APOPTOSIS (PROGRAMMED CELL DEATH)

Organizers: John A. Cidlowski and J. John Cohen

March 5-11, 1995; Tamarron, Colorado

Plenary Sessions *Page*

Poster Sessions

Apoptosis (Programmed Cell Death)

Physiological Regulation of Apoptosis

B8-001 INTRACELLULAR AND EXTRACELLULAR SIGNALS IN PROGRAMMED CELL DEATH AND APOPTOSIS, Richard A. Lockshin,[1] Theresa Latham,[1,2], Jana Jochová,[1,2] and Zahra Zakeri[2]. [1]Department of Biol. Sci., St. John's University, Jamaica NY 11439 and [2]Department of Biology, Queens College and Graduate Center of CUNY, Flushing, NY 11367.

The programmed cell death that characterizes metamorphosis of mammalian secretory tissues and many insect tissues, such as those of muscle and labial gland (salivary gland) of the tobacco hornworm *Manduca sexta* and *Drosophila* involves heavy lysosomal activity in the cytoplasm and late destruction of the nucleus. Nevertheless, it is possible, by pulse-field electrophoresis and *in situ* labeling, to detect early fragmentation of DNA into pieces approximately 50 kbp in size. It seems unlikely that this fragmentation, unless it disrupts specific genes, is sufficient to activate the death process. We have therefore been following metabolism of the cells during the period in which death is triggered. There is a sharp drop in total protein synthesis, coupled with a gradually-expanding erosion of the rough endoplasmic reticulum. During this period, ATP levels and respiration of the cells reflect the total protein in the cells, suggesting that there is no major restriction on available energy. The synthesis of some proteins is immediately and totally suppressed, while for others synthesis gradually drops, and others are transiently or permanently up-regulated. This specific up-regulation survives through the third day, at which time the cell is heavily vacuolated and energy resources, which have persisted, finally drop. We have cloned one of a few genes that are sharply up-regulated as cell death begins. Partial sequence of this gene has not yet revealed homologies to other known genes or motifs. As *Manduca*, starts metamorphosis, lysosomes in the labial gland migrate from a basal to a more central position, and in *Drosophila* they become more prominent. In *Drosophila*, fluorescence visualization of the cytoskeleton by antibodies and phalloidin revealed early changes in both microfilaments and microtubules. These rearrangements suggest that more general alteration in intracellular substructure or trafficking characterizes the transition to the beginning of programmed cell death. These several lines of inquiry are beginning to generate an overall picture of the signaling, reception of signal, and initiation of response, which we will discuss in greater detail .

B8-002 GENE EXPRESSION ASSOCIATED WITH DELAYED NEURONAL DEATH IN TRANSGENIC RODENTS, James I. Morgan and Tom Curran, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

Particularly during development, cell death appears to be the result of a genetic program, some elements of which have been elucidated in nematode worms and fruit flies. In mammals, there are also instances of developmental cell death that can be prevented or attenuated by blocking translation or transcription. This has lead to a search for the genes that might be involved in the programmed death of vertebrate cells as well as for potential homologs of genes that control cell death amongst invertebrate species.

If gene expression does play a role in cell death then there must be mechanisms that couple the signals that trigger death to the transcriptional machinery of the cell. Attractive candidates for participation in this process are genes of the cellular immediate-early class. These genes are rapidly induced by many types of stimuli and frequently encode transcription factors that provide the potential to regulate further, target genes. Furthermore, expression of two cellular immediate-early genes, *c-fos* and *c-jun*, have been shown to be associated with moribund cells in vivo.

Besides massive programmed cell death in the developing nervous system, there are neuropathological states that lead to a loss of neurons in the adult brain. For instance, neurons in the penumbra of a cerebral infarction undergo a delayed death that exhibits some of the molecular (DNA laddering) and morphological (condensed chromatin) hallmarks of apoptosis. In our studies, kainic acid was administered to adult rats and mice at doses that cause a highly selective and delayed loss of neurons in the limbic system. Many of these dying neurons exhibit the characteristics of apoptosis. Using the kainic acid model we have established that there is a biphasic pattern of expression of several cellular immediate-early genes (cIEGs). Using mice and rats that are transgenic for *c-fos* and *c-jun* linked to β-galactosidase we show that the second peak of immediate-early gene expression is exclusively localized to the vulnerable neurons in this model. Data will be presented using various biochemical approaches to characterize the transcription factor complexes that are uniquely associated with neuronal death.

B8-003 PROGRAMMED CELL DEATH: SOCIAL CONTROLS AND MECHANISM, M.C. Raff, B. Barres, J. Burne, H. Coles, M. Jacobson, and Y. Ishizaki, MRC Laboratory of Molecular Cell Biology, University College London, London WC1E 6BT, United Kingdom.

We have been exploring the possibility that all mammalian cells are programmed to kill themselves unless they are continuously signalled by other cells not to do so. Some cells clearly operate in this way, but it is uncertain how general this death-by-default mechanism is. Oligodendrocytes and their precursors work in this way. They need signalling molecules secreted by other cells to avoid undergoing programmed cell death (PCD) *in vitro*. About 50% of newly formed oligodendrocytes normally die in the developing rat optic nerve, and this death can be inhibited by the experimental delivery of exogenous survival factors, suggesting that it may reflect a competition for limiting amounts of survival factors. Rat lens epithelial cells and chondrocytes can survive for weeks when cultured at high density in the absence of exogenous signalling molecules, but they undergo PCD when cultured at low density; conditioned medium from high-density cultures promotes the survival of the cells in low-density cultures, suggesting that these cells require signals from other cells of the same type to avoid PCD. There is large-scale cell death in the normal developing rat kidney and much of it can be inhibited by treatment with EGF or IGF-1, suggesting that it may reflect a lack of adequate survival factors. A variety of cell types, but not blastomeres, undergo PCD when treated with a high concentration of staurosporine, even in the presence of protein synthesis inhibitors, suggesting that in most cells all the protein components of the death programme are in place. PCD can occur in the absence of a nucleus, mitochondrial respiration, atmospheric oxygen, or active cdc-2 kinase. We conclude that the death programme is probably mediated by a cytoplasmic regulator that acts in parallel on multiple organelles, but the nature of the regulator is unknown.

Cell Surface Activation of Apoptosis

B8-004 FAS-BASED T CELL-MEDIATED CYTOTOXICITY, Pierre Golstein, Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9, France (together with F. Vignaux, V. Depraetere, A. Anel, A.-M. Schmitt-Verhulst, E. Vivier, B. Malissen in Marseille and with S. Nagata et al. - Osaka - and D. Kägi et al. - Zürich and Basel)

Two distinct mechanisms have been demonstrated in T cell-mediated cytotoxicity, a perforin-granzyme B-granule exocytosis one and a Fas-based one. The latter involves direct membrane interactions between a Fas ligand at the effector cell surface and Fas at the target cell surface. Fas transduces in the target cell a signal leading to its death. Using both perforin knock-out mice (established in Zürich / Basel) and Fas-based reagents, in several cytotoxicity models we found both mechanisms and no third one, strongly suggesting that the Fas-based and perforin-based mechanisms account for all of the T cell-mediated cytotoxicity detectable in short-term in vitro assays. To investigate the possibility that Fas-based immune regulation (evidenced by the deregulated autoimmune lpr / gld phenotype) and Fas-based T cell-mediated cytotoxicity are causally related, we explored the latter in activated lymphocyte populations, and found that Fas-based cytotoxicity can lead to the lysis of *syngeneic* activated lymphocytes, consistent with the possibility of its participation in the down-regulation of immune responses, and more generally offering a model of socially controlled, direct membrane-mediated cell death. To investigate the induction of antigen-specific Fas-based T cell-mediated cytotoxicity, we used as model systems either uncloned MLC cells, or T cell clones, or T cell hybridomas, which we stimulated through their TCR/CD3 complex with appropriate antibodies. We could thus dissect out experimentally the induction of Fas-based cytotoxicity from its execution, and study some of the properties of this induction.

B8-005 **BIOLOGICAL SIGNIFICANCE OF CELL SURFACE APOPTOSIS ANTIGEN FAS,**

Shin Yonehara [1], Yoshiko Nishimura [2], Kazuhiro Sakamaki [1], Yoshiki Yamasaki [2], Kazuhiko Nakamachi [2], and Shuji Kishi [2], [1] Institute for Virus Research, Kyoto University, Kyoto 606-01, Japan, and [2] Pharmaceutical Basic Research Laboratories, JT Inc., Yokohama 236, Japan.

Fas antigen (Fas) is a cell surface receptor protein which mediates apoptosis. We have prepared both anti-human and mouse Fas mAbs with associated apoptosis-inducing activity. Fas and its natural ligand are cell surface proteins which belong to the NGF/TNF receptor family and TNF family, respectively. Mice carrying the *lpr* and *gld* mutations, both of which suffer from a SLE-like autoimmune disease, have defects in the Fas and the Fas-ligand gene, respectively, suggesting that Fas system has an important role in the elimination of autoreactive immunocytes. We have reported the involvement of Fas in negative selection/clonal deletion of superantigen-reactive human thymocytes and mouse peripheral T cells. We prepared transgenic mice which over-express mouse Fas under the control of T cell-specific *lck* promoter. In thymus of these mice with gain of the function of Fas, number of double-positive and CD4-single positive thymocytes decreased although number of other thymocytes were same as that of wild-type mice, suggesting the involvement of Fas system in the differentiation and selection of double-positive and CD4-single positive thymocytes. Ogasawara *et al.* reported that intraperitoneal administration of hamster anti-mouse Fas mAb into mice caused severe damage of the liver and killed the mice within 3-6 h (*Nature*, 364: 806, 1993). On the contrary, administration of our prepared hamster anti-mouse Fas mAb, RK-8, which shows strong apoptosis-inducing activity both *in vitro* and *in vivo*, never kill adult mice. Histrogical analyses indicated severe damage of the thymus, moderate damage of the spleen and liver, and weak damage of the heart. More detailed analyses of RK-8-administrated mice are now in progress. To analyze the signal transduction mechanism of apoptosis *via* Fas, Fas and Fas-associated proteins were identified by immunoprecipitation on human immature T cell line HPB-ALL. We identified Fas with Mr 48kd, Fas-associated cell surface protein with Mr 42kd, and several Fas associated intracellular proteins. We purified some of these proteins and more detailed analyses are now in progress.

Signaling: Intracellular Pathways

B8-006 REGULATION OF T CELL APOPTOSIS VIA T CELL RECEPTORS AND STEROID RECEPTORS, Makoto Iwata[1], Yong Zhao[1], Yoshiharu Ohoka[1], Yuzuru Tozawa[1], Rieko Iseki[1], Mutsumi Mukai[1], Kazuki Sato[2], and Minesuke Yokoyama[2], [1]Project Research Center and [2]Division of Fundamental Research, Mitsubishi Kasei Institute of Life Sciences, Machida-shi, Tokyo, 194 Japan.

The regulation of apoptosis in immature thymocytes during positive and negative selection events is dependent on signaling through the T cell receptors (TCR) and some of the accessory molecules, such as CD4, CD8, and LFA-1. The intracellular signals essential for positive selection, however, still remain to be resolved partly due to the lack of a good in-vitro model system. Glucocorticoid hormones at their physiological peak levels induce apoptosis in immature thymocytes in vitro, especially in murine and rat cells. Thus, it is conceivable that the immature thymocytes, that are to be positively selected, are rescued not only from spontaneous apoptosis but also from glucocorticoid-induced apoptosis in vivo. We have previously shown that proper stimulation through TCR/CD3 inhibits glucocorticoid-induced apoptosis in thymocytes in vitro. Costimulation via CD4, CD8, or LFA-1 facilitated the inhibitory activity, whereas costimulation via CD2, CD28, Thy-1, or H-2^d failed to do so. FK506, an immunosuppressant, canceled the inhibitory activity. Since FK506 is known to inhibit the activation of calcineurin (CN), a Ca^{2+}/calmodulin-dependent protein phosphatase, the apoptosis-inhibiting activity may involve CN activation. Its involvement was further suggested by using T hybridoma cells transfected with a mutant CN catalytic subunit having Ca^{2+}/calmodulin-independent, constitutive phosphatase activity. Their sensitivity to glucocorticoid-induced apoptosis was markedly reduced. We have previously suggested that glucocorticoid-induced apoptosis in murine thymocytes involves the activation of Ca^{2+}-independent (novel) protein kinase C (nPKC)-ε but not that of Ca^{2+}-dependent (classical) protein kinase C (cPKC)* or an early increase in the intracellular Ca^{2+} level. On the contrary, the apoptosis-inhibitory activity appears to involve the activation of cPKC-α and/or -β as well as CN activation. Indeed, the inhibitory activity was mimicked by a proper combination of ionomycin, a calcium ionophore, and thymeleatoxin, an activator of cPKC-α and -β but not that of nPKC. The activation of CN and cPKC also appears to be involved in positive selection in a fetal thymus organ culture system. These results suggest that the signals for the apoptosis-inhibitory activity are closely related to the essential signals for positive selection.

*Iwata, M., Iseki, R., Sato, K., Tozawa, Y., Ohoka, Y. 1994. *Int. Immunol. 6: 431-438.*

B8-007 p53-MEDIATED APOPTOSIS - IMPORTANCE OF THE CELLULAR CONTEXT. Ygal Haupt, Rebecca Haffner, Eyal Gottlieb, Sheldon Rowan and Moshe Oren. Dept. of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel.

The p53 protein contributes to induction of apoptosis by a variety of signals. Studies on DNA tumor viruses suggest a link between p53 and pRB, the product of the retinoblastoma susceptibility gene, in controling cell growth and apoptosis. We examined the role of pRB in the control of p53-mediated apoptosis in HeLa cells, in which the activities of p53 and members of the pRB family are compromised by the expression of HPV E6 and E7 proteins. Transient overexpression of wild type (wt) p53 in HeLa cells induced apoptotic cell death. Importantly, co-expression of functional pRB resulted in significant protection of HeLa cells from p53-mediated apoptosis, without interfering with the transcriptional activity of wt p53. These results suggest that pRB, and possibly other pRB-related proteins, play a major role in the decision whether cells respond to activated p53 by undergoing growth arrest or apoptosis. Our findings provide a direct link between these two tumor suppressors in the control of cell growth and cell death.

In addition to its central role in DNA damage-triggered apoptosis, p53 can also contribute to the death of hematopoietic cells deprived of survival factors. IL3-dependent DA-1 cells, containing wt p53, undergo relatively rapid apoptosis following IL3 withdrawal. When the activity of the endogenous wt p53 is blocked through introduction of a negative dominant p53 miniprotein, DA-1 cells survive longer without IL3, implying that p53 plays an important role in their apoptotic response to survival factor withdrawal. Nevertheless, IL3 withdrawal does not enhance transcription of p53 target genes, suggesting either that the transactivation function of p53 is dispensable for this process. or that the relevant p53 target genes are already constitutively active even prior to IL3 removal. Unlike DA-1 cells, which originate in a murine lymphoma, non-transformed myeloid 32D cells do not exhibit a significant dependence on wt p53 for efficient induction of apoptosis by IL3 deprivation. However, such dependence becomes apparent in 32D cells stably transduced with oncogenic v-*raf* (kindly provided by J. Cleveland). This observation suggests that the role of p53 in the regulation of apoptosis may be more prominent in transformed cells than in their normal counterparts, thereby providing a possible explanation for the preferential loss of p53 function during advanced stages of certain tumor progression scenarios.

B8-008 SIGNAL TRANSDUCTION PATHWAYS IN T LYMPHOCYTE APOPTOSIS, Sten Orrenius, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

A variety of treatments can trigger apoptosis in thymocytes and T lymphocytes by signal transduction mechanisms, which are either unknown or only partly identified. In some instances, these pathways appear to share common components. Thus, the p53 protein has been found to play an important role in thymocyte apoptosis triggered by DNA damage, whereas intracellular Ca^{2+}elevation seems to mediate apoptosis in several experimental models, including thymocytes treated with anti-CD3 antibodies, glucocorticoids, irradiation and certain immunotoxicants. In fact, treatment of thymocytes with Ca^{2+} -elevating agents (i.e. ionophores, SERCA-ATPase inhibitors, etc.) can reproduce all features of apoptosis after a lag phase of 1.5 to 2 hours.

The critical target(s) for Ca^{2+} in apoptotic cells remains to be identified. It is conceivable that elevation of the intracellular Ca^{2+} level might promote apoptosis by directly stimulating the enzymatic activities of proteases, phospholipases and/or endonucleases responsible for mediating cellular demise in apoptosis; activation of the Ca^{2+} -dependent neutral protease calpain has recently been shown to be involved in glucocorticoid- and irradiation-induced thymocyte apoptosis. Alternatively, Ca^{2+} rises may activate intracellular signal transduction pathways involving protein kinases and/or phosphatases that could regulate downstream effectors of apoptosis via postranslational modification. The observation that calmodulin antagonists can interfere with apoptosis supports this interpretation.

Subpopulations of thymocytes exhibit different sensitivity to apoptosis-inducing agents. Although the immature $CD4^+CD8^+$ thymocytes are highly sensitive to apoptosis induced by glucocorticoids and irradiation, they are less sensitive than peripheral T lymphocytes to apoptosis triggered by intracellular Zn^{2+} chelation. The latter is Ca^{2+} -independent and also insensitive to inhibitors of protein synthesis. The finding that peripheral T lymphocytes are resistant to induction of apoptosis by Ca^{2+}-elevating agents is in accordance with this observation. Further, antibodies to the APO-1/Fas surface antigen trigger apoptosis in JURKAT cells. This process involves both high-molecular-weight and internucleosomal chromatin fragmentation and is associated with extensive nuclear disintegration. Like apoptosis induced by Zn^{2+} chelation in lymphocytes, Fas-mediated signalling does not appear to involve Ca^{2+} or to require ongoing protein synthesis.

The possible relationship between oxidative stress and apoptosis has attracted increased interest recently. Our studies have shown that induction of apoptosis in thymocytes is associated with decreased intracellular levels of antioxidants and, conversely, that it can be inhibited by various oxygen radical spin traps. This protection is observed with different inducers of apoptosis, suggesting that redox modulation may play a central role in the regulation of apoptosis in both Ca^{2+} -dependent and Ca^{2+} -independent systems. The level at which redox modulation of apoptosis may occur is not known, although the recently discovered sensitivity of various transcription factors to redox regulation offers an attractive possibility which, however, awaits experimental support.

B8-009 IDENTIFICATION OF GENES THAT REGULATE APOPTOSIS IN T CELLS, Barbara A. Osborne[1], Sallie W. Smith[1], Kelly A. McLauglin[1], Lisa Grimm[1], Richard A. Goldsby[2], Zheng-Gang Liu[1] and Lawrence Schwartz[1], 1University of Massachusetts, Amherst, MA 01003 and [2] Amherst College, Amherst, MA 01002

Engagement of the T cell receptor on immature T cells induces apoptosis. To identify genes that regulate this process, we have constructed a library from dying thymocytes. By subtractive screening strategies, we have identified several genes that are either induced or repressed during cell death. Two of these genes, *nur77* and *egr-1*, encode zinc finger transcription factors previously identified as immediate early genes induced in response to mitogens. The role of these genes in apoptosis will be discussed. Another gene, *apt-4*, is expressed 4 hours following the induction of apoptosis in thymocytes. This gene appears to be a member of a gene family and we have isolated a related gene that is expressed in living cells and is repressed in cells that are in the process of dying. Additionally, we have demonstrated a requirement for the p53 tumor suppressor gene in the induction of apoptosis in thymocytes by ionizing radiation. However p53 does not play a role in cell death induced by either glucocorticoids or T cell receptor engagement. Taken together these data indicate that there are at least two distinct pathways, one that requires p53 and another, independent of p53.

Recent data from our lab support a role for molecular oxygen in apoptosis in T cells. When cells are cultured in the absence of oxygen, induction of apoptosis by either T cell receptor engagement or ionizing radiation is significantly inhibited. The data indicate a requirement for molecular oxygen in at least two cell death pathways in T lymphocytes. These conclusions are supported by the observation that N-acetyl-cysteine, a potent anti-oxidant, acts to inhibit apoptosis induced through the T cell receptor as well as by ionizing radiation.

Apoptosis Genes: Genetic Approaches

B8-010 GENETIC CONTROL OF PROGRAMMED CELL DEATH IN THE NEMATODE *CAENORHABDITIS ELEGANS*, H. Robert Horvitz, Howard Hughes Medical Institute, Department of Biology, MIT, Cambridge, MA 02139.

Of the 1090 nuclei generated during normal development of the *Caenorhabditis elegans* hermaphrodite, 131 undergo programmed cell death. We have characterized developmentally, genetically and to some extent molecularly the roles of 10 genes that function in all 131 of these programmed cell deaths. Three of these genes control the onset of the death process, six act in the phagocytosis of dying cells by their neighbors, and one functions in the digestion of the DNA of cell corpses. Three additional genes specify which cells will or will not express this cell death program. Some of these genes show structural and functional similarities to genes that act in cell death in vertebrates. For example, the nematode gene *ced-9*, which protects against programmed cell death in *C. elegans*, is similar to the human oncogene *bcl-2*, which also protects against cell death. The nematode gene *ced-3*, which causes cell death in *C. elegans*, is similar to the human gene that encodes the enzyme interleukin 1-beta converting enzyme, which can cause the programmed death of mammalian cells. These findings suggest that molecular mechanisms responsible for programmed cell death are broadly conserved among organisms as diverse as nematodes and humans.

