicting the Myc induced apoptotic pathway during productive proliferative cycles. Transcriptional activation of eIF-4E by Myc itself may serve a negative feedback role in this salvage pathway.

PATHOGENESIS OF THE BIRTH DEFECT SACRAL AGENESIS

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Sacral agenesis is a major congenital defect in which the vertebral column is truncated below the level of the lumbar spine. Apart from various degrees of osseous agenesis in the sacral and coccygeal regions, patients may also suffer from multiple anomalies involving genitourinary, gastrointestinal and nervous systems, resulting in stillbirth and neonatal death. Sacral agenesis is especially common in babies born to mothers with diabetes mellitus, and also occurs in cases of excess intake of vitamin A during pregnancy. Sacral agenesis develops during the 5th and 6th weeks of human pregnancy, when the tail-bud undergoes differentiation to form various types of tissues of the sacral and coccygeal regions. To obtain information on the cellular and molecular basis of development of sacral agenesis, we employed a mouse model of sacral agenesis in which retinoic acid (RA), a vitamin A derivative, causes truncation of the vertebral column at the sacral level. The vertebral and genitourinary malformations in the mouse model are, in many respects, very similar to those observed in human.

When 100 mg/kg RA was injected intraperitoneally into mice at various time points between 9.25 and 10.25 days of pregnancy (equivalent to the 5th week of human development), various degrees of sacral agenesis were induced in the offspring, with effects more severe when RA was applied earlier. Histological examination showed massive cell death in the tail bud region after 24 hours of RA treatment and the dead cells were cleared within a few hours. To determine whether the tail bud cells underwent apoptosis or necrosis, total DNA was extracted from about fifty tail buds of embryos at every six hours interval after RA administration and then separated on an agarose gel. A characteristic DNA ladder pattern, as a result of internucleosomal cleavage, was found 24 hours after RA treatment, indicating that apoptosis occurred in the tail bud. In situ staining with the terminal transferase-mediated nick end labelling (TUNEL) method of transverse sections taken from the tail buds 24 hours after RA treatment also showed that apoptotic cells were present throughout the tail bud. Our results suggested that apoptosis was induced by the RA treatment in the tail bud. Since the sacral and caudal structures are derived from the tail bud mesenchyme, excessive death of cells in the tail bud may account for failure of development of these structures, resulting in sacral agenesis.

ENDOPLASMIC RETICULUM $Ca2^+$ POOL DEPLETION IS CONCOMITANT WITH LOW SERUM ACTIVATED APOPTOSIS IN EARLY STAGE, PRENEOPLASTIC SYRIAN HAMSTER EMBRYO CELLS

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The multistage process of neoplastic progression requires uncoupling of signal transduction pathways involved in proliferation and apoptosis in such a manner that a proliferative advantage is acquired in the transformed cell. Utilizing a cellular system consisting of four Syrian hamster embryo cell lines, each of which depict a different stage of progression, it was shown that two preneoplastic cell lines, stage I sup+ cells (sup+I) and stage II sup- cells (sup-II) displayed contrasting phenotypes. The sup+I cells underwent apoptosis at a high rate in low serum, whereas the later stage sup-II cells were resistant to apoptotic signals. In the current study, we used the calcium indicator fura-2 and fluorescent microscopy to measure and compare intracellular free calcium concentrations, $[Ca2^+]_i$, between sup+I and sup-II cells when placed in 0.2% serum. The results indicate that $[Ca2^+]_i$ levels were lower in logarithmically growing sup+I cells ($\sim$100nM) in contrast to $\sim$260nM in the sup-II cells. The $[Ca2^+]_i$ was reduced in the sup+I cells upon removal of serum to $\sim$82nM, while the level in sup-II cells remained relatively constant. To determine if a reduction in endoplasmic reticulum (ER) calcium concentrations was associated with the activation of apoptosis, we evaluated ER $Ca2^+$ levels by measuring thapsigargin releasable $Ca2^+$. Thapsigargin inhibits the ER CaATPase causing a rapid release of ER $Ca2^+$ that can be measured by fura-2 in the cytosol. After 4h in low serum, the thapsigargin releasable $Ca2^+$ (or ER $Ca2^+$) in sup+I cells was 45nM as compared to sup-II cells at 190nM. The data show that thapsigargin releasable $Ca2^+$ was depleted only in the cells that undergo apoptosis. Further, these cells can be rescued from apoptosis by incubation in high extracellular $Ca2^+$ and concomitant with rescue, ER $Ca2^+$ pools were not depleted. We propose that depletion of ER $Ca2^+$ pools contribute to the apoptotic process and that this may be one mechanism through which a preneoplastic cell may be eliminated from the organism.

Title : **Methional derived from 4-methylthio-2-oxobutanoate is a cellular mediator of apoptosis in BAF3 lymphoid cells.**

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ABSTRACT

4-methylthio 2-oxobutanoic acid, an intermediate in the methionine salvage pathway is a metabolite which is situated at the cross roads of two important cellular phenomena. On one hand, it can alleviate the methionine dependence of transformed cells and on the other, it is the precursor of methional which is a potent inducer of apoptosis in a BAF3 murine lymphoid cell line which is interleukin-3 (IL3)-dependent. Cultures treated for 8 h with methional in the presence of IL3 show extensive DNA double-strand breaks on flow cytometric analysis, increases in DNA fragmentation as measured by the amount of non-sedimentable DNA present in the 30 000 *g* supernatant of cell lysates and the typical laddering pattern of multiples of 180 bp seen upon agarose gel electrophoresis. No such features of apoptosis were found in cells treated with 4-methylthio 2-oxobutanoic acid or propanal, suggesting that the simultaneous presence of the methylthio group on the propanal moiety is essential for apoptosis to take place. Methional is further metabolized in cells by two reactions : oxidation via aldehyde dehydrogenase to methylthio propionic acid or β-hydroxylation to malondialdehyde. The formation of malondialdehyde from methional in vitro by chemical hydroxylation under the conditions of the Fenton reaction provides a mechanism for the β-hydroxylation which takes place in vivo. During apoptosis induced by IL3 deprivation, the ratio of 2,4-DNPH MDA to 2,4-DNPH methional is 0.94 in cells in $IL3^-$ medium compared with 0.54 in cells in $IL3^+$ medium. These results support a role of cellular methional and malondialdehyde in apoptosis.

CHARACTERIZATION OF THE ASSOCIATION BETWEEN THE E1B 19K PROTEIN AND LAMIN A/C

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Adenovirus E1A and E1B oncoproteins play an important role in regulating apoptosis during the infection of human cells and the transformation of rodent cells. E1A proteins initiate cell proliferation, leading to the accumulation of p53 and induction of apoptosis. The E1B 19K protein functions indirectly to overcome apoptosis, thereby sustaining productive infection or efficient transformation. E1B 19K shares functional and partial sequence homology to the Bcl-2 protooncogene

In an attempt to understand the mechanism of inhibition of apoptosis by E1B 19K, interacting cellular proteins were isolated using the yeast two-hybrid system. One such interacting cloned obtained from screening a Hela cDNA library was designated Bp2, and is identical to human lamin A, a major component of the nuclear lamina. It has been previously established by this lab that the E1B 19K protein co-localizes and copurifies with the nuclear lamina in infected and transformed cells. Interestingly, during E1A induced apoptosis lamin degradation is observed before cell viability is lost. Association of E1B 19K with lamins may prevent lamin breakdown and therefore the nuclear events associated with apoptosis.

The association between E1B 19K and lamin A/C has been further characterized and the regions of binding mapped. The minimal region of lamin A/C required for interacting with E1B 19K in the two-hybrid system resides between amino acid residues 252-390 which includes part of the α-helical domain. This has been confirmed with *in vitro* binding assays using lamin deletions made as GST fusion proteins. The recriprocal binding region of E1B 19K maps to amino acids 19-57. Further, this interaction is specific as Bcl-2 does not bind lamin A/C under the same conditions.

The observation that lamins are degraded during E1A induced apoptosis directs to the involvement of proteases. The role of ICE/*ced*-3 family of cysteine proteases during apoptosis has been previously documented. ICE itself does not play a role as the expression of *crm* A, a potent inhibitor of ICE, does not block E1A induced apoptosis during adenovirus infection of Hela cells or transformation of BRK cells. However, the possibility that one of the other ICE family members is involved still exists.

CLONING AND FUNCTIONAL ANALYSIS OF A NOVEL PROTEIN WHICH BINDS TO THE p55 TNF RECEPTOR DEATH DOMAIN

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The majority of the known effects of tumour necrosis factor (TNF) are mediated by p55 TNFR (TNFR1), one of two distinct cell surface receptors for this cytokine. Mutagenesis of TNFR1 has identified a death-domain of approximately 80 amino-acids in the cytoplasmic protein of the receptor which is essential for signalling cell death. Recently a number of cytoplasmic proteins have been identified which bind to this region of TNFR1 - these appear to be involved in transmitting signals for both cell death and NF-kB activation.

Using the yeast-two hybrid system, we have identified a new member of this protein family, designated wsl-1, which binds specifically to the TNFR1 death domain. This protein is highly homologous (48% identity) to TNFR1, has a very restricted tissue distribution and is capable of inducing activation of NF-kB even in the absence of exogenous TNF, when transfected into mammalian cells. The tissue distribution of wsl-1 is significantly different to the TNFR1 binding protein TRADD, which may suggest that this emerging protein family is involved in cell-specific signalling through TNFR1.

IDENTIFICATION OF CELL DEATH GENES IN *DROSOPHILA*,

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A *Drosophila* gene reaper (*rpr*) appears to exert a central control function for the initiation of programmed cell death White et al., Science 264:677, 1994). The *rpr* gene is normally expressed in cells fated to die 1 to 2 hours prior to their actual degeneration, and ectopic expression of *rpr* is sufficient to induce cell death in many types of cells. Previous work showed that in flies lacking *rpr* activity, the cell death machinery is intact. Therefore, it is likely that *rpr* acts upstream of the molecular effectors of cell death. To understand the entire pathway leading to cell death, from the selection of the doomed cell to the molecular effectors of the killing process, we have initiated a genetic screen in a search for genes that interact with *rpr*. A collection of fly strains, in which each strain carries a distinct chromosomal deletion, was examined for an effect on *rpr*-induced cell death in the eye. Together, this collection uncovers ~60% of the *Drosophila* genome. Of the 70 strains tested so far, six strains exhibited a suppression of *rpr*-induced cell death in the eye, while one showed an enhancement of the process. We initially ruled out the possibility that the genes included in the selected regions were acting on the regulatory elements directing *rpr* expression to the eye. Interestingly, cell death that is normally observed during embryogenesis appears reduced in some the selected suppresser strains. Furthermore, ectopically-induced embryonic and larval cell death is also reduced in some of the suppressers. Our goal is to identify and characterize the specific *rpr*-interacting genes that are responsible for these biological effects.

MACROPHAGE RECOGNITION OF "POST-APOPTOTIC" CELLS: A LAST LINE OF DEFENCE AGAINST TISSUE INJURY?

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A number of phagocyte receptors have been implicated in recognition of apoptotic cells including $\alpha_v\beta_3$/CD36/thrombospondin; phosphatidylserine (PS) receptors; lectin-like receptors; class A scavenger receptors; and ABC transporter proteins. We wondered whether these different recognition mechanisms might be ordered in a hierarchy of "back-ups", such that the cell dying by apoptosis may be recognisable to phagocytes by a number of different mechanisms at different times before it finally swells and disintegrates. In particular, we were interested in "post-apoptotic" cells which despite remaining intact and shrunken had lost the ability to exclude trypan blue, since in co-culture experiments in vitro inhibition of phagocytosis resulted in accumulation of such cells but no detectable leakage of intracellular contents until several hours later.

We modelled "post-apoptotic" cells by heat shocking apoptotic human neutrophils. Recognition of such cells was studied in standard phagocytosis assays using mouse bone marrow-derived macrophages and thioglycollate-elicited murine peritoneal macrophages. The former employ $\alpha_v\beta_3$ in recognition of apoptotic cells, while the latter use PS receptors. In neither case did inhibitors of the appropriate recognition mechanism block uptake of post-apoptotic PMN, but phagocytosis was inhibited in each case by 0.1 mMol of colchicine. Fucoidan (1 mg/ml) had no effect on recognition of apoptotic PMN, but in both cases inhibited uptake of post-apoptotic PMN by ~70%. Dextran sulfate also inhibited, but this was non-specific as uptake of opsonised red cells was also inhibited. Although these data suggested involvement of macrophage (Mø) Class A scavenger receptors, only weak inhibition was seen with specific inhibitors such as polyinosinic acid and 2F8 mAb (gift of Drs S Gordon and N Platt). We conclude that "post-apoptotic" but intact neutrophils, are specifically recognised by macrophages using receptors for polyanionic structures which probably differ from classical scavenger receptors. This may represent a last line of phagocytic defence for clearance of cells dying by apoptosis.

THE ACTIVITY OF DIPEPTIDYLPEPTIDASE 1Y (AS CD26) IN THE LIVING AND DEAD MOUSE THYMOCYTES.

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The bulk of works discussed current understanding of biochemical and molecular mechanisms of apoptosis, a widespread phenomenon in a myriad of physiological and pathological processes (Schwartzman, Cidlovski, 1993). But the role of dipeptidylpeptidase 1Y (the enzyme, which has been identified as leukocyte antigen CD26, playing an important part in the lymphocyte activation) has not been investigated in the processes of cell death.

We evaluated the activity of dipeptidylpeptidase 1Y (DP 1Y) histochemically according to the method of Loida (1982) in the living and dead thymocytes, separated by the method of Davidson, Parish (1975). The enzyme activity was higher in the living cells in comparings with dead. Some statistical parameters of the enzyme distribution also differed in these two cell populations. The deviation of DP 1Y of living cells was rather higher than in dead. Coefficients of asymmetry and excess did not stay constant in the above mentioned cells. Entropy of DP 1Y of dead cells was too lower in comparings with living one.

So DP1Y takes part not only in the processes of activation and proliferation of the lymphocyte, but also in the cell death.

THE INTEGRATED CONTROL OF CELL PROLIFERATION AND PROGRAMMED CELL DEATH (APOPTOSIS) BY ONCOGENES

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The spontaneous outgrowth of more rapidly proliferating mutant cells is a source of continuous neoplastic risk to multicellular organisms. Nonetheless, metazoans require proliferation of their component cells in order to differentiate, maintain and repair their morphologies. There thus exists a dynamic natural selective tension between an organism and its component cells. Metazoans must somehow permit proliferation of their component cells yet a the same time rigorously suppress outgrowth of neoplastic variants.

The c-*myc* proto-oncogene encodes an essential component of the cell's proliferative machinery and its deregulated expression is implicated in most neoplasms. Intriguingly, c-*myc* is also a potent inducer of apoptosis in cells deprived of growth factors or subjected to cytostatic drugs. Myc-induced apoptosis is dependent upon the level at which c-Myc is expressed, and deletion mapping shows that regions of c-Myc required for apoptosis overlap with regions necessary for co-transformation, autoregulation, inhibition of differentiation, transcriptional activation and sequence-specific DNA binding. Moreover, induction of apoptosis by c-Myc requires association with c-Myc's heterologous partner, Max. All of this strongly implies that c-Myc drives apoptosis through the transcriptional modulation of target genes.

Two simple models can be invoked to explain the induction of apoptosis by c-Myc. In one, death arises from conflict generated by the inappropriate or unscheduled expression of c-Myc under conditions that would normally promote growth arrest. In this "Conflict" model, induction of apoptosis is a not a normal function of c-Myc but a pathological manifestation of its deregulation. The other model holds that induction of apoptosis is a normal obligate function of c-Myc that is usually suppressed by the action of specific survival factors. We term this the "Dual Signal" model - one signal activates both mitogenesis and apoptosis, the second is required to block cell death.

Our studies show that c-Myc-induced apoptosis in fibroblasts is specifically suppressed by the insulin-like growth factors and PDGF independently of, growth state, position within the cell cycle, or whether apoptosis is triggered by low growth factors or by DNA damage. These results favour the Dual Signal model.

c-Myc-induced apoptosis is also suppressed by expression of the *bcl*-2 proto-oncogene and we have recently identified novel members of the *bcl*-2 family, some of which trigger apoptosis rather than suppress it. c-Myc-induced apoptosis also requires expression of p53.

THE E1B 19K GENE PRODUCT FUNCTIONS AS AN APOPTOSIS INHIBITOR BY INTERACTING WITH AND INHIBITING THE P53-INDUCIBLE AND DEATH-PROMOTING BAX PROTEIN.

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Regulation of programmed cell death (apoptosis) by the adenovirus E1A and E1B oncogenes is important for sustaining a productive infection in human cells and for transforming rodent cells. The E1A proteins initiate cellular proliferation which causes p53 accumulation and apoptosis. The E1B gene encodes functions to suppress p53-dependent apoptosis, one of which is the E1B 19K protein that is the adenovirus functional equivalent and homologue of Bcl-2. Expression of the E1B 19K or Bcl-2 proteins permits E1A expression and subsequent growth deregulation to occur unimpeded by cell death. Without inhibition of p53-dependent apoptosis by E1B or Bcl-2, transformation of rodent cells is rare and premature death of productively infected human host cells impairs virus yield. In cells driven into apoptosis by E1A and p53, E1B 19K and Bcl-2 protein expression prevent apoptosis but the growth arrest function of p53 remains intact. Thus, the apoptotic and growth arrest functions of p53 are separable, and Bcl-2 expression may represent a cellular mechanism for controlling the activity of p53.

p53 is a transcription factor that can both activate and repress transcription. Mutant p53 containing a crippled activation domain is unable to induce apoptosis. p53 must therefore induce apoptosis by either activating the transcription of death genes such as *bax,* or by repressing the transcription of survival genes such as *bcl-2*. Bax mRNA and protein levels were dramatically elevated by conformational change of p53 to the wild-type form. Furthermore, *bax* expression induced apoptosis in cells expressing mutant p53 indicating that Bax acts downstream of p53 and is alone sufficient to induce apoptosis.

To establish the mechanism of regulation of apoptosis, E1B 19K interacting cellular proteins have been identified using the yeast two-hybrid system. One 19K binding protein is Bax, the previously identified functional antagonist of Bcl-2. E1B 19K expression prevents Bax-induced apoptosis suggesting that the 19K protein acts similarly to Bcl-2 by binding to the same death promoter Bax to prevent apoptosis. Thus p53 transcriptionally activates *p21/WAF-1* and *bax* which then activates pathways for both growth arrest and apoptosis, respectively. Bcl-2 or E1B 19K proteins bind to and inhibit the action of Bax, blocking apoptosis and thereby permitting p21/WAF-1-dependent growth arrest.