Enzymology of Apoptosis

B8-011 FORMATION OF LARGE MOLECULAR WEIGHT FRAGMENTS IS A KEY COMMITTED STEP IN THYMOCYTE APOPTOSIS Gerald M. Cohen, Howard Fearnhead, Marion MacFarlane and Xiao-Ming Sun. Medical Research Council Toxicology Unit, Hodgkin Building, The University of Leicester, P.O. Box 138, Lancaster Road, Leicester, LE1 9HN, United Kingdom.

Apoptosis has been best characterised biochemically by the cleavage of chromatin at internucleosomal regions to yield a classical DNA ladder pattern. Recently we and others have shown that this internucleosomal cleavage of DNA is preceded by the formation of large fragments of 30-50 and 200-300 kilobase pairs in length. These fragments may represent cleavage of chromosomal domains such as supercoiled loops and rosettes. Large molecular weight fragments of DNA, particularly of 30-50 kilobase pairs in length, are formed in a preapoptotic population of thymocytes at an early but committed stage of apoptosis but prior to internucleosomal cleavage. These fragments are absent in normal thymocytes and their formation is dependent on protein synthesis. The formation of these fragments is associated with the condensation of chromatin abutting the nuclear membrane, which is recognised as one of the earliest ultrastructural signs of apoptosis. Studies using several protease inhibitors also interfere with the induction of apoptosis in thymocytes at different stages of the apoptotic process. Some protease inhibitors appear to act at a very early stage of apoptosis blocking the formation of large kilobase pair fragments of DNA. The results suggest the involvement of a preexisting protease present in thymocytes which is important for the induction of apoptosis.

Anti-Apoptosis Genes

B8-012 ANTI-APOPTOSIS GENES AND VIRUS-HOST INTERACTIONS, Rollie Clem, Lois Miller*, Christopher Karp, Emily H.-Y. Cheng, Michael Veliuona, Jennifer Lewis, Diane Griffin, and Marie Hardwick. Neurovirology Laboratories, Johns Hopkins University School of Medicine, Baltimore, MD 21287-7681 and *Departments of Genetics and Entomology, The University of Georgia, Athens, GA 30602-2603.

Because of the widespread nature of apoptosis, it is perhaps not surprising that viruses have evolved strategies to counteract or exploit this important cellular function. A rapidly growing number of viruses have been found to trigger apoptosis in a wide variety of both invertebrate and vertebrate cells. Both large DNA viruses as well as smaller RNA viruses have been found to cause apoptosis, and a number of DNA viruses also express gene products which are able to block apoptosis. The ability to block apoptosis appears to be an important step in the life cycle of the baculoviruses, large DNA viruses which infect insects. Infection of insect cells by the prototype baculovirus, *Autographa californica* nuclear polyhedrosis virus, normally does not result in apoptosis due to the expression of a viral gene known as *p35*. Loss of function mutations in the *p35* gene result in viruses which induce apoptosis upon infection, with severe effects on replication both in cell culture and insects. The *p35* gene has been shown to block apoptosis not only in insect cells but in *C. elegans* and mammalian cells as well, suggesting that the function of *p35* is widely conserved. At least two other baculoviruses contain members of a second baculovirus gene family which can also block apoptosis in insect cells and can functionally substitute for *p35*. These genes, known as *iap* genes, exhibit no detectable homology to *p35* but instead encode motifs suggesting interactions with DNA, including a ring finger similar to that encoded by the mammalian *mdm2* gene.

In contrast to the situation with baculoviruses, induction of apoptosis by the positive strand RNA virus Sindbis virus (SV) is not detrimental to viral replication, at least in the context of short term replication in vitro. SV induces apoptosis during replication in a wide variety of cultured cells, and SV-induced apoptosis can be blocked by the cellular *bcl*-2 and *bcl*-X genes in a cell line specific manner. This protection can be overcome by neurovirulent virus strains, and the ability to overcome *bcl*-2 or *bcl*-X mediated protection has been mapped to a single amino acid change in the E2 structural glycoprotein. The ability to induce apoptosis in the presence of protective genes such as *bcl*-2 or *bcl*-X correlates with the neurovirulence of SV strains in mice, suggesting that susceptibility of neuronal cells to virally induced apoptosis may be an important determinant of alphavirus virulence and pathogenesis in vivo. Studies are underway to determine the mechanisms by which SV induces apoptosis and the relationship between E2 and the ability to overcome *bcl*-2 or *bcl*-X mediated protection.

Recognition of Apoptotic Cells

B8-013 RECOGNITION OF APOPTOTIC CELLS BY PROFESSIONAL AND AMATEUR PHAGOCYTES

Valerie A. Fadok, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843-4474.

The process of apoptosis is now widely recognized as critical to the regulation of normal tissue growth. To maintain normal tissue integrity and function, apoptotic cells must be removed prior to their lysis. Removal of apoptotic bodies avoids the damaging sequelae associated with release of toxic or inflammatory intracellular contents. Removal prior to lysis implies that there are specific surface changes on the apoptotic cells which signal the phagocyte to engulf it. Apoptotic corpses are not only removed by professional phagocytes (macrophages) but by amateur phagocytes, including epithelial cells, fibroblasts, other tissue cells, and in tumors by viable neoplastic cells.

At least three distinct mechanisms can be utilized by macrophages to recognize apoptotic cells. One of these, the recognition of exposed phosphatidylserine (PS), appears to be mediated in a receptor-like manner, because phagocytosis can be inhibited in a dose-dependent and stereospecific manner by phosphatidylserine and its chemical derivatives. In mice, the ability to recognize PS was found to be associated with inflammatory macrophages. Mouse bone marrow-derived macrophages (BMDM), although capable of phagocytosis, were not inhibited by PS; however, the ability to recognize PS was induced in BMDM by treatment with particulate glucan, a stimulus known to induce several characteristics of the inflammatory phenotype.

How does the amateur phagocyte recognize apoptotic cells? Very little about these mechanisms are known. I have recently begun to study this phenomenon in a new system, the spontaneously regressing Sinclair swine cutaneous melanoma. In vivo, phagocytosis of apoptotic bodies is accomplished by both macrophages and neoplastic cells. Established cell lines from this tumor were induced to undergo apoptosis with protein synthesis inhibitors, calcium ionophores, or TGF beta; the apoptotic populations were subsequently fed to viable melanoma cells and to macrophages. Phagocytosis by the melanoma cells appeared to be less efficient than phagocytosis by macrophages; however, preliminary data suggested that these amateur phagocytes could also recognize exposed PS. Clearly much more work is required. Studying phagocytosis of apoptotic cells will further our understanding of normal tissue remodeling and perhaps provide clues to unraveling the mysteries of some of the chronic inflammatory disorders.

B8-014 VITRONECTIN RECEPTOR AND CD36: A HIGH CAPACITY MECHANISM FOR PHAGOCYTE UPTAKE OF APOPTOTIC CELLS, John Savill[1], Yi Ren[1], Dyfed Evans[1], Jeremy Hughes[1], Roy Silverstein[2], Ian Dransfield[3] and Chris Haslett[3], [1]University Hospital, Nottingham UK, [2]Hematology/Oncology, Cornell University Medical School, New York, and [3]Rayne Laboratory, Edinburgh University Medical School UK.

Swift recognition, uptake and degradation by phagocytes is the normal fate awaiting apoptotic cells and bodies *in vivo*. We have studied mechanisms by which phagocytes recognize apoptotic cells as "unwanted", concentrating initially on the "professional" high-capacity phagocyte, the macrophage. Employing the human monocyte-derived macrophage (Mø) as a model, we investigated recognition of senescent neutrophils undergoing constitutive apoptosis, a system emphasising the potential of apoptosis as an "injury-limiting" cell disposal system operating to promote resolution of inflammation (*J Clin Invest 83: 865*). *Specific inhibition by aminosugars (J Clin Invest 84: 1518) suggested involvement of a Mø integrin, which* was characterized by mAb inhibition and positive selection by flow cytometry as the αvβ3 "vitronectin" receptor (Nature 343: 170). Further mAb inhibition studies indicated that Mø αvβ3 co-operated with Mø CD36 to bind thrombospondin 1 (TSP), which "bridged" the Mø to unknown, protease resistant sites on the apoptotic cell (J Clin Invest 90: 90: 1513). Mø also use this system to ingest apoptotic lymphocytes (Akbar et al, J Exp Med *in press*), and elements of this mechanism have been implicated in recognition of apoptotic targets by murine bone-marrow derived macrophages (J Immunol 149: 4029), and by "semi-professional" phagocytes such as fibroblasts (Hall et al, J Immunol *in press*) and glomerular mesangial cells (Baker et al, J Clin Invest *in press*). However, such phagocytes express much lower levels of surface CD36 than Mø, and have a lower phagocytic capacity. This talk will focus on unpublished data showing that CD36 gene transfer specifically confers "professional" phagocytic capacity for uptake of apoptotic neutrophils, lymphocytes and fibroblasts upon poorly phagocytic CD36-ve cells of various types by a mechanism requiring co-expression of αvβ3 and availability of TSP. This could be an approach to protecting healthy tissue by "tailoring" local phagocytic capacity to cope with increased loads of apoptotic cells which could be a potentially hazardous by-product of future therapies designed to eliminate cells by apoptosis.

B8-015 TRANSBILAYER LIPID MOVEMENTS AND THE RECOGNITION OF APOPTOTIC CELLS, Patrick Williamson[1], Bret Verhoven[1], Deepti Pradhan[2], and Robert A Schlegel[2], [1]Department of Biology, Amherst College, Amherst, MA 01002, [2]Department of Biochemistry and Molecular Biology, Penn State University, University Park, PA 16802.

Apoptosis is assumed to represent a common mechanism resulting in the orderly demise of cells in response to a variety of different signals. In lymphocytes, the characteristics of this common pathway have been largely defined by morphological studies, with the exception of degradation of the nuclear chromatin. We have begun to characterize the common pathway by identifying the order of identifiable events during activation-induced and hormone-induced apoptosis in thymocytes and in the DO11.10 thymoma cell line. To expand our molecular understanding of events during apoptosis, we have exploited information about the regulation of transbilayer lipid asymmetry to explore the events which underly the phosphatidylserine (PS)-dependent recognition of apoptotic thymocytes by activated macrophages. In particular, we have examined the regulation of the enzyme which normally prevents PS appearance on the surface of cells, the aminophospholipid translocase, and found that this activity is down regulated several hours before the onset of cell lysis. In addition, we have obtained evidence for the nearly simultaneous activation of a nonspecific pathway for transbilayer lipid movement, a pathway which is capable of permitting PS on the internal leaflet of the plasma membrane to reach the external surface. The current status of the molecular characterization of these two pathways and the signals which control them will be reported.

Apoptosis/Cancer/Aids

B8-016 APOPTOSIS OCCURS IN BYSTANDER CELLS AND NOT IN PRODUCTIVELY INFECTED CELLS OF HIV-INFECTED LYMPH NODES, T. H. Finkel[1], G. Tudor-Williams[2], N. K. Banda[1], M. F. Cotton[1], & A. Kupfer[1], [1]National Jewish Center, Denver, CO, [2]Molecular Histology Laboratory, Inc., Gaithersburg, MD.

Although more than ten years have passed since the identification of human immunodeficiency virus-1 as the cause of AIDS, we do not yet know how HIV-1 kills its primary target, the CD4 T cell. Prominent theories include, direct killing of infected CD4 T cells by cytopathic viral RNA, transcripts or proteins, and indirect killing of uninfected CD4 T cells by autoimmune mechanisms, cytokines, superantigens, or apoptosis. We and others have shown a significant increase in the percent of apoptotic cells among the circulating CD4 (and CD8) T cell populations of HIV-seropositive adults and children, and an increase in the frequency of apoptosis with disease progression in infected children. In order to determine relative contributions of direct and indirect killing in HIV infection, we analyzed lymph node sections of 30 infected adults and children. Each section was analyzed for HIV infection by RNA *in situ* hybridization and for apoptosis by nick translation of fragmented DNA. Some sections were also analyzed for surface expression of CD3 or CD4 by immunohistochemical staining. The sections were analyzed and quantified by digital confocal microscopy, using a fluorescent filter to enhance color differences between the two markers. Interestingly, although there was a correlation between the amount of apoptosis and productive infection, apoptosis was noted in bystander cells and not in the productively infected cells themselves. These data suggest that while uninfected cells are killed during HIV infection, productively infected cells are relatively resistant to apoptosis.

What are the implications of a productive infection that does not induce apoptotic cell death? Recently, several viral gene products that inhibit cellular apoptosis have been identified. Interestingly, these genes are encoded by viruses known to cause persistent, non-cytolytic infection. We hypothesize that HIV encodes a protein that inhibits apoptosis of its host cell. This would obviously be of selective advantage to the virus. Thus, we suggest that HIV may not kill the cell it infects, but rather uses the infected cell as a factory to produce new virus. In addition, we hypothesize that the predominant mechanisms of cell death in HIV are indirect. Certainly, there are host and viral factors (stage of disease, treatment, viral strain) that may impact these results. We are currently expanding our study of apoptosis and HIV infection to address these issues.

Support: AMFAR 770188-11-PF & 02270-16-RG, NIH AI-30575A, AI35513, & AI29903A. Acknowledgements:Molecular Histology Laboratory, Inc., Drs. B. Mueller, P. Pizzo, UCHSC ACTU

B8-017 THE SIGNIFICANCE OF SPONTANEOUS AND RADIATION-INDUCED APOPTOSIS IN THE GASTROINTESTINAL TRACT. Christopher S. Potten, CRC Department of Epithelial Biology, Paterson Institute for Cancer Research Christie Hospital (NHS) Trust, Wilmslow Road, Manchester, M20 9BX, UK.

Apoptosis occurs at a low level spontaneously in the small intestinal crypts of mice. The levels can be dramatically raised by a variety of cytotoxic agents including radiation. The apoptosis induced by radiation, isopropylmethane sulphonate, bleomycin and adriamycin, and four mutagenic chemicals as well as the spontaneous apoptosis, shows some specificity for the stem cell position in the small intestinal crypts. Radiation and the chemical mutagens target cells in the mid region of the colonic crypt and are therefore more likely to be damaging dividing transit cells than stem cells. p53 expression is elevated at the same time and the same positions in these two regions of intestine, but does not appear to be elevated in the cells undergoing apoptosis. Bcl-2 expression is largely absent in the small intestine, but is expressed, albeit weakly, at the stem cell position (at the base of the crypt) in the colon. Spontaneous apoptosis is observed in p53 null mice which also develop normally suggesting that developmental apoptosis is also normal. However, radiation induced apoptosis is completely absent at these early times post-irradiation. These observations suggest that the spontaneous apoptosis is p53-independent and it is hypothesised that in the small intestine, this process is part of the homeostatic mechanisms maintaining stem cell numbers. The spontaneous apoptosis has been linked with the production of extra stem cells that would result from occasional symmetric divisions in stem cells that mostly divide asymmetrically. This is supported by preliminary data which show an association between the rate of proliferation and the levels of apoptosis. The apoptosis induced by cytotoxic agents is p53 dependent, although the apoptotic bodies themselves have not been observed to express p53 protein. The function of p53 expressing cells at early times after damage remains to be elucidated. Bcl-2 is a survival gene which may prevent cells from undergoing apoptosis. It has been suggested that apoptosis in the small intestine is part of the protective mechanism against carcinogenesis in the stem cells of the small intestine which rarely develops cancer, effectively removing any cells that possess genetic damage. In the large bowel, this mechanism is not effective possibly as a consequence of the action of bcl-2. Thus stem cells may persist in this tissue with genetic damage resulting from misrepair or no repair which would result in a higher cancer risk.

Apoptosis, Reproduction, Development, Aging

B8-018 APOPTOSIS, AGING AND LONGEVITY: THE LESSON OF CENTENARIANS AND DOWN'S SYNDROME, Claudio Franceschi[1], Daniela Monti[1], Giovannella Baggio[2], Sabrina Donazzan[2], Maria Grazia Franceschini[1], Paolo Negro[1], Roberto Azzi[1], Stefano Salvioli[1], Miriam Capri[1], Walter Malorni[3], Roberto Rivabene[3], Maria Paola Giulietti[4], Galina Kalashnikova[1], Andrea Cossarizza[1]. [1] Dept. Biomedical Sciences and [4] School of Dentistry, Univ. Modena, Italy; [2] Inst. Internal Medicine, Univ. Padua, Italy; [3] Dept. Ultrastructures, Istituto Superiore di Sanità, Rome, Italy.

Apoptosis is an ancestral, basic mechanism to eliminate heavily damaged or virus-infected cells, and has a particular importance in the physiopathology of the immune system. Moreover, we and others have stressed the intriguing relationship between the molecular mechanisms of cell proliferation and cell death (1). For all these reasons, it can be predicted that apoptosis plays a major role in the aging process. We have tested this hypothesis by assessing the propensity to undergo apoptosis in peripheral blood mononuclear cells (PBMC) from healthy subjects of different ages (from young people to centenarians) and patients affected by Down's syndrome (DS), a syndrome of accelerated aging. Apoptosis was induced by 2-Deoxy-D-ribose (D-Rib) in quiescent PBMC, as described (2). Our major finding was the inverse correlation between propensity to apoptosis and age of the donor. Centenarians did not escape this trend, and showed the highest resistance to D-Rib-induced apoptosis. By using a new cytofluorimetric method we recently described to measure mitochondrial membrane potential at the single cell level (3), we found that mitochondria are involved in the early phases of apoptosis, and that mitochondria from aged donors and centenarians are highly resistant to apoptotic stimuli. PBMC from DS subjects showed a slightly increased resistance to apoptosis, in comparison to age-matched control. This age-related increased resistance to apoptosis may help in avoiding cell loss during aging, but the price to pay for such a favourable aspect might be the accumulation with age of transformed or mutated cells. However, the fact that the highest resistance to apoptosis was observed in healthy centenarians, the best example of successful aging, suggests that this phenomenon might help in reaching the extreme limit of human life with a well preserved immune system (4-11).

1. Monti D. *et al.*, Ann. N.Y. Acad. Sci 673: 70-82, 1992; 2. Barbieri D. *et al.*, Biochem. Biophys. Res. Commun. 201: 1109-1116, 1994; 3. Cossarizza A. *et al.* Biochem. Biophys. Res. Commun. 197: 40-45, 1993; 4. Franceschi C. *et al.*, Ann. N.Y. Acad. Sci 663: 4-16, 1992; 5. Monti D. *et al.*, Ann. N.Y. Acad. Sci 663: 250-261, 1992; 6. Kirkwood T.B.L. and Franceschi C., Ann. N.Y. Acad. Sci 663: 412-417, 1992; 7. Paganelli R. *et al.*, Clin. Exp. Immunol. 90: 351-354, 1992; 8. Cossarizza A. *et al.*, J. Immunol. Res. 4: 118-126, 1992; 9. Sansoni P. *et al.*, Blood 80: 2767-2773, 1993; 10. Mariotti S. *et al.*, Lancet 339: 1506-1508, 1992; 11. Mariotti S. *et al.* J. Clin. Endocr. Metab. 177, 1130-1134, 1993.

B8-019 HORMONAL REGULATION OF GONADAL CELL APOPTOSIS, Aaron JW Hsueh, H Billig, SY Chun, T Shikone, SY Hsu, S Moran, K Eisenhauer, Division of Reproductive Biology, Dept. of Gyn/Ob, Stanford University Medical School, Stanford, Calif. 94305-5317

In the human ovary, only 400 out of the 400,000 follicles found at puberty onset will ovulate, the rest of them (>99%) undergo a degenerative process called atresia. We have found that apoptotic cell death is the underlying mechanism of follicle atresia (1). Using a DNA 3'-end labeling method to quantify apoptotic DNA fragmentation and an in situ method to localize affected cells, we found that treatment of rats with gonadotropins and estrogens in vivo prevents ovarian granulosa cell apoptosis whereas treatment with androgens and GnRH induces apoptosis (2,3). Preovulatory follicles undergo spontaneous apoptosis under serum-free culture in vitro whereas treatment with FSH, LH/hCG, IGF-I, EGF/TGF-alpha, basic FGF or GH prevents apoptosis. In contrast, treatment with IGF binding protein partially blocks gonadotropin and GH actions, suggesting a mediator role of endogenous IGF-I in follicle survival (4). Using the in situ approach, DNA fragmentation was also found only in granulosa cells of atretic follicles. Because proteins of the bcl-2 gene family are involved in apoptotic cell death in lymphatic and other tissues, we have identified bcl-2 and bax mRNAs in the ovary. We have also over-expressed the human Bcl-2 protein in the mouse ovary by using the inhibin-alpha gene promoter to drive the transgene, and the phenotype of these transgenic animals are under study. In the testis, up to 75% of the germ cells are lost during normal spermatogenesis and testis regression can be induced by gonadotropin withdrawal and prevention of testis descent to elevate testis temperature. We have found that treatment with FSH, LH and androgens prevents hypophysectomy-induced increases in testis cell apoptosis (5). In addition, experimentally-induced cryptorchidism increases testis cell apoptosis. Although specific hormonal receptors are found mainly in somatic cells of the testis, in situ analysis indicated that only germ cells at different stages of development are undergoing apoptosis. Thus, analysis of gonadal cell apoptosis allows the identification of different apoptotic and survival factors for gonadal cells. Because the primary target cell types undergoing degeneration differ in the ovary (granulosa cells) and the testis (germ cells), the refined gonadal models provide the opportunity to elucidate the molecular mechanisms underlying the hormonal regulation of apoptosis in both somatic and germ cells.