HUMAN CYTOMEGALOVIRUS IE1 AND IE2 PROTEINS BLOCK APOPTOSIS

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As much as 60% of the adult population is seropositive for human cytomegalovirus (HCMV). It is an important pathogen because it causes birth defects and a variety of human diseases, especially in immunocompromised individuals, such as chemotherapy patients, AIDS patients and organ transplant patients. HCMV encodes two major immediate-early genes, IE1 and IE2. IE2 is a transcriptional regulatory protein. It may interact with tumor-suppresser gene, p53, and be involved in coronary restenosis. The function of IE1 remains unclear.

Apoptosis plays an important role in viral infection. Infected cells may undergo apoptosis to protect against virus spread. Some viruses encode anti-apoptotic proteins to prolong the survival of the host cells and maximize viral production. HCMV-infected human fibroblasts are resistant to the induction of apoptosis by superinfection with a mutant adenovirus lacking the E1B 19 kDa gene that normally causes an E1A protein-mediated apoptotic response. Two HCMV gene products were identified that block apoptosis. The IE1 and IE2 proteins each inhibit the induction of apoptosis by tumor necrosis factor α or by the E1B 19 kDa-deficient adenovirus but not by irradiation with ultraviolet light. These two proteins also cooperate with the adenovirus E1A proteins to transform baby rat kidney cells, presumably by blocking apoptosis. Our results suggest a new physiological role for the IE1 and IE2 proteins in the HCMV replication cycle.

Activation of novel oncogenic functions of Epstein Barr Virus BHRF1 and Bcl-2 proteins that efficiently overcome p53-induced apoptosis and result in cell proliferation. Paul Theodarakis, Erik Uhlmann, Cleta D'Sa Eipper, T. Subramanian and G. Chinnadurai. Institute for Molecular Virology, St. Louis University Medical Center, 3681 Park Avenue, St. Louis, MO 63110.

The tumor suppressor protein, p53, induces either growth arrest or apoptosis, depending on cell type. Apoptosis by p53 can be potentiated by oncogenes such as adenovirus E1A, *c-myc* and E2F. Primary rat kidney (BRK) cells, transformed by adenovirus E1A and a *ts* p53 mutant (p53val135) undergo total apoptosis when the p53 protein assumes *wt* conformation at 32.5°C. This apoptotic cell death can be suppressed by anti-apoptosis proteins such as EBV BHRF1, adenovirus E1B 19 kDa and Bcl-2. We examined the effect of a library of EBV BHRF1 mutants on the apoptosis of BRK-E1A/p53val135 cells at 32.5°C. These studies revealed four distinct domains essential for anti-apoptosis activity and a fifth, regulatory domain. A number of mutants within the regulatory domain efficiently suppressed apoptosis. Interestingly, despite expression of high levels of functionally *wt* p53, the regulatory domain mutants allowed cells to proliferate efficiently at 32.5°C, in contrast to extended cell survival (without proliferation) conferred by *wt* BHRF1 protein. These results raise the possibility that such mutational activation of the BHRF1 protein may play a direct role in oncogenesis. Consistent with this prediction, we have observed that mutants within the regulatory domain exhibit enhanced *in vitro* transforming activity. Mutagenization of the region of human Bcl-2 protein corresponding to the BHRF1 regulatory domain also confers such anti-apoptosis, proliferation and *in vitro* transforming activities. Thus far, it has been believed that anti-apoptosis genes such as Bcl-2 and BHRF1 promote oncogenesis by extending cell survival. Our present results suggest that these genes can play a role in oncogenesis by both inhibiting apoptosis and facilitating cell proliferation. We will also discuss the potential mechanisms for such mutational activation.

BACULOVIRUS ANTI-APOPTOSIS PROTEIN P35 INHIBITS THE INTERLEUKIN -1β CONVERTING ENZYME (ICE) AND ITS HOMOLOG ICH-2

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ICE is the cysteine protease that generates the active form of the proinflammatory cytokine IL-1β from its inactive precursor. Invertebrate and mammalian homologs of this enzyme, CED-3, ICH-1, ICH-2 and CPP32 are thought to play essential roles in apoptosis. Coexpression of the baculovirus anti-apoptotic protein p35 blocks ICE-induced apoptosis in insect cells. The ability of ICE to process pro-IL-1β in COS cell transfectants is also blocked by cotransfection with cDNA that encodes p35. Preincubation of recombinant p35 with purified human ICE inhibits the proteolytic activity of the latter, as measured with a tetrapeptide substrate. Inhibition of enzymatic activity correlates with the appearance of two cleavage fragments of p35 that form a stable complex with ICE. Similar results are obtained with the human ICE homolog ICH-2. Co-expression of p35 in bacterial cells prevents the autoproteolytic activation of ICE from its precursor form. We propose that p35 is a specific inhibitor of ICE-family cysteine proteases and that such activity accounts for the anti-apoptotic effects of this insect virus protein.

SUPPRESSION OF APOPTOSIS BY BACULOVIRUS P35: IDENTIFICATION OF P35 FUNCTIONAL DOMAINS.

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The baculovirus protein P35 prevents programmed cell death that is induced by a variety of signals in diverse organisms (insects, nematodes, and mammals). Thus, it is likely that this insect virus-encoded death regulator blocks a highly conserved cell death signal or activity. We have examined the molecular mechanism of P35 function by using site-directed mutagenesis to identify protein domains required to prevent virus-induced apoptosis. A systematic mutational analysis using Ala-Ser insertions and charged-to-alanine substitutions revealed two charged regions (CHR1 and CHR2) within the N-terminal half of P35 that were essential for anti-apoptotic activity. CHR1 (amino acid residues #17-27) may mediate P35 oligomerization since it is contained within $p35^{1-76}$, a truncation of *p35* that dominantly interferes with P35 activity. In addition, mutation of CHR1, but not CHR2, disrupted P35-P35 interaction as shown by a yeast two hybrid assay. CHR2 (residues #54-100) contains a predicted amphipathic α-helix with a potential interleukin-1β-converting enzyme (ICE)-like protease cleavage site, DQMDG (P4-P1′). Substitution of the P1 and P4 aspartic acid residues with alanine caused loss of P35 anti-apoptotic activity. Pulse-chase and immunoprecipitation analysis of baculovirus-infected cells also revealed that P35 is proteolytically processed to produce polypeptide fragments with a size expected for cleavage within CHR1. Thus, P35 cleavage is correlated with anti-apoptotic activity. These data are consistent with a model whereby P35 oligomerization and P35 interaction with an ICE-like protease within infected insect cells are required for suppression of virus-induced apoptosis.

SEMLIKI FOREST VIRUS INDUCED DEATH OF VERTEBRATE NEURONS

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Viral infections of the central nervous system cause diseases ranging from acute fatal to chronic progressive and include polio, rabies, measles and HIV-associated encephalopathy. Viruses also have been implicated in the aetiology of several neurodegenerative disorders such as ALS or Motor Neuron disease, Multiple Sclerosis and Parkinsonian syndromes. Understanding in detail how viruses may contribute to such disorders is hampered by a lack of sufficient information on the neurobiology of virus and host cell interaction. For example, a virus may cause neuron or glial cell destruction as part of its natural replicative life-cycle, or it may induce changes in host-cell phenotype due to underlying changes in gene expression in response to viral infection. Certain neurotropic viruses are virulent in embryonic, neonatal and postnatal stages of animal development but avirulent in adults. The replication of the A7(74) strain of Semliki Forest virus (SFV), a small positive stranded RNA virus, is complete and productive in embryonic and neonatal mouse neurons, but is restricted in neurons of adult mice. This is associated with a change from widespread to focal infection of the CNS and determines death or survival for infected individuals. SFV also infects oligodendrocytes and the restricted infection of the A7(74) strain in older mice is associated with foci of inflammatory demyelination. The related alphavirus Sindbis, can kill cells by programmed cell death but it has been shown that a persistent infection can be maintained by expression of the anti-apoptotic gene *bcl-2*.

To elucidate how and why SFV infection kills neurons we are initially characterising the mechanism of death using low density mouse primary cultures enriched for sensory neurons. The suppression of programmed cell death in these neurons during embryogenesis and *in vitro* requires NGF. Virus-induced death of these neurons appears similar to the cell death induced by NGF deprivation and possesses the morphological hallmarks of apoptosis, though the temporal sequence of events following addition of the virus is distinct from those caused by NGF deprivation. DNA breakages in dying neurons can be detected using labelled dUTP (TUNEL). SFV proteins and TUNEL reactivity colocalise within a small percentage of cells. The role of proteases in effecting the death of neurons is being investigated.

More mature neurons appear less susceptible to virus induced death. This may represent intrinsic molecular changes in the regulation of, or the implementation of the death mechanism. Alternatively, the virus stimulated production of interferons acting in an autocrine/paracrine manner may mediate a protective effect on neurons. This is being investigated using neuronal cultures prepared from interferon receptor knock-out mice. The change in susceptibility may ultimately contribute, in the absence of a virus-specific immune response in the animal, to the establishment of a persistent viral infection.

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IDENTIFICATION OF AN ANTI-APOPTOTIC GENE IN THE CHICKEN ADENOVIRUS CELO.

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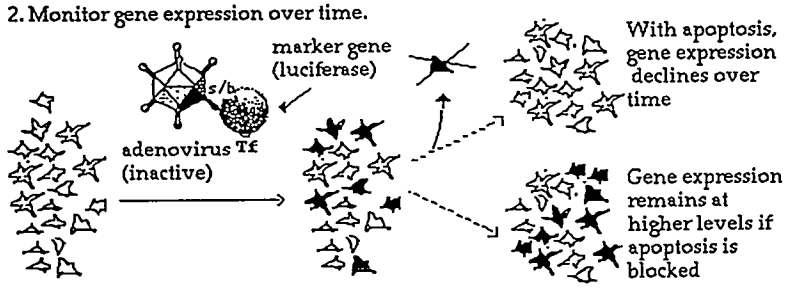
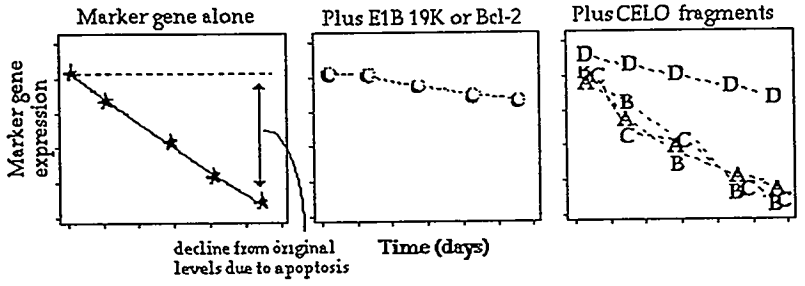
We have demonstrated that the decline in cell viability induced by adenovirus-augmented transfection in primary human fibroblasts can be prevented by expression of the anti-apoptotic genes E1B 19K or Bcl-2. This forms the basis of a simple screen for anti-apoptotic genes: co-transfect a luciferase expression plasmid with a test gene and look for an enhancement in long-term gene expression. Considering our previous experience with the avian adenovirus type 1 (CELO virus) we are screening for anti-apoptotic genes in the CELO virus. The CELO virus genome was subcloned onto bacterial plasmids and individual plasmids bearing CELO virus sequences were co-transfected with a marker gene into primary fibroblasts. We have found enhancement of long-term gene expression with plasmids bearing a portion of the CELO genome. Sequencing and deletion analysis of this region has identified a single large open reading frame with no homologies to the known anti-apoptotic genes. We are further characterizing this region.

A screen for anti-apoptotic genes

1. Transfect into primary fibroblast a marker gene alone, or in the presence of an anti-apoptotic gene or inthe presence of a test gene (e.g. CELO virus fragments).
2. Monitor gene expression over time.





IDENTIFICATION OF BIOCHEMICAL AND MOLECULAR DISTINCTIONS BETWEEN P53-MEDIATED GROWTH ARREST AND APOPTOSIS.

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Analysis has been made of the murine embryo fibroblast cell line Val 5, which contains the temperature-sensitive p53 mutant valine 135 as its only p53 allele, as well as a cell line derived from Val 5 (Vm10), that additionally contains an overexpressed c-myc gene. Importantly, when Val 5 cells are shifted to the permissive temperature of 32 degrees, these cells undergo a well-characterized G1 growth arrest; in contrast, temperature-shift induction of wt p53 in Vm10 cells induces a uniform and dramatic entry into apoptosis. The availability of these two cell lines provides a unique reagent for the analysis of potential differences in p53 activities during growth arrest and apoptosis.

This investigation has involved an analysis of these two cell lines for differences in the levels and activities of various cyclins, cyclin-dependents kinases, and cdk inhibitors, after wt p53 induction. Briefly, we have found that the p53-induced cdk inhibitor p21/waf1 is induced and likely functions as a cyclin/cdk inhibitor identically during both growth arrest and apoptosis. In fact, thus far the only distinction between cyclin/cdk activities during p53-mediated growth arrest and apoptosis that we have detected involves differences in the activity of the cyclin B/cdc2 complex.

Finally, we have also analyzed these two cell lines, using the differential display procedure, for differences in gene expression during p53-mediated growth arrest and apoptosis. In particular, due to recent evidence that p53-mediated repression of gene expression may be influential in apoptosis induction, we have identified three genes whose expression is repressed in response to wt p53. The characterization and possible functions of these three genes in p53-mediated apoptosis will be discussed.

CYCLIN KINASES, p21 AND APOPTOSIS. **Rati Fotedar,* Jody Flatt,† Patrick Fitzgerald,† Howard Brickner,† Neshat Sadatmandi,† Tristan Rousselle,* Sunita Gupta,* Dominique Cannela,* Helen Messier† Shailaja Khastilba,† & Arun Fotedar.†**
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We have investigated the role of cell cycle regulators in T cell death. Synchronously cycling T cell hybridomas undergo programmed cell death after activation of the T cell receptor. We have shown that activated T cells exit the cell cycle at G2/M and undergo apoptosis. T cell death is accompanied by persistent and elevated levels of cyclin B kinase activity (Fotedar, et al 1995). Cyclin B specific antisense suppresses activation induced T cell death but does not suppress IL2 gene induction in the same cell line. These results suggest that a persistent elevation of cyclin kinases is required for activation induced T cell death.

To further examine the relationship between cell cycle regulators and apoptosis, we have analyzed the effect of p21 on T cell death. p21 is an inhibitor of cyclin kinase inhibitor but it also associates with PCNA. We have specifically examined the effect of the N terminal domain of p21 which binds cdk-cyclin and inhibits cyclin kinase activity, on activation induced cell death in transient transfection assays. We have also examined the effect of inducers of cyclin kinase inhibitors such as TGFβ-1 on activation induced cell death in T cell hybridomas. Finally, we have generated p21 transgenic mice in which the thymus specific promoter lck drives the expression of the transgene. Using these mice, we have determined the effect of p21 transgene on cell death induced by different apoptotic stimuli in quiescent thymocytes. The implications of our observations in current apoptotic paradigms will be discussed.

ALTERED G1/S-PHASE CONTROL CAUSES APOPTOSIS IN RB-DEFICIENT MOUSE EMBRYONIC FIBROBLASTS

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The retinoblastoma protein (pRb) has a growth suppressive function attributed to its control of the G1/S-phase transition. Mouse embryonic fibroblasts (MEF) from Rb or p53 knock-out mice were used to determine the specific contributions of Rb and p53 to cell proliferation or survival. MEFs with two inactivated Rb alleles (Rb-/-) entered S-phase in the presence of the DHFR inhibitor Mtx. The DHFR expression levels were elevated in Rb-/- cells, demonstrating in culture that release of the E2F transcroptional activator from pRb was responsible S-phase entry. Rb-/- cells, similar to p53-/-, but not $Rb^{+/+}$ or $Rb^{+/-}$ MEFs entered S phase after γ-irradiation. Since the response to γ-irradiation is p53-dependent, this demonstrates a functional link between p53 and Rb in a common cell cycle pathway which controls S-phase entry.

Treatment with antineoplastic, DNA damaging, drugs caused accumulation of p53 in the nuclei of all MEFs, regardless of their Rb genotype. However, p53 induction had a different physiological outcome in the different MEFs, resulting in G1/S arrest in Rb+/- or Rb+/+ cells, and apoptosis in Rb-/- MEFs. We are presently analyzing the levels of Bcl-2, Bcl-x, Bag-1, and Bax in cells with different genotypes. Generally, there was a lower level of the cell survival proteins, Bcl-2, Bcl-x and Bag-1 in Rb-/- MEFs. The kinetics of protein induction differed at specific times after drug, or radiation, treatment in cells with different genotypes and a higher ratio of cell death versus cell survival proteins was observed in Rb-deficient cells. Most significantly, p53-/- MEFs induced Bcl-2 protein after γ-irradiation, demonstrating that Bcl-2 activation is prevented in cells with functional p53, as suggested earlier by *in vitro* experiments (1). Loss of p53-mediated repression of Bcl-2 would explain why inactivation of p53 or over-production of Bcl-2 so frequently result in tumors. Our results suggest the necessity for a concerted action of Rb and p53 for controlling cell survival in addition to their demonstrated role in cell proliferation control.

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DIFFERENTIAL EXPRESSION OF CELL SURVIVAL AND CELL DEATH FACTORS DURING MULTISTAGE TUMORIGENESIS

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Transgenic mice (Rip1-Tag2) expressing the SV40 large T oncoprotein under control of a rat insulin promoter reproducibly develop ß cell tumors in the pancreatic islets. Tumor development involves at least four discernible stages: 'normal' islets in which all ß cells express the oncogene; hyperplastic islets with a high incidence of proliferating ß cells; angiogenic islets, which elicit the growth of new blood vessels; and solid tumors. Analysis of the apoptotic incidence at the various stages of tumor development has revealed that cell death increases between the 'normal' and hyperplastic stages, and further increases to a peak in the angiogenic stage, followed by a subsequent drop in end-stage tumors. Interestingly, clonal cell lines from tumors have been found to exhibit varying propensities toward apoptotic death under low serum conditions, suggesting heterogeneity in the ability of cells within tumor cell populations to resist apoptotic death signals. We have previously shown that the cytokine/survival factor insulin-like growth factor II (IGF-II) is up-regulated concomitant with the hyperproliferative switch. Rip1-Tag2, Igf-2$^{-/-}$ mice develop smaller tumors with a five-fold increase of apoptotic cells compared to wild-type. Apoptosis in cultured tumor cells can be reduced by the addition of IGF-I or IGF-II, supporting a role for IGFs in promoting tumor cell survival. Surprisingly, however, overexpression of IGF-II in the early stages of tumorigenesis in Rip1-Tag2, Rip-Igf-2 double transgenic mice does not significantly reduce the apoptotic incidences, implicating additional factors in the regulation of apoptosis. We have now analyzed the expression of other known apoptotic regulatory genes in the pathway, namely bcl-2, bcl-x, bax, bad, bag-1 and bak. The death effector bax is low to non-detectable in normal non-transgenic islets, but is clearly up-regulated and consistently expressed during tumorigenesis. The survival factor bcl-x, in contrast, is expressed in normal islets and detectable throughout the pathway, and is up-regulated in the final tumor stage. Notably, expression of bax and bcl-x do not appear to be regulated by IGF-II. The analysis of bad, bag-1 and bak is ongoing, as are experiments to assess the functional contribution of these factors to tumor cell proliferation, survival and apoptosis.