1. Endocrine Review Dec. 94; 2. Endocrinology 133:2204, 93; 3. Endocrinol 134:245, 94; 4. Endocrinology Nov. 94; 5. Molecular Endocrinology 7:643, 93.

B8-020 PROGRAMMED CELL DEATH IN *DROSOPHILA*, Hermann Steller[1], Julie Agapite[1], Megan Grether[1], Anne-Francoise Lamblin[1], and Kristin White[2], [1]Howard Hughes Medical Institute, Depts. of Brain and Cognitive Sciences and Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, [2]Massachusetts General Hospital, Charlestown, MA 02129.
During *Drosophila* development, large numbers of cells undergo natural cell death. Even though the onset of these deaths is controlled by many different signals, most of the dying cells undergo common morphological and biochemical changes that are characteristic of apoptosis in vertebrates (1). We have surveyed a large fraction of the *Drosophila* genome for genes that are required for programmed cell death by examining the pattern of apoptosis in embryos homozygous for previously identified chromosomal deletions. A single region on the third chromosome (in position 75C1,2) was found to be essential for all cell deaths that normally occur during *Drosophila* embryogenesis (2). We have cloned the corresponding genomic DNA and isolated a gene, *reaper*, which is capable of restoring apoptosis when reintroduced into cell death defective deletions. 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. This gene is also rapidly induced upon X-ray irradiation, and deletions that include *reaper* offer significant protection against radiation-induced apoptosis. Therefore, multiple signalling pathways for the activation of apoptosis converge onto the *reaper* gene. Finally, ectopic expression of *reaper* under the control of the hsp70 heat shock promoter induces high levels of ectopic cell death in transgenic embryos, and high levels of *reaper* expression in the developing retina cause eye ablation. More recently, we have identified a second gene, *head involution defective (hid)*, that maps near *reaper* and appears to play a similar role for the induction of apoptosis. *hid* mutant embryos have decreased levels of cell death and contain extra cells in the head. Like *reaper*, expression of *hid* is sufficient for the induction of apoptosis. Furthermore, the ability of *hid* to kill does not depend on *reaper* function and vice versa, indicating that both genes can at least partially substitute for each other. We conclude that both *reaper* and *hid* encode important control functions for the activation of apoptosis.

1) Abrams, J., White, K., Fessler, L., and Steller, H. (1993). Programmed Cell Death During *Drosophila* Embryogenesis. Development **117**, 29-43.

2) White, K., Abrams, J., Grether, M., Young, L., Farrell, K., and Steller, H. (1994). Genetic Control of Cell Death in *Drosophila*. Science **264**, 677-683.

Neural Apoptosis

B8-021 RELATIONSHIP BETWEEN NEUROPROTECTIVE EFFECTS OF NEUROTROPHINS AND APOPTOTIC-ANTI-APOPTOTIC MECHANISMS, Hans Thoenen, Benedikt Berninger, Eero Castrén, Jonathan Cooper, Dan Lindholm, Theologos Michaelidis, Yoko Ogha, Georgios Tzimagiorgis, Department of Neurochemistry, Max Planck Institute for Psychiatry, Am Klopferspitz 18A, D-82152 Martinsried, Germany.

Neurotrophic factors, in particular the members of the nerve growth factor (NGF) gene family named neurotrophins, have been shown to protect specific populations of neurons (expressing the appropriate signal-transducing receptors) and even to restore impaired neuronal functions resulting from a great variety of mechanical or chemical insults and also genetically determined disorders. Although great progress has been made in the understanding of the details of the signal-transduction mechanisms subsequent to the interaction of neurotrophins with their receptors, it is not known which are the key mechanisms responsible for the neuroprotective actions of neurotrophins. We have studied the expression, cellular localization and developmental changes *in vivo* of a series of apoptotic-anti-apoptotic molecules (e.g. bcl-2, bcl-x, bax, and ICE) together with their regulation by neurotrophins *in vitro*. These investigations have been complemented in transgenic mice by tissue-specific overexpression and gene-targeting.

Late Abstracts

MOLECULAR GENETIC ANALYSIS OF THYMOCYTE APOPTOSIS, Frank Flomerfelt, Mark Chapman, Dave Askew and Roger Miesfeld, Departments of Biochemistry and Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721. To better understand the molecular basis of glucocorticoid-regulated thymocyte apoptosis, we have investigated apoptosis in a well-characterized murine T cell thymoma cell line called WEHI 7.2. This cell line is essentially diploid in chromosome content, expresses surface markers consistent with it being derived from immature thymocytes (CD4+, CD8+) and has very low levels of Bcl-2. WEHI 7.2 cells are induced to undergo apoptosis in response to the synthetic glucocorticoid, dexamethasone, and cAMP and gamma irradiation; apoptosis induced by these agents can be blocked by ectopic expression of human Bcl-2 in WEHI 7.2 cells. Interestingly, WEHI 7.2 cells do not express the CED-3 homologue, ICE, a recently characterized cysteine protease, nor do WEHI 7.2 cells appear to be protected from steroid-induced apoptosis by expression of the viral crmA gene, a potent inhibitor of ICE. Taken together, these results suggest that mouse thymocytes may contain other members of the CED-3 family, for example NEDD-2/ICH-1, or that the thymocyte apoptotic pathway may be different than what has been described for cells in neural lineages. We have focused our efforts on identifying primary glucocorticoid-induced target genes, and apoptotic "effector" genes, in WEHI 7.2 cells using two approaches First, we have isolated a panel of apoptotic-deficient (Apt-) WEHI 7.2 mouse thymoma cell lines by chemical mutagenesis and selection in media containing dexamethasone. As shown by heterokaryon cell fusion studies, these Apt$^-$ mutants contain non-glucocorticoid receptor, recessive mutations in genes that represent multiple complementation groups. Apt- mutations block apoptosis induced by dexamethasone, c-AMP treatment, and gamma irradiation, before the point where Bcl-2 exerts its protective effect. We propose that different signals share a common apoptotic pathway, and that the induction of apoptosis involves multiple precommitment steps that can be blocked by recessive mutations. Second, we have used subtraction cDNA cloning and mRNA differential display to isolate rapidly-induced glucocorticoid-regulated transcripts. These studies have led to the characterization of several genes that are induced in WEHI 7.2 cells within 2 hours of steroid-treatment. We are currently characterizing the transcription of these genes in response to other inducers of thymocyte apoptosis, and by examining their expression pattern in the Apt- mutants. In addition, we have begun to develop strategies to utilize functional complementation assays as a means to isolate genes that are mutated in the Apt- cell lines.

CYTOLYTIC T CELL CYTOTOXICITY IS MEDIATED THROUGH TWO LYTIC PATHWAYS: PERFORIN AND FAS , Bente Lowin, Michael Hahne, Chantal Mattmann and Jürg Tschopp, Institute of Biochemistry, University of Lausanne, CH-1066 EPALINGES, Switzerland.

The recent generation of perforin knock-out mice (ref.1,2) has demonstrated a crucial role for the pore-forming perforin in cytolytic T lymphocyte (CTL)-mediated cytolysis. Perforin-deficient mice failed to clear lymphocytic choriomeningitis virus *in vivo*, yet substantial killing activity still remained in perforin free CTLs *in vitro*, indicative of the presence of (a) further lytic pathway(s). Fas is an apoptosis signaling-receptor molecule on the surface of a number of different cells. Here we report that both perforin- and Fas ligand-deficient CTLs show impaired lytic activity on all target cells tested. The killing activity was completely abolished when both pathways were inactivated by using target cells from Fas receptor-deficient *lpr* mice and perforin-free CTL effector cells. Fas ligand-based killing activity was triggered upon T cell receptor occupancy and was directed to the cognate target cell. Thus, two complementary, specific cytotoxic mechanisms are functional in CTLs, one based on the secretion of lytic proteins and one which depends on cell surface ligand-receptor interaction.

1. Kägi, D., *et al. Nature* **369**, 31-37 (1994).
2. Lowin, B., Beermann, F., Schmidt, A. & Tschopp, J. *Proc. Natl .Acad. Sci. USA* , in press (1994).

THE GENES THAT CONTROL PROGRAMMED CELL DEATH: FROM WORM TO MAMMAL. Masayuki Miura, Valeria Gagliardini, Lin Wang, Louise Bergeron, Pierre-Alain Fernandez, Rocco J. Rotello and Junying Yuan Cardiovascular Research Center, Massachusetts General Hospital-East, 149 13th St. Charlestown, MA 02129; and Department of Medicine, Harvard Medical School, Boston, MA 02115

The genetic control of programmed cell death has been well elucidated by the work on programmed cell death in the nematode *C. elegans*. Recessive loss-of-function mutations in *ced-3* and *ced-4* block all programmed cell death that occurs during *C. elegans* hermaphrodite development, suggesting that the functions of *ced-3* and *ced-4* are required for cells to die. The *C. elegans* CED-3 protein is 503 amino acids in length and contains a serine-rich central region of about 100 amino acids. The non-serine-rich portions of the CED-3 protein are similar to human interleukin-1β (IL-1β) converting enzyme (ICE), a cysteine protease that can cleave the inactive 31 kd precursor of IL-1β to generate the active cytokine. The similarity between CED-3 and ICE suggests not only that CED-3 might function as a cysteine protease but also that ICE might function in programmed cell death in vertebrates. To examine if overexpression of *Ice* will induce programmed cell death, we designed a transient expression system which uses *E. coli lacZ* gene as an indicator of gene expression. We found that expression of the murine *Ice* cDNA fused with the *E. coli lacZ* gene in Rat-1 cells induced programmed cell death. The cowpox virus gene *crmA* encodes a 38 kD protein that can specifically inhibit ICE activity . To test if *Ice* plays a role in neuronal cell death, we microinjected *crmA* expression construct into DRG neurons and removed trophic factor (NGF and serum). We found that expression of *crmA* in DRG neurons can effectively blocked the cell death induced by trophic factor removal. Microinjection of *bcl-2* can also protect the DRG neurons from death induced by trophic factor removal. Microinjection of both *bcl-2* and *crmA* expression constructs do not provide further protection than either *crmA* or *bcl-2* alone, suggesting that *bcl-2* and *crmA* may prevent neuronal cell death in a single pathway.

Given the fact that a single important gene in a simple organism often evolved into a multigene family in more complex organisms, we searched for additional members of *Ice/Ced-3* family in vertebrates. A third member of ICE/CED-3 family, *Nedd-2*, was identified from a cDNA isolated by Kumar et al. as a mRNA expressed mostly during early mouse embryonic brain development and down-regulated in adult brain. Since many neurons die during early embryonic brain development, we wanted to know if *Nedd-2* is also a cell death gene. We isolated and characterized the human *Nedd-2* gene, which we renamed *Ich-1* (Ice and ced-3 homolog). We found that the human *Ich-1* cDNA encodes a protein much longer than the mouse *Nedd-2* and shows sequence similarities to ICE and CED-3 proteins along their entire sequence. 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. Our results suggest that *Ich-1* may play an important role in both positive and negative regulation of programmed cell death in vertebrate animals.

Poster Session 1

B8-100 AFRICAN SWINE FEVER VIRUS (ASFV) GENE 23-NL ENCODES A 23 KDA NUCLEAR PROTEIN THAT DELAYS VIRAL-INDUCED APOPTOSIS, C.L. Afonso, A. Brun, S. Dhume, J.G. Neilan, T. Burrage, G.F. Kutish, and D.L. Rock, Plum Island Animal Disease Center, USDA, ARS, Box 848, Greenport, NY 11944

The function of ASFV gene 23-NL is unknown; however, its similarity to a myeloid differentiation factor (MyD116) and the HSV neurovirulence associated gene ICP34.5 suggests a possible role in viral virulence or host range (J Virol 66:5586). Monospecific antibodies to 23-NL specifically immunoprecipitated a protein of 23 kDa in ASFV infected macrophages. Immunofluorescence studies reveled cell-type-specific intracellular localization; in infected Vero cells, p23 was present in both the cytoplasm and nucleus, while in infected macrophages, protein was restricted to the cell cytoplasm. 23-NL containing recombinant vaccinia and baculoviruses (Bv) were constructed to study gene function. Expression of p23 in SF21 insect cells infected with a 23-NL recombinant Bv resulted in significant increases in cell viability at 72, 96 and 120 h post infection (PI) which were accompanied by a decrease in cellular DNA fragmentation. Similar observations were made for cells of myeloid origin infected with a p23 expressing vaccinia recombinant. Significant increases in cell viability at 24, 48 and 72 h PI over that observed for a recombinant containing the 23-NL gene in the antisense orientation were observed. These observations, of increased cell viability accompanied by a reduction in cellular DNA fragmentation in p23 containing virus infected cells, suggest that the protein prevents or significantly delays viral-induced apoptosis.

B8-101 LATENT EPSTEIN-BARR VIRUS BLOCKS ONLY ONE OF THE PATHWAYS LEADING TO APOPTOSIS, Martin J. Allday[1,2], Alison Sinclair[2], Gillian Parker[1,2] and Paul J. Farrell[2], Department of Medicine[1] and Ludwig Institute for Cancer Research[2], St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG, UK.

Lymphoblastoid cell lines expressing the full complement of EBV latent genes are very sensitive to DNA damaging agents such as cisplatin. The response includes a rapid accumulation of the tumour suppressor protein p53 and induction of the cellular genes *mdm2* and *WAF1/p21*. Although the levels of Bcl2 protein and Bax mRNA appear unaltered by this activation of p53, within 24 hours the majority of cells undergo apoptosis. In contrast, Burkitt's lymphoma cell lines carrying p53 mutations did not respond to equivalent doses of cisplatin by either apoptosis or growth-arrest. Primary resting B cells showed no response to cisplatin and even after drug treatment, p53 remained undetectable; only after infection with transcriptionally active virus was there evidence of p53 induction. Thus in EBV-immortalised B cells there must be two distinct pathways ending in apoptotic death; EBV, through Bcl2, can rescue cells from apoptosis induced by factor withdrawal (Gregory et al, 1991, Nature 349; Henderson et al, Cell 65) but it does not appear to protect cells responding to DNA damage. As B cells become activated and immortalised by EBV they are sensitised to the p53-mediated response but, unlike other tumour viruses, EBV leaves this critical defence pathway unimpaired.

B8-102 TH1/TH2 CYTOKINES AND THE REGULATION OF PROGRAMMED T-CELL DEATH IN AIDS AND PARASITE INFECTION. Jean Claude Ameisen, Jérôme Estaquier, INSERM U 415, Institut Pasteur, 59019 Lille, France

We have proposed that the pathogenesis of AIDS may be due to the abnormal induction of programmed cell death (PCD), a process that could be related to an inhibitory effect of pathogenic lentiviruses on the capacity of accessory cells to provide appropriate cosignals to activated CD4+ T cells (1). Cellular immunity is mediated by the CD4+ T helper (TH)1-cell subset, and a TH1 to TH2 cell shift has been shown to have deleterious consequences in the pathogenesis of several chronic infectious diseases. It was proposed that a TH1 to TH2 cell shift may also account for cellular immunity defects in AIDS (2). We have investigated the possible involvement in T-cell PCD of two cytokines secreted by accessory cells, IL-10 and IL-12, that play an important role in the regulation of the CD4+ TH1/TH2 cell balance. We have explored the possibility that cytokine-mediated T-cell PCD may participate in two pathological models that both lead to impaired CD4+ TH1-cell function: HIV infection, that also leads *in vivo* to CD4+ TH-cell depletion, and a murine model of *Schistosoma mansoni* parasite infection, that induces a well characterized TH1 to TH2 switch, but does not lead to CD4+ TH-cell depletion.

Our results indicate that the progressive loss of CD4+ TH1-cell function in HIV-infected persons is not related to a TH1/TH2 shift, but to the fact that activation of the CD4+ TH1 cells results in their rapid deletion by PCD. CD4+ T-cell PCD is prevented *in vitro* by anti-IL-10 antibodies, or by the addition of recombinant IL-12, suggesting that defective IL-12 secretion by monocytes from HIV-infected persons (3) leads to IL-10-mediated CD4+ T-cell PCD. In the *S. mansoni* infection model, similar IL-10-mediated T-cell PCD is observed, with the important difference that T-cell PCD occurs in the presence of an increasing secretion of TH2 cytokines, including the TH2-cell growth factor IL-4, and of an increasing number of TH2 cells, i. e. in the context of an effective TH1 to TH2 shift.

These findings suggest that cytokine-mediated TH1-cell PCD may represent a general mechanism of TH1-cell down-regulation. In contrast to several chronic infectious diseases, in which a loss of CD4+ TH1 cells is compensated by a concomitant expansion of CD4+ TH2 cells, HIV infection may represent a particular case of abortive TH1 to TH2 switch in which CD4+ TH1-cell PCD occurs in the absence of IL-4 secretion, and therefore of any compensatory expansion of CD4+ TH2 cells. Such an abortive TH1 to TH2 switch could participate in the progressive CD4+ T-cell dysfunction and depletion leading to AIDS.

1. *Immunol Today* 1991,12:102; 2. *ibid.* 1993,14:107; 3. *J Exp Med* 1994,179:1361

B8-103 BCL-2 AND THERMOTOLERANCE COOPERATE IN CELL SURVIVAL. Robin L. Anderson, Tim Gabriele and A. Strasser*. Peter MacCallum Cancer Institute, St. Andrews Place, East Melbourne, 3002 and * Walter and Eliza Hall Institute, Royal Parade, Parkville, 3050, Victoria, Australia.

The protein product of the oncogene *bcl-2* is an effective inhibitor of apoptosis in mammalian cells, including that caused by exposure to heat. Hyperthermia in the temperature range of 41° to 45°C induces apoptotic death in many cell types. Transient resistance to heat, known as thermotolerance, is induced by mild heat pre-treatment and can inhibit apoptosis induced by subsequent more severe heat exposure. This protection is thought to be mediated by the expression of increased amounts of heat shock proteins (HSP) which act as chaperones to prevent heat-induced denaturation and to repair heat damaged proteins. We set out to test whether there was any relationship or cooperation between *bcl-2* expression, heat shock proteins and/or the thermotolerant state in inhibiting the process of apoptosis and in promoting cell proliferation after heat stress.

Three murine cells lines, a myeloid line, a T cell hybridoma and a B cell lymphoma, were stably infected with a retrovirus containing a human *bcl-2* construct. The presence of Bcl-2 conferred resistance to heat shock in all three lines with decreased numbers of cells entering apoptosis. The induction of thermotolerance also increased resistance to heat, but much more so in the lines containing *bcl-2*. The results suggest that *bcl-2* and the thermotolerant state act via independent mechanisms to inhibit apoptosis and provide evidence that two separate pathways can cooperate to promote cell survival.

To examine directly the role of HSP in apoptosis, we are testing whether over-expression of one major HSP, HSP70, can substitute for the thermotolerant state in blocking heat-induced apoptosis and in enhancing the effect of *bcl-2*. The cell lines mentioned above are being transfected with a construct containing human HSP70 under the control of a constitutive promoter. In one cell line, we have isolated several clones that express high levels of human HSP70 and have preliminary evidence of protection from apoptosis.

B8-104 PREVENTION OF PROGRAMMED CELL DEATH IN NERVE GROWTH FACTOR DEPENDENT PC12 CELLS BY OVEREXRESSION OF THE V-CRK ONCOGENE. Raymond B. Birge[1], Robert H. Glassman[1,2], Hideasburo Hanafusa[1] and Barbara L. Hempstead[2]. [1]Laboratory of Molecular Oncology, Rockefeller University [2]Department of Hematology-Oncology, Cornell Univ. Medical College.

The survival and differentiation of immature neuroblasts is essentially regulated by the supply of neurotrophic factors, the best characterized of which is nerve growth factor(NGF). Nerve growth factor mediates these effects by binding its receptor, p140 trkA, a receptor-type tyrosine kinase. Adaptor proteins, which consist of src homology 2 and 3 (SH2 and SH3) domains, link receptor tyrosine kinases such as trk A with downstream cytosolic effector proteins and are likely integral components of NGF signaling pathways. To further investigate how SH2 and SH3 containing proteins influence growth factor signaling, survival, and differentiative pathways in neurons, we stably overexpressed the oncogenic form of the adaptor protein crk, p47gag-crk, in rat pheochromocytoma (PC12) cells. The v-crk transfected cells display neurite outgrowth in response to both NGF and epidermal growth factor (EGF), unlike parental cells which do so only when exposed to NGF. Furthermore, the transfectants are dramatically spared from cell death upon withdrawl of NGF; this survival effect is augmented by the addition of EGF to the media following NGF removal.To evaluate the roles of the individual SH2 and SH3 domains in survival signaling, we stably expressed two v-Crk SH2 point mutants, H294R and R273N, in PC12 cells. These cells lines (R2-vcrk and N9-vcrk) differentiate in NGF, but with a significant delay compared with wild type PC12 cells, and are no longer protected from apoptosis after growth factor removal. Through serial dilutions, we isolated subclones of v-CrkPC12 cells which do not differentiate in response to NGF but maintain elevated v-Crk levels and normally associated v-Crk binding proteins. These "nonresponders"(NR cells) survive in the presence of NGF but quickly undergo apoptosis after NGF withdrawl. As assessed by cell counting and ^{3}H-thymidine, the NR cells also proliferate rapidly in NGF compared with native and v-crk PC12 cells.This data affirms that one can dissociate survival and differentiative signaling pathways; however, as also shown in the R2- and N9-vcrk PC12 cells, protection against apoptosis in growth factor free conditions may correlate with the rate of neurite outgrowth after NGF addition. Studies are underway to assess cell cycle parameters in native and v-Crk-expressing cells to study this mechanism in more detail. These data highlight the importance of tyrosine kinase pathways in survival signaling .