G1 PHASE REGULATION OF T-CELL RECEPTOR ACTIVATION-DEPENDENT APOPTOSIS

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Strong activation of cycling T-cells through the T-cell receptor (TCR) leads to rapid programmed cell death. The speed and specificity of this response suggest that cell cycle control may be important in TCR activation-dependent apoptosis. To test this hypothesis, the effects of TCR signaling on cell cycle progression were studied, using the Jurkat T-cell lymphoma cell line as a model system. Activation of TCR in Jurkat cells results in programmed cell death and is largely complete by 24 hours. This time course coincides closely with the doubling time of these cells. The kinetics of TCR stimulation on asynchronous Jurkat cells was analyzed for the phosphorylation state of the retinoblastoma protein (pRb), cyclin-dependent kinase 2 (cdk2) activity, and DNA content. pRb changes gradually from a primarily hyperphosphorylated (inactive) to a hypophosphorylated (active) form after stimulation and persists in the apoptotic cells. Cdk2 activity is correspondingly decreased following stimulation, with kinetics slightly lagging the detection of hypophosphorylated pRb. Flow cytometric analysis (FACS) for DNA content from TCR stimulated asynchronous cells suggests that cell death is preferentially occurring from the G1 phase of the cell cycle and not from S, G2 and M phases. These results suggested that passage through a G1 check point proceeded activation of the apoptotic degradation machinery. To test this notion further, S phase Jurkat cells labeled with BrdU from an asynchronous population were followed after TCR stimulation. The BrdU positive cells were found to cycle through G2 and M phase, accumulate in G1, then undergo apoptosis. In addition, replating of TCR stimulated elutriated late G2/M phase cells results in progression through early G1 followed by a late G1 phase apoptosis. Taken together, these results are consistent with a late G1 phase check point occurring prior to apoptotic commitment.

FUNCTION OF THE PITSLRE PROTEIN KINASES DURING THE CELL CYCLE AND AS EFFECTORS OF APOPTOSIS

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The PITSLRE protein kinases are members of the $p34^{cdc2}$ gene family whose function appears to be linked to apoptosis. Multiple physiological stimuli that trigger apoptosis in T cells results in the induction of a PITSLRE protein kinase isoform, p50, and a concomitant increase in enzyme activity. This isoform is generated by specific proteolysis of multiple, larger PITSLRE isoforms, and it can be blocked by specific protease inhibitors, such as TPCK and the viral gene product *crmA*. We have now shown that expression of only the smaller, processed PITSLRE isoforms (p58 and p50) can induce apoptosis in heterologous cells, whereas expression of larger PITSLRE isoforms (p65, p90 and p110) does not. Site-directed mutagenesis of several potential ICE-like cleavage sites in the PITSLRE molecule that could give rise to the p50 isoform is being performed, as well as mapping of the in vivo cleavage product(s), to determine whether this class of protease acts directly upon these kinases. Finally, we have determined that the largest PITSLRE isoform, p110, is normally localized in the nuclear matrix with other spliceosome components, and may play a role in regulating RNA processing/transcription during the cell cycle and cell death. The RNA-binding protein RNPS1 associates with the p110 PITSLRE kinase in vivo, and may be crucial to its function. Additionally, RNPS1, as well as other DNA/RNA binding proteins that interact with the PITSLRE kinases, are rapidly down-regulated following physiological signals that trigger apoptosis in T cells.

UBIQUITIN-REGULATED DEGRADATION OF THE P53 AND P27 PROTEINS

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The regulated degradation of key intracellular proteins is achieved through the conjugation of a small MW 7,000 protein called ubiquitin, to a target substrate. A biochemical cascade that involves enzymes called ubiquitin-conjugating enzymes (UBCs or E2s) and ubiquitin-ligases (E3s) is responsible for the specific ubiquitination of a given target in response to a given stimulus.

Proteins that have been shown to be degraded via ubiquitination include cyclin B, p53, c-jun, c-myc and others. We recently became interested in studying the degradation of p53 in human cervical carcinoma cells, with the aim of developing p53 degradation inhibitors that would cause tumor cells to enter apoptosis. As previously shown by Howley and colleagues, in the presence of a cellular protein, E6AP, the viral oncoprotein E6 binds and induces the degradation of p53 in cells infected with HPV (human papilloma virus) of the serotypes 16 and 18. We have cloned cDNA's encoding several human ubiquitin-conjugating enzymes and have shown that UBC4 is the enzyme responsible for inducing p53 degradation in HPV infected cells. Using antibody and antisense microinjection we show that UBC4 and E6AP are both required for p53 degradation in HPV-cancer cells, and that in fact E6AP accepts ubiquitin from UBC4 and then transfers it to p53. We also find that the microinjected HPV-infected cells enter apoptosis, presumably as a result of the unbalance created by the up-regulation of p53 in cells that have lost a functional pRb, due to its binding to HPV-E7. Our results open the possibility of targeting the biochemical reactions involved in p53 degradation for inhibitors that would specifically cause HPV-infected tumor cells to enter apoptosis.

We have also studied the regulation of the cyclin-dependent kinase inhibitor p27 by the ubiquitin pathway. p27 accumulates in quiescent cells, eg. lymphocytes, and it rapidly disappears upon growth factor stimulation of these cells. We have discovered that p27 is indeed targeted for ubiquitination and degradation as cells exit quiescence. Using an inhibitor of the proteasome we find that p27 is ubiquitinated in living cells. The rate of p27 ubiquitination and degradation is significantly higher in actively growing cells compared to quiescent cells, while its rate of synthesis remains constant. We found that human UBC2 (Rad6) and UBC3 (Cdc34) are both capable to induce p27 ubiquitination in vitro, in the presence of a cellular fraction that presumably supplies an E3 function. We believe that inhibitors of ubiquitin-mediated degradation might become of use as therapeutic agents. These agents could be used to specifically inhibit the degradation of certain intracellular proteins that are involved in proliferative diseases such as cancer.

THE INS AND OUTS OF PROGRAMMED CELL DEATH M.C. Raff, B. Barres, J. Burne, H. Coles, M. Jacobson and Y. Ishizaki, M. Weil. MRC Laboratory of Molecular Cell Biology, University College London, London WC1E 6BT, U.K.

Programmed cell death(PCD) is the process where cells activate an intrinsic death programme and thereby kill themselves. It is only recently that it has been recognized to be a fundamental property of animal cells. In my talk I will review evidence for the following three tentative conclusions: (1) All nucleated mammalian cells are capable of undergoing PCD and constitutively express all the protein components required to execute the death programme. (2)All mammalian cells may be programmed to kill themselves unless they are continuously signalled by other cells not to do so. (3) PCD can occur in the absence of a nucleus, mitochondrial respiration, atmospheric oxygen, or active cdc-2 kinase, suggesting that it is mediated by a cytoplasmic regulator that acts in parallel on multiple organelles; although the nature of the regulator is unknown, there is increasing evidence that it may be a cysteine protease of the Ced-3/ICE family.

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THE ATP BINDING CASSETTE TRANSPORTER ABC1 IS REQUIRED FOR THE ENGULFMENT OF APOPTOTIC BODIES.

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ATP binding cassette transporters define a family of proteins with strong structural similarities , conserved across evolution and able to translocate a variety of substrates across cell membranes. A few mammalian members of the family are known but the knowledge of their molecular function is still scanty. We report here a morphological and functional study on the recently identified mammalian ABC transporter ABC1. Its distribution during embryonic development correlates spatially and temporally with the occurrence of programmed cell death and appears to be directly related to the presence of macrophages engaged in the engulfment and clearance of cell corpses. The presence of ABC1 on engulfing macrophages has been evidenced in two well known models of PCD, the interdigital web during the sculpting of digits and thymic apoptosis induced by corticosteroids. Moreover, ABC1 transporter appears to be required during the engulfment process since the ability of macrophages to ingest apoptotic bodies is severely impaired after antibody-mediated steric blockade of ABC1.

Finally, ABC1 appears to be strikingly conserved across evolution since a structural homolog could be identified in C.elegans genome.

LOSS OF RETINOBLASTOMA TUMOR SUPPRESSOR GENE FUNCTION INDUCES CELL DEATH IN THE CENTRAL NERVOUS SYSTEM IN A p53-DEPENDENT MANNER AND IN THE SENSORY GANGLIA IN A p53-INDEPENDENT MANNER.

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The retinoblastoma and p53 tumor suppressor genes are inactivated in a wide variety of human tumors and several DNA tumor viruses have evolved viral oncoproteins which functionally inactivate both pRb and p53. Furthermore, mutations in Rb and p53 cooperate in the formation of certain tumor types in the mouse. In response to certain stresses and depending on cell type and growth factor environment, p53 can induce growth arrest or programmed cell death. Recent work has suggested that activated E2F-1 and wild-type p53 cooperate to induce apoptosis and this may explain how mutations in Rb and p53 cooperate in the genesis of some tumor types.

We have examined the role of p53 in mediating cell death in the nervous system of Rb deficient mouse embryos and have concluded that loss of Rb induces programmed cell death in the central nervous system by a p53-dependent mechanism but by a p53-independent mechanism in the sensory ganglia. Mice carrying a targeted disruption of the Rb gene die between days 13.5 and 14.5 of gestation exhibiting extensive cell death in the fetal liver, the ocular lens and the nervous system. Programmed cell death plays a critical role in the development of the normal nervous system. However, the extent of apoptosis occurring in the nervous system of Rb deficient mice is much more extensive than that seen in wild-type mouse embryos. Abnormal cell death occurs in the forebrain, hindbrain, spinal cord and sensory ganglia and is associated with aberrant proliferation of supposedly post-mitotic neurons. Cell death in the central nervous system is accompanied by increased levels of the p53 tumor suppressor gene product and increased expression of the p53-target gene, $p21^{WAF-1/CIP-1}$. Consistent with these observations, loss of p53 gene function rescues cell death in the central nervous system of Rb mutant embryos. However, loss of p53 does not rescue death in the sensory ganglia of these mice indicating that apoptosis in Rb mutant embryos occurs by a p53-dependent mechanism in the central nervous system and by a p53-independent mechanism in the sensory ganglia. These results underscore the importance of cell-type in determining the nature of any functional interaction between Rb and p53.

INACTIVATION OF RB FAMILY MEMBER OR AN RB RELATED PROTEIN BY SV40 T ANTIGEN(LT) IN EPITHELIAL CELLS RESULTS IN MASSIVE APOPTOSIS AND MESENCHYME LIKE CONVERSION. Cecile Martel[1], Eric Batsché[1], Francis Harper[2] and Chantal Crémisi[1].[1]INSERM U180 45 rue des St[s]-Pères, ParisV.[2]Laboratoire ultrastruture du noyau, CNRS, Villejuif, France.

LT transformation has been linked to an increase in cell proliferation. In renal MDCK epithelial cells, the mitogenic effect of LT is followed by a mesenchyme conversion and a massive apoptosis which can affect at confluence 50% of the cells. Cell death and mesenchyme conversion were dependent on the activity of LT to bind RB or an RB related protein, since MDCK cells expressing LT mutants unable to bind RB did not die and behaved like the parental cells. These results suggest that RB(or an related protein) is not only essential for the epithelial phenotype, but is also linked to the signals leading to programmed cell death (PCD) in epithelial cells.
This PCD occured in presence of serum, 5% or 10% FCS. Importantly apoptosis was dependent on cell density and intercellular contacts.
In our system, the serum had a dual effect, (i) a survival effect stimulating proliferation and preventing apoptosis and (ii) an apoptotic effect inhibiting proliferation and increasing cell death. These two effects were expressed sequentially during cell growth. Moreover, the deprivation of serum induced a slight increase in apoptosis(10%) and a decrease in cell proliferation. These results showed that there is a correlation between proliferation and increased survival and inversely an increase of PCD with a down regulation of proliferation. These observations were reinforced by the simultaneously rescue of apoptosis and cell proliferation by EGF and TPA treatment of MDCK(LT). PDGF, which had no mitogenic effect on epithelial cells, was unable to prevent apoptosis, whereas we have shown, that it completely rescued apoptosis in fibroblasts overexpressing c-myc. Again EGF, which has no mitogenic effect on fibroblasts, had no effect on PCD in these cells. Cell viability could also be restored by BCL2.
Recently we have demonstrated that c-myc is highly and constitutively expressed in epithelial cells(1) and that RB transvactivates c-myc independently of the E2F factor(2). RB inactivation in epithelial cells also led to a down regulation of c-myc and EGF and TPA treatment simultaneously prevented apoptosis and restored high c-myc expression. In order to get an insight into the functional relation between RB, c-myc and apoptosis in these cells, MDCK(LT) cells were retransformed with RB and c-myc. Results will be presented and discussed. It should be noted that our system mimics the steps occurring during metanephric development in vivo; massive apoptosis and mesenchyme conversion(3,4).
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APOPTOSIS AND REMODELLING OF MAMMARY GLAND TISSUE DURING INVOLUTION PROCEEDS THROUGH p53 INDEPENDENT PATHWAYS

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Mammary gland involution is a physiologic process which follows lactation and results in the rapid disappearance of the entire lobulo-alveolar compartment. Coincident with the onset of involution, milk protein gene expression ceases and alveolar cells undergo programmed cell death (PCD). In mammary epithelial tissue culture cells *in vitro*, both p53 dependent and p53 independent apoptosis pathways have been identified. We investigated whether p53 induces apoptosis during mammary gland involution *in vivo* and participates in tissue remodelling. Towards this end, we examined the process of involution in the presence and absence of functional p53 in two mouse models. First, in transgenic mice which express SV40 T-antigen specifically in mammary tissue during pregnancy. Secondly, mice were analyzed which carried non-functional p53 alleles in their germline. Mammary gland whole mount and histological analyses revealed that involution and remodelling, with the concommitant disappearance of the lobulo-alveolar structures, proceeded normally in the absence of functional p53. The number of mammary alveolar cells undergoing PCD in the two models and in mice exhibiting functional p53 was similar. In addition, the absence of functional p53 did neither alter the involution related pattern of bax (death inducer) gene expression, nor the ratio of RNAs encoding bcl-x_S (death inducer) to bcl-x_L (survival inducer).

NEURONAL CELL DEATH FOLLOWING CALCINEURIN ACTIVATION IN XENOPUS EMBRYOS

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Elevated levels of intracellular calcium, mediated by glutamate-sensitive calcium channels, play an important role in neuronal cell death during brain trauma and stroke. The pathways by which this elevated calcium induces cell death is unknown, although several calcium-activated enzymes such as CAM-kinase II, nitric oxide synthetase, and calcineurin are potential functional participants.

Calcineurin is a calcium/calmodulin-activated protein phosphatase that is found at high concentrations in the brain, and has been implicated in development of long-term synaptic depression. To test the effect of calcineurin activity during embryonic development, the mRNA encoding both the wild-type or constitutively active mutant form of calcineurin was injected into fertilized, single cell embryos of Xenopus laevis, and the developmental program was followed. While overexpression of the wild-type, calcium-dependent calcineurin had no effect on the normal development of Xenopus embryos, those expressing the constitutively active mutant showed severe defects restricted to the neural tube and related structures of the central nervous system, and ultimately died around stage 40. Significantly, the neural tube and related structures of the CNS in affected embryos show massive cell death detected by TUNEL staining, suggesting that these neurons undergo apoptotic cell death. The ability of calcineurin to induce neuronal cell death is remarkably similar to the effects of calcineurin expression in mammalian fibroblasts, where it causes apoptotic cell death which is blocked by the oncoprotein Bcl-2 and growth factors, especially bFGF.

We are presently assaying the interactions between calcineurin, Bcl-2, and bFGF in the regulation of neuronal cell death during Xenopus development. In addition, we hope to determine the role of calcium channels such as the NMDA (N-Methyl-D-Aspartic acid) receptor in regulating the activitiy of calcineurin and other calcium-activated enzymes that in turn may function in neuronal development and survival.

PEPTIDE INHIBITORS OF THE INTERLEUKIN-1B CONVERTING ENZYME (ICE) FAMILY PREVENT MOTONEURON PROGRAMMED CELL DEATH *IN VIVO* AND *IN VITRO*.

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Programmed cell death (PCD) during development serves to: 1) remove deleterious cells; 2) remove transiently needed tissues; 3) match populations of interacting cells. This latter process has been most extensively studied in the embryonic chick spinal cord where 50% of the developing motoneurons are lost. In order to characterize the molecular mechanisms that mediate this PCD, we have employed an *in vitro* primary culture system where cells plated in the absence of trophic support die by apoptosis. in this study, we have examined the role of the ced-3/ICE family of proteases. These proteases have been implicated in the regulation of PCD in both invertebrates and vertebrates. Peptide inhibitors of ICE arrest the programmed cell death of motoneurons *in vitro* as a result of trophic factor deprivation. Furthermore, when the peptide inhibitors are administered to chick embryos *in ovo* during the period of naturally occurring motoneuron death, this death is inhibited. In addition, interdigital cells that normally die during development are also rescued in animals treated with ICE inhibitors. Taken together, these results provide the first *in vivo* evidence that ICE or an ICE-like protease plays a regulatory role not only in vertebrate motoneuron death but also in the developmentally regulated deaths of interdigital cells. We have begun to clone ICE family members from the embryonic chick and have identified two putative ICE homologs. One of these genes appears to be constitutively expressed in most tissues examined. The other is expressed in the developing brain, but not adult, suggesting that it may be developmentally regulated.

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EARLY GENE EXPRESSION AND TRANSCRIPTION FACTORS ACTIVATION IN RAT THYMOCYTES UNDERGOING APOPTOSIS AFTER γ IRRADIATION

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Previous data from our laboratory suggest that the same molecular pathways are shared in the early steps of cell proliferation and cell death. Indeed, we observed that some oncogenes - like c-fos, c-jun, c-myc - and transcription factors (TFs) - like AP-1 and NFkB - involved in cellular proliferation are induced also in thymocytes undergoing apoptosis after dexamethasone treatment (Grassilli et al, BBRC, 188: 1261, 1992; Sikora et al, BBRC, 192: 386,1993; Sikora et al, BBRC, 197: 709, 1993; Desiderio et al., Cell Growth Diff., 6: 505, 1995). In order to investigate if the same set of genes and TFs is involved in different types of apoptosis and to extend our knowledge about the TFs activated during the apoptotic process we characterized the early molecular events occuring in rat thymocytes undergoing cell death after γ irradiation.

By means of Northern blot we observed that c-fos mRNA was induced as soon as 0.25 hs after treatment, peaked at 0.5 hs and then slowly down-regulated, reaching basal levels within 8 hs. c-jun mRNA accumulated more transiently, peaking at 0.25 hs and becoming undetectable within 2 hs. Accordingly, a peak of AP-1 DNA-binding activity was recorded, by means of EMSA, 1-2 hs after thymocytes irradiation. As far as the expression of another couple of TFs coding genes is concerned we observed that c-myc induction reached its maximum at 0.5 hs while the expression of its partner, i.e. max, was constitutive. Concomitantly we showed that MYC/MAX DNA-binding activity peaked at 0.5 hs. Finally, we extended our observations to E2F, a TF whose transcriptional activation during the mid-late G1 phase of the cell cycle is known to allow the progression into the S phase. We observed that the low basal level of E2F DNA-binding activity present in untreated thymoctes rapidly increased reaching a peak 2 hs after the exposure to the apoptogenic stimulus.

On the whole our data suggest that the same set of genes and TFs is involved in different types of apoptosis and reiforce the hypothesis that the early phases of cell proliferation and apoptosis share the same molecular events. Moreover, the precocious E2F activation support the idea that apoptosis could be an abortive mitosis, i.e. could result from an abnormal passage through the cell cycle due to the deregulated expression of normal cell cycle components.

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INCREASED EXPRESSION OF THE ANTI-APOPTOSIS GENE, BCL-2, IN S49 "DEATHLESS" MURINE LYMPHOMA CELLS.

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Wildtype S49 murine lymphoma cells are killed after exposure to high levels of cAMP (dibutyryl cAMP; db cAMP). Mutant S49 cells known as "deathless" (D^-) have been characterized as resistant to the cytolytic effects of elevated cAMP. In D^- cells, growth is inhibited with increased cAMP levels, yet cells remain viable, and both phosphodiesterase and cAMP-dependent kinase activities are normal (Lemaire and Coffino, 1977, Cell 11:149). We have analyzed expression levels of genes which influence apoptosis in the mutant and parent S49 cell lines, using reverse-transcriptase polymerase chain reaction for quantitation. The *bcl-2* gene, characterized as repressing apoptosis, has significantly increased RNA expression in the D^- cells of at least 5 fold over the level in the normal S49 cells. Two other genes which may function as regulators of apoptosis, *bcl-x* and *bax*, were found to have unchanged RNA expression levels in the mutant cell line. The high expression of the *bcl-2* gene may play an important role in the inhibition of cAMP-induced cell death in the D^- murine lymphoma cell line.

FAP-1 A PROTEIN TYROSINE PHOSPHATASE THAT ASSOCIATES WITH FAS.

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Fas (CD95/AP0-1) is a member of the Tumor Necrosis Factor Receptor (TNF-R) family that controls a poorly understood signal transduction pathway leading to cell death via apoptosis. A protein tyrosine phosphatase (PTPase), FAP-1, capable of interacting with the cytoplasmic domain of Fas was identified by a yeast two-hybrid screen. FAP-1 binds specifically to the cytosolic domain of Fas but not TNF-RI, TNF-RII, or CD40. The carboxyl-terminal 15 amino-acids of Fas are necessary and sufficient for interaction with FAP-1. This 15 amino-acid region has previously been shown to function as a negative regulatory domain that impairs Fas-mediated cytotoxicity. FAP-1 expression is highest in tissues and cell lines that are relatively resistant to Fas-mediated cytotoxicity. Gene transfer-mediated expression of FAP-1 in Jurkat T-cells partially abolished Fas-induced apoptosis. These data indicate that FAP-1 is a negative regulator of Fas-induced pathways that lead to cell death.

ROLE OF PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY IN THE REGULATION OF APOPTOSIS.

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Various hemopoietic cytokines have been used to investigate the role of phosphatidylinositol 3-kinase (PI3K) in the inhibition of apoptosis. Recent studies from this laboratory have shown that a number of cytokines can inhibit programmed cell death (PCD), or apoptosis, in murine MC-9 cells, including IL-3, GM-CSF and IL-4. Each of these can also activate the enzyme PI3K. Our finding that IL-4 can activate PI3K, but is unable to activate p21ras and its downstream targets such as MAP kinase, allows us to dissociate these two major signalling pathways in hemopoietic cells. In contrast to IL-3 and GM-CSF, IL-4 acts only as a survival factor, rather than a growth factor, on MC-9 cells. Therefore, activation of PI3K may play an important role in hemopoietic cells as the major pathway by which apoptosis is inhibited. We have made use of the selective activity of wortmannin and LY294002 as inhibitors of PI3K to test this hypothesis. In cells deprived of cytokine, the characteristic fragmentation of DNA seen in cells undergoing apoptosis was observed. As expected, incubation with IL-3, IL-4, IL-5, GM-CSF or SLF, at optimal doses, inhibited DNA fragmentation. Incubation with cytokines in the presence of PI3K inhibitors resulted in DNA fragmentation in cells incubated with IL-3, IL-4 or SLF. Cells incubated with GM-CSF were unaffected, and there was a partial effect on cells in the presence of IL-5, even when the inhibitors were used at much higher doses. The concentrations of wortmannin and LY294002 required to induce DNA fragmentation in the presence of the other cytokines correlated with the ability of the inhibitors to block PI3K in cells.

The difference in the effects observed with IL-3 and GM-CSF was surprising, since these two cytokines are known to activate similar signalling pathways. Several cell lines, both mouse and human, showed the same effect. We next tested whether there was a difference in effect on the p21ras-MAP kinase pathway. While wortmannin was able to partially inhibit activation of MAP kinase by IL-3 or GM-CSF, the inhibitory effect was the same in each case. LY294002 also partially inhibited MAP kinase activation. However, these effects appeared to be due to modulation of enzymes other than PI3K, based on the concentrations of inhibitors required. These findings provide the first demonstration that PI3K serves as an important signal transduction component in maintaining hemopoietic cell viability. In addition, the p21ras signalling pathway alone is unable to inhibit apoptosis, but GM-CSF can inhibit apoptosis by activating one or more additional signalling events that bypass the need for PI3K.

Effect of Radiation on the Survival of Undifferentiated and Differentiated Embryonal Carcinoma Cells.

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Pluripotent embryonal carcinoma cells, P19, derived from murine teretocarcinoma, proliferate continuously in culture. They can be induced to differentiate into a variety of different cell types. When exposed to retinoic acid at a concentration of $5X10^{-7}$, the cells differentiate into neurons and astrocytes. Exposure of these undifferentiated and differentiated cells to various doses of gamma radiation (400-1000 rads) showed that the differentiated cells are more resistant to apoptosis than undifferentiated cells as seen from the growth curves of both, 12 to 72 hrs after irradiation. Flow cytometric analysis show that both undifferentiated and differentiated cells upon irradiation show a G2 block 12 hrs after irradiation; however, in differentiated cells the release of G2 block starts after 24 hrs and the cells start cycling. In the undifferentiated cells the release of G2 block leads to most of the cells going into apoptosis.

It has been previously shown that there is a high level of p53 and low level of Rb in undifferentiated P19 cells and that this pattern reverses upon differentiation. The present results thus indicated that the high level of p53 protein does not protect undifferentiated embryonal carcinoma cells from apoptosis.

RAPID REGULATION OF TROPHIC FACTOR EXPRESSION IN DENTATE GRANULE CELLS INDICATES A ROLE FOR ENDOGENOUS PROTECTION IN AN APOPTOSIS-PRONE NEURONAL POPULATION.

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Only few neuronal populations in the adult rodent brain have been convincingly shown to undergo apoptotic cell death. Granule cells of the dentate gyrus in the hippocampaus are outstanding in this respect as they succumb with ultrastructural symptoms of apoptosis after withdrawal of adrenal hormones (Sloviter et al., J.Comp.Neurol. 330: 337, 1993). Trophic factors are natural antagonists of programmed cell death. Their persistance in the adult brain suggests a continued need for such antagonism to cell death programs.

In the presented series of experiments, granule cells were analysed in status epilepticus and brain ischemia using in situ hybridization for mRNA on brain sections and immunohistochemistry for study of cell damage. Following induction of status epilepticus using high dose pilocarpine in rats, mRNA upregulation is found for brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) within 1-6 h after onset of seizures. After a brief period of reversible forebrain ischemia induced in adult rats by four-vessel occlusion, BDNF and GDNF mRNA are upregulated within 3 h. Interestingly, the mRNA coding for neurotrophin-3 (NT-3) is downregulated under both conditions. Granule cells did not show cell death in seizures or ischemia when studied with immunohistochemical markers.

Seen together, rapid upregulation of trophic factors BDNF, NGF and GDNF in acute pathological conditions indicates a mechanism of autoprotection for granule cells, which otherwise are prone to apoptosis in adrenal hormone withdrawal.

BCL-X_{ES}, A NEW MEMBER OF THE BCL-X FAMILY; POTENTIAL FUNCTION IN REGULATING APOPTOSIS IN HUMAN PROMYELOCYTIC HL60 CELL LINE.

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Bcl-x_L and bcl-x_S are dominant regulators of apoptotic cell death (Boise, l.H. et al; Cell 74, 597-608, 1993). We have identified and cloned a novel form of the bcl-x family, which we named bcl-x_{ES}, from the null-p53 human promyelocytic HL60 cell line. RT-PCR detection studies indicated that Bcl-x_{ES} is expressed in a series of human cancer cell lines and RNAse protection assays confirmed the presence of this mRNA in human HL60 cells. Bcl-x_{ES} cDNA is an open reading frame of 360 nucleotides coding for an expected protein of 120 amino acids with a calculated MW of approximately 13.4 kDa. Bcl-x_{ES} derived amino acid sequence shared some but not all consensus binding domains with bcl-2, bax, bcl-x_L and bcl-x_S. In contrast with bcl-x_S, bcl-x_{ES} lacks the binding domain for the cellular proteins nip-1, -2 and -3 but contains consensus sequences shared with bax proteins included in the BH2 domain. These differences suggest differential binding affinities and function of bcl-x_{ES} in regulating apoptosis. Further expression and transfection studies are underway to assess the importance of bcl-x_{ES} in regulating apoptosis. HL60 cells are hypersensitive to apoptosis induced by a variety of stimuli and Bcl-x_{ES} may play a role in this phenomena.

EVIDENCE OF APOPTOSIS IN TERMINALLY DIFFERENTIATED CHONDROCYTES OF THE CHICK GROWTH PLATE

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The chondro-osseous junction has been the subject of considerable scrutiny, especially in terms of the fate and role of the terminally differentiated chondrocyte. Although it has been proposed that these cells change their phenotype and survive in the epiphysis, possibly as osteoblasts, evidence from a number of other studies suggests that chondrocytes may undergo programmed cell death.We found that we could label cells in the proliferative and the hypertrophic region of the proximal tibial growth plate of the chick with ApopTag™ . Most of the chondrocytes in the hypertrophic region were labeled by the reagent; in contrast, few proliferative chondrocytes were stained by the end labeling procedure. Both agarose and pulsed field electrophoresis were used to confirm that there was fragmentation of chondrocyte DNA. Alkaline gel electrophoresis indicated that there was more fragmentation of DNA from hypertrophic cells than from proliferative chondrocytes. Further evidence in support of apoptosis was provided by electron microscopic observation of cells in the hypertrophic region of the growth plate. We noted that many of the cells in this region of the growth plate appeared to be undergoing programmed cell death, since their nuclei contained condensed chromatin. Finally, we used flow cytometry to analyze chondrocytes isolated from the proliferating and hypertrophic regions of the growth plate for apoptosis. Dual parameteric flow cytometric contour plots of Hoechst and 7-amino-actinomycin D fluorescence showed that about 8% of cells in the plate were apoptotic. Most of these cells were in hypertrophic cartilage. In summary, the results of the investigation indicated that chondrocytes terminate their life history by apoptosis. While it is possible that the terminal labeling studies may over estimate the number of cells undergoing this event, the data lends credence to the view that cells are removed from the epiphysis through apoptosis. If this is the case, then chondrocytes probably enter the terminal phase of their life history as fully functioning cells and genomic and/or local environmental conditions provide termination signals that initiate events that lead to programmed cell death.

p53-INDEPENDENT APOPTOSIS INDUCED BY MUTANT ADENOVIRUS THAT LACKS E1B-19kDa

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Adenovirus mutants that fail to express E1B-19kDa protein can cause premature death of infected cells by inducing apoptosis. The viral gene responsible for inducing apoptosis has been mapped to E1A. Experiments with a temperature-sensitive p53 mutant have suggested that wild-type p53 is required for E1A-induced apoptosis (Debbas and White, 1993, Genes Dev. 7, 546-554).

Based on this information, one would predict that E1B-19kDa-deficient adenovirus mutants should fail to induce apoptosis in cells lacking p53. In this report, we show that this is not the case. When an adenovirus mutant (Ad5dl337) that does not encode E1B-19kDa protein was used to infect cells lacking endogenous p53, apoptosis was still induced. Infected cells displayed morphological features characteristic of apoptotic cell death, and cellular DNA, as well as viral DNA, was degraded into an oligonucleosomal "ladder" pattern. Apoptosis induced by this mutant virus can be blocked by E1B-19kDa protein, as shown by infection with wild-type adenovirus. The induction of apoptosis is not cell type-specific, because all the p53-null cell lines we have tested, including human Saos-2, mouse 10(1), and primary embryo fibroblast cells from p53-knockout mice, underwent apoptosis when they were infected with Ad5dl337. In addition, the rates of cell death progression in embryo fibroblast cells are similar whether they are derived from p53-knockout mice and from normal mice when they are infected with Ad5dl337. E1A is responsible for this p53-independent apoptosis, because an E1A/E1B-19kDa double mutant adenovirus failed to induce apoptosis in p53-minus cells.

Taken together, these data suggest that the induction of apoptosis by mutant adenovirus requires adenovirus E1A but can occur in a p53-independent manner.

HUMAN LYMPHOCYTES UNDERGO PROGRAMMED CELL DEATH FOLLOWING EXPOSURE TO MERCURIC COMPOUNDS.
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The goal of this investigation was to examine the immunotoxic properties of organic (MeHgCl, EtHgCl and PhHgCl) and inorganic ($HgCl_2$) mercuric compounds on human T-cells. Cells were incubated with varying concentrations of mercurial compounds and viability was assessed by propidium iodide. Minimal reduction in cell viability was noted 1-4 hr after treatment. However, after exposure to mercurials for 24 hr, cell death was apparent. Electron microscopic assessment of the cells revealed early nuclear alterations characterized by hyperchromaticity, nuclear fragmentation and condensation of nucleoplasm. Cells were further analyzed by flow cytometry; an increase in cell volume was observed as well as evidence of DNA degradation. The latter was demonstrated by changes in Hoechst fluorescence patterns and by use of the TUNEL assay. Also, there was a concomitant increase in intracellular levels of calcium.

To further explore the mechanism of cell death, we measured the glutathione-glutathione disulfide ratio and the energy charge ratio. We found that mercury induced depletion of glutathione without changing the energy charge ratio. Moreover susceptibility to the cytotoxic effects of mercury was directly related to intracellular glutathione levels. Finally, evidence is provided for the role of free radicals in mercury induced cell death.

We conclude that these alterations are consistent with the notion that mercuric compounds initiate cytotoxic changes in human lymphoid cells in a manner consistent with programmed cell death.

ALTERNATIVE PATHWAYS OF ACTIVATION-INDUCED APOPTOSIS IN T-CELLS AND B-CELLS . Yufang Shi and David W. Scott, Immunology Dept., Holland Laboratory for the Biomedical Sciences, American Red Cross, Rockville, MD 20855.

Using both B-cell and T-cell lines, we have previously reported that crosslinking of antigen-specific receptors can lead to programmed cell death via apoptosis. In WEHI-231 and CH31 B-lymphoma cells, we have shown that membrane IgM ligation must occur in early G1 in order to cause growth arrest in late G_1 and subsequent apoptosis, but in the A1.1 T-cell hybridoma, apoptosis is cell cycle-independent. Herein, we summarize additional data that the pathways to apoptosis differ in T and B cells. For example, crosslinking of the T-cell receptor causes the upregulation of the Fas ligand and autonomous Fas-dependent death. Recent data in B-cell lymphomas suggest that anti-IgM-induced cell death is Fas-independent. In addition, we will show that dexamethasone and anti-IgM synergize in causing growth arrest and apoptosis, a result which contrasts markedly with apoptosis in T-cell hybridomas and thymocytes. That is, dexamethasone and anti-CD3 individually signal apoptosis, but together antagonize each other. Similar results contrasting T-cell and B-cell apoptosis were obtained with TGF-β. Furthermore, myc is required for apoptosis in T-cell hybridomas, whereas loss of myc is critical in death of B-cell lines. These data will be discussed in terms of the implications of T-cell and B-cell deletional tolerance mechanisms. (Supported by USPHS grant CA-55644 and the American Red Cross.)

STUDY OF CELL DEATH INDUCED BY HYPOXIA: APOPTOTIC PROCESS INHIBITED BY *BCL-2* AND PROTEASE INHIBITORS

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The proto-oncogene *bcl-2* and the *bcl-2*-related gene *bcl-x* prevent apoptotic cell death induced by various treatments. Although a mechanism has been proposed that involves Bcl-2 activity on reactive oxygen species (ROS), we find that expression of Bcl-2 or Bcl-xL prevents cell death induced by withdrawal of oxygen (hypoxia), which drastically decreases the net formation of oxygen free radicals and does not increase oxidized lipid, protein or DNA. Furthermore, neither ROS scavengers nor inhibitors of these scavengers affect the cell death, regardless of the expression of Bcl-2 or Bcl-xL. Thus, we suggest that Bcl-2 or Bcl-xL exerts an anti-cell death function by a mechanism other than through regulation of ROS activity.

The widely held notion that hypoxia kills cells in a necrotic fashion does not appear to be consistent with the inhibition of hypoxic cell death by Bcl-2 and Bcl-xL. Using electronmicroscopy and confocal and non-confocal fluorescence microscopy, we have found that hypoxia induces both necrosis and apoptosis, the ratio of which varies considerably in different cell types. Bcl-2 and Bcl-xL predominantly prevent apoptosis induced by hypoxia in a dose-dependent manner. Since ROS do not seem to represent a common mediator of apoptosis, we analyzed the possible involvement of proteases, which do appear to be common mediators. Overexpression of the *crmA* gene and an ICE inhibitor, inhibited hypoxia-induced apoptosis, suggesting the involvement ICE-like proteases in hypoxia-induced apoptosis. Effects of bcl-2, bcl-xL and protease inhibitors on hypoxia-induced necrotic process is under rigorous investigation.