B8-105 Role of Apoptosis in Anticancer Drug Resistance, Jeanine Boesen, Kees Nooter, Ton Boersma, Robert Oostrum, Herman Burger, and Gerrit Stoter. Dept. Medical Oncology, DdHK/ AZR, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

Drug resistance is still a major problem in the chemotherapeutic treatment of cancer. So far, for the majority of human cancers, specific mechanisms which can explain clinical drug resistance have not been identified. A new approach to get insight in this phenomenon is to study how cells are killed by cytotoxic drugs via the triggering of the apoptosis pathway. Cellular genes have been identified, whose expression can inhibit apoptosis and which are of importance for drug resistance due to their expression in human tumours.

Since expression of oncogenic RAS genes is a frequent observation in human cancer, we investigated the effects of the c-H-RAS oncogene on anticancer drug-induced apoptosis. Apoptosis induced by a 2-hour exposure to doxorubicin (1 uM) was measured by in situ nick translation and flow cytometry in a rat cell line (R2T24) stably transfected with the c-H-RAS oncogene, and in a control cell line (R2NEO). Both cell lines had nearly identical growth characteristics, including cell doubling time, distribution over the cell cycle phases, and plating efficiency in soft agar. Anticancer drug exposure of the R2NEO cells leads to massive induction of apoptosis. After 48 hrs 2 populations of cells can be distinguished, a population with an increased number of DNA strand breaks and a population of cells with a sub G1 DNA content. After 72 hrs the apoptosis process is completed, as no more viable cells are found. In contrast, R2T24 cells expressing the c-H-RAS oncogene, showed significantly less apoptosis; 72 hrs after exposure to doxorubicin 70% of the cells is still viable. Similar results were found for all concentrations tested (0.1, 0.3 and 1 uM doxorubicin). However the inhibition by RAS is not an 'all or none' phenomenon. No difference was observed in intracellular drug uptake between the 2 cell lines. In clonogenic assays, doxorubicin induced approximately 3 to 5 fold less cytotoxicity in the R2T24 cells than in the R2NEO cells. From our data it can be concluded that RAS-transfected cells are still sensitive to drug induced apoptosis, but the process is delayed and less severe.

B8-106 LYSOPHOSPHATIDYLCHOLINE RESCUES CELLS FROM APOPTOSIS INDUCED BY ANTINEOPLASTIC PHOSPHOLIPIDS, Kevin P. Boggs, Charles O. Rock, and Suzanne Jackowski, Biochem. Dept., St. Jude Children's Research Hospital, Memphis TN 38101

ET-18 (1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine) is an anti-neoplastic phospholipid that is currently in phase II clinical trials, although its mechanism of action has not been established. A colony-stimulating factor 1-dependent cell line was used to study the relationship between ET-18-induced apoptosis and its interference with phospholipid metabolism. ET-18 inhibits *de novo* phospholipid synthesis at the CTP:phosphocholine cytidylyltransferase (CT) step *in vivo*. Kinetic analysis of delipidated, baculovirus-expressed CT showed that ET-18 was a competitive inhibitor with respect to lipid activators of the enzyme. ET-18 treatment caused the translocation of CT from the soluble fraction to cellular membranes, providing *in vivo* evidence that ET-18 interacts with the lipid binding domain of CT. Exposure to ET-18 at the G1/S boundary led to accumulation of cells in G2 phase, whereas ET-18 treatment in early G1 phase prevented entry into S phase. In both cases, ET-18 triggered apoptosis. Exogenous lysophosphatidylcholine (LPC) was also a potent inhibitor of membrane phospholipid synthesis at the CT step. However, LPC treatment did not result in growth arrest or apoptosis since it circumvented the requirement for *de novo* phospholipid biosynthesis by being directly acylated to form phosphatidylcholine. LPC treatment prevented the G2 cell cycle block induced by ET-18, although ET-18-treated cells still arrested in G1 phase in the presence of LPC. LPC completely blocked the onset of ET-18-dependent apoptosis. Our data suggest that ET-18 inhibits *de novo* phospholipid biosynthesis at the CT step by acting as a nonmetabolizable LPC analog, and that diminished capacity to produce membrane phospholipids triggers programmed cell death. (Supported by GM45737)

B8-107 MECHANISMS OF ACTIVATION INDUCED CELL DEATH IN HUMAN B CELL LYMPHOMA. POSSIBLE INVOLVEMENT OF NAK-1, THE HUMAN NUR77 HOMOLOGUE

Kurt Bommert, Markus Y. Mapara, Pamela Weinmann, Peter Daniel, Ralf Bargou, and Bernd Dörken.
Max Delbrück Center for Molecular Medicine, Robert Rössle Str. 10,Berlin-Buch and Free University of Berlin, University Medical Center Rudolf Virchow, Robert Rössle Klinik, Department of Internal Medicine Lindenbergerweg 80, Berlin-Buch.

Activation induced cell death (AICD) is an important form of cell death occurring in different mammalian tissues. The main feature of this form of apoptosis is its dependence on *de-novo* transcription. We established a human cell line model for the analysis of apoptosis induced by crosslinking of surface IgM (sIgM). Using the human EBV negative Burkitt lymphoma cell line BL41, which is highly sensitive to sIgM mediated apoptosis and a resistant subclone B5, we are able to identify events occurring after the crosslinking of sIgM, wich are specific for the induction of sIgM mediated apoptosis. BL41 and its resistant subclone B5 display similar levels of sIgM and both cell lines reveal a comparable increase in intracellular Calcium after triggering of sIgM. Thus, it is likely that the surface IgM complex in the resistant subclone is still functional. Hence, the inability of subclone B5 to undergo apoptosis appears to be an event more dowstream within the sIgM signaling cascade. Recently, it could be demonstrated that the orphan steroid receptor nur77 is crucial for CD3 mediated apoptosis in murine T-cell hybridomas. We studied the expression of NAK-1, the human nur77 homologue, during the induction of sIgM apoptosis in BL41 and the apoptosis resistant subclone B5. Interestingly, NAK-1 mRNA expression was rapidly induced in BL41 after sIgM crosslinking. In contrast, the resistant subclone B5 did not show any NAK-1 mRNA expression. Using gel retardation assays we were able to show that NAK-1 specific DNA binding activity was observable after sIgM triggering in BL41 cells but not in subclone B5. In conclusion, NAK-1, the human nur77 homologue, appears to play a pivotal role during sIgM mediated apoptosis in human B cells, thus indicating that AICD in B cells might use similar signaling mechanims as in T cells.

B8-108 DELINEATION OF APOPTOSIS PATHWAYS IN THE T CELL LINE JURKAT, Jannie Borst[1], Margaret Critchlow[2] and Gwyn T. Williams[2] [1]The Netherlands Cancer Institute, Amsterdam, The Netherlands; [2]Keele University, Keele, U.K.

The T-ALL line Jurkat represents the thymocyte differentiation stage, where T cell selection occurs. Triggering of the T cell receptor (TCR) on Jurkat induces apoptosis. In addition, Jurkat expresses the apoptosis-inducing receptors Apo-1/fas (CD95) and TNFR1. The cell line is resistant to glucocorticoid-induced death, but undergoes apoptosis upon treatment with the topoisomerase inhibitor etoposide, as well as upon X-irradiation. We are interested to know whether the various signalling pathways leading to cell death in Jurkat have common aspects. Ultimately, we aim to identify proteins required for apoptosis-induction by multiple stimuli, or proteins conferring resistance to multiple apoptosis signals. To this end, we have derived 10 variant subclones from the Jurkat line, which are resistant to CD95-mediated apoptosis (JA series). These JA clones have been monitored for their susceptibility to apoptosis induced by anti-CD3 mAb, phytohaemagglutinin (PHA), etoposide and X-irradiation. Whereas anti-CD3 mAb induces intracellular Ca^{2+} mobilization in all JA clones, indicating that TCR-proximal signalling is intact, 6 out of 10 clones are resistant to anti-CD3-induced death. Though PHA is known to bind to the TCR, the PHA-induced signal differs from the anti-CD3-induced signal in that only 2 out of 10 JA clones are resistant. Interestingly, the majority (8 out of 10) JA clones are fully resistant to etoposide, while 2 clones display wild type sensitivity. Wild type clones undergo a cell cycle arrest in G2 prior to undergoing apoptosis. The etoposide resistant JA clones display this G2 arrest, but do not die. At least 4 etoposide resistant clones are fully sensitive to X-irradiation induced death, indicating that etoposide and X-irradiation pathways are at least partially distinct. The Jurkat parental line contains 25% wild type p53 alleles as determined by an assay that reads out p53 transcriptional activity in yeast (Van 't Veer et al., NCI). The p53 status of the JA clones will be reported.

This inventory thusfar indicates that CD95-, TCR and etoposide-induced apoptosis pathways are partially distinct but can have common aspects. To identify gene products regulating these common aspects, the JA clones will be analysed by differential cDNA display as a preliminary for a complementation cloning procedure.

B8-109 APOPTOTIC PHENOTYPE INDUCED BY HYPEROSMOTIC CONDITIONS IN IMMATURE THYMOCYTES, Carl D. Bortner and John A. Cidlowski, Department of Physiology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599

A major physical event which occurs in all apoptotic cells is cell shrinkage and we wished to determine if this event was sufficient to initiate apoptosis. To investigate this possibility, S49 immature thymocytes which undergo apoptosis in response to a variety of agents, were cultured in hypertonic medium. We increased the normal osmolality (approximately 300 mOsm) of tissue culture medium to either 550 or 800 mOsm using either mannitol, sucrose or NaCl. These hyperosmotic conditions led to a profound killing of lymphocytes within 5 to 7 hours of incubation. DNA fragmentation, morphology, and flow cytometric analysis were performed on all cells to determine the mode of cell death. Hyperosmotic conditions led to a rapid induction of apoptosis in S49 cells which exhibited both morphological (cell shrinkage, nuclear condensation, and apoptotic body formation) and biochemical (internucleosomal DNA cleavage) characteristics of apoptosis. Interestingly, the response to hyperosmotic conditions was shown to be independent of protein synthesis as addition of 1 uM cyclohexamide to the hypertonic medium failed to prevent the apoptotic process. These data suggest that in S49 cells, the machinery of apoptosis is already present and can be activated by the physical shrinkage of these cells to induce the apoptotic process. This work is supported by DK 32078.

B8-110 CHARACTERIZATION OF NECROTIZING ARTERITIS IN MICE TRANSGENIC FOR BCL-2, Sarah K. Bronson, Elizabeth G. Plaehn, Kimberly D. Kluckman, Andrea M. Vaillancourt, John R. Hagaman, J. Charles Jennette, and Oliver Smithies, Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27599

Insertion of a single-copy, single position bcl-2 transgene into the HPRT locus by gene targeting in embryonic stem cells has led to the establishment of mouse lines expressing near physiological levels of murine bcl-2 under the direction of a chicken ß-actin promoter.

Mice express the transgene in a variety of tissues including, but not limited to, bone marrow, brain, spleen, thymus, heart and reproductive tissues. Light microscopy of adult tissues after about 6 months of age reveals marked lymphoid hyperplasia including extensive expansion of splenic white pulp germinal centers and renal periarterial lymphoid infiltrates. A sampling of animals has revealed severe necrotizing arteritis in approximately 20% of the transgenics. The arteritis was characterized by segmental fibrinoid necrosis with associated infiltration by neutrophils and mononuclear leukocytes. Lesions have been detected in the kidney, heart, and skeletal muscle. Preliminary serologic studies suggest the presence of circulating autoantibodies, including anti-neutrophil cytoplasmic autoantibodies, which are associated with necrotizing arteritis in humans. Analysis of these animals may further our understanding of arteritis and the role of bcl-2 in the induction of this disease.

B8-111 ANALYSIS OF THE HUMAN p55-TNF RECEPTOR CYTOPLASMIC DOMAIN USING RECOMBINANT FUSION PROTEINS. Alexander M. Castellino and Moses V. Chao, Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021.

Tumor necrosis factor (TNF) is a potent cytokine which is involved in cellular cytotoxicity. Its action is mediated by two receptors, p55 and p75. Binding of TNF to these transmembrane receptors elicits a series of biochemical responses which lead to cell death. Since the receptors have no apparent biochemical activity it is assumed that TNF signal transduction is transmitted to other cellular proteins through direct interactions with the receptor. From cell culture experiments and targeted mutations of the p55-TNF receptor, signaling by TNF is believed to be principally mediated by this receptor. To identify molecules which interact with the human p55-TNF receptor several GST-fusion proteins have been generated. These contain amino acids sequences 183 to 435, 212 to 435, 340 to 435, 183 to 341 and 212 to 341 from the transmembrane and cytoplasmic domain of the p55-receptor. These proteins have been made in large quantities in soluble form. Using immunoprecipitation of lysates prepared from metabolically labeled cells and screening of cDNA expression libraries, cellular proteins specifically interacting with these receptor domains and which may be involved in TNF signal transduction are being characterized.

B8-112 THE ROLE OF RETINOIC ACID IN CELL DIFFERENTIATION AND CELL DEATH OF EMBRYONIC STEM CELLS, Castro-Obregón, S. and Covarrubias, L. Instituto de Biotecnología, UNAM. Apdo. Postal 510-3, Cuernavaca, Morelos 62271, México.

The embryonic stem (ES) cells represent a powerful system to study the cellular events that occur during early development. We have developed an *in vitro* system which allows to observe either cell death or neuron differentiation in response to retinoic acid (*all-trans* RA). When the ES cells are grown as an aggregate, condition that induces the formation of embryoid bodies, cell death is observed in response to RA even in the presence of fetal calf serum (FCS). However, when aggregation was avoided, neurons appeared, as demonstrated by morphology and immunocytochemystry against neurofilaments, only when RA was present; this process was prevented by FCS. These results show that ES cells have the potential to undergo cell death as consequence of RA induced differentiation.

ES cells grown in the absence of FCS showed undifferentiated morphology but, interestingly, cell death was observed if β-mercaptoethanol (β-ME) was removed from the culture medium; internucleosomal DNA degradation, a characteristic of apoptosis, was observed under these conditions. βME also favored neuron differentiation survival since more differentiated cells and longer processes were observed than in its absence. Our results support that β-ME, a reductant agent, is required for both undifferentiated and differentiated ES cell survival, which is in accordance to several results indicating that the oxidative stress is involved in the mechanism of cell death. We proposed that ES cells maintain activated the cell death program and require exogenous signals to suppress it; these signals can be overcome by β-ME and neither feeder cells nor serum can block the cell death pathway.

This work was supported by CONACyT and DGAPA.

B8-113 VACCINIA VIRUS INFECTION INDUCES APOPTOSIS WHICH CAN BE PREVENTED BY BCL-2

Wen Chang, Che-Sheng Chung and Chi-Hong Bair, Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan, R.O.C

Vaccinia virus (VV) infection of Chinese hamster ovary (CHO) cells induces apoptosis. DNA laddering could be detected as early as 4 hours post infection. Cells treated with cytosine arabinoside (ara-C) still induce apoptosis after VV infection indicating viral DNA replication is not necessary for triggering apoptosis signal. However, VV inactivated by UV treatment fails to trigger apoptosis though virus adsorption to cells was not affected. These data suggest that some early events prior to DNA replication are necessary and sufficient to induce apoptosis in VV-infected cells. It could be either at viral early gene expression or at the uncoating step of viral cores. Experiments are in progress to understand the mechanism.

Recombinant VV vectors expressing bcl-2 gene in both sense and antisense orientations were constructed and used to infect CHO cells. Apoptosis was inhibited in cells infected by bcl-2-VV and not by wild type or antisence bcl-2-VV. Several cellular genes such as p53, NF-kB and c-myc have been reported to induce apoptosis and could be targeted by bel-2. Possible involvement of these cellular genes in VV-induced apoptosis is currently investigated.

B8-114 A POSSIBLE G-PROTEIN ACTIVATING DOMAIN IN CELL-DEATH MOTIFS OF LANR, FAS/APO-I AND TNFR-I, Barbara S. Chapman, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446

The neurotrophin receptor (LANR), lymphotoxin receptors (TNFR-I, TNFR-II and TNFRrp), the Fas/Apo-I antigen and the CD40 antigen have been implicated in inducing cell death by apoptosis. All possess extracellular domains composed of repeating units that characterize a growing family of cell surface glycoproteins. However, little or no homology has been detected in the intracellular domains of these molecules, except for an "apoptosis motif" near the C-termini of TNFR-I (55 kDa) and Fas/Apo-I. None of the receptors contains a kinase domain, ruling out substrate phosphorylation as a primary consequence of interaction with their ligands. This study uses molecular modeling to investigate the possibility that one group of these cell-death receptors signals through heterotrimeric G-proteins. Sequence alignment reveals 32 residues in LANR homologous to the center of the "apoptosis motif" of TNFR-I and Fas/Apo-I. Analysis of secondary structure shows each of the three homologous domains has a strong propensity to form alpha helical structure. In helical wheel representations all three peptides potentially form amphipathic helices at their carboxyl termini. The amphipathic helix in LANR was previously shown to be homologous to the G-protein-activating peptide mastoparan (36% identity, 13/14 conservative substitutions). Molecular design software (SYBYL, Tripos Associates) was used to build models of the three receptor apoptosis domains and a model of mastoparan. The three death-receptor models are composed of two subdomains, one containing the conserved Glu and Trp residues known from mutagenesis studies to be critical for apoptotic function. The second subdomain, an amphipathic helix, resembles the mastoparan structure. The receptor models are strikingly alike, especially on the hydrophobic face, and more similar to each other than to mastoparan. Since non-polar surfaces of peptides from the hepta-spanning receptors are thought to regulate selectivity among G alpha subunits, the nearly identical appearance of these death-receptor peptides would be consistent with associations to a common G-protein. Molecular modeling reveals strong structural homologies within apoptosis motifs of three cell death-mediating receptors, where amino acid sequence homologies are weak. Biochemical studies could be guided by the structural details in these models to explore a G-protein-coupled signaling mechanism for members of the LANR/TNFR family of cell death receptors.

B8-115 STIMULATION OF NEUTROPHIL APOPTOSIS BY TNFα: DIFFERENTIAL EFFECT COMPARED TO OTHER PRIMING AGENTS, Edwin R. Chilvers, Joanna Murray, Alison M. Condliffe and Chris Haslett, Respiratory Medicine Unit, University of Edinburgh, EH8 9AG, UK.

Neutrophil (PMN) apoptosis has been proposed as a potential mechanism underlying the removal of PMNs from an inflammatory focus. The ability of a wide variety of pro-inflammatory peptides (eg. GM-CSF, LPS and C5a) to impair the rate of PMN apoptosis *in vitro* suggests that such agents may act not only in a priming capacity, to enhance secretagogue-mediated superoxide (O_2^-) generation and the release of histotoxic enzymes, but also serve to increase the longevity of these cells by delaying apoptosis. In an extension of this work, we have sought to examine whether this hypothesis holds true for all priming agents, in particular TNFα which has been reported to induce apoptosis in a number of immortal cell lines. The ability of TNFα to prime human peripheral blood PMNs was confirmed by demonstrating dramatically enhanced fMLP-induced O_2^- formation in cells pre-exposed for 30 min to 0.05-50ng/ml rh-TNFα (mean ± SEM O_2^- in nmoles/10^6 cells/10 min: control 0.6 ± 0.1, 0.5ng/ml TNFα 0.9 ± 0.1, 100nM fMLP 3.9 ± 0.3, TNFα + fMLP 18.4 ± 2.3). Although incubation of PMNs with TNFα for 20 hr appeared to inhibit apoptosis, more detailed time-course studies demonstrated its ability to stimulate apoptosis at earlier times.

	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	20 hr
control	2.5 (0.6)	2.8 (0.8)	1.4 (0.2)	1.6 (0.1)	2.0 (0.3)	2.0 (0.1)	50.4 (4.6)
+TNFα (25ng/ml)	2.7 (0.7)	12.0 (0.5)	16.0 (0.6)	17.1 (0.2)	22.6 (2.3)	24.4 (6.6)	40.9 (4.3)

(Values represent % apoptosis (SEM), n=6, 2 separate experiments)

No such effect was observed with priming concentrations of fMLP, PAF, $InsP_6$, LPS, LTB_4 or GM-CSF. TNFα did not affect cell recovery or viability and there was no decrease in TNFα concentration (ELISA assay) between 0 and 20 hr. Furthermore, the effect of TNFα at 6 hr was completely abrogated by pre-incubation with mouse anti-human TNFα neutralising antibody and had an EC_{50} (2.8 ng/ml) very similar to that observed for enhanced O_2^- formation. These data suggest that TNFα has a distinctive (bimodal) effect on PMN apoptosis not apparent with other priming agents. This is the first biological agent identified as being able to stimulate early PMN apoptosis. Such an effect *in vivo* may promote PMN clearance from an inflamed site.