DNA FRAGMENTATION INDUCED BY PROTEASE ACTIVATION IN APOPTOTIC HUMAN LEUKEMIA HL60 CELLS FOLLOWING TREATMENT WITH THE TOPOISOMERASE I INHIBITOR, CAMPTOTHECIN : CELL-FREE SYSTEM STUDIES

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We studied the role of proteases in apoptosis using a cell-free system prepared from a human leukemia cell line, HL60. HL60 cells are extremely sensitive to a variety of apoptotic stimuli including DNA damage induced by the topoisomerase I inhibitor, camptothecin. We measured DNA fragmentation induced in isolated nuclei by cytosolic extracts using a filter elution assay. Cytosol from camptothecin-treated HL60 cells induced internucleosomal DNA fragmentation in nuclei from untreated cells. This fragmentation was suppressed by serine protease inhibitors (DCI, TPCK, TLCK). Serine proteases (trypsin, endoproteinase Glu-C, chymotrypsin A, and proteinase K) and papain by themselves induced DNA fragmentation in naive nuclei. This effect was enhanced in the presence of cytosol from untreated cells. Cysteine protease inhibitors (E-64, leupeptin, Ac-YVAD-CHO [ICE inhibitor]) did not affect camptothecin-induced DNA fragmentation. The apopain/Yama inhibitor, Ac-DEVD-CHO and the proteasome inhibitor, MG 132 were also inactive both in the cell-free system and in whole cells. Interleukin-1b converting enzyme (ICE) or human immunodeficiency virus (HIV) protease failed to induce DNA fragmentation in naive nuclei. Together, these results suggest that DNA damage activates cytosolic serine (or unknown cystein) protease(s) which in turn activate(s) nuclear endonuclease(s) during apoptosis in HL60 cells.

ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IN CHICK SYMPATHETIC NEURONS UNDERGOING DOPAMINE INDUCED APOPOTOSIS

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Cultured chick embryo sympathetic neurons undergo programmed cell death (PCD) in response to physiological concentration of Dopamine (DA) (1). In order to screen for mRNA species that are selectively associated with neuronal degeneration caused by DA, we applied the Differential Display Method to chick embryo post-mitotic sympathetic neurons in culture. RNA extracted from DA-treated and untreated cultures was subjected to RT-PCR, using variable combinations of arbitrary defined sequences as primers. Qualitative changes in mRNA expression were observed on sequencing gels, and cDNAs of interest were cloned and sequenced. We isolated and characterized a set of mRNA molecules that were selectively induced by DA, as well as a set of molecules that exhibit decreased expression during the programmed cell death. mRNA molecules were grouped into three categories: (1) those identical to already known genes, although their relevant role in the apopotic process is still unknown; (2) those showing significant homology to known proteins, but their cDNA have not been isolated from chicken, nor their role in apopotosis established; or (3) new apopototic-related genes. One of the genes induced by DA was a chicken homologue to the mouse T-complex protein-1 (TCP-1). TCP-1 belongs to the chaperonine family of heat shock protein HSP60, which are induced by oxidative stress. The possible relevance of HSP induction in neurons undergoing apopotosis is of much interest since it has been suggested that neuronal degeneration in Parkinson's disease is linked to an apopototic process. Our result provide information on the molecular events underlining this degenerative process.

Ref: (1) Neuroscience Letters 170 (1994 p. 136-140

PROGRAMMED CELL DEATH INDUCED BY THE CHIMERIC ONCOPROTEIN E2A-PBX1

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The chimeric transcriptional regulator E2A-PBX1 was isolated from the breakpoint of the t(1;19) translocation and has been shown to transform lymphoblasts in a transgenic mouse model. The chimeric protein contains two amino terminal transactivation domains of the E2A protein fused to the carboxy terminal portion of PBX1 containing its DNA-binding homeodomain. The chimeric E2A-PBX1 protein functions *in vivo* as a potent transcriptional activator on reporter genes containing consensus PBX binding sites.

In addition to controls on cellular proliferation, regulation of cell death also plays an important role in homeostasis and neoplastic transformation. Programmed cell death or apoptosis is an important physiologic regulator during embryogenesis, tissue involution, and maturation of the immune system. We have determined that expression of E2A-PBX1 leads to extensive apoptotic death of lymhoid cells.

Upon conditional expression of this oncogene using a metal responsive promoter, we have discovered that the pre-B lymphocyte cell line REH undergoes rapid and complete apoptosis by 72 h following induction of E2A-PBX1 as demonstrated by morphological analysis and typical intranucleosomal DNA degradation. E2A-PBX1 protein production is correlated directly with the resulting apoptosis as witnessed by titration of the inducible metal and western blot analysis. The apoptotic effect of the chimeric protein can be suppressed by expression of the apoptosis inhibitory gene product BCL-2.

Current structure/function analysis of E2A-PBX1 should shed light on apoptotic effector regions of the molecule addressing the issue of whether E2A-PBX1 needs to activate target genes to induce apoptosis. Furthermore, western and northern blot analysis of the tumor supressor and apoptosis promoting protein p53 during E2A-PBX1 expression in these cells will ascertain whether this molecule acts in the effector route of cell death. The results obtained from this model system will elucidate the mechanisms by which homeodomain proteins modulate cell death/survival and should allow for a clearer understanding of the complex interrelationships which exist between cell proliferation and cell death.

Metamorphosis and the *B.thuringiensis* δ-endotoxin induce programmed cell death in insect tissues.

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During metamorphosis, many of the larval tissues of holometabolous insects are replaced by adult counterparts. We have examined two such tissues, the salivary gland of *Drosophila melanogaster* and the midgut of the mosquito, *Culex pipiens*, in order to determine the role of programmed cell death in the removal of the larval tissues. We have characterized programmed cell death morphologically and biochemically, and have found that it appears similar to the program of cell death found in mammalian cells.

The δ-endotoxins, derived from different strains of *Bacillus thuringiensis*, are toxic to many species of insects and have become extremely useful and specific pesticides. The mechansims of action of δ-endotoxins, however, are poorly understood. Although the toxins bind with high affinity to midgut receptors, it is not clear how the toxins effect the death of target insects following ingestion. We show that one δ-endotoxin, isolated from *B.t.* var. *israelensis*, effectively initiates programmed cell death in the midgut cells of the mosquito, and that this programmed cell death follows the kinetics and concentration dependence of the toxin's lethality, suggesting that programmed cell death is the significant, lethal effect of the toxin. Toxin-induced cell death has also been characterized morphologically and biochemically, and appears similar to the program of cell death found in metamorphosing cells. Toxin-induced cell death may thus mimic the normal death of midgut cells that takes place during metamorphosis. A better understanding of toxin-induced cell death may lead to the design of safer, more effective pesticides; at the same time, further studies, including pesticide resistant insects, may allow a better understanding of conserved, molecular mechanisms of apoptosis.

PROENKEPHALIN REPRESSES OR INDUCES APOPTOSIS ACCORDING TO SUBCELLULAR LOCATION AND IS DYSREGULATED IN TRANSFORMED CELLS.

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Proenkephalin is a molecule traditionally perceived as a neuropeptide precursor, but which may in addition have a more widespread fundamental role. It is expressed in developing tissues of mesodermal origin as well as in many mature non-neural cell types such as fibroblasts and lymphocytes. An unexpected finding was its existence as a nuclear protein in embryonic fibroblasts and undifferentiated myoblasts where it is responsive to external growth arrest and differentiation signals. Removal of the signal peptide sequence is sufficient to confer a nuclear fate to transfected proenkephalin (Boettger and Spruce, 1995, J. Cell Biol. In Press).

Proenkephalin exists predominantly as a nuclear protein in proliferating non-transformed Swiss 3T3 cells but is revealed in the cytoplasm at high cell density. During spontaneous transformation proenkephalin relocates to the cytoplasm and becomes cell density-independent. Proenkephalin is expressed in a range of malignant cell lines including HeLa cervical carcinoma and MDA 468 breast carcinoma lines, in which there is a two to three fold increase in overt apoptosis on treatment with anti-proenkephalin monoclonal antibodies whose epitopes have been mapped using phage display libraries (Boettger et al., 1995, J. Mol. Biol. 247, 932-946). This suggests that transformed cells are at least partially dependent on proenkephalin for their survival.

We have shown that high density 3T3 cells resist oxidant damage predominantly due to the effect of cell-cell contact rather than soluble factors. Proenkephalin is upregulated in 3T3 cells that have received a sublethal oxidative injury in a cell density-dependent manner. Cell lines in which proenkephalin is constitutively and inducibly overexpressed resist apoptosis induced with hydrogen peroxide and staurosporine; in these lines the transfected gene product is localised exclusively in the cytoplasm. Cell viability was compared with non-transfected cells using MTT assays, phase contrast microscopic examination of cell morphology, and apoptotic cell scores by propidium iodide staining. Whereas overexpression of cytoplasmic proenkephalin represses apoptosis, we have data which indicate that proenkephalin targeted to the nucleus induces death (Dewar et al., in preparation). Thus, proenkephalin mediates death or survival according to its subcellular destiny.

We propose that cytoplasmic proenkephalin participates in a pathway which mediates cell density-dependent repression of apoptosis, and that dysregulation of such a pathway may occur as a step in oncogenesis.

PHYLOGENETIC CONSERVATION OF APOPTOTIC NUCLEOSOMAL LADDERS WITH BLUNT, 5'-PHOSPHORYLATED DNA ENDS.

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A widely accepted hallmark of apoptosis is the generation of nucleosomal DNA "ladders". However, to date, ladders have only been observed in vertebrates, either following pharmacological manipulation, or during the physiological, hormone-induced regression of an entire tissue, when large numbers of cells undergo synchronous cell death. Ladder formation has yet to be reported amongst invertebrates, even though programmed cell death (PCD) often appears apoptotic by morphological criteria, and recent genetic analyses have indicated that signaling molecules regulating apoptosis are evolutionarily conserved. In the present study, nucleosomal ladders were detected by a ligation-mediated polymerase chain reaction (LMPCR), that selectively amplifies DNA fragments which are blunt and 5'-phosphorylated at both ends. LMPCR detection of ladders markedly increased in the dexamethasone treated mouse thymus, a widely accepted model of apoptosis. In addition, the amplification of ladders directly correlated with an independent measure of thymocyte cell death, the *in situ* end labeling of fragmented DNA. Nucleosomal ladders were detected in healthy, adult mouse organs, including thymus, spleen, liver, kidney, intestine and brain. Here, ladder amplification directly correlated with the reported extent of physiological cell turnover; more ladders were detected in tissues such as thymus and intestine, than in tissues with slower turnover such as brain. For the first time, nucleosomal ladders were observed in invertebrate species *in vivo*, in a wide range of multicellular organisms including *Drosophila melanogaster* (fruit fly), *Manduca sexta* (tobacco hawkmoth), *Anthopleura xanthogrammica* (sea anemone) and *Leucetta losangelensis* (sponge). Ladders were never amplified from the unicellular eukaryotes *Dictyostelium discoedium* (slime mold) and *Saccharomyces cerevissiae* (yeast), indicating that nucleosomal DNA fragmentation is not induced by our methods of DNA isolation. These results suggest that PCD in multicellular organisms frequently involves apoptotic mechanisms responsible for degrading nuclear DNA. Moreover, since the majority of nucleosomal DNA fragments have blunt, 5'-phosphorylated ends, it seems likely that these mechanisms are phylogenetically conserved. [supported by the Klingenstein Fund and the UC Tobacco-Related Disease Research Program]

A DOMINANT NEGATIVE MYB PROTEIN INDUCES APOPTOSIS IN T CELLS

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The c-Myb protein has been implicated as a key regulator of hematopoietic growth and differentiation. In previous work, we converted c-Myb, a transcription activator, into an active repressor, by fusing the Engrailed repressor domain to the Myb DNA binding domain, and showed that when targeted into the T cell lineage, this dominant negative allele, termed MEnT, severely inhibited thymopoiesis in transgenic mice. We have now made an inducible version of MEnT by fusing it to a modified estrogen receptor hormone binding domain. The resulting chimeric protein is tightly regulated, becoming active only in the presence of 4-hydroxy-tamoxifen. T cell lines have been established in which endogenous Myb protein can be inhibited by activation of the inducible MEnT. In contrast to earlier observations using antisense oligonucleotides to block c-Myb expression, we see no perturbation of the cell cycle when MEnT is turned on; instead, cells undergo programmed cell death, as measured by visual inspection, reduced cell counts, DNA laddering, and the appearance of a sub-G1 peak by flow cytometry. Apoptosis can be partially inhibited by addition of IGF-1 to the culture medium, and we are currently looking for any changes in the expression of bcl-2 family members upon induction of MEnT. Preliminary evidence from the MEnT transgenic mice indicates that the severe disruption of T cell development observed in these animals is also due to inappropriate apoptosis. Taken together, these data point to a new role for Myb proteins as protectors against programmed cell death.

ANALYSIS OF DEATH PROGRAM IN CHICK LIMB BUD

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Cell death occurs at specific regions during the development of amniote limbs. Such cell death is essential for shaping the normal form of a limb. Our goal is to clarify the cascades leading to cell death and to identify death genes.

We have been used interdigital cell death in chick limb buds as an experimental system. We have previously found that (1) Appropriate treatment of embryos with BrdU (5-bromodeoxyuridine) specifically modified the developmental fate of cells destined to die. Cells in interdigital necrotic zone (INZ) were rescued from death(Dev. Growth & Differ. 25, 381-391) (2) Cell cycle and cell death were closely related events and there existed the critical S-phase in relation to cell death, which corresponded to the most sensitive time of BrdU effect (Dev. Growth & Differ. 30, 261-270) . (3) Biochemical examination of genomic DNA of INZ cells revealed the existence of DNA ladders, indicating that dying cells in limb morphogenesis would undergo apoptosis. We could distinguish cells in the apoptotic process by in situ labeling of DNA breaks in nuclei (TUNEL method) (Exp. Cell Res. 215, 234-236).

We will report some recent progress to elucidate the death program in INZ. First, the use of multiparametric microfluorometries (Exp. Cell Res.186, 6-14) coupled with TUNEL method enabled us to measure the extent of DNA breaks in individual nuclei even in very early death process that may precede apparent morphological changes. Second, organ culture system for cell death in INZ has been established. Interdigital tissues of day $6^2/3$ were cultured for 24hrs and showed massive cell death. On the contrary, when tissues treated with BrdU in vivo were organ-cultured, the number of dead cells was much reduced. It was suggested that this system reflected the developmental events. Using the system, we found that de novo synthesis of proteins was necessary for INZ cells to die. While RNA necessary for suicide was found to be already transcribed in day $6^2/3$ embryo. Third, using subtractive hybridization method, we have cloned three death-related cDNAs. The analysis of their expression pattern is in progress.

ACTIVATION OF AN ICE-LIKE ACTIVITY OF PROTEASOME IN P19 EC CELLS DURING THE INDUCED DIFFERENTIATION.

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P19 embryonal carcinoma cells can differentiate multipotentially into myogenic, neuronal and glial cells upon treatment with retinoic acid. During the treatment of retinoic acid, the differentiation inducer, cells which did not commit to differentiate died with cytoplasmic contraction and packaging of cellular components. DNA fragmentation was observed by agarose gel electrophoresis of genomic DNA extracted from retinoic acid-treated P19 cells. We determined an interleukin 1β-converting enzyme (ICE)-like proteinase activity in P19 cells by using a fluorogenic substrate, Ac-Tyr-Val-Ala-Asp-MCA. During the apoptotic process, an ICE-like activity in P19 cells was activated about 7-fold by the retinoic acid-treatment. This activity was inhibited by N-ethylmaleimide and Ac-Tyr-Val-Ala-Asp-CMK but not by E-64, EDTA, PMSF nor amastatin. However, the activity was not affected by the viral ICE-inhibitor, *crm*A product. The ICE-like activity in P19 cells eluted as a single peak just after void volume by gel filtration. The active fraction was also containing a Suc-Leu-Leu-Val-Tyr-MCA degrading activity. We could not find any ICE-like activity at molecular-mass of 30-50 kDa. These results suggested that the ICE-like activity in P19 cells was caused by the multicatalytic proteinase, proteasome, and this ICE-like activity was stimulated during the retinoic acid-induced differentiation in P19 cells.

The v-Rel protein blocked apoptosis and degradation of IκB, suggesting that processing and activation of NFκB are including apoptotic process. Proteasome is considered as a key enzyme for the activation of NFκB. Therefore, proteasome may play a crucial role in the apoptosis. In fact, the accumulation of the activated form of NFκB, p50, was observed in P19 cells by the treatment of retinoic acid. We conclude that the activation of proteasome may involved in the apoptotic process in the retinoic acid-induced cell death in P19 cells.

TEMPORAL CHANGES IN CHROMATIN STRUCTURE, INTRACELLULAR CALCIUM AND POLY (ADP-RIBOSE) POLYMERASE DURING SINDBIS VIRUS-INDUCED APOPTOSIS OF NEUROBLASTOMA CELLS

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Sindbis virus (SV) induces apoptosis in most vertebrate cells but the mechanism is unknown. To gain insight into this mechanism the nature and time course of intracellular changes related to programmed cell death were studied in SV-infected mouse neuroblastoma cells. New virus production began at 6 hours after infection and reached a peak at 24 hours. Staining DNA with Hoechst 33342 demonstrated changes in chromatin structure beginning 6 hours after infection. Chromatin changes were cell cycle-dependent affecting cells in G_0/G_1 and G_2/M, but not S phase. Changes were not dependent on increases in intracellular Ca^{2+} and occurred more rapidly in the absence of extracellular Ca^{2+}. These nuclear changes were accompanied by activation of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) resulting in increased consumption of NAD which was apparent by 10 hours after infection. SV-induced apoptosis also involved the proteolytic cleavage of PARP. This cleavage was detectable at 18 hours after infection approximately the same time that DNA fragmentation was apparent by agarose-gel electrophoresis. We conclude that SV-induced apoptosis of neuroblastoma cells is dependent on viral replication, is not dependent on a rise in intracellular Ca^{2+} and is accompanied by activation of PARP and or protease that cleaves PARP.

HIGH RATE OF APOPTOSIS IN BREAST CANCER IS ASSOCIATED WITH MITOSIS, P53 ACCUMULATION, AND REDUCED BCL-2.