This work was supported by The Wellcome Trust and MRC(UK).

B8-116 **MECHANISMS AND REGULATION OF FAS AND TUMOR NECROSIS FACTOR RECEPTOR-MEDIATED CELL DEATH** M. V. Clement and I. Stamenkovic Departement of Pathology, Massachussetts General Hospital and Harvard Medical School and Pathology Research, Charlestown Navy Yard, Boston Massachussetts 02129

Fas and tumor necrosis factor receptors (TNFR), p55 and p75, are currently known as the major cell surface transducers of cytotoxic signals. However, it is still unclear wether they trigger the death signal along the same or distinct pathways.To study the mechanism that regulates Fas and TNFR-mediated cell death, we have developed fusion proteins composed of the extracellular domain of the B cell-associated antigen CD40 and the transmembrane and intracellular domains of Fas, TNFRp55, and TNFRp75, or the extracellular domain of the TNFRp55 and the transmembrane and intracellular domains of Fas. All fusion protein were stably expressed in two human melanoma cell lines (MC and M14) that are deficient in Fas and TNFRs.Transfectants were tested for responses to stimulation by a soluble form of the CD40 ligand, CD8-gp39 or recombinant TNFα. Our results show that all fusion proteins induce cell death upon engagement by specific ligand. However, the cytotoxic signal kinetics and sensitivity to cyclohexamide were dictated by the intracellular domain, raising the possibility that Fas, TNFRp55, and TNFRp75 might operate via different pathways. While maximal Fas-dependent cell death occurred within hours, maximal TNFr-associated cytotoxicity was observed 2-3 days after engagement by CD8-gp39. In presence of cyclohexamide, suceptibility to cell death of MC40-Fas, MC40-TNFp55 and M14TNF-Fas following triggering by soluble gp39 or recombinant TNFα was increased, resulting in maximal cell death within 6 hours for Fas and 24 hours for TNFrp55 while kinetics of cell death triggered by TNFRp75 were not altered. To address the possible correlation between the level of fusion protein expression and the rapidity of cell death following engagement by ligand, eight independent MC40-Fas and seven M14TNF-Fas transfectants clones were tested for their response to respectively CD8-gp39 or recombinant TNFα. Remarkably, resistant MC and M14 transfectant clones were the ones expressing a high level of the fusion protein whereas intermediate level expressors proved to be the most sensitive to fas-mediated cytotoxicity. Moreover, we found that double CD40-Fas and TNFR-Fas fusion protein transfectants were resistant to stimulation by both TNF and CD8-gp39, while being sensitive to triggering by either ligand alone. These results raise the possibility of the existence of an ideal phenotype which may constitute a novel regulatory mechanism of Fas and TNFR function. Finally, we have observed that Fas and TNFR-mediated cell death is differentially regulated by oxygen free radicals.

B8-117 EMBRYONIC STEM CELL SPECIFIC FEATURES OF THE CELLULAR RESPONSE TO DNA-DAMAGING AGENTS. Sula W. Corbet, Sarah Gledhill, Alan R. Clarke, Colin C. Bird and Andrew H. Wyllie, CRC Laboratories, Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh, Scotland.

We have used a variety of wild type embryonic stem (ES) cell lines and ES cell lines rendered homozygous for a targeted inactivation of the p53 gene by high G418 selection to investigate the induction of apoptosis in response to the DNA-damaging agents γ-radiation, UV light and the topoisomerase II inhibitor, etoposide. In wild type cell lines, we observed a strong and rapid induction of apoptosis following exposure to UV and etoposide but γ -radiation induced a reduced and much delayed response. This unexpected result led us to investigate the mutation rate at the HPRT locus in the surviving cells. Preliminary results show no increase in this rate as compared to untreated controls. Considering the high level of radiation (up to 10 Gray) to which these cells were subjected, this suggests an extremely efficient repair mechanism, at least in response to certain types of DNA damage. This appears to be specific to ES cells. That embryonic stem cells are inherently more genetically stable than many other cell types is indicated by results we have obtained which show that both p53 deficient and p53 wild-type ES cell lines maintain a relatively normal karyotype at high passage. Analysis of p53 deficient stem cells revealed a dose-dependent induction of apoptosis following exposure to UV light. The p53 dependency of the response to etoposide was less well defined, consistent with results previously obtained from thymocytes.

B8-118 ROLE OF CELL CYCLE AND PKC ISOENZYME ACTIVATION IN PHORBOL ESTER INDUCED APOPTOSIS IN PROMYELOID U937 CELLS, Elizabeth M. Deacon, Judit Pongracz, Dianne McHattie, Janet M. Lord, Department of Immunology, University of Birmingham, Birmingham, B15 2TT, U.K.

We have investigated the role of PKC isoforms in the regulation of both myeloid cell differentiation and apoptosis using the human leukaemic promyeloid cell line U937. Treatment of U937 cells with a number of phorbol esters (DOPPA, DOPP and TPA) showed that DOPP and TPA effectively induced differentiation and apoptosis at the concentrations used (5-50nM), whereas DOPPA induced only apoptosis. DOPP and TPA activated and downregulated several PKC isoenzymes (α, β and δ) whereas DOPPA predominantly activated PKC-β. We conclude that the dominant activation of PKC-β can activate the apoptotic program but is insufficient for differentiation. In addition, only a proportion of the U937 cells (20-25%) were induced to undergo apoptosis at any one time, hence the role of cell cycle in this phenomenon was also investigated. Following the method of Gorczyca *et al* (1993), U937 cells were treated with DOPPA (5-100nM) and harvested at 1, 4, 24, 48 and 72hrs. The cells were fixed, permeabilised and *in situ* end labelled using TdT and Digoxigenin-11-dUTP. Labelling was visualised using an anti-digoxigenin-FITC labelled antibody after which propidium iodide (PI) was added. PI and fluorescein fluorescence was analysed on a FACScan to relate apoptosis with cell cycle. Preliminary results indicate that DOPPA treatment induces apoptosis preferentially in the G1 phase of cell cycle. Furthermore, analysis of PKC isoenzyme expression in U937 cells at different stages of the cell cycle revealed that expression of PKC-β was highest in G1 cells. We conclude that DOPPA is able to induce apoptosis in promyeloid cells at the G1 phase of cell cycle and that this effect is mediated via the activation of PKC-β. This phorbol ester may therefore be of therapeutic benefit, particularly in the treatment of myeloid leukaemia.

B8-119 IDENTIFICATION OF NOVEL GENES INDUCED DURING NEURONAL CELL DEATH, Mohanish Deshmukh, Robert S. Freeman, Eugene M. Johnson, Jr., Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110

Extensive programmed cell death (PCD) occurs during development of the nervous system. Our goal is to understand the molecular mechanism of neuronal PCD. We study the death of rat sympathetic neurons upon deprivation of their trophic factor, nerve growth factor (NGF). NGF-depravation-induced death of rat superior cervical ganglion occurs largely over a period of 24-48 hours after NGF removal and has many of the hallmarks of apoptosis. In addition, inhibitors of RNA and protein synthesis can completely suppress this neuronal death, indicating a requirement for gene expression.

Recently, our laboratory has provided evidence that specific genes are induced during neuronal PCD. These include cyclin D1, *c-fos*, *fos B*, *c-jun*, *jun B*, *c-myb* and *NGF-IA*. To provide additional support for the hypothesis that PCD involves the expression of specific genes as part of a "death program", we have employed the PCR-differential display technique to identify novel genes that are induced in dying neurons. We compared mRNAs prepared from neurons deprived of NGF for 10-20 hours with those prepared from control, non-deprived neurons. In our initial screen, we have identified one induced message, termed DD1-x, whose expression increases 15- to 20-fold in dying neurons. Its expression, which peaks between 10 and 12 hours after NGF withdrawal, precedes the commitment point of death in these neurons by 5-7 hours and thus is a good candidate "death program" gene. The sequence of DD1-x is identical to sequences from a recently cloned PDGF-inducible gene termed SM-20 (Wax, S. D. *et al.* J. Biol. Chem. 269: 13041-13047, 1994).

We are currently screening more combinations of the forward and reverse primers in the PCR-differential display scheme to identify additional genes that are induced in dying neurons. Candidate cell death genes identified by this approach will be tested for their potential role in PCD.

This research is supported by the Washington University Alzheimer's Disease Research Center.

B8-120 PHOSPHORYLATION EVENTS DURING cAMP AND CALCIUM MEDIATED APOPTOSIS IN LYMPHOCYTES, Diane R. Dowd, Kristine Yates-Siilata, Susan A. Gurwitch, and Merideth Kamradt; E.A. Doisy Department of Biochemistry and Molecular Biology; St. Louis University Health Sciences Center; St. Louis, MO 63104

Apoptosis during hematopoietic development is an effective means to regulate the number of blood cells in the organism. The WEHI7.2 murine lymphoma cell line is one model system to study apoptosis in lymphocytes because it is programmed to die when exposed to increases in intracellular Ca^{2+} or cAMP. These agents activate two different signaling pathways, however, these pathways may share steps involving common phosphorylation events. When WEHI7.2 cells were treated with forskolin (an activator of adenylyl cyclase), we observed transcriptional activity from a cAMP-response element (CRE) linked to a heterologous reporter construct. Western blot analysis with an antibody specific for the phosphorylated isoform of the cAMP response element binding protein, CREB, demonstrated that forskolin treatment enhanced the level of phospho-CREB as expected. Presumably, this effect occurs via the cAMP/PKA-mediated activation of CREB. CRE-dependent transcription and CREB phosphorylation also was detected when the WEHI7.2 cells were treated with the calcium ionophore A23187. Moreover, A23187 induced transcription from a CRE in a concentration-dependent manner which correlated with the level of apoptotic cell death caused by this agent. We propose that a shared step in the apoptotic pathways initiated by calcium and cAMP is the phosphorylation of CREB, and this transcriptional activation may be necessary for apoptosis to occur.

B8-121 APOPTOSIS-ASSOCIATED ALTERATION OF NEUTROPHIL FUNCTION/PHENOTYPE Ian Dransfield and Christopher Haslett, Dept. of Medicine, University of Edinburgh, Edinburgh. U.K.
During inflammatory responses, removal of neutrophils and their injurious contents from inflamed sites represents an important factor determining whether inflammation resolves or progresses to a chronic inflammatory state. Neutrophils undergo apoptosis leading to recognition and phagocytosis by macrophages via a novel mechanism that does not trigger release of macrophage proinflammatory mediators. In order to further understand the functional consequences of neutrophil apoptosis we have examined apoptosis-induced alterations in expression and function of key neutrophil surface molecules and have recently reported that reduction in surface expression (>90%) of the FcγRIII (CD16) is temporally associated with apoptosis (Dransfield et al. (1994) J. Immunol. 153:1254). Similar marked changes in expression of a number of other surface molecules was not observed suggesting that in addition to morphological and nuclear changes, there may be specific alterations in expression of surface receptors. Apoptotic neutrophils show reduced capacity for adhesion via integrins (CD11b/CD18) to fibrinogen and to E-selectin. Examination of expression of neutrophil adhesive molecules revealed reduced expression of L-selectin/selectin ligand (sialyl $Lewis^x$), whereas increased levels of CD11b/CD18 and CD11c/CD18 integrins was found. Thus, selectin-mediated adhesion may be regulated by control of surface expression of receptors/ligand, possibly as a result of altered protease/sialidase activity. In contrast, modulation of integrin-mediated adhesion may involve functional uncoupling of receptors as revealed by dramatic reduction in expression of the integrin activation epitope recognized by mAb 24 on the surface of the apoptotic cell. The reduced adhesive potential of the apoptotic neutrophil may serve to limit release of its histotoxic contents and reduce inappropriate tissue injury.

B8-122 T CELL-MEDIATED, FAS-DEPENDENT APOPTOSIS AND CANCER, Richard C. Duke, David Poticha, and Paul B. Nash. Division of Medical Oncology, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262

Fas is a member of the tumor necrosis factor (TNF) receptor superfamily which includes the TNF receptors, the low affinity nerve growth factor receptor, and the CD27, CD30, CD40, and OX40 molecules. These cell surface glycoproteins act as signaling molecules and are involved in cell activation, growth and survival. Fas is expressed on activated T and B cells, many leukemia and lymphoma cells, and several types of cancers. Cross linking of Fas on certain tumor cells with IgM anti-Fas antibody, or via a ligand for Fas (Fas-L) which is present on cytotoxic T lymphocytes (CTL), induces apoptotic cell death.

Mice which genetically lack expression of Fas (encoded by the *lpr* gene) or Fas-L (encoded by the *gld* gene) are unable to eliminate activated lymphocytes and develop a progressive lymphoproliferative disease associated with autoimmunity and immunodeficiency. The similarities between the *lpr/gld* disease and human lymphoproliferative disorders suggest a common mechanism.

We have found that murine CTL express Fas-L when they are triggered by interaction with cells bearing their specific antigen. In order to be killed, the target cells must express functional Fas, *and* either syngeneic MHC or allogeneic MHC (if the CTL are alloreactive). In the context of human leukemia/lymphoma, we have found that human CTL can kill target cells bearing human Fas. We have examined a number of freshly isolated human leukemia cells for expression of membrane and soluble Fas using flow cytometry and cytotoxicity assays. We have also generated and characterized CTL from normal individuals and leukemia patients. The results of our studies will be discussed as they relate to human cancers.

Supported by the USPHS-NIH (AI29953 and AI07405), the Pauline A. Morrison Charitable Trust, the Cancer League of Colorado, and the Milheim Foundation for Cancer Research. RCD is a Lymphoma Research Foundation of America Fellow.

B8-123 CD40 STIMULATION AUGMENTS APOPTOSIS IN CARCINOMA CELL LINES, Aristides G. Eliopoulos, Chris W. Dawson, Michael J. O. Wakelam and Lawrence S. Young, CRC Institute for Cancer Studies, University of Birmingham Medical School, Birmingham B15 2TH, UK.
CD40 is a transmembrane protein with homology to a family of cell-surface glycoproteins that includes the receptors for nerve growth factor, tumour necrosis factor (TNF), FAS/APO-1 and the T-cell antigen OX40. CD40 is predominantly expressed on follicular dendritic cells, mature B cells and Burkitt lymphoma cell lines where antibody to CD40 or CD40 ligand (CD40L) has been shown to protect against apoptosis. CD40 is also expressed in nonhemopoietic tumour cell lines such as the EJ bladder carcinoma, the A2780 ovarian and Caski cervical carcinoma cells. In these cases we have found that cross-linking of CD40 by the G28.5 mAb confers a significant suppression of cell growth which correlates with the levels of CD40 expressed on the cell membrane and this effect can be augmented by pre-treatment with IFNγ. We obtained similar results when Rat-1 fibroblasts, which do not express CD40, were transfected with a CD40 cDNA plasmid. Furthermore, we have found that G28.5 enhances the apoptosis-inducing effects of TNFα and various chemotherapeutic agents such as *cis*-platin and adriamycin and partially reverses the resistance of certain carcinoma cell lines to these drugs.

B8-124 MECHANISMS OF SUPPRESSION OF APOPTOSIS IN HETEROKARYONS. Rosemary B. Evans-Storms and John A. Cidlowski, Department of Physiology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, N.C. 27599. Apoptosis (programmed cell death) is responsible for selective elimination of cells and has been implicated in the development of cancer. This form of cell death is responsible for chemotherapy and radiation-induced regression of tumors. Many tumors become resistant to such treatment, and it is of paramount importance to determine how such resistance arises. The machinery required for apoptosis appears to be constitutively present in a repressed form in murine thymocytes and cell lines derived from this tissue, and may be present in such a form in other cell types. Activation of apoptosis may therefore represent a default pathway which may be followed by cells in the absence of proliferative signals. Several genes which encode suppressors of apoptosis have been identified, and it is reasonable to believe that others exist. We have chosen to use cell fusion technology to study whether suppressors of apoptosis are able to diffuse and whether their activity is dominant. The fusion partners used are murine BW5147 thymoma cells (which undergo apoptosis after treatment with glucocorticoids) and murine HTC hepatoma cells (which are resistant to induction of apoptosis by glucocorticoids). After formation of heterokaryons from these cell types using standard polyethylene glycol techniques and growth on plastic multiwell slides, we examine the response of these cell types to various inducers of apoptosis in the presence of one another. Apoptotic nuclei are measured by the formation of membrane-bound vesicles containing DNA. These data indicate that the presence of HTC cells in heterokaryons can inhibit induction of apoptosis in BW5147 cells. These results are in contrast to those generated by others using murine thymoctes fused with fibroblasts or gliosarcoma cells which suggest that the presence of one cell type in a heterokaryon has no effect on the ability of another cell present in the same heterokaryon to undergo apoptosis. Our data imply that a diffusable factor present in HTC cells is able to suppress apoptosis in murine thymoma cells, and that this is a dominant function. This work is supported by NIH DK32078, CA09156, and CA65049.

B8-125 A LATE-ACTING PROTEIN KINASE IS INVOLVED IN SIGNALLING FOR GLUCOCORTICOID- AND EPIPODOPHYLLOTOXIN-INDUCED APOPTOSIS. Kerin L. Fresa, Ingo F. Georgoff, Stanley Cohen and Xiaodan Ye, Department of Pathology and Laboratory Medicine, Hahnemann University, Philadelphia, PA 19102

We have previously shown that apoptosis induced in mouse thymocytes by dexamethasone or teniposide (VM-26) could be inhibited by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) and sangivamycin, but not by N-(2-guanidinoethyl)-5-isoquinoline-sulfonamide, suggesting a central role for protein kinase C in this process. However, apoptosis was not blocked by EGTA and no increase in cytosolic Ca^{++} was observed during apoptosis induced by either agent, suggesting that the kinase was Ca^{++}-independent.

In the present study, we demonstrate that the kinase involved in this process acts relatively late in the apoptotic pathway. Addition of 10μM sangivamycin to thymocyte cultures up to two hours after addition of either inducer resulted in virtually complete inhibition of apoptosis. Addition of sangivamycin at 3 or 4 hours after addition of inducer resulted in partial inhibition of apoptosis.

We also demonstrate here that induction of apoptosis in mouse thymocytes by dexamethasone or VM-26 is associated with *de novo* phosphorylation events. Cells were loaded with ^{32}P-orthophosphate and whole cell lysates were prepared at various times after addition of inducer. Phosphoproteins were separated by two dimensional polyacrylamide gel electrophoresis and autoradiograms analysed by computerized image analysis. Induction of apoptosis by VM-26 was associated with increased phosphorylation of two intracellular protein species (Protein 1: m.w.=22.9kDa, pI=5.11; and Protein 2: m.w.=22.9kDa, pI=4.98), when compared to untreated cells. Phosphorylation of these protein species was maximal at one hour after addition of VM-26, and was somewhat reduced two hours after addition of VM-26. Similar results were seen in cells treated with dexamethasone. These results suggest that the late activation of a calcium-independent, PKC-like enzyme and phosphorylation of a limited number of intracellular protein substrates may represent common events in apoptosis of mouse thymocytes induced by a variety of agents.

B8-126 THE ROLE OF HSP90 IN THE REGULATION OF APOPTOSIS IN MYELOMONOCYTIC CELLS. Joanna Galea-Lauri, Andrew J. Richardson, David S. Latchman, and David R. Katz. Departments of Immunology and Molecular Pathology, UCL, 46 Cleveland Street, London, W1P 6DB, UK.

The morphological and biochemical features of apoptosis are well characterised, including cell shrinkage, plasma membrane blebbing, chromatin fragmentation, DNA cleavage and formation of apoptotic bodies. Several gene products play a role in regulating this process, including tumour suppressor genes (e.g. rb-1), oncogenes (e.g. c-fos, c-jun, c-myc and bcl-2), and cysteine proteases (e.g. IL-1β converting enzyme). Most of these studies are on T and B cells. In contrast, very little is known about the control of apoptosis in monocytes and macrophages. In this study, therefore, we have investigated the mechanism of apoptosis in this lineage, using the monoblastoid cell line U937 as a starting point. In these cells apoptosis can be induced by serum deprivation, UV-b irradiation and treatment with cycloheximide (Cx, >10ug/ml), and by a combination of tumour necrosis factor α (TNFα) and Cx (1.0ug/ml) at doses which when used singly are ineffective. We used U937-derived transfectants which either hyper- or hypo-express the heat shock protein hsp90 in the same assays. When treated with TNFα + Cx (low dose), cells which over-express hsp90 have a higher frequency of apoptosis than control cells. In contrast, cells which under-express hsp90 show the opposite effect: there is less apoptosis. Similar findings were observed using high doses of Cx (75ug/ml). With UV-b, however, there was no difference between the transfectants and the parental cells. These findings suggest that hsp90 does not protect against apoptosis, as might be expected from the conventional view of hsp function in general. Rather, excess hsp90 may bind to other proteins inside the cell which have anti-apoptotic protective function.

B8-127 VASCULAR SMOOTH MUSCLE CELL PROGRAMMED CELL DEATH: INTERACTION OF NITRIC OXIDE AND ANGIOTENSIN II, Gary H. Gibbons and Matthew J. Pollman, Falk Cardiovascular Research Center, Stanford University, Stanford, CA 94305

Alterations in vascular wall structure are thought to be mediated by the interplay of autocrine-paracrine factors which balance growth stimulation and inhibition of the constituent vascular smooth muscle cell (VSMC). Locally generated vasoactive substances appear to modulate vascular cell growth as well as vessel tone. However, little is known about how these substances may influence programmed cell death (PCD) or apoptosis in the VSMC. Nitric oxide (NO), a vasodilator, has been shown to be a growth-inhibitor of VSMC by a cGMP-mediated mechanism. In contrast, the vasoconstrictor angiotensin II (Ang II), exerts a growth stimulatory effect on VSMC. Therefore, we tested the hypothesis that the control of PCD in VSMC is regulated by the balance of the antagonistic autocrine factors NO and Ang II. In our initial experiments, we established that rabbit aortic smooth muscle cells grown to a near-confluent state in 10% serum exhibit little evidence of PCD. However, incubation in serum-free media induced PCD as evidenced by the characteristic internucleosomal cleavage of DNA assayed by end-labeling and gel electrophoresis. The induction of PCD during serum withdrawal was further verified and quantitated by analysis of cellular chromatin condensation using a fluorescent DNA-binding dye (Hoechst 33342). The addition of Ang II (10-1000 nM) to the serum-free media markedly inhibited the activation of PCD in a dose-responsive fashion. In contrast to the anti-apoptotic effect of Ang II, NO induced PCD in a dose-dependent fashion which could be mimicked by the administration of cGMP analogues. The addition of Ang II directly antagonized NO-induced apoptotic death. These findings suggest that VSMC number is determined not only by the balance between cell growth and inhibition but additionally by PCD that is regulated by the interaction between Ang II and NO. The regulation of PCD may be an important determinant of vascular lesion structure in atherogenesis or hypertension.

B8-128 INCREASED T CELL PROGRAMMED CELL DEATH (PCD) MAY PREDISPOSE TO INSULIN-DEPENDENT DIABETES MELLITUS (IDDM), Bruce M. Gill and Terry L. Delovitch, Autoimmunity Group, John P. Robarts Research Institute, London, Ontario, Canada N6G 2V4

Thymic and peripheral CD4+ T cell proliferative unresponsiveness induced by TCR crosslinking is detectable both at insulitis and IDDM onset in nonobese diabetic (NOD) mice, and is mediated by a decrease in IL-2 and IL-4 production. Exogenous IL-4, but not IL-2, completely restores NOD T cell responsiveness. Our findings suggest that, in NOD mice, Th2 cells may need a higher threshold of activation and may be more sensitive to PCD than Th1 cells. This possibility was tested initially by analyzing the relative sensitivity of NOD T cells to activation-induced PCD (AIPCD). We found that NOD thymic and peripheral T cells have a greater propensity to TCR-dependent AIPCD than do corresponding T cells from some IDDM-resistant strains examined. NOD and BALB/c thymocytes were stimulated with an anti-CD3 plus IL-2 for 48 hr and then with IL-2 or IL-4 for a further 72 hr before treatment with either dexamethasone (Dex) or Ceramide (Cer). Low spontaneous PCD was noted in both NOD and BALB/c thymic T cell blasts, and IL-4 elicited slightly less T cell AIPCD than IL-2. NOD T cell blasts were more sensitive to Dex-induced PCD than BALB/c T cell blasts. Dex-induced about 2- to 3-fold more PCD in IL-2 treated NOD and BALB/c T blasts than IL-4 treated NOD and BALB/c blasts. Thus, IL-4, in comparison to IL-2, protects T cells from Dex-induced PCD. Similarly, NOD splenic T cells were found to be more susceptible to spontaneous and AIPCD than BALB/c splenic T cells. Both IL-4 and IL-2 reduced Cer-activated PCD in NOD splenic T cells, with IL-4 being considerably more effective than IL-2. These data support the notion that IL-4 specifically rescues peripheral Th2 cells from TCR-dependent AIPCD, and that IL-2 is ineffective in these cells. Thus, IL-4 provides a stronger survival signal for NOD peripheral T cells than does IL-2, consistent with our hypothesis that IL-4 potentiation of Th2 cell proliferation and function may be protective from IDDM. Whereas AIPCD of autoreactive effector Th1 cells may generate and maintain self tolerance, our data raise the novel idea that deficient activation and increased AIPCD of regulatory Th2 cells that protect from IDDM in NOD mice may elicit the breakdown of tolerance and be causal to IDDM onset in NOD mice. (Supported by JDFI and MRC of Canada)

B8-129 ANTIGEN PRESENTATION BY ASTROCYTES PRIMES RAT T-LYMPHOCYTES FOR APOPTOTIC CELL DEATH: A MODEL FOR T-CELL APOPTOSIS IN VIVO, R. Gold [1], M. Schmied [2], H.P. Hartung [1], H. Wekerle [3], K.V. Toyka [1], H. Lassmann [2]; [1] Department of Neurology, Clinical Research Unit for Multiple Sclerosis, Julius-Maximilians-Universität Würzburg, [2] Research Unit for Experimental Neuropathology, Austrian Academy of Sciences, Vienna; [3] Department of Neuroimmunology, Max Planck Institute for Psychiatry, Martinsried

Apoptosis is a major control mechanism for inflammatory T-lymphocytes in experimental autoimmune encephalomyelitis (EAE). Cellular or humoral factors mediating T-cell apoptosis in the nervous system have not yet been identified. Data from other autoimmune disorders suggest a role of tissue-specific factors. In this study we have characterized apoptotic cell death resulting from the interaction between autoimmune T-cells and astrocytes and its modulation by steroid hormones. We compared time kinetics of T-cell activation by γ-IFN treated astrocytes from neonatal Lewis rats and professional antigen presenting cells (APC) from thymus or spleen. Apoptosis was asssesed by nuclear morphology and confirmed by molecular labeling techniques and agarose gel electrophoresis. With both types of APC upregulation of IL 2- and transferrin-receptor expression was observed as assayed by FACS analysis. ^{3}H-thymidine incorporation of MBP- and P2-specific T-cell lines declined after 48 hours with astrocytes only. A similar suppressive effect was observed when T-cells were cocultured with thymic APC and astrocytes, but it disappeared when astrocytes were separated by a transwell system. On day three after astrocyte activation 10 - 30 % T-cells disclosed morphological signs of apoptosis. Glucocorticosteroids and estrogen specifically enhanced T-cell apoptosis (60-90%) when added on day 3 in the astrocyte system, but not at earlier time points of T-cell activation or when professional APC were used. Pregnenolon, Lipocortin-1, and TGF-γ had no consistent effect. Local antigen presentation may render T-cells susceptible for elimination by apoptosis. Some corticosteroids can enhance this process.

B8-130 **Consequences Of Bcl-2 Overexpression In Olfactory Receptor Neurons Of Transgenic Mice** Michael D. Hayward*, Christopher Bocchiaro, James I. Morgan, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

The active elimination of cells, termed programmed cell death, is a process that often requires ongoing protein and RNA synthesis; suggesting the involvement of particular gene products. Indeed several studies have implicated specific proteins in the cell suicide program. For example, the product of the proto-oncogene, *bcl-2*, blocks programmed death in cells of the lymphoid and other lineages. We wished to determine whether *bcl-2* could prevent cell death in neurons *in vivo*. To this end we have constructed a line of transgenic mice in which *bcl-2* overexpression has been specifically targeted to olfactory receptor neurons using the Olfactory Marker Protein (OMP) promoter. Our choice of this strategy lies in the fact that mature olfactory neurons degenerate and are replaced by the proliferation and differentiation of precursor neuroblasts with a half-life of approximately thirty days. In addition, these neurons can be induced to undergo premature death either by depriving them of their target, the olfactory bulb, or by exposing them to a number of chemicals and viruses. Therefore, we wished to determine whether *bcl-2* expression could alter these properties of olfactory receptor neurons. In parallel, we have been examining the olfactory neuroepithelium of transgenic mice harboring Fos-lacZ and Jun-lacZ transgenes following unilateral removal of the olfactory bulb. Previous studies have indicated that expression of Fos and Jun may be associated with neuronal death and regeneration.

B8-131 GLUCOCORTICOID - INDUCED APOPTOSIS OF HUMAN LEUKEMIC CELLS IS CAUSED BY THE REPRESSIVE FUNCTION OF THE GLUCOCORTICOID RECEPTOR, Arno Helmberg, Nathalie Auphan, Carme Caelles and Michael Karin, Department of Pharmacology, Program in Biomedical Studies, Center for Molecular Genetics, School of Medicine, University of California, San Diego, La Jolla, CA 92093-0636

Glucocorticoid-induced apoptosis, a phenomenon that may account for the therapeutic effects of glucocorticoids in various diseases including leukemia, depends on the presence of glucocorticoid receptor, but the events leading from the activated receptor to cell lysis are not understood. Here, we establish a cell system designed to study this aspect of glucocorticoid-induced apoptosis. We show that an activation-deficient glucocorticoid receptor mutant is as effective as the wild-type receptor in inhibition of interleukin-2 production, inhibition of c-myc expression, repression of AP-1 activity and induction of apoptosis. Furthermore, we show that retinoic acid can also induce apoptosis in these cells through the retinoic acid receptor, whose repressive functions but not target site specificity are similar to those of the glucocorticoid receptor. Therefore, the primary effect of the glucocorticoid receptor in glucocorticoid-mediated apoptosis correlates with transcriptional repression rather than activation and could be mediated by interference with the physiological regulation of other transcription factors required for cell survival.

B8-132 CD40L-CD40 INTERACTION INDUCES RESISTANCE TO p55TNF RECEPTOR MEDIATED APOPTOSIS, Sigrun Hess, Hans Smola, Anne Rensing-Ehl, Gert Riethmüller and Hartmut Engelmann, Institute of Immunology, University of Munich, Goethestr. 31, 80336 Munich, F.R.G.

The role of CD40 in transformed cell lines and carcinomas was investigated. CD40 and the p55 tumor necrosis factor receptor (p55TNFR), both belonging to the same family of proteins, do not only share similarties in their extracellular but also cytoplamic domains. Therefore we examined whether the structural conservations would also imply similarities in their functions.

In contrast to TNFR stimulation, CD40 had no cytotoxic effect in any of the tested cell lines. However, CD40 prestimulation with CD40L or anti CD40 antibodies induced almost complete resistance to TNF mediated apoptosis in a CD40 transfected human fibroblast cell line. Maximal resistance was achieved after CD40 stimulation with 2 μg/ml of polyclonal anti CD40 antibodies. A comparable resistance effect after prestimulation of the p55TNFR had been described before (D. Wallach, 1984), indicating that the structurally related receptors CD40 and p55TNFR may also share common functions. Our findings suggest a common resistance pathway for both receptors as resistance was induced with very similar kinetics. This is the first evidence for an antiapoptotic function of CD40 in a cell type other than B-lymphocytes.

The resistance signalling pathway via the CD40L-CD40 axis is under current investigation.

B8-133 LOSS OF SURFACE PROTEINS INCLUDING COMPLEMENT REGULATORY MOLECULES FROM APOPTOTIC NEUTROPHILS. Jane Jones & B.Paul Morgan, Dept. Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff, UK.

Human neutrophils express a number of proteins which protect them from lysis by homologous complement. The purpose of this study was to examine the expression of four of these molecules on neutrophils aged in culture:-CD59 antigen, CD55 (decay accelerating factor; DAF) which are both anchored to the membrane via glycosyl phosphatidylinositol(GPI), and the two transmembrane proteins CD35 (CR1) and CD46 (membrane cofactor protein). Overnight incubation of normal neutrophils in culture medium containing 10% autologous serum induced morphologically distinct apoptotic changes in 30-50% of the cells. Neutrophils removed from culture at 20 hours and stained with antibodies to CD35 and CD46 showed one fluorescent peak, while cells stained with CD55 or CD59 showed two. The cells in each peak were sorted and those expressing higher levels of CD55 or CD59 were morphologically normal (99%) whilst those expressing lower levels were predominantly apoptotic (>90%). This observation was also extended to cells labelled for CD16. This phenomenon was not restricted to GPI-anchored molecules as aged neutrophils stained for the transmembrane proteins CD11a, CD11b and CD18 could also be sorted into bright non-apoptotic and dim apoptotic populations. The relevance of these surface changes on apoptotic neutrophils, with particular reference to complement susceptibility, is under investigation.

B8-134 PHENOBARBITAL MODULATES THE TYPE OF DEATH OF RAT HEPATOCYTES IN LOW-SERUM MEDIUM, William K. Kaufmann and Chia Chiao, Department of Pathology and Linebergher Comprehensive Cancer Center, University of North Carolina at Chapel Hill, 27599-7295

A transformed rat hepatocyte line was isolated which displays promoter-dependent proliferation. Clonal expansion requires the presence in culture medium of liver tumor promoters such as phenobarbital or DDT. Cell proliferation also is growth-factor dependent as population expansion slows and the mitotic fraction falls by 75% when the serum concentration in medium is reduced from 10% to 0.5%. After 4 days of incubation in low-serum medium, hepatocytes degenerate and die. The type of cell death is modulated by phenobarbital. In the presence of phenobarbital, death in low-serum medium is by apoptosis as indicated by light and electron microscopy of apoptotic bodies and by nucleosomal laddering of DNA. In contrast, in the absence of PB, death is by necrosis with vacuolar degeneration of endoplasmic reticulum and mitochondria preceding plasma membrane lysis. Preliminary analysis of oncogene expression indicated that *c-myc* mRNA remained at near control levels even as cell division was arrested in low-serum medium. These hepatocytes, therefore, appear to resemble transformed fibroblasts with altered regulation of *c-myc* gene expression. In the presence of phenobarbital, the conflict between the stimulus to arrest cell division in low serum and the *c-myc*-driven stimulus to divide appears to trigger apoptosis. In the absence of phenobarbital in serum-sufficient and low-serum medium, the transformed hepatocytes appear to revert to a state of finite *in vitro* proliferative lifespan, then degenerate and die by membrane lysis. Supported by PHS grant CA42765.

B8-135 CYCLIC LYMPHOMA REGULATED BY APOPTOTIC CELL DEATH, Yael Kaufmann, Amira Many, Gideon Rechavi, Orna Mor, Ziva Mark, Frida B. Simoni and Bracha Ramot, Institute of Hematology, Chaim Sheba Medical Center and Sackler School of Medicine, Tel Aviv University, 52621 ISRAEL.

A patient with a unique mantle cell lymphoma whose course was characterized by cycles of acute phases alternating with spontaneous remission episodes was observed. During acute phases the patient experienced generalized lymphadenopathy, hepatosplenomegaly, leucocytosis and trombocytopenia. These bouts regressed after 1-2 weeks without treatment. **Fifteen** such cycles were documented during the last 50 months. The alternate nature of this disease seemed to offer a unique opportunity to reveal natural mechanisms regulating remission. The neoplastic blast-like cells of the acute phase expressed surface IgM, IgD and CD5 and constituted 95% of the peripheral blood lymphocytes. During clinical remission a small population of the lymphoma cells was still observed. Two clones with different rearranged heavy chain genes appeared consistently at the recurrent acute phases. During remission only one of these clones (named **acute**) regressed considerably. The second clone was relatively stable, suggesting that proliferation and decay of the acute clone cells determine the cyclic nature of the disease. The lymphoma cells from the acute phase, but not the remission phase, underwent spontaneous apoptosis. This process was enhanced by inhibitors of mRNA and protein synthesis. No molecular alterations were detected in the bcl-2 and p53 genes of the lymphoma cells. Presumably, apoptosis of the lymphoma cells is regulated by preexisting, short-lived factor(s) yet to be identified.

B8-136 FAS ANTIGEN AND APOPTOSIS OF HIV- INFECTED CELLS

;KOBAYASHI N[1], OKA S[2] and NAKANISHI Y[3].
[1]Department of Virology and Parasitology, School of Medicine,Yamaguchi University. Japan.[2]Institute for Medical Sciences, Tokyo University, Japan. [3]Faculty of Pharmaceutical Sciences, Kanazawa University, Japan.

Recently several reports have been published that apoptosis is the key mechanism of CD4 cell depletion in AIDS patients (Terai et al. J. Clin. Invest. 87.1991, Crawford et al. Virology 185. 1991, Ameisen and Capron Immunol Today 12. 1991, Nirmal et al J. Exp. Med.. 176. 1992, Meyaards et al. Science 257, 1992, Gougeon and Montagnier, Science,260.1993). However, the mechanisms of CD4 cell death in AIDS patients are not fully understand yet. We previously reported that tumor necrosis factor (TNF) selectively kill HIV-infected cells (Med. Microbiol. Immunol. 177, 181, 1988, J. Virol. 63, 2504, 1989). We investigated the mechanism of HIV- infected cell's death by TNF and cleared that TNF kills HIV-infected cells by apoptosis. We also cleared that HIV-infected cells are susceptible to anti-Fas monoclonal antibody (Proc. Natl. Acad. Sci. USA 87, 9629, 1990). We cleared that HIV infection induces Fas gene in HIV-infected cells. Recently cDNA of Fas antigen was moleculary cloned and it was cleared that Fas antigen mediate cellular apoptosis (Ito et al. Cell, 66. 233.1991). Fas antigen is transmembrane glycoprotein and is a novel member of the TNF receptor family. Extensive studies on apoptosis in context of Fas antigen were conducted in mouse system (Fukunaga et al. Nature 356, 314.1992, Ogasawara et al. Nature 364, 806.1993), however, little is known in human system. Expression of Fas antigen on CD45 positive human T cell was reported (Miyawaki et al. J. Immunol. 179, 3753.1992) which was upregulated when human peripheral blood mono-nuclear cells (PBMC) were stimulated by PHA and cultured in the presence of rIL-2 (Owen-Scaub et al. Cellular Immunol. 140,197.1992). We examined whether peripheral blood mono-nuclear cells (PBMC) of HIV infected individual express Fas antigen on their cell surface or not. We found that Fas antigen are expressed on 20-30% of normal lymphocytes, while augmentation of Fas antigen expression was observed in 67% (34/51) of HIV infected patints. Asymptomatic carriers of HIV exhibit lower activation (46%) than those seen in ARC (83%) and AIDS 100%). We found that PBMC of HIV-infected patients were sensitive to anti-Fas monoclonal antibody, while PBMC of un-infected patients were resistant to anti-Fas antibody. Our finding strongly suggest that HIV- infected cells were killed in vivo by apoptosis through Fas antigen activation.

B8-137 APOPTOSIS, BUT NOT NECROSIS, OF INFECTED MONOCYTES IS COUPLED WITH KILLING OF INTRACELLULAR BCG,

Pairote Laochumroonvorapong, Anthony Molloy, Gilla Kaplan, Dept. of Cellular Physiology and Immunology, The Rockefeller University, New York, NY 10021.

Apoptosis has been implicated in resistance to viral infections and perturbations of the apoptotic mechanism are implicated in the pathogenesis of both viral infections and malignancies.

We have examined the effect of killing of host monocytes infected with bacillus Calmette-Guerin (BCG) on the viability of the intracellular mycobacteria. Peripheral blood monocytes were infected *in vitro* with a single bacillus per cell and maintained in culture for 6-8 d to allow the bacilli to replicate. Replicating viable BCG were found singly in perinuclear vacuoles bounded by tightly apposed lipid bilayers. Monocytes were then exposed to toxic mediators that induced killing of cells as evaluated by ^{51}Cr release into the culture medium. Both hydrogen peroxide (H_2O_2) (an inducer of cell necrosis) and adenosine triphosphate (ATP) (an inducer of cell apoptosis) treatment killed infected monocytes. H_2O_2-induced necrotic killing had no effect on BCG viability. ATP-induced cell death was accompanied by DNA fragmentation and nuclear condensation typical of apoptotic cell death. Apoptosis was associated with a swelling of the phagocytic vacuoles which became multibacillary. A reduction of BCG viability as enumerated by colony-forming units was observed in ATP-treated cultures.

At the present, we are investigating the role of apoptosis *in vivo* and the identification of a more physiological mediator of apoptosis in the control of mycobacterial infection.

B8-138 MEMBRANE EXPRESSED HIV ENVELOPE GLYCOPROTEINS ARE A POWERFUL INDUCER OF CELL DEATH IN UNINFECTED CD4+ TARGET CELLS,

A.G. Laurent-Crawford, E. Coccia*, B. Krust and A.G. Hovanessian. Institut Pasteur, Dept. of AIDS and Retroviruses, 75724 Paris, France. *Istituto Superiore di Sanità, 00161 Roma, Italy.

We have previously reported that HIV-infection of CD4+ T cells in culture results in the induction of cell killing by apoptosis. This cytopathic effect is observed during infection with syncytium-inducing or non-syncytium-inducing HIV isolates. Apoptosis is triggered by the interaction of cell membrane expressed HIV envelope glycoprotein gp120-gp41 heterodimer with the CD4 receptor. Here we demonstrate an experimental model for the induction of apoptosis independent of HIV infection, using transiently transfected HeLa cells with the HIV-1 *env* gene as effector cells and the $CD4^+$ T cell line MOLT4-T4 cells as target cells. Single amino acid point mutations of the envelope products that affect the binding to CD4 or the fusion process, abrogate the capacity of the gp120-gp41 complex to induce apoptosis. Interestingly, a point mutation in the V3 loop which inhibits fusion without affecting CD4 binding, also results in the abrogation of apoptosis. These observations indicate that the induction of apoptosis is an intrinsic property of the cell membrane-expressed gp120-gp41 complex, and thus should be considered as one of the functions of HIV *env* gene products. In view of these results and the correlation between depletion of CD4 lymphocytes and viral burden, it is possible to suggest that the killer capacity of HIV envelope may play a major contribution to the CD4+ cell depletion occuring during the progression towards AIDS.