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Anticancer drugs can induce apoptosis. Products of the *bcl*-2 gene family are important mediators of cellular sensitivity to apoptotic cell death. Bcl-2, Bcl-X, Bax and Mcl-1 probably have a role in normal cyclical breast development. In a previous study on tumor specimens from a series of 423 node-negative, premenopausal breast cancer (BC) patients we found that immunostained Bcl-2 strongly correlated with presence of estrogen and progesteron receptors, but inversely correlated with immunostained p53 protein and proliferative activity. Evaluating the value of Bcl-2 as a predictive factor for responsiveness to a combination of 5-FU, Adriamycin, and Cyclophosphamide (FAC) we found that, in contrast to p53, Bcl-2 levels did not predict response to adjuvant chemotherapy.

We now have extented this study to evaluate (i) the rate of apoptosis in cancers with high versus low Bcl-2 expression, (ii) the correlation between Bcl-2 expression and specific mutations in the p53 gene, and (iii) the intra- and intertumor heterogeneity of Bcl-2 and other cell death-related proteins in primary tumors and their metastases in relation to a large variety of tumor cell characteristics.

From the above series a subset of 57 tumors (including 41 ductal carcinomas) was selected, in which Bcl-2 expression was either absent (24) or very high (33). Bcl-2 and p53 were determined by immunohistochemistry on paraffin sections. Apoptotic cells and bodies were visualized using *in situ* end labeling (ISEL) of DNA strand breaks and were scored semi-quantitatively. The mitotic index (MI) was determined by counting the number of mitoses per 10 high power fields.

In general, a strong correlation was observed between mitosis and apoptosis ($p<0.0001$) and high Bcl-2 expression was strongly associated with low rate of both apoptosis ($p<0.0001$) and mitosis ($p<0.0001$). Apoptotic activity correlated strongly with (mutated) p53 protein accumulation ($p<0.0006$), also in a subset of 23 tumors with high MI ($p<0.006$). This would suggest that in BC, p53 protein accumulation is associated with a phenotype in which a loss of cell cycle control is related to down-regulation of *bcl*-2 and high rate of apoptosis. Because this phenotype was found to be relatively refractory to treatment with FAC, one might hypothesize that wild-type p53 is an absolute requirement in the pathway leading to FAC-induced apoptosis, and that the status of the Bcl-2 checkpoint is of minor importance in case wild-type p53 is lacking.

We also present data on the relation between specific p53 mutations and Bcl-2 expression and on immunostaining patterns of Bcl-2, Bcl-X, Bax and Mcl-1, comparing primary tumors with regional and distant metastases.

SEPARATION OF TWO BRANCHES OF MOLECULAR EVENTS ALONG THE APOPTOTIC PATHWAY IN T CELL HYBRIDOMAS

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Apoptotic cell death induced in T cell hybridomas proceeds through loss of plasma membrane lipid asymmetry (generating a phagocyte recognition signal), DNA fragmentation, cell shrinkage and cell lysis. Using high concentrations of Ca^{2+} ionophore, two distinct branches in this common molecular pathway of death were identified. When intracellular Ca^{2+} levels are elevated, loss of lipid asymmetry and cell shrinkage occur immediately, implying that the machinery necessary for these events is fully assembled in normal cells. In contrast, DNA degradation and cell lysis, also induced by elevating intracellular Ca^{2+}, do not occur immediately. Rather, both of these events commence at about the same time as they normally do during activation-induced apoptosis, suggesting that the cell must assemble the machinery necessary for these molecular events. Despite this evidence suggesting two separately controlled branches, during activation-induced apoptosis cell shrinkage and cell lysis occur at almost precisely the same time, underscoring the linkage and coordination between these two branches of the apoptotic pathway in T cell hybridomas.

IDENTIFICATION OF A C-JUN AMINO TERMINAL KINASE ACTIVITY IN RAT SYMPATHETIC NEURONS FOLLOWING WITHDRAWAL OF NERVE GROWTH FACTOR.

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Sympathetic neurons from neonatal rats are dependent on NGF for their survival both *in vivo* and in culture. When NGF is withdrawn from these neurons they die by apoptosis (a form of programmed cell death). In addition to NGF several other factors are also known to sustain survival of rat sympathetic neurons in culture (e.g. ciliary neurotrophic factor, CNTF; leukaemia inhibitory factor, LIF; a cell permeant analogue of cAMP, CPTcAMP). The molecular mechanism(s) responsible for the induction of apoptotic cell death following withdrawal of neurotrophic support remain undefined. In the present study we have investigated whether the recently identified stress-activated protein kinases play a role in the induction of neuronal cell death. When NGF was withdrawn from sympathetic neurons following a 1 hr incubation, there was a time-dependent increase in c-jun amino terminal kinase activity (assessed by the solid phase kinase assay using immobilised c-jun (1-193) GST protein as substrate). This kinase activity became apparent after 3 hrs of NGF deprivation and continued to increase up to 9 hrs. Continuous culture in the presence of NGF efficiently suppressed JNK activity as did a readdition of NGF following prolonged culture in NGF-free medium. Similar effects on suppression of JNK activity were also observed with CPTcAMP. By contrast, CNTF, a factor able to sustain only short term survival of rat sympathetic neurons (3-4 days) was found to enhance JNK activity in a time- (3-12 hrs) and dose-dependent (0.1-100 ng/ml) manner. Additionally, CNTF-induced JNK activity was attenuated by a subsequent addition by NGF. Studies aimed at establishing a relationship between JNK activation and induction of apoptosis will be presented.

REAPER INDUCES APOPTOSIS IN SF-21 CELLS AND BACULOVIRUS P35 AND IAP GENES BLOCK ITS ACTION

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The reaper gene (*rpr*) of *Drosophila melanogaster* was originally identified genetically as an effector for the initiation of apoptosis in a number of embryonic cells (1). We have found that the IPLB SF-21AE lepidopteran cell line undergoes apoptosis when transfected with *rpr*. Apoptosis was evident from cell surface blebbing, nuclear fragmentation observed by DAPI staining, and DNA oligonucleosomal ladder formation. To assess the position of *rpr* in the apoptotic pathway, we determined whether several known anti-apoptotic genes can block apoptosis upon co-transfection with *rpr*. Cotransfection of *rpr* with cowpoxvirus *crm*A or baculovirus *p35,* which encode inhibitors of aspartate-specific proteases (2,3,4), reduced *rpr*-induced apoptosis three-fold and eight fold respectively. Thus, we infer that *rpr*-induced apoptosis proceeds through a pathway involving the activation of an aspartate-specific protease probably belonging to the ICE family. Baculovirus genes belonging to the "inhibitor of apoptosis" (*iap*) class were also able to block *rpr*-induced apoptosis but not ICE-induced apoptosis and therefore the *iaps* appear to act at a point upstream of cysteine protease activation. No synergism was observed upon co-transfection of *rpr* and the *p32* form of the ICE gene. Mammalian *bcl-2*, *bag-1* or a combination of *bcl-2* and *bag-1* did not block *rpr*-induced apoptosis in this system. We will discuss our site-directed mutational analysis of *rpr*.

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ACTIVATION OF NUCLEAR FACTORS RESPONDING TO cAMP IN NEURONAL CELL DEATH. Vyas S, Michel P. P, [1]Foulkes N.S, [2]Faucon Biguet N, [1]Monaco L, [1]Sassone-Corsi P, Agid Y.
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In NGF-differentiated PC12 cells, chronic treatment with calcium ionophore A23187 results in degenerative changes followed by cellular death that is time- and concentration-dependent. PC12 cells co-differentiated with NGF and dBcAMP in the presence of serum become irreversibly dependent on these factors for survival and undergo programmed cell death (PCD) when both factors are withdrawn. In both cases, we observed prolonged induction of c-*fos* that precedes and correlates with cell death. c-Fos immunoreactivity was also seen in the primary mesencephalic cultures undergoing cell degeneration upon A23187 treatment.
To understand the signalling mechanisms involved in c-*fos* induction, we examined binding of the regulatory proteins at the CRE and SRE elements of the c-*fos* promoter. These two major regulatory sites mediate c-*fos* induction in response to calcium, cAMP and NGF. In NGF-differentiated PC12 cells, binding to a CRE in the c-*fos* promoter is induced during calcium-triggered degeneration. Phosphorylation of CREB/CREMτ transactivator proteins was also observed during this time period. In co-differentiated NGF/dBcAMP cells, deprivation of these factors did not lead to changes in CRE binding activity, however there was specific phosphorylation of CREB/CREMτ preceding cell death. During cell death induced by either calcium toxicity or trophic deprivation, the SRE binding activity did not change, although a decrease was observed during chronic A23187 treatment. Analysis of the levels of CRE regulatory proteins showed decrease in CREB during the period of significant cell death whereas CREMτ levels remained unaltered. Overall, our results suggest that c-*fos* plays a role in neuronal cell death, the mechanism of c-*fos* induction involves CRE transactivating proteins. These proteins may also have additional transactivating functions during cell death.

AUGMENTED EXPRESSION OF THE FAS-ENCODING GENE AND THE INDUCTION OF APOPTOSIS IN INFLUENZA VIRUS-INFECTED HELA CELLS

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HeLa cells die within two days after influenza virus infection, which event is accompanied by cytoplasmic shrinkage, chromatin condensation, and DNA fragmentation, collectively referred to as apoptosis. We found that the amount of Fas, a cell surface receptor for an apoptosis signal, increases at the mRNA level in the virus-infected cells prior to their death by apoptosis. This suggests that Fas and its ligand are involved in the apoptosis of influenza virus-infected cells. When we cloned and analyzed the promoter region of the human Fas-encoding gene, we found that nucleotide sequences for the binding of NF-IL6, also known as C/EBPß, were repeated eight times in the 5'-end region of the gene, spanning from -1360 to +320. This region directed the expression of a downstream marker gene when introduced into HeLa cells, and the activity of the Fas gene promoter was stimulated about twofold upon influenza virus infection. The Fas promoter was also activated by NF-IL6 in co-transfection experiments. Moreover, the DNA-binding activity of NF-IL6 increased after infection with the virus, whereas the amount of NF-IL6 seemed unchanged. The results suggest that NF-IL6 is activated upon influenza virus infection through post-translational modification and the modified factor stimulates the transcription of the human Fas gene.

NORMAL P53 FUNCTION CONFERS RESISTANCE TO TAXOL® BY INDUCTION OF G1 ARREST

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The molecular basis by which proliferating normal cells resist cytotoxicity and transformed cells show increased sensitivity to antineoplastic agents remains unclear. The anticancer agent paclitaxel (TAXOL) has shown encouraging results in cancers refractory to DNA damage-based therapy. Paclitaxel is not known to cause DNA damage but rather has been shown to stabilize tubulin polymerization resulting in arrest in mitosis and cell death from apoptosis. Normal fibroblasts made to have nonfunctional p53 either by expression of the SV40 large T antigen, which complexes with p53, or expression of human papillomavirus (HPV) type 16 E6, which targets p53 for degradation, showed 6-8 fold increased cytotoxicity by paclitaxel compared to parental cells, suggesting a role for p53 function in resistance to paclitaxel. This was confirmed with primary embryo fibroblasts from p53 null mice which showed a 6-fold increase in paclitaxel toxicity relative to fibroblasts with wild type p53, excluding effects due to expression of viral oncogenes. HPV16 E6 mutants varying in their ability to cause p53 loss directly correlated p53 decline with increased G_2 arrest and increased paclitaxel-induced apoptosis. At the IC_{50} of the sensitive cells, resistant cells with functional p53 progressed through mitosis and arrested in G_1 after paclitaxel exposure, coincident with increased p53 and $p21^{cip1,waf1}$ protein levels. This may indicate that in addition to DNA damage, p53 responds to inappropriate microtubule organization, and initiates protective checkpoint control. Micronucleation and DNA fragmentation were preferentially observed in cells without functional p53 indicating paclitaxel-induced apoptosis was p53 independent. These results are in contrast to those linking p53 loss of function with tumor cell resistance to DNA damaging anti-cancer agents and indicate that tumor cells lacking p53 may be selectively targeted by this class of tubulin polymerizing agents.

THE HPVE7 PROTEIN INTERFERES WITH P53-INDUCED APOPTOSIS

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Ectopic expression of wild type p53 using a temperature sensitive construct (ts p53) triggers apoptosis in the v-*myc* retrovirus-induced p53-negative T cell lymphoma line J3D. Constitutive bcl-2 expression inhibits p53-induced apoptosis. Here we show that more than 90% of the ts p53 transfected J3D cells were arrested in the G1 phase of the cell cycle at 18 hours after induction of wild type p53 expression by temperature shift to 32°C. At least 80% of the cells remained viable at this time. After 30 hours at 32°C, around 50% of the cells had died by apoptosis while most of the remaining cells were still alive in G1, indicating that p53-induced G1 arrest preceded apoptosis. Expression of both WAF1 and *bax* mRNA was induced by wild type p53 in both the ts p53 and ts p53/*bcl*-2 transfected cells. The kinetics of G1 cell cycle arrest at 32°C were similar in both the ts p53 and the ts p53/*bcl*-2 double transfectants. Thus *bcl*-2 has no effect on wild type p53-induced G1 arrest and does not interfere with wild type p53-mediated transactivation of WAF1 and *bax* during wild type p53-induced G1 arrest/apoptosis. In order to test whether or not p53-induced apoptosis was dependent on p53-induced G1 arrest, we transfected the J3D-ts p53 cells with an HPVE7 expression vector. In comparison with ts p53 transfected J3D cells, only about 50% of ts p53/HPVE7 transfected cells were arrested in G1 after 18 hours at 32°C. In addition, ts p53/HPVE7 cells were more resistant to p53-triggered apoptosis than ts p53 transfected cells at 32°C. Thus, a partial relief from p53-induced G1 arrest by overexpression of HPVE7 correlates with delayed p53-triggered apoptosis, indicating that p53-induced G1 arrest is a prerequisite for p53-induced apoptosis in the J3D cells.

CD40 AND SIG RECEPTOR CROSS-TALK AT THE LEVEL OF CREB/ATF AND RESCUE OF WEHI-231 B CELLS FROM APOPTOSIS

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WEHI-231 B cells undergo apoptosis in response to sIgM cross-linking, thereby constituting a model for tolerance induction. Apoptosis is blocked by engagement of the CD40 receptor with CD40L, a membrane protein of activated T cells. Because cAMP raising agents enhance sIgM-triggered cell death in WEHI-231 B cells, we explored the possibility that changes in CREB/ATF transcription factor complexes are associated with receptor-specific regulation of apoptosis. Cross-linking of sIgM strongly stimulated phosphorylation of CREB/ATF-1 whereas engagement of CD40 did not. Moreover, CD40 ligand inhibited CREB/ATF-1 phosphorylation during co-stimulation of WEHI-231 B cells in conjunction with anti-IgM, demonstrating cross-talk between these two receptors at the level of CRE-binding proteins. These results were accurately reflected by transcriptional activation, in that expression of a CRE-dependent reporter gene was induced by treatment of transiently transfected WEHI-231 B cells by anti-IgM alone, but not by CD40L alone, and the concurrent presence of CD40L substantially inhibited reporter gene expression induced by anti-IgM. These observations suggest that the CREB/ATF family of transcription factors may play a role in sIgM-induced apoptosis and in the cross-talk between CD40 and sIgM that leads to modulation of cell death. CD40L may rescue WEHI-231 from anti-IgM-mediated apoptosis through the modulation of gene expression regulated through CRE sites.

THE SEARCH FOR PROTEINS INTERACTING WITH THE CELL DEATH GENES *reaper* AND *hid*

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We are using *Drosophila melanogaster* as a system for identifying novel genes in the apoptotic pathway. A collection of chromosomal deletions representing 50% of the genome was previously screened for defects in embryonic cell death. This screen yielded *reaper* and *hid* which appear to play an important role in *Drosophila* cell death. Both *reaper* and *hid* are located in a deletion that is required for all cell death during embryogenesis. Both genes are expressed in dying cells and ectopically expressing either gene in the deletion background partially restores the cell death phenotype. Both *reaper* and *hid* are novel proteins, but *reaper* shares limited homology with the Fas death domain.

To identify other genes involved in the cell death pathway and to understand how *reaper* and *hid* might function, we are screening an embryonic and an imaginal disc *Drosophila* library for proteins that interact with *reaper* and *hid* using the yeast two-hybrid system. We isolated several clones that interact with *reaper* and some that interact with *hid*. Interestingly, some of the isolated clones appear to interact with both *reaper* and *hid*. At present, we are characterizing the genes encoding for these interactors. We are also screening the same libraries with other known cell death genes including the baculovirus protein p35. Characterizing the involvement of the proteins that interact with *reaper* and *hid* in the cell death pathway has the potential of greatly enhancing our understanding of apoptosis in general.

RECOGNITION OF APOPTOTIC LYMPHOCYTES BY MACROPHAGES: MULTIPLE RECEPTORS FOR A COMPLEX SIGNAL

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Apoptotic lymphocytes must be recognized and phagocytosed by macrophages prior to cell lysis to prevent inflammatory responses. The exposure of phosphatidylserine (PS) on the lymphocyte surface generates a signal for recognition, but the context in which PS is recognized differs in activated vs unactivated macrophages: PS vesicles inhibit phagocytosis of apoptotic lymphocytes by activated, but not unactivated, macrophages; whereas erythrocytes with PS on their surface inhibit uptake by unactivated, but not activated, macrophages. The tetrapeptide RGDS, as well as cationic amino acids and sugars, specifically inhibits phagocytosis of apoptotic lymphocytes by unactivated lymphocytes, implicating an integrin-mediated mechanism; N-acetylglucosamine specifically inhibits uptake by activated macrophages, implicating a glycoprotein-mediated mechanism. The monoclonal antibody 61D3 inhibits uptake by both activated and unactivated macrophages, suggesting that it recognizes a common component of the recognition machinery, such as the PS receptor. These results indicate that apoptotic lymphocytes display a complex signal for recognition and that activated and unactivated macrophages share an ability to recognize PS, but differ in other aspects of their receptor function.

MECHANISMS CONTROLLING EXPRESSION OF A SIGNAL FOR RECOGNITION OF APOPTOTIC LYMPHOCYTES

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The apoptotic program leads not only to cell death, but to recognition and elimination of apoptotic cells by phagocytosis. The program must therefore include events which generate signals that provoke recognition. In lymphoid cells, one such signal is the exposure of phosphatidylserine (PS) on the apoptotic cells' surface. Measurement of transbilayer movements of fluorescent phospholipid analogs indicate that PS is actively removed from the surface of lymphoid cells by the aminophospholipid translocase. This process is downregulated in apoptotic cells at a time preceding DNA degradation, cell shrinkage and fragmentation (zeiosis), and cell lysis. At the same time, a mechanism which mediates transbilayer lipid randomization (scrambling) is upregulated, bringing PS to the cell surface. This coordinate regulation of transbilayer lipid movements is observed upon induction of apoptosis by several agents, indicating that it is part of the common pathway of cell death.

JNK, BUT NOT MAPK, ACTIVATION IS ASSOCIATED WITH FAS-MEDIATED APOPTOSIS IN HUMAN T CELLS

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Fas is a cell surface molecule that is expressed on a wide array of cell types and triggers apoptosis. While in most situations Fas ligation activates a cascade of programmed cell death, on resting T lymphocytes it can costimulate proliferation with the T-cell antigen receptor (TCR)/CD3 complex. This incongruity suggests that Fas may engage signaling events that overlap with those used by proliferation cues. We observe that in the human T cell line, Jurkat, and in human peripheral blood lymphocytes (PBL) Fas stimulation does not signal by the Ras/Raf-1/mitogen-activated protein kinase (MAPK) pathway, nor by increased intracellular calcium. Rather, Fas ligation strongly activates Jun kinase (JNK). This activity, as well as Fas-induced apoptosis, is blocked by increased levels of cAMP. The balance between proliferation and apoptosis by Fas triggering of T lymphocytes may therefore reflect a signaling ratio between TCR activation of the Ras/Raf-1/MAPK pathway versus JNK activation by Fas.

STRUCTURE AND EXPRESSION OF THE *FAS/APO-1* GENE IN HUMAN LYMPHOMAS

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FAS/APO-1 [CD95] is a membrane glycoprotein belonging to the TNF/NGF receptor family, and which can trigger apoptosis in some lymphoid cell lines. We analyzed the possible occurrence of rearrangements and/or allelic loss of the *FAS/APO*-1 gene in a representative series of 101 human lymphomas, including Hodgkin's disease (HD) and non Hodgkin's lymphoma (NHL) cases. The rationale for this analysis relied on two observations : (1) the frequent occurrence, in some lymphoma subtypes, of chromosome 10 alterations encompassing the chromosomal localization of *FAS/APO*-1, and (2) the demonstration of structural alterations of the *FAS/APO*-1 gene in mouse *lpr* mutants, which present with a generalized lymphoproliferative disease. In this retrospective study, we performed *FAS/APO*-1 gene dosage and analysis using Southern blotting. No alterations of *FAS/APO*-1 were observed in the 31 HD cases ; allelic loss of *FAS/APO*-1 was observed in 3 out of the 70 T-cell and B-cell NHL cases tested. Two cases with different clinical, phenotypical and histological presentations showed a rearrangement of this gene, whereas a third case showed amplification.

The pattern of FAS/APO-1 expression was determined by immunohistochemistry (IHC) combined with Northern blotting. A strong FAS/APO-1 immunostaining, predominantly located in the neoplastic cells, was observed in 25 out of 27 (92 %) HD cases, and in 8 out of 8 (100 %) CD30-positive anaplastic large cell lymphoma (ALCL) cases. In contrast, positive FAS/APO-1 immunostaining was only observed in 22 out of 41 (53 %) CD30-negative NHLs. Northern blot analysis detected variable amounts of the *FAS/APO*-1 transcript in the IHC-positive samples. These results thus (1) suggest a possible hyperexpression of FAS/APO-1 in HD and ALCL, and (2) indicate that *FAS/APO*-1 alterations only occur at a low frequency in human lymphomas.

RADIATION INDUCES p53-MEDIATED ACTIVATION OF PROLIFERATING CELL NUCLEAR ANTIGEN EXPRESSION.

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The preponderance of data indicates that the cell cycle regulatory activities of p53, a DNA damage-inducible protein, are related to its function as a transcription factor. Our previous results indicated that the gene encoding the proliferating cell nuclear antigen, PCNA, is a target for transcriptional regulation by p53. These observations suggest a cellular response to DNA damage whereby p53 induces expression of an essential DNA repair protein, PCNA.

To examine p53-mediated regulation of PCNA expression, a rat embryo fibroblast cell line, CREF cells, was transfected with PCNA promoter-CAT constructs possessing or lacking sequences that bind wild-type p53 *in vitro*. Exposure of the transfected cells to γ-radiation activated expression of PCNA-CAT via sequences that correlate with a p53-binding site. Moreover, a dominant interfering of p53 mutant repressed PCNA-CAT expression in a p53-binding site-dependant manner. p53-binding site mediated activation of an associated promoter is not unexpected, but it seems likely that transcriptional regulation of a DNA replication and repair protein by p53 would correlate closely with p53-mediated regulation of the cell cycle and cell viability.

To test the regulation of PCNA expression by p53 in a rodent model of lung injury, p53 and PCNA were examined by immunohistochemistry in the airways of mice irradiated with 8 grays of γ-radiation and sacrificed at various times post-exposure. Expression of p53 and PCNA was virtually undetectable in the airway cells of unirradiated control animals. In irradiated animals, the percentage of airway cells (mostly Clara cells of the small bronchioles) that were positive for p53 immunostaining peaked at 16 to 24 hours and returned to undetectable levels within 72 hours. Similarly, PCNA immunostaining peaked at 16 to 24 hours and returned to low basal levels at 72 hours. Detection of both p53 and PCNA in the same population of irradiated pulmonary cells by co-immunofluorescence suggests that the two proteins are coordinately expressed. These observations are consistent with our model wherein p53 regulates the PCNA promoter, and these events could be critical in repair of DNA damage in pulmonary cells. In agreement with this observation, our preliminary findings suggest that PCNA expression is not induced in the lungs after irradiation of p53-deficient mice.

THE INDUCTION OF HEPATOMA CELL APOPTOSIS BY *C-MYC* REQUIRES ZINC AND IS MEDIATED BY OXIDATIVE STRESS.

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Following liver injury from the hepatotoxin galactosamine, delayed and prolonged *c-myc* expression is associated with apoptotic cell death. To determine the function of *c-myc* in hepatic cell death, the human hepatoma cell line HuH-7 (WT) which constitutively expresses *c-myc*, was compared with stably transfected clones of HuH-7 which overexpress sense (S-myc) or antisense *c-myc* (AN-myc) RNA with zinc treatment, resulting in increased or decreased Myc protein respectively. In serum-free medium (SFM), all three cell types survived for 4-5 days before undergoing necrotic cell death. In SFM with 37.5 μM zinc, however, both WT and S-myc cells died rapidly while AN-myc cells did not. The percentage of surviving WT, S-myc, and AN-myc cells at 6 h was 82%, 61%, and 94% respectively, and by 24 h 19%, 13%, and 72% respectively. Fluorescent and electron microscopy of WT and S-myc cells showed that death occurred by apoptosis, but it was not associated with DNA fragmentation. This zinc effect could not be explained by effects of *c-myc* on metallothionein gene expression, and all three cell lines were equally sensitive to toxicity from copper and cadmium. Apoptosis in SFM plus zinc was not inhibited by mitogens. Cell survival 6 h after the addition of SFM plus zinc to S-myc cells (52 $\pm$ 4%) was unaffected by supplementation with EGF (49 $\pm$ 5%), FGF (45 $\pm$ 6%), or TGF-α (48 $\pm$ 5%), and significantly decreased by IGF-I (35 $\pm$ 3%). Combinations of these growth factors also failed to inhibit apoptosis. Investigations of other serum components revealed that the degree of cell death in all three cell lines was profoundly affected by the concentration of glutamine. In S-myc cells survival decreased from 48 $\pm$ 3% in SFM with 2 mM glutamine to 11 $\pm$ 5% in SFM lacking glutamine. This effect was specific for glutamine since no difference in cell survival was seen when the amino acids serine or asparagine were removed from the medium. Since glutamine can be metabolized to glutamate, a precursor of the intracellular antioxidant glutathione (GSH), this finding suggested that the cellular state of oxidant stress may trigger apoptosis. When S-myc cells were pretreated with N-acetyl cysteine to increase GSH levels, survival after 24 h in SFM plus zinc increased from 14 $\pm$ 5% to 58 $\pm$ 9%. Similarly treatment with catalase to metabolize the reactive oxygen intermediate H_2O_2 increased survival to 62 $\pm$ 7%. Thus, unlike other cell types, hepatoma cell apoptosis from *c-myc* overexpression in SFM was dependent on zinc. Oxidative stress, and not a deficiency of growth factors, is crucial to this commitment to apoptosis.

BILIRUBIN INDUCES APOPTOSIS OF CULTURED CEREBELLAR GRANULE AND PURKINJE NEURONS.

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Although a well known neurotoxin neither the cellular nor molecular mechanisms underlying bilirubin (Br) neurotoxicity are known. Unconjugated Br can traverse the blood-brain barrier easier than bound Br causing hyperbilirubinemia-induced kernicterus in infants. We now report that Br induced neurotoxicity of cultured cerebellar neurons is associated with the biochemical and morphological features of apoptosis. Additionally, a dramatic dose dependent activation of NF-κB is apparent by gel shift analysis. Fetal rat cortical neurons, however, are refractory to higher Br concentrations with no increase in activated NF-κB. Inhibitors of NF-κB activation, TPCK and TLCK, as well as antisense to NF-κB (p65) block both Br induced apoptosis as well as NF-κB activation in cerebellar neurons. Nuclear protein levels of p53 were unaffected by exposure to Br. These data suggest that activation of NF-κB is a proximal signal mediating Br-induced apoptosis in cultured cerebellar neurons. Br-induced neuronal apoptosis may be the cause of the neurodegenerative changes associated with kernicterus.

INVESTIGATIONS OF INTERACTIONS BETWEEN MEMBERS OF THE BCL-2 PROTEIN FAMILY USING YEAST TWO-HYBRID SYSTEM.

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Interactions of the Bcl-2 protein with itself and other members of the Bcl-2 family, including Bcl-X-L, Bcl-X-S, Mcl-1 and Bax were explored using a yeast two-hybrid system. Fusion proteins were created by linking Bcl-2 family proteins to a LexA DNA binding protein or a B42 trans-activation domain. Protein-protein interactions were examined by expression of these fusion proteins in *S. cerevisiae* carrying a lacZ gene under control of a LexA operator. Using this approach, evidence for Bcl-2 protein homo-dimerization was obtained. Bcl-2 also interacted with Bcl-X-L and Mcl-1, as well as the dominant inhibitors Bax and Bcl-X-S. Bcl-X-L displayed the same pattern of the combinatorial interactions with Bcl-2 family proteins as Bcl-2. Our preliminary attempts to map the region within Bcl-2 that are necessary for homodimerization suggest a head-to-tail model for this protein-protein interaction (Sato, T. *et al.*, *PNAS*, **91**, 9238, 1994; Hanada, M. *et al.*, *JBC*, **270**, 11962,1995). Furthermore, the demonstration that a LexA/Bax protein has a lethal phenotype in yeast suggests that Bax is a cell death effector whose activity is neutralized by Bcl-2 and Bcl-X-L, and raises possibilities for exploiting yeast for identification of novel genes that participate in or regulate the Bax death pathway. The candidate genes isolated by yeast two-hybrid will be discussed.

TRANSCRIPTIONAL ACTIVATION PLAYS A ROLE IN THE INDUCTION OF APOPTOSIS BY TRANSIENTLY-TRANSFECTED WILD-TYPE P53

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The p53 tumor suppressor gene is a frequent target of genetic alterations in human cancer. The protein was shown to function as a transcription factor: it can up-regulate certain genes by interacting with specific DNA elements, or down-regulate other genes that do not have those DNA elements by other mechanisms. The levels of p53 protein increase in response to DNA damage, resulting in growth arrest in G1, presumably to permit DNA repair, or in apoptosis. Other signals may also promote p53-mediated apoptosis. We have recently reported that transiently-transfected wt p53 is capable of inducing apoptosis in certain transformed cell lines. We demonstrated by quantitative analysis using flow cytometry that apoptosis was restricted to the population expressing wt, but not mutant, p53. In the present study we use this model system to analyze the functional domains of p53 in the induction of apoptosis. Several constructs expressing mutations or deletions in the C-terminal oligomerization domain, the N-terminal transactivation domain or the central DNA-binding domain were introduced into HeLa cells, and the ability of the expressed proteins to induce apoptosis was evaluated. All the functional domains were found to be necessary for the induction of apoptosis. In addition, cycloheximide and actinomycin D inhibited p53-mediated apoptosis in these cells. We therefore conclude that p53 acts in this cell system, at least in part, as a transcriptional activator in the induction of apoptosis.

FOCAL RELEASE OF INTRACELLULAR ZINC VISUALIZED WITH A ZINC FLUOROPHORE ZINQUIN: A NEW EARLY CYTOPLASMIC MARKER OF APOPTOSIS

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Although apoptosis is well-defined by morphological criteria, there are relatively few biochemical markers available to assist in the identification and/or quantification of apoptosis in cells or tissues and in the delineation of early, intermediate and late phases of the process. DNA fragmentation is an early marker of nuclear changes in apoptosis. However, this process occurs only partially or not at all in some types of cell and is not essential for cell death since cells devoid of nuclei still undergo apoptosis in response to exogenous stimuli. There is clearly a need to identify and study the cytoplasmic regulators of apoptosis and the biochemical events that they mediate. Deprivation of cellular zinc in vitro or in vivo triggers both cytoplasmic and nuclear apoptosis suggesting that zinc is an important regulator. While studying levels of intracellular free or loosely bound (labile) zinc in lymphoid cells using the zinc-specific fluorophore Zinquin, it was observed that the occasional apoptotic cells in the preparations fluoresced intensely with Zinquin, suggesting a marked increase in intracellular labile zinc [1]. The Zn flux appeared to occur early in the process, prior to changes in membrane permeability, and to involve release of zinc from intracellular pools not available to Zinquin rather than enhanced uptake from the medium. This phenomenon has now been observed in a wide variety of cells. The cytoplasmic release of zinc is localized to foci and, in degenerating embryonic blastomeres, coincides spatially and temporally with the cytoplasmic fragmentation into vesicular structures. The possible role of oxidative stress in the zinc release and the implications for regulation of apoptotic enzymes by zinc will be discussed.

1. Zalewski PD, Forbes IJ, Seamark RF, Borlinghaus R, Betts WH, Lincoln SF and Ward AD (1994) Flux of intracellular labile zinc during apoptosis (gene-directed cell death) revealed by a specific chemical probe, Zinquin. *Chemistry & Biology* **1**, 153-161.

CYTOPLASMIC AND NUCLEAR PROTEASES IN THYMOCYTE APOPTOSIS.

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The requirement of proteolysis for both induction and perpetuation of apoptosis now is well documented. Recent studies in our and other laboratories have shown that inhibitors of serine proteases (TPCK, TLCK and DCI) can block apoptotic chromatin cleavage (formation of 50 kbp DNA fragments and subsequent DNA 'laddering') and morphological changes characteristic of apoptosis in both intact cells and isolated nuclear preparations. Another inhibitor of serine proteases, PMSF, as well different inhibitors of cysteine proteases (leupeptine, antipain, E64, calpain inhibitors I and II) revealed the lack of protection from MPS- and etoposide-induced apoptosis in rat thymocytes. However, most of these inhibitors (except the calpain inhibitors) are general inhibitors of serine and cysteine proteases rather than specific inhibitors of any known proteases.

In the present work we used highly selective protease inhibitors in an attempt to estimate the precise role of ICE-like protease and Ca^{2+}-regulated nuclear scaffold-associated serine protease (CRP) activities in apoptosis. Our data show that the two ICE-like inhibitors can prevent morphological and biochemical changes characteristic of apoptosis induced in rat thymocytes by MPS or etoposide. These inhibitors were only effective when added before the addition of apoptosis-inducing agents. Measurement of ICE-like activity using a membrane impregnated with a specific substrate for ICE-like enzyme has shown that, in thymocytes undergoing apoptosis, the activity of this enzyme is increased and peaks at 1 h after treatment. Incubation of cells with the ICE-like inhibitors prevented enzyme activation. An inhibitor of CRP prevented lamin breakdown, chromatin fragmentation and apoptotic morphological changes in thymocytes treated with MPS, but not with etoposide. Our findings have provided evidence for the involvement of multiple proteases in the initiation and progression of thymocyte apoptosis. ICE-like proteases are critically involved in the early phase of both MPS- and etoposide-induced apoptosis in thymocytes, and proteolytic degradation of nuclear lamins by the scaffold-associated CRP is an obligatory component of MPS-induced apoptosis. The nature of the signal(s) leading to the activation of the ICE-like protease(s) and CRP is currently unknown but subject to ongoing studies.

AN ICE-LIKE PROTEASE IS A COMMON MEDIATOR OF APOPTOSIS IN THYMOCYTES AND THP.1 CELLS.

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Ced-3 is essential for cell death in *Caenorhabditis elegans* and is homologous to the mammalian interleukin-1β converting enzyme (ICE). The present study investigated the possible role of ICE and ICE homologues as general mediators of apoptosis. It was demonstrated that apoptosis can be induced in both immature rat thymocytes and THP.1 cells, a human monocytic tumour cell line, by a number of mechanistically diverse stimuli. In thymocytes, apoptosis was induced by the glucocorticoid dexamethasone, the DNA topoisomerase II inhibitor etoposide and the microsomal Ca^{2+}-ATPase inhibitor thapsigargin. In THP.1 cells, apoptosis was induced by etoposide, thapsigargin, the protein synthesis inhibitor cycloheximide and the protein kinase inhibitor staurosporine. The induction of apoptosis by all these stimuli was completely abrogated when cells were pretreated with an ICE-like protease inhibitor with a broad specificity, but not by more specific ICE inhibitors. The ICE-like protease inhibitor blocked apoptosis at an early stage preventing all the morphological and biochemical changes we have measured including cleavage of poly (ADP-ribose) polymerase, formation of large kilobase pair fragments (30-50 and 200-300 kbp) and internucleosomal cleavage of DNA. These results suggested that an ICE homologue(s) rather than ICE itself may act as a common mediator of the induction of apoptosis. The induction of apoptosis by all these stimuli was inhibited in the thymocytes but enhanced in THP.1 cells in the presence of the trypsin-like protease inhibitor, Nα-tosyl-L-lysinyl chloromethyl ketone (TLCK). These results also highlight the role of TLCK target as an important regulator of apoptosis in some systems

SURVIVAL AND DEATH OF PRELYMPHOMATOUS B-CELLS FROM N-MYC/BCL-2 DOUBLE TRANSGENIC MICE CORRELATES WITH THE REGULATION OF INTRACELLULAR CA^{++} FLUXES

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Coexpression of the proto-oncogenes *c-myc* and *bcl-2* under the control of the immunoglobulin enhancer Eμ provokes the rapid development of primitive lymphoid tumors in transgenic mice. In the present study we show that the *myc* familiy members *N-myc* and *L-myc* also cooperate with *bcl-2* in oncogenesis and can provoke the development of more mature pre-B, B and T cell type lymphomas. The analysis of prelymphomatous B-cells from single Eμ *N-myc* and *bcl-2-Ig* transgenic animals and from young, tumor free, double transgenic Eμ *N-myc/bcl-2-Ig* mice revealed that Eμ directed expression of *N-myc* leads to very rapid apoptosis after explantation and culturing compared to B-cells from normal mice. As expected, B-cells from *bcl-2-Ig* transgenics were protected to a certain degree from apoptosis. Strikingly however, B-cells from Eμ *N-myc/bcl-2-Ig* double transgenic animals were found to be almost completely resistant towards a number of different apoptotic stimuli. Furthermore, after induction of apoptosis with H_2O_2, B-cells from Eμ *N-myc* animals reach levels of intracellular free Ca^{++} concentrations that are comparable to B-cells from normal mice, whereas B-cells from *bcl-2-Ig* or Eμ *N-mcy/bcl-2-Ig* double transgenic mice show no increase of intracellular Ca^{++} concentrations after stimulation with H_2O_2. These findings suggest that the inhibition of apoptosis conferred by *bcl-2* and the induction of apoptosis mediated by *N-myc* are both correlated with the proper regulation of intracellular Ca^{++} fluxes. We hypothesize therefore that the regulation of intracelluar Ca^{++} concentrations represent one important parameter in the oncogenic cooperation between *bcl-2* and *N-myc*.