1. A.G. Laurent-Crawford et al. (1991) Virology 185:829-839.
2. A.G. Laurent-Crawford et al. (1993) AIDS Res. & Hum. Retroviruses 9:761-773.
3. Rey-Cuillé et al. (1994) Virology 202:471-476.

B8-139 APOPTOSIS INDUCED BY BISTRATENE A IN HL60 CELLS INVOLVES THE SELECTIVE ACTIVATION OF PKC-δ AND ITS ASSOCIATION WITH NUCLEAR LAMINS AND DNA REPLICATION SITES,

Janet M. Lord[1], Bernadette Garrone[2], Gareth Griffiths[1] and Dianne Watters[2]. [1]Department of Immunology, Birmingham University, Birmingham B15 2TT, UK and [2] Department of Chemistry, Queensland Institute of Medical Research,Brisbane 4006, Australia.

HL60 cells were treated with the polyether Bistratene A (Bis A) and assessed for induction of apoptosis. 50 nM Bis A significantly increased the number of apoptotic cells, from control values of 9.2 ± 0.4% to 36.2 ± 2.0 % ($p<0.02$). Bis A has previously been shown to activate PKC, but cannot produce all the effects of TPA on HL60 cells. Bis A may therefore be restricted in its ability to activate PKC isoenzymes. Using translocation and autophosphorylation of PKC isoenzymes as a marker of their activation, we have shown that Bis A activates only PKC-δ in HL60 cells. Indirect immunostaining and confocal microscopy revealed that PKC-δ was located in the cytoplasm and nucleus of control cells. Treatment with Bis A translocated PKC-δ to the nuclear envelope and to discrete intranuclear sites. Dual labelling studies revealed that PKC-δ staining was coincident with DNA replication sites within the nucleus and with nuclear lamin B at the nuclear envelope. These data suggest that the activation of PKC-δ and its association with nuclear structures may play a role in the induction of apoptosis by Bis A in HL60 cells. We have also previously shown that the induction of apoptosis in the promonocyte cell line U937, by the phorbol ester Doppa, involves the activation of PKC-β and its rapid association with nuclear structures (Pongracz et al., 1994). The selective activation of PKC-β and δ and their translocation to nuclear and perinuclear sites, may therefore represent a mechanism central to the induction of apoptosis by a variety of agents.

B8-140 EXPRESSION OF INSULIN -LIKE GROWTH FACTOR 1 RECEPTOR INHIBITS APOPTOSIS IN IL-3-DEPENDENT CELLS, Rosemary O'Connor, Thomas Chittenden, Yimao Liu, Cynthia Ferris, Walter Blattler, and Braydon Guild, ImmunoGen, Inc., 148 Sidney St., Cambridge, MA 02139.

Many mammalian cells, including hemopoietic cells, neuro-endocrine cells, and endothelial cells are dependent on signals from other cells and their environment to survive. Absence of these survival factors causes death by apoptosis. This suggests that survival factors may act to suppress an endogenously active cell death program. Such a concept is supported by the observation that specific cytokines, principally insulin-like growth factor 1 (IGF-1), can inhibit apoptosis induced by forced expression of the oncogene *c-myc* in serum-starved Rat-1 fibroblasts. IGF-1 is abundant in fetal bovine serum used for cell culture, its receptor (IGF-1R) is expressed on most cells, and it is an important growth factor for fibroblasts.

To further investigate the role of IGF-1 as a survival factor we expressed the IGF-1R in a cell line Fl5.12, which is dependent upon the exogenously added cytokine, IL-3, for proliferation and survival. Fl5.12 cells die rapidly by apoptosis when IL-3 is removed, even in the presence of fetal bovine serum. A cDNA clone encoding the human IGF-1R under the control of the SV40 early promoter was introduced into Fl5.12 cells and receptor expression on the resulting stable cell lines was quantitated with an anti-human IGF-1R antibody. The ability of the IGF-1R to contribute to survival or proliferation was assessed by removing IL-3, adding IGF-1, and monitoring viable cell number over time. Under these conditions cell survival was greatly enhanced in cells expressing the receptor compared with mock-transfected control cells, and the degree of survival correlated with the level of IGF-1R expression. Interestingly, IGF-1 could not replace IL-3 as a mitogen for Fl5.12/IGFR transfectants, and appeared to be acting uniquely as a survival factor. This system can be used to study the role of the IGF-1R in the inhibition of apoptosis, and its potential interaction with other proteins known to regulate cell death in these cells.

B8-141 *IN VIVO* RESISTANCE TO GLUCOCORTICOID-INDUCED APOPTOSIS IN RAT THYMOCYTES. Nicklas B.E. Oldenburg and John A. Cidlowski, Department of Physiology The University of North Carolina at Chapel Hill, Chapel Hill, N.C. 27599

Glucocorticoid(GC)-induced lymphoid cell death represents the activation of programmed cell death, apoptosis. Rat thymocytes are profoundly sensitive to GC-induced apoptosis and have served as a model system for studying apoptosis. When rats are injected with dexamethasone (dex) thymocyte cell number decreases and internucleosomal DNA degradation is detected until 72 hours after treatment. The population of lymphocytes that remains within the thymus gland 72 hours after *in vivo* steroid administration maintains intact DNA and has been referred to as corticoresistant. The molecular basis for this resistance to GCs was investigated. Rats were adrenalectomized and treated 72 hours with dex to produce a population of thymocytes resistant to GC-induced apoptosis. Thymus weight was reduced 79% and cell number reduced 97.5% when compared to the control animals. Re-injection of equivalent doses of dex into previously treated animals failed to demonstrate any evidence (morphology or chromatin degradation) of apoptosis in thymocytes, suggesting that cells remaining in the gland are indeed apoptosis resistant. Since clinically demonstrated cases of GC resistance have been shown to result from altered receptor proteins, GC receptor characteristics of the resistant thymocytes were examined by measuring ^{3}H-dex binding and compared to immature GC sensitive thymocytes. Receptor density and binding affinity was very similar in the resistant thymocytes versus the controls. Western blot analysis of whole cell lysates from GC resistant and sensitive thymocytes demonstrated equivocal levels of GC receptor. The effect of dex on ^{3}H-Lysine incorporation was studied to determine if GC receptors functioned properly. Cells resistant to GC-induced apoptosis were equally inhibited by dex indicating both populations of cells have functional GC receptors. To evaluate if *in vivo* GC-resistant thymocytes have any capacity to undergo apoptosis, *in vitro* studies using the calcium ionophore A23187 were performed. Resistant cells were isolated and treated with either dex, the calcium ionophore A23187, or PBS. Internucleosomal cleavage of chromatin into oligonucleotide fragments was demonstrated in resistant thymocytes treated with A23187 indicating that these cells are capable of undergoing apoptosis. *In vivo* dex resistant thymocytes also exhibited internucleosomal cleavage of chromatin in response to dex *in vitro*. suggesting that *in vivo* some endogenous substance or cell-to-cell contact confers resistance to GC-induced cell death that does not persist *in vitro*.

B8-142 DETERMINANTS OF ENDOTHELIAL CELL PROGRAMMED CELL DEATH: INTERACTION OF TRANSFORMING GROWTH FACTOR-β_1 (TGF-β_1) AND THE EXTRACELLULAR MATRIX, Matthew J. Pollman, Sanjay K. Khurana and Gary H. Gibbons, Falk Cardiovascular Research Center, Stanford University, Stanford, CA 94305

Programmed cell death (PCD) or apoptosis is now recognized as a critical determinant of tissue architecture. It is postulated that changes in vascular structure involve an alteration in the homeostatic balance between cell growth and physiologic cell death. The regulation of vascular cell growth and differentiation involves the interaction between autocrine-paracrine growth factors within the context of the extracellular matrix microenvironment. We tested the hypothesis that the interplay between growth factors and cell-substrate interactions that regulate vascular cell growth and differentiation also regulates PCD. Bovine aortic endothelial cells that were grown on standard tissue culture glass or polystyrene to a near-confluent state in 10% calf serum exhibited little evidence of PCD. However, incubation in serum-free media induced PCD as evidenced by the characteristic internucleosomal cleavage of DNA assayed by end-labeling and gel electrophoresis. The induction of PCD during serum withdrawal was further verified and quantitated by analysis of cellular chromatin condensation using a fluorescent DNA-binding dye (Hoechst 33342). In contrast to the anti-apoptotic effect of serum, TGF-β_1 induced PCD in a dose-dependent fashion at concentrations as low as 100 pg/ml. In contrast to PCD induced by serum withdrawal, treatment with 10% calf serum failed to prevent TGF-β_1-induced apoptotic death. However, cells plated on collagen I were rescued from both serum withdrawal and TGF-β_1-induced apoptotic death. These findings suggest that endothelial cell number is determined by a balance between cell growth and PCD that is regulated by the interaction between growth factors within the context of the extracellular matrix. The regulation of PCD may be an important determinant of vascular lesion structure in atherogenesis or angiogenesis.

B8-143 The Role of p53 in c-Fos-induced Apoptosis

G. A. Preston, T.T. Lyon, Y. Yin and J.C. Barrett. Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

To investigate further earlier indications that c-Fos may play a role in apoptosis in our system, preneoplastic Syrian hamster cell lines expressing a transfected hormone-dependent c-Fos-ER chimeric protein (Superti-Furga et al., 1991) were placed in serum free conditions in the presence of activated c-FosER (addition of 1ug/ml estradiol). This resulted in apoptosis in sup+I/FosER transfectants similar to the sup+I parental cells although at an accelerated rate. However, sup-II/FosER cells also died by the process of apoptosis which is in sharp contrast to the sup-II parental cells that were resistant to apoptosis in serum free conditions. The data indicate that the *c-fos* product has a causal relationship in apoptotic cell death in our cellular system.

As a continuation of these studies, two human tumor cell lines expressing the c-FosER construct were generated. RKO(p53$^{+/+}$)/FosER cells could be induced to undergo apoptosis in serum free medium in contrast to the parental cell line. For comparison, H1299(p53$^{-/-}$)/FosER cells could not be induced to undergo apoptosis by over-expression of c-FosER. Restoration of normal p53 function in H1299 cells, using a p53ER construct, resulted in p53-induced apoptosis and western blot analysis showed increased levels in of endogenous c-Fos in these cells. Therefore, c-Fos-induced apoptosis requires a normal p53 function.

Implications are that the role of c-Fos in apoptosis is one of a transrepression function rather that a transactivation function based on two observations: c-Fos-induced apoptosis does not require new protein synthesis and v-Fos (impaired in transrepression activities) cannot substitute functionally for c-Fos to induce apoptosis.

Coexpression of Bcl-2 protein with c-FosER can override c-Fos-induced apoptosis in a manner similar to that reported for Bcl-2 blocking p53-dependent apoptosis.

B8-144 ENHANCEMENT OF THE LPR SYNDROME BY A BCL-2 TRANSGENE. Elizabeth A. Reap, P. Anne Wolthusen, Philip L. Cohen, and Robert A. Eisenberg, Department of Medicine, University of North Carolina, Chapel Hill, NC 27599-7280.

Mice homozygous for the lpr gene develop autoantibodies and massive lymphadenopathy. The lpr gene encodes a defective form of the apoptosis-inducing receptor, Fas. Mice bearing the anti-apoptosis bcl-2 transgene (tg) also have a defect in apoptosis resulting in an abnormal survival of a population of mature B cells. Since multiple mechanisms controlling programmed cell death may contribute to systemic autoimmunity, the effect of the bcl-2 transgene on the lpr model of SLE was examined by crossing bcl-2 immunoglobulin transgenic and B6/lpr mouse strains. Four experimental groups of mice were obtained: lpr homozygous, bcl-2 + (lpr, tg +); lpr homozygous, bcl-2 - (lpr, tg -); lpr heterozygous, bcl-2 +; and lpr heterozygous, bcl-2 -. Enhancement of lymphadenopathy was seen in lpr, tg + mice compared to lpr, tg - littermate controls. Spleen and lymph node weights were higher in lpr, tg + mice (0.719 g, 3.192 g, respectively) compared to lpr, tg - mice (0.261 g, 0.743 g, respectively). At five months of age, the absolute numbers of double negative T cells in lymph nodes were significantly increased ($p<.05$) in lpr, tg + mice (2×10^9) compared to lpr, tg - littermates (3×10^8). Total numbers of lymph node and spleen cells were also significantly higher in lpr, tg + mice versus lpr, tg - mice($p<.05$). The absolute numbers of CD4 and CD8 positive T cells were increased in the lpr, tg + mice versus lpr, tg - mice, while the absolute numbers of splenic IgM^+ B cells were not different between lpr, tg + mice and lpr, tg - littermate controls. Two month old lpr, tg + mice had six-fold more IgG anti-chromatin autoantibodies than lpr, tg - mice, although levels were similar at four months. Levels of total IgG and IgM were similar between the two groups of mice at 2, 3, and 4 months of age. Lpr heterozygote tg + and - littermate controls had no autoantibodies and no lymphadenopathy. These results suggest that double negative T cells normally have a limited lifespan, and their survival is enhanced by the bcl-2 transgene such that lymphadenopathy is markedly exacerbated. This illustrates that defects in separate apoptosis mechanisms may combine to produce enhanced pathological effects.

B8-145 Cold induced recruitment of brown adipose tissue; possible role of apoptosis in the readaptation to thermoneutral temperature

Stefan Rehnmark, Anders Jacobsson and Jan Nedergaard
The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, S-106 91 Stockholm, Sweden

In order to increase the total capacity for thermogenesis during acclimation to cold, brown adipose tissue is recruited. The recruitment process includes transcriptional activation of several genes, an increased number of mitochondria, and ultimately the total number of brown fat cells are increased. Similarly, when a cold adapted animal is returned to thermoneutrality, there is an abrupt cessation of the thermogenic activity within the tissue and a new adaptation process starts. The adaptation to a warm environment includes decreasing the thermogenic capacity and eventually the mitochondrial content of the tissue is decreased. Upon prolonged living at thermoneutral temperature the brown adipose tissue mass is decreased.

Brown adipose tissue constitutes a valuable model for studying physiological regulation of the balance between cell proliferation and cell death.

We are currently studying the expression of bcl-2 and p53 in brown adipose tissue during different physiological conditions (e.g. cold exposure and adaptation to thermoneutral temperature), and also whether programmed cell death is involved in the reacclimation to thermoneutral temperature.

B8-146 THREE FUNCTIONAL SOLUBLE FORMS OF THE HUMAN APOPTOSIS-INDUCING FAS MOLECULE ARE PRODUCED BY ALTERNATIVE SPLICING. Giovina Ruberti, Giusy Fiucci, Giuliana Papoff and Isabella Cascino, Department of Immunobiology, Institue of Cell Biology C.N.R, Viale Marx, 43 -00137 Roma, Italy.

Fas/Apo1 molecule is an apoptosis-signaling cell surface antigen belonging to the Tumor Necrosis Factor Receptor family. To investigate the possibility that soluble forms of the Fas receptor are expressed in human cells we analyzed Fas mRNA transcripts obtained from activated peripheral mononuclear cells of healthy donors and from human tumor cell lines. We identified and characterized three human mRNA Fas variants: FasTMDel, FasDel2 and FasDel3. To determine whether the three transcripts were derived by alternative splicing, the Fas genomic intron/exon organization of the regions surrounding the deleted sequences was analyzed in Fas clones isolated from a human genomic library. Expression of the transcripts was studied in COS cells transiently transfected with the FasTMDel, FasDel2 and FasDel3 cDNAs. Immunocytochemical and in vitro apoptosis inhibition studies suggest that the transcripts are expressed as soluble Fas proteins that may play a functional role in the regulation of apoptosis.

B8-147 DETECTION OF HUMAN FAS-LIGAND EXPRESSION USING A FAS-Ig FUSION PROTEIN. David M. Sansom*, Neil McHugh and Yusef Patel. Bath Institute for Rheumatic Diseases, Trim Bridge, Bath, BA1 1HD,UK, Schools of *Pharmacy and Pharmacology, and Postgraduate Medicine, University of Bath, BA2 7AY.

The Fas antigen (CD95) is a cell surface molecule expressed on of a variety of cell types including T lymphocytes. It has been established that binding to the Fas antigen by some monoclonal antibodies as well as by its defined ligand induces apoptosis in several cell types, however there is evidence that under certain circumstances Fas may provide signals which enhance T cell responses.

Support for the importance of the Fas antigen within the immune system comes from the MRL/*lpr* and *gld* mice in which mutations in the Fas gene and its ligand respectively lead autoimmune disease. Thus it is clear that the integrity of both Fas and Fas-ligand plays a major role in regulation of lymphocyte function. Functional studies suggest however that the loss of self tolerance is not associated with defects involving the selection of T cells in the thymus. Instead it appears that Fas maybe involved in activation driven suicide of activated mature T cells whereby T cells from *lpr* or *gld* mice are resistant to anti CD3 induced suicide The above situation is also consistent with other observations associated with mature, activated T human cells. It is clear from a number of studies that activated T cells become increasingly prone to TCR induced apoptosis supporting a role for this pathway in the apoptotic cell death of activated T cells

In order to study Fas-ligand interactions, we have produced a chimeric protein which expresses the extracellular portion of the human Fas molecule fused to the constant domains and hinge region of mouse IgG (Fas-Ig).Using this molecule we have assessed Fas-Ig binding to various cell types in peripheral blood, reflecting the expression of the Fas-ligand. Our data suggests that the Fas-ligand is expressed on resting and activated T cells. However professional antigen presenting cells such as monocytes or B lymphoblastoid cell lines do not show expression. Preliminary data using this protein also suggest that Fas-Ig may inhibit PHA driven cell death in T cell clones and suggests that apoptosis in this system may result from Fas-Fas-ligand interactions.

B8-148 Ig-α AND Ig-β CAN INDEPENDENTLY SIGNAL GROWTH ARREST AND APOPTOSIS IN TRANSFECTED B-CELL LYMPHOMAS. David W. Scott and Xiao-rui Yao, Department of Immunology, Jerome H. Holland Laboratory, American Red Cross, Rockville, MD 20855

We have previously established a model system in murine B-lymphoma cells, in which cross-linking of surface IgM induces growth arrest and subsequent cell death by apoptosis. Signal transduction via membrane IgM requires the physical association of membrane immunoglobulin with the accessory Igα/Igβ heterodimer. The cytoplasmic tails of Igα and Igβ both contain conserved sequence motifs shared by a number of signal transducing molecules. These motifs involved in the specific interaction between the receptor complex and SH2 domain-containing protein tyrosine kinases. It is not known, however, whether Igα and Igβ may jointly or individually transduce the signals for growth arrest and apoptosis. To analyze the independence of Igα or Igβ in the inhibitory signaling pathways, we transfected chimeric cDNA constructs encoding the extracellular and transmembrane domains of CD8 with the cytoplasmic tails of Igα, Igβ, or control IgG2a (γ2a), into the CH31 B-cell lymphoma. This procedure results in equivalent surface expression of chimeric proteins. When the cells bearing CD8:Igα or CD8:Igβ were incubated with anti-CD8, growth arrest and apoptosis were observed, whereas parental cells or the cells expressing a CD8:γ2a protein were not growth arrested by anti-CD8. These findings demonstrate that cytoplasmic tails of Igα and Igβ are both functionally active domains, that they are capable of transducing signal independently and that heterodimer formation is not required for apoptosis induction. The similarities and differences between Igα and Igβ signaling pathways, and their interaction with *src*-like protein tyrosine kinases, will be presented. (Supported by NIH grants CA55644 and AI29691)

B8-149 **Transcriptional activation of the matrix metalloproteinase gene *stromelysin-3* is associated with thyroid hormone-induced cell death during amphibian metamorphosis**, Yun-Bo Shi, Danielle Patterton, and William Par Hayes*. Laboratories of Molecular Embryology and *Developmental Neurobiology, NICHD/NIH, Bethesda, MD 20892.

Amphibian metamorphosis systematically changes every single tissues of a tadpole and is totally controlled by thyroid hormone. In particular, the tadpole intestine is a simple tubular organ consisting of predominantly a single layer of primary epithelium. It undergoes a drastic remodeling during metamorphosis to form a complex structure with multiple epithelial folds. This process involves complete degeneration of the primary epithelium through specific death of the epithelial cells. At the same time, secondary epithelial cells proliferate and migrate to replace the primary epithelial cells. The connective tissue and muscle also develop extensively during this process. To investigate the potential roles of extracellular matrix remodeling during this process, we have cloned and characterized in detail the *Xenopus* stromelysin-3 gene. We found that the stromelysin-3 gene was activated at the beginning of intestinal remodeling and then repressed by the end and this same expression pattern was observed when premetamorphic tadpoles were induced to metamorphose precociously by thyroid hormone. The activation of its expression by thyroid hormone is an immediate early event since it is independent of new protein synthesis. More importantly, this activation precedes cell death in the epithelium. Furthermore, *in situ* hybridization showed that the gene was expressed only in the developing connective tissue. These results suggest that the *Xenopus* stromelysin-3 may participate in the remodeling of the extracellular matrix, which would in turn mediate proper cell-cell and cell-matrix interactions during larval epithelial degeneration and adult tissue development. The potential role of stromelysin-3 in influencing apoptosis is also in agreement with the observation that stromelysin-3 gene is expressed during the period of limb morphogenesis and tail resorption when cell death takes place.