APOPTOTIC PERIPHERAL T CELLS PRODUCE A 17 KD PROTEIN ANTIGENICALLY SIMILAR TO INTERLEUKIN-6

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By utilizing intracellular staining methodology and flow cytometry, we examined the production of IFNγ, IL-2, IL-4, IL-6, IL-10, and TNFα by human peripheral blood T lymphocytes (PBTs) activated with PMA plus ionomycin, PHA, or cross-linking of the T cell receptor with anti-CD3 antibody. Of the six cytokines examined, we observed that only the T cells that stained with an anti-IL-6 antibody showed a selective loss of the activation marker CD69 with concurrent decrease in cell size. By coupling the intracellular staining with the terminal transferase (TdT) assay for DNA strand breaks, we showed that a majority of the IL-6+ T cells were undergoing apoptosis and that maximum apoptosis and production of IL-6 both occurred at days 2 and 3 after activation. Western blot analysis of these activated PBTs with anti-IL-6 reagents demonstrated a predominant 17 kD band distinct from the 26-30 kD mature glycosylated forms of IL-6. Apoptotic and live PBTs were sorted by flow cytometry using the dyes Hoechst 33342 and 7-AAD and Western blot analysis of these populations showed that the 17 kD protein was preferentially present in the apoptotic T cells. The striking correlation of p17 production with apoptosis provides an excellent marker for following the apoptotic process in human peripheral T cells. Purification of this protein for sequence analysis is underway to determine whether the 17 kD molecule is an alternate form of IL-6 or a novel protein, either of which may be involved in T cell apoptosis.

LENS FIBER CELL APOPTOSIS AND CATARACTS IN IRBP-E7 TRANSGENIC MICE LACKING THE P53 GENE.

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We wish to discern the molecular controls of apoptosis in the eye. The human papillomavirus type 16 E7 protein binds to the retinoblastoma tumor suppressor gene product pRb, and its related proteins p107 and p130, effectively eliminating their function. Transgenic mice, expressing E7 under the control of the interstitial retinol binding protein (IRBP) promoter, develop apoptosis of the immature lens fiber cells and form cataracts by post-natal day 1 (P1). This impact on lens cells is in addition to the apoptosis of the developing photoreceptors (E16-neonatal period) as they begin to differentiate (see Howes et al, Genes and Development, 8:1300, 1994). By P10, most lens fiber cells become necrotic and lyse. When IRBP-E7 mice are bred to p53 knockout mice (p53-/-) and back crossed, the resulting offspring are generated: E7+/p53+/- mice have apoptosis of lens and photoreceptors. E7-/p53+/- or E7-/p53-/- mice have normal eyes while E7+/p53-/- mice develop cataracts which do not differ significantly from those of E7+/p53+/+ mice. The apoptosis of their photoreceptors is slightly delayed (see Phipps et al, this meeting). This result differs from Pam and Griep (Genes and Devel. 1994) who made α-crystallin-E7 transgenic mice and Morganbesser et al (Nature 1994) who generated RB-/-/p53-/- embryos and showed a profound reduction of lens apoptosis in the absence of the p53 gene. The IRBP-E7/p53-/- transgenic mice also develop tumors of the photoreceptor layer resembling undifferentiated retinoblastomas of humans as well as ciliary body tumors, pigment epithelial (PE) carcinomas and poorly differentiated PE tumors by 2 months of age. Thus, expression of E7 in lens cells and photoreceptor precursors around the time of their last division (under the control of the IRBP promoter) triggers apoptosis in both photoreceptors and lens even in the absence of p53. The lack of p53 allows some of the photoreceptor precursor cells to enter mitosis and become malignant even in a setting of an apoptotic retina. Supported by EY 6891 (DSP), EY 9213 (JJW) and EY 10092 (JJW & DSP).

LOSS OF THE P53 TUMOR SUPPRESSOR GENE PROTECTS NEURONS FROM KAINATE-INDUCED CELL DEATH

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The tumor suppressor gene, p53, has been associated with apoptosis. A possible role for p53-related modulation of neuronal viability has been suggested by the finding that p53 expression is increased in damaged neurons in models of ischemia and epilepsy. Further, in transgenic mice with p53 null genes, ischemia-induced cell damage is reduced compared to the wild type. In the present study we evaluated the possibility that reduced p53 expression (in "knockout" mice) reduces cell damage in models of seizure activity normally associated with well-defined patterns of cell loss.

Subcutaneous injection of kainic acid (35 mg/kg), a potent excitotoxin, induced comparable seizures in both mice deficient in p53 (-/-) and in the corresponding wild-type mice (+/+). Using a silver stain for neurodegeneration, in animals sacrificed 7 days after kainate injection, we found that the majority (68%) of wild-type mice (+/+, n=16) exhibited well-defined patterns of cell loss in the hippocampus, which ranged from mild to severe. The extensive cell loss observed in (+/+) mice involved many subfields of the hippocampus, including CA1, CA3, the hilus and the subiculum. The presence of degenerating neurons was confirmed using an in situ tailing technique to detect DNA fragmentation. In marked contrast, sixty-four percent of the p53 -/- mice (n=14) displayed no signs of cell damage; in the remaining p53 -/- mice, damage was mild or moderate, but never severe. The cells most consistently affected in (-/-) mice were in CA3b of the dorsal hippocampus, where a small region of pyknotic neurons were seen. Remarkably, damage was restricted exclusively to the hippocampus of (-/-) mice, whereas, damaged neurons were also observed in the amygdala, piriform cortex, cerebral cortex, striatum and thalamus of (+/+) mice following kainate treatment. The pattern and extent of damage in mice heterozygous for p53 (+/-, n=16) was identical to that seen in wild-type (+/+) mice, suggesting that a single copy of p53 is sufficient to confer neuronal vulnerability. These results suggest that p53 may influence viability in multiple neuronal subtypes and brain regions following excitotoxic insult. Furthermore, these data are consistent with the hypothesis that p53 may play a critical role in the modulation of irreversible neuronal damage.

CANCER CELLS DERIVED FROM SOLID TISSUES REQUIRE BCL-2/BCL-X_L FOR SURVIVAL AND TUMOR FORMATION IN VIVO.

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Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x_S*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills human carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. Cells infected with the *bcl-x_S* adenovirus but not control virus expressed high levels of the Bcl-x_S protein that localized to membranes of cytoplasmic organelles and perinuclear region. Dying cells infected with the *bcl-x_S* adenovirus morphologically resembled cells undergoing apoptosis. Cell death occurred in cancer cells expressing wild-type, mutated or no p53 protein. To assess if cancer cells expressing Bcl-x_S are capable of forming tumors in vivo, 5×10^6 human RKO colon cancer cells were infected with *bcl-x_S* adenovirus or control β-galactosidase virus. Uninfected cells or RKO infected with the control virus formed tumors in seven out of ten and two out of five injected nude mice respectively. However, RKO cells infected with the *bcl-x_S* adenovirus did not form tumors in any of the 15 nude mice injected with such cells. In contrast to cancer cells derived from epithelial tissues, human hematopoietic progenitor cells and primitive cells capable of repopulating immune-deficient SCID mice were refractory to killing by the *bcl-x_S* adenovirus. These results suggest that Bcl-2 family members are required for survival of cancer cells derived from solid tissues. Furthermore, our observations indicate that strategies such as the *bcl-x_S* adenovirus designed to disrupt the Bcl-2 family pathway may provide novel therapeutic approaches to cancer treatment.

PROGRAMMED DEATH (APOPTOSIS) OF CD4+ T CELLS IN AIDS: INVOLVEMENT OF FAS (CD95/APO-1) AND MODULATION BY TH1/TH2 CYTOKINES.

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We have proposed that the pathogenesis of AIDS may be related to the inappropriate induction of programmed cell death through HIV1-mediated interference with cell signaling, and that modulation of cell signaling may therefore have therapeutic implications in HIV-infected persons.

The depletion of CD4+ T cells in AIDS is preceded by early functional defects of CD4+ TH1-cell-mediated immunity. Quantification of cytokine secretion by immunoassays, and of cytokine mRNA expression by competitive RT-PCR, indicated that this progressive loss of CD4+ TH1-cell-mediated immunity is not related to a TH1 to TH2 cytokine switch, but to the fact that TCR stimulation of CD4+ TH1 cells results in their rapid death by apoptosis. Apoptosis of CD4+ T cells from HIV-infected persons, but not of CD8+ T cells, was prevented in vitro by antibodies to IL-10 or to IL-4, two TH2 cytokines that downregulate TH1-cell responses, or by the addition of rIL-12, a cytokine that upregulates TH1 functions, suggesting that normal levels of TH2 cytokines sensitize CD4+ TH1 cells for T-cell receptor (TCR)-induced apoptosis in the absence of sufficient levels of IL-12 secretion.

The regulation of the expression of the Fas and Fas ligand molecules, and of the sensitivity of the T cell to Fas ligation-mediated death, represents a major mechanism in the control of T-cell survival and T-cell death in response to activation. We observed that CD4+ and CD8+ T cells from HIV-infected persons expressed high levels of the Fas molecule. In contrast to T cells from controls, T cells from HIV-infected persons were highly sensitive to Fas ligation by agonistic anti-Fas antibodies or recombinant Fas ligand, which induced apoptosis in a percentage of T cells similar to that induced by TCR stimulation. The addition of an antagonistic antibody to the Fas receptor had a preventive effect on TCR-induced apoptosis of CD4+ T cells from HIV-infected persons, but not of CD8+ T cells. As TCR-induced apoptosis, Fas-mediated apoptosis of CD4+ T cells, but not of CD8+ T cells, was prevented by the addition of rIL-12 and enhanced by the addition of IL-10.

These findings indicate that T cells from HIV-infected persons are in an activation stage that couples the Fas molecule with transduction signals leading to death and renders them highly sensitive to Fas ligand-mediated death. In addition, they indicate that TCR stimulation leads to the upregulation of the Fas ligand in the CD4+ T cells, and that cytokines modulate CD4+ T cells' death by regulating their sensitivity to Fas ligation. These findings have potential implications for the understanding of AIDS pathogenesis and the design of immunotherapy strategies.

FAS LIGAND DEFICIENT DONOR T CELLS FAIL TO PRODUCE ACUTE GRAFT VERSUS HOST DISEASE (GVHD) AND INSTEAD INDUCE LUPUS-LIKE AUTOIMMUNITY IN THE PARENT-INTO-F1 (P->F1) MODEL. Charles S. Via[1], Andrei Shustov[1] Jorn Drappa[2], Keith Elkon[2,1] [1]VA Medical Center and Univ. of Maryland School of Medicine, Baltimore, MD 21201; [2]Hospital for Special Surgery, NY, NY

The injection of parental strain T lymphocytes into unirradiated F1 mice results in either: a) lymphocytopenia and immunodeficiency (acute GVHD) or b) lympho-proliferation and a lupus-like disease (chronic GVHD). Donor anti-host cytotoxic T lymphocytes (CTL) play a prominent role in mediating the cytopenic picture of acute GVHD and their absence in chronic GVHD contributes to the lymphoproliferation. Based on the observation that Fas ligand (FasL) contributes to CTL activity and that FasL is massively upregulated in another murine model of lupus (MRL/lpr), we asked whether FasL mediated apoptosis was important in mediating acute GVHD in the parent -> F1 model.

B6C3F1 recipients were either uninjected or injected i.v. with 50 x 10^6 spleen cells from normal C3H/HeJ (HeJ) or FasL deficient C3H/gld/gld (gld) donors. Analysis of HeJ->F1 mice at two weeks demonstrated findings typical of acute GVHD i.e., substantial engraftment of donor CD4+ and CD8+ T cells, lymphocytopenia and anti-host CTL activity. In contrast, gld->F1 mice exhibited lymphoproliferation, autoantibody production and no anti-host CTL activity consistent with the development of chronic GVHD. Flow cytometric analysis confirmed that the number of HeJ donor CD4+ and CD8+ T cells transferred was nearly identical to that of gld donor cells transferred. Donor cells from gld mice did not differ from HeJ mice in their in vitro production of IL-2 in response to host alloantigen (H-2^b) or in their ability to upregulate activation markers such as CD44 and CD69, indicating that gld donor cell anergy can not explain our results. These data indicate that: 1) Fas ligand has a major role in the developemnt of anti-host CTL activity and in mediating the cytopenic aspects of acute GVHD, and 2) activation of T cells in the absence of functioning FasL favors a T helper rather than a cytotoxic T cell response which may result in autoimmunity.

NEW SUBSTRATES FOR THE ICE-LIKE PROTEASES DURING APOPTOSIS IDENTIFIED BY HUMAN AUTOANTIBODIES.

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Many of the autoantigens targeted in systemic lupus erythematosus are clustered in surface blebs of apoptotic cells. Limited proteolysis of autoantigens can reveal previously cryptic epitopes to the immune system, and thereby break tolerance to self. Since the apoptotic interleukin 1ß-converting enzyme (ICE)-like proteases cleave two known autoantigens, poly (ADP-ribose) polymerase and U1-70k, we addressed whether other autoantigens clustered in this novel microenvironment are similarly cleaved during apoptosis. By immunoblotting lysates of control and apoptotic human cells with high titer human autoantisera, we demonstrated that five other autoantigens are specifically cleaved during apoptosis. One of these autoantigens has been identified as an important protein kinase involved in signal transduction. The time-courses of all cleavages are identical, and occur co-incident with the onset of morphologic apoptosis. Furthermore, all cleavages share the same inhibition characteristics, which strongly implicate an ICE-like activity(ies). Since ICE itself is unable to cleave these molecules, it is likely that the apoptotic ICE homologs are the responsible proteases.

We propose that cleavage of these autoantigens targets them for an autoimmune response by revealing immunocryptic fragments in a pro-immune apoptotic setting. Human autoantibodies will therefore be valuable tools with which to identify the mechanistically important proteolytic steps along the apoptotic pathway.

APOPTOSIS IN GLOMERULONEPHRITIS: IN VITRO DEFINITION OF MESANGIAL CELL SURVIVAL FACTORS

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Glomerulonephritis is characterised by recruitment of leukocytes and proliferation of resident cells. During resolution, excess cells are cleared by apoptosis - eg we have found that mesangial cell apoptosis is the major mechanism for resolution of glomerular hypercellularity in experimental mesangial proliferative glomerulonephritis, a condition in which antibody-mediated glomerular injury results in inflammation and an increase in number of glomerular cells which is spontaneously reversible (J Clin Invest 1994; 94:2105). We speculate that failure to delete mesangial cells by apoptosis may be a hitherto unrecognised mechanism promoting persistent glomerular hypercellularity and later scarring with loss of organ function. Prompted by exogenous cytokine control of oligodendrocyte and Rat-1 fibroblast survival, we investigated whether cytokines were capable of providing survival signals to cultured glomerular mesangial cells (GMC) from humans and rats.

We studied three in vitro models of GMC apoptosis - (i) serum starvation for 8 hours; (ii) exposure to cycloheximide for 8 hours; and (iii) exposure to etoposide. Single recombinant human cytokines were assessed for their effect upon GMC apoptosis in these systems. While rat GMC proved more susceptible to apoptosis than human GMC, comparable results were obtained with both cell types. Apoptosis initiated by all three classes of stimuli was partially inhibited (to between 40 and 60% of the normal "apoptotic signal") by IGF I at 100 ng/ml, IGF II at 25 ng/ml and bFGF at 10 ng/ml. PDGF-BB did not inhibit apoptosis, nor did EGF or TGFβ. Cytokines exhibiting survival factor activity did not appear to affect GMC expression of either Bcl-2 or Bax as assessed by quantitative immunofluorescence flow cytometry, but the possibility remains that other endogenous survival factors of the Bcl-2 family might be involved. Nevertheless, inhibition of apoptosis induced by 50 μmol of cyclohixamide strongly implies that new protein synthesis is not required.

We conclude that GMC, a mesenchymal cell type which in disease adopts a myofibroblast-like phenotype, is susceptible to apoptosis, and exogenous IGF I, IGF II, and bFGF are candidate GMC survival factors. In glomerulonephritis their expression may result in undesirable persistence of expanded mesangial cell populations, with the ultimate result that glomerular function is lost because of excessive extracellular matrix deposition and subsequent scarring.

C/EBPβ INDUCED HEMATOPOIETIC DIFFERENTIATION AND APOPTOSIS

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To investigate the role of C/EBPβ in hematopoietic cell growth and differentiation, we constructed a conditional form of the protein by fusing it to the hormone binding domain of the estrogen receptor. Activation of the chimeric protein in a stably transfected multipotent hematopoietic progenitor cell line resulted in the up-regulation of myeloid- and eosinophilic markers and in the downregulation of immature markers.

In addition to the onset of differentiation, cell death was induced with typical apoptotic features. A comparison between the effects of different C/EBPβ mutants showed a correlation between the apoptotic potential of C/EBPβ and its gene induction activity. This indicates that C/EBPβ activates genes involved in the apoptotic pathway. Interestingly, apoptosis could be completely inhibited by the papilloma virus protein E7 but not by E6 or the Myb oncogene suggesting a participation of Rb-type proteins in C/EBP mediated apoptosis.

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