B8-150 **B Cell antigen Receptor (BCR)-mediated apoptosis:importance of accessory molecules-CD19 and CD22- and of sIgM cross-linking.** Aimé **Vazquez,** Nadia **Chaouchi,** Pierre **Galanaud** & Corinne **Leprince. INSERM U131**, 32 rue des Carnets 92140 Clamart, France.

Engagement of the B cell antigen receptor (BCR) can induce a suicide pathway in various B cell types. Earlier studies showed that anti-IgM mAb treatment triggers apoptotic death in the Burkitt lymphoma-derived cell line, Ramos. We show that two B cell surface molecules - CD19 and CD22 - which have been reported to interact either functionally or structurally with the BCR also stimulate cell suicide when sufficiently aggregated, both in the Ramos and EBV-infected Ramos AW cell lines. In conditions of lower cross-linking, both molecules enhance the apoptotic response induced by a suboptimal dose of anti-IgM mAb in Ramos cells, reinforcing the notion that CD19 and CD22 may be involved in the death pathway and modulate Ag-induced B cell apoptosis. Similar conclusions were obtained with human tonsillar B cells which enter the death program upon treatment with cross-linked anti-IgM, -CD19 or -CD22 mAb. These results indicate that Ag-induced B cell suicide may affect mature B cells in the periphery and may be regulated *via* the interaction of CD19 and/or CD22 with their respective ligand(s). Early tyrosine phosphorylations were analyzed by Western-blotting. The biological outcome of these various treatments - cell survival or death - could not be related to any detectable new tyrosine phosphorylated substrate, further questioning the biochemical basis of apoptosis signaling.

B8-151 EPITHELIAL-DERIVED INTERLEUKIN-1 MEDIATES CORNEAL TISSUE ORGANIZATION THROUGH INDUCTION OF FIBROBLAST APOPTOSIS, Steven E. Wilson, Hu-Guang He, Jian Weng, Qian Li, Mark Vital, Eric L. Chwang, Department of Ophthalmology, Univ. Texas Southwestern Med Center, Dallas, Texas 75235.

Development, maintenance of tissue morphology, and wound healing are controlled by various forms of communications between cells. Epithelial-stromal interactions are undoubtedly important in the regulation of these fundamental mechanisms where epithelial and connective tissues conjoin in the formation of an organ such as the cornea or skin. The results of this study demonstrated that epithelial scrap wounds produced apoptosis in underlying fibroblasts (keratocytes) within the corneal stroma. IL-1 alpha and beta are produced by corneal epithelial and endothelial, but not keratocyte, cells in the cornea. IL-1 stimulates apoptosis of corneal fibroblasts in vitro. Micro-injection of IL-1 into the central cornea results in keratocyte apoptosis and redistribution. IL-1 may have a similar role in skin. These studies suggest that the IL-1 cytokine-receptor system is an important modulator of tissue organization and response to injury in cornea and skin. It is hypothesized that systems involving IL-1, or other mediators, modulate epithelial-stromal interactions that are involved in similar tissue homeostatic functions in other organs. Supported by Research to Prevent Blindness, EY09379, and EY10056.

B8-152 **TRANSFORMING GROWTH FACTOR ß INHIBITS APOPTOSIS IN HUMAN TUMOR CELLS TREATED WITH Fas ANTIBODY**

Thomas S. Winokur and Jeff Ryan. Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35233

Fas is an antibody directed against a cell surface receptor originally identified on human lymphocytes. Binding of the antibody or stimulation of the receptor by its natural ligand results in apoptotic cell death. Subsequent studies have identified the receptor on the surface of numerous human tumor cell lines although not all cell lines undergo apoptosis when treated with Fas antibody. Fas antigen and antibody are structurally related to tumor necrosis factor and its receptor although it appears that there is considerable divergence in their mode of action. Many cell lines which express both Fas and TNF receptor respond only to one agent and several agents which protect against TNF induced cytotoxicity are ineffective in protecting against Fas. Transforming growth factor-ß is a peptide growth factor with many actions including control of cell proliferation and extracellular matrix production. In hepatocytes, in some myeloid tumor lines and in T- lymphocytes TGFß induces apoptosis. In many cell lines TGF-ß protects cells against TNF induced cell death. We have investigated whether TGF-ß can protect against Fas induced apoptosis.

Design: Cell lines were treated with Fas antibody(UBI) with or without added TGF-ß1. After an appropriate interval cells were stained with a combination of acridine orange and propidium iodide. This allows identification of apoptotic cells and nonviable cells. Time course and dose response curves were generated.

Results: TGF-ß is able to protect tumor cell lines of diverse lineage from the apoptosis induced by Fas antibody. The mechanism of protection is undetermined.

Conclusion: TGF-ß produced by tumors may protect them from Fas induced apoptosis resulting in a selective growth advantage. This may also interfere with therapeutic efforts directed at the FAS antigen.

B8-153 DIFFERENT SUSCEPTIBILITY TO DEXAMETHASONE-INDUCED APOPTOSIS IN POPULATIONS OF FIBRONECTIN ADHERENT AND NONADHERENT HUMAN THYMOCYTES. Marina B. Zaitseva*, Christopher F. Mojcik#, David Stephany@ and Hana Golding*. *Laboratory of Retroviruses, CBER, FDA, @Laboratory of Molecular Structure and #Laboratory of Immunology, NIAD, NIH, Bethesda, MD, 20892.

Thymocytes are sensitive to steroid-induced cell death at various stages of differentiation. At the same time triggering of the TcR-CD3 complex can protect thymocytes from steroid-mediated cell death. To investigate the correlation between the susceptibility to steroid-induced apoptosis and the stage of the thymocytes differentiation we used human thymocytes separated on the basis of constitutive adhesion to fibronectin (FN) into population of adherent and nonadherent thymocytes. The nonadherent population is more mature, express high level of CD3 and in contrast to adherent population, which express low level of CD3, can respond to activation with anti-CD3 plus FN. (D. Salomon et al., 1994).

RT-PCR showed that freshly isolated unseparated, FN adherent and FN nonadherent thymocytes express the same level of bcl-2 mRNA, but in FN adherent thymocytes bcl-2 mRNA is downregulated after overnight culture in media alone. Apoptosis was detected by terminal deoxy-nucleotidyl transferase and DNA ladder methods. We demostrate that less mature thymocytes population (FN adherent) is most sensitive to the steroid-induced apoptosis compared to more mature FN nonadherent and unseparated thymocytes population. FN nonadherent thymocytes can be rescued from steroid-induced apoptosis by culture on the anti-TcR antibody. Thus, pretreated nonadherent thymocytes have lower level of dexamethasone-induced cell death. The level of protection correlates with elevated expression of the bcl-2 mRNA in FN nonadherent thymocytes after activation of their TcR with antibody. The data presented suggest that signalling through TcR could lead to the activation of the survival signal bcl-2 thus providing protection of the thymocytes from negative selection via steroid-induced apoptosis.

B8-154 MONOCYTE-DERIVED MACROPHAGES PROVIDE APOPTOTIC SIGNALS TO T LYMPHOCYTES THROUGH CELL-CONTACT MECHANISM, Katsuhiro Zen, Junichi Masuda, Chiya Kosaka, Toshiyuki Sasaguri, Kentaro Shimokado, Takeo Abumiya, Akio Ishida, Tomoyuki Yamakawa, Jun Ogata. National Cardiovascular Center Research Institute, Osaka 565, Japan

Purpose: Macrophages play an important role in recognition and ingestion of cells undergoing apoptosis. In the present study, we examined roles of monocytes/macrophages in induction of apoptosis in human T lymphocytes. **Methods:** Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by a density centrifugation method. Purified T lymphocytes were prepared from PBMCs using a nylon wool column. Cells were cultured in RPMI1640 containing 10% FCS with or without staphylococcal enterotoxin B (SEB) or phytohemagglutinin (PHA). DNA content of cultured cells were measured with a flow cytometer after propidium iodide staining, and hypodiploid cells with fragmented nuclei (<2c) were defined as cells undergoing apoptosis. **Results:** Apoptosis was elicited by SEB or PHA after 4 day-culture in unfractionated PBMCs, and cell population undergoing apoptosis was revealed to be CD3-positive by immunofluorescence staining. In purified T lymphocyte preparations, apoptosis was not induced by SEB or PHA, but was induced when the cells were co-cultured with monocyte-derived macrophages adhering to plastic dishes. To determine whether macrophages provide signals through secretion of soluble factors or direct cell-contact mechanism, the purified T lymphocytes and monocyte-derived macrophages were co-cultured in split-well chambers with microporous membrane (pore size: 0.4 µm). Placing T lymphocytes in the insert well physically separated from the adhering macrophages resulted in a complete loss of apoptosis induced by SEB and PHA. **Conclusion:** Monocyte-derived macrophages provide signals for activation-induced apoptosis of T lymphocytes through cell-contact mechanism.

B8-155 **B LYMPHOCYTES FROM THE BURSA OF FABRICIUS OF CHICKENS PROVIDE A MODEL SYSTEM FOR ANALYSIS THE APOTOTIC PATYWAY.** **Donald L. Ewert, James DuHadaway, Decker Beck, and Louise Showe The Wistar Institute, Philadelphia, PA.**

Normal B lymphocytes when removed from the environment of the bursa of Fabricius rapidly undergo apoptosis when placed in medium at 37°C. Within 6hr about 90% of the cells are apoptotic as evidenced by nuclear condensation, end labeling of fragmented DNA, or by detection of nucleosomal size fragments of DNA. Also, in vitro grown cell lines derived from retroviral transformed bursal cells can also be induced to undergo apoptosis by depravation of serum. We have used both of these systems to analyze factors that regulate apoptosis: expression of the bcl-2 proto-oncogene, infection with herpesvirus, and cell-substrate contact. Apoptosis of the dispersed bursal cells can be partially inhibited by forcing the cells into close contact with other bursal cells or onto a solid surface coated with fibronectin, but not other integrin molecules. This result suggests that the initial trigger that starts the apoptosis cascade in these immature B cells involves the breaking of a specific cell-cell interaction.

The effect of *bcl-2* expression on the development of B lymphocytes was also examined in chicks infected at hatch with a retroviral vector containing the human bcl-2 gene (RCASBPbcl-2). A population of B cells developed in the cortex of the bursal follicles which expressed the human bcl-2 protein. These cells were arrested at G_1/G_0 of the cell cycle, refractory to induction of apoptosis, and were refractory to mitgenic stimuli. This immature population of bcl-2+ B cells infiltrated most of the peripheral lymphoid tissues, About 10% of the RCASbcl-2 chickens developed a B lymphoblasic leukemia. The pathology associated with aberrant expression of bcl-2 is similar to that observed in B cell chronic lymphocytic leukemia.

We have also observed that a common herpesvirus of chickens, the Marek's, virus can inhibit apoptosis of bursal derived B cell lines This observation suggests a mechanism for the enhancement of retroviral induced B cell tumors in chickens that are co-infected with the Marek's virus.

Thus multiple components of the apoptotic pathway are amenable to testing in this system.

Poster Session 2

B8-200 ANALYSIS OF THE CYTOLYTIC PROCESSES INDUCED BY DIFFERENT BIOLOGICALLY ACTIVE SUBSTANCES AT TRANSFORMED CELL LINES

Elena Yu. Blishchenko, Irina I. Mirkina, Olga A. Mernenko, Daulet K. Satpaev and Andrey A. Karelin, *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, GSP-7, 117871, Moscow, Russia*

Cytolytic activities of neurotoxin II from venom *Naja naja oxiana*, tubocurarin, substance P and TNF were studied by Trypane blue inclusion method at L929 and K562 tumor cells. Cytotoxicity of each substance was determined within 1-24 hour at concentration range of 10^{-5}-10^{-16} M. The mechanism of DNA fragmentation was analyzed by agarose gel electrophoresis.

Cytolytic characteristics of these factors were shown to have several common aspects as follows. Each of these substances induced 5-6 different cytolytic processes within these dynamic and concentration ranges at both cell lines. All factors mediated both short-term (developing within 1-7 hours of incubation) and long-term (which required more than 8 hours for their realization) cytolytic processes. Contribution of different cytolytic processes depended primarily on concentration of cytolytic agent. At least the following pathways of target genome fragmentation were demonstrated for these substances:

1. Necrosis, which was not accompanied with DNA fragmentation;
2. Apoptosis, which was characterized by large-scale (5-50 kbp) DNA fragmentation preceding internucleosome cleavage;
3. Apoptosis, which resulted primarily in 200 bp fragmentation.

The major differences in cytolytic characteristics were due to the mechanisms of cell death associated with long-term processes and depended both on target cell type and the nature of cytolytic agent.

The nature of the multiplicity of the cytolytic processes will be also discussed.

B8-201 SUPERANTIGEN-INDUCED LETHAL SHOCK IN Vβ8.2 T-CELL RECEPTOR TRANSGENIC MICE IS REGULATED BY FAS.

Carl K. Edwards, III[*], Constance M. Cullen[†], Peter F. Bonventre[†], Horst Bluethmann[‡], and John D. Mountz[¶], [*]Department of Immunology, MMDRI, Cincinnati, OH 45215, [†]Univ. of Cincinnati College of Medicine, Cincinnati, OH 45267, [‡]Pharm. Res. Gene Tech., F. Hoffmann-LaRoche, Basel, Switzerland, and [¶]Department of Medicine, Univ. of Ala-Birmingham and the Birmingham VAMC, Birmingham, AL 35294.

There is a 1000-fold greater susceptibility to SEB-induced septic shock in MRL-*lpr/lpr* mice. Anti-Ia[k] and anti-Vβ8, but not anti-H2K[k] protected Vβ8 TCR transgenic and non-transgenic MRL-*lpr/lpr* mice from lethal SEB-induced septic shock. The contribution of the expression of class II molecules to induction of septic shock was also demonstrated by a 1000-fold greater resistance to SEB-induced septic shock in C57BL/6 MHC class II A[b0]/A[b0]-deficient-+/+ mice compared to control C57BL/6-+/+ mice. The Tg93 mice are exquisitely sensitive to TSST-1-induced shock when compared to conventional mice. Recombinant TSST-1 (rTSST-1) administered to Tg93 mice at doses as low as 0.1 μg/mouse resulted in 100% lethality. In contrast, nontransgenic mice heterozygous at the *lpr* locus (Vβ8.2/0;*lpr*/+) survived doses 100 times higher ($P<0.01$). Also, a rTSST-1 mutant at amino acid 135 was not toxic in this model. Amplification by RT-PCR of splenic cDNAs with Vβ3 and Vβ15 specific primers revealed expression of TSST-1 reactive Vβ elements. In both cases, SAG-induced shock can be inhibited at several levels including blocking of cell surface molecules and at the level of TNF-α transcription. We conclude that the sensitivity of Tg93 mice to SEB or to rTSST-1 in the presence of low amounts of TSST-1 reactive T-cells suggest a critical role of functional FAS for resistance to SAG-induced shock.

B8-202 CD4 T CELL DEPLETION BY APOPTOSIS IN HUMAN PBL-SCID MICE INFECTED WITH MACROPHAGE-TROPIC AND T CELL-TROPIC HIV-1 STRAINS, Jacqueline M. Glynn, and Donald E. Mosier, Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037

HIV-1 infection of hu-PBL-SCID mice (severe combined immunodeficient mice reconstituted with human peripheral blood lymphocytes) leads to CD4 T cell depletion. As previously described, the macrophage-tropic strain HIV-1_{SF162}, causes more extensive T cell depletion than the T cell-tropic strain HIV-1_{SF33}. Substantial evidence from the literature suggests that the CD4 T cell depletion observed in AIDS may be occurring by apoptosis, which is a form of cell death that is characterized by nuclear condensation and DNA fragmentation. In order to examine whether the CD4 depletion observed in HIV-1-infected hu-PBL-SCID mice occurs by apoptosis, human cells were recovered by peritoneal lavage from animals which had been infected with macrophage-tropic or T cell-tropic HIV-1 isolates at equal viral burden. These cells were analyzed by flow cytometry and the TUNEL assay (which measures DNA strand breakage as a marker for apoptosis). Tissue sections from these animals were also analyzed using an *in situ* assay for apoptosis. Preliminary results suggest that there is a correlation between CD4 depletion and levels of apoptosis observed in the hu-PBL-SCID model. Experiments are currently underway to determine whether direct infection of cells is necessary for the induction of apoptotic cell death or whether indirect mechanisms contribute to T cell depletion.

B8-203 THE ROLE OF INSULIN LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPS) IN REGULATING ACTIVE CELL DEATH IN REGRESSING RAT PROSTATE AND MAMMARY GLAND. R. Sean Guenette and Martin Tenniswood, W. Alton Jones Cell Science Center, Lake Placid, NY 12946

Apoptosis or active cell death (ACD) plays an important role in development, tissue homeostasis, and growth regulation. ACD of terminally differentiated secretory epithelial cells of the normal prostate and lactating mammary gland requires the induction of several secondary thanatogens, including among others,TRPM-2 (clusterin), tissue transglutaminase, and cathepsin B. To identify other genes that may be involved in this process we have used a lateral cross screening strategy to identify genes that are expressed in both regressing prostate and mammary glands. One of the cDNAs that we have cloned (RSG-8) is the rat homolog of insulin like growth factor binding protein 5 (IGFBP-5). Northern analysis shows that the mRNA for IGFBP-5 is induced *de novo* during ACD in regressing prostate and mammary gland induced by hormone ablation, reaching maximal levels between 4 and 6 days after hormone ablation, before returning to undetectable levels by day eight. *In situ hybridization shows that the induction of the IGFBP-5 mRNA is localized to the* secretory epithelial cells of both the prostate and mammary gland. These cells are known to undergo ACD during tissue regression. In contrast to IGFBP-5 mRNA, the steady state levels of IGFBP-2 mRNA, which is also localized to the epithelial cells of the glands, do not alter significantly during regression in either tissue. The IGFBPs are known to modulate the response of cells to the IGF growth factors in both a positive and negative fashion. Our data suggests that the survival of the secretory epithelial cells of the prostate and mammary gland is dependent on the interaction of Insulin like growth factor (which is synthesized in the stroma) with its cognate epithelial receptor. IGFBP-2 appears to modulate this interaction. We hypothesize that the induction of ACD in terminally differentiated epithelial cells in tissues such as the prostate and breast may be due to the attenuation of IGF-1 mediated survival signals as a result of the induction of IGFBP-5. This binding protein appears to sequester the growth factor away from its cognate receptor, inducing ACD. Thus the induction of IGFBP-5 may be a very early step in the cascade of events that leads to ACD. Further studies into the potential role of the IGFBPs in modulating growth and/or the induction of ACD in prostate and mammary gland should lead to a better understanding of ACD and the treatment of malignancies in these tissues.

(Supported by the Medical Research Council of Canada and the National Cancer Institute of Canada)

B8-204 Fas induced cell death by incomplete proliferation signals via Ras and Rac2

E.Gulbins*, K.Schlottmann#, G.Busch*, B.Brenner+, A.E.Busch*, K.M.Coggeshall# and F.Lang*
I.Institute of Physiology, University of Tuebingen, 72076 Tuebingen, Germany +Dep. of Pediatrics, University of Heidelberg, 69210 Heidelberg, Germany #Dep.of Microbiology, Ohio State University, 43210 Ohio, USA

Activation of lymphocytes or hepatocytes via the Fas receptor results in programmed cell death, however the intracellular signaling cascade mediating programmed cell death is not known. Previously we have shown a ceramide release and Ras activation upon cellular anti-Fas stimulation (E.Gulbins et al., submitted). Inhibition of Ras by transient expression or microinjection of transdominant inhibitory Ras or electroporation of Ras-neutralizing antibodies inhibited cell death in Jurkat, indicating an important role of Ras in Fas induced apoptosis. Here, we demonstrate an activation of the small G-protein Rac2 (measured by increase of GTP-binding and translocation of Rac2 to the TritonX100 insoluble fraction) and an intracellular H_2O_2 synthesis in Jurkat cells after stimulation with Fas (2μg/ml). Cellular apoptosis, Rac2 activation and H_2O_2-synthesis were blocked by transient expression of transdominant inhibitory N17Ras, suppression of Rac2-expression with antisense molecules or inhibition of the Rac2-function by electroporation of Botulinus C3-exoenzyme. Similarly, N-acetylcysteine, a radical scavenger inhibited Fas and exogenous H_2O_2 induced cell death. Since incomplete proliferation signals seem to be a key event in apoptosis we tested whether the activation of the Ras, Rac2 and H_2O_2 pathway after Fas is accompanied by an inhibition of other signaling pathways involved in cell proliferation. Treatment of cells via the Fas-receptor abolished the stimulatory effect of anti-TCR/CD_3 on IP_3-synthesis and Ca^{2+}-release. Our results suggest that Fas induces programmed cell death by incomplete cell activation via Ras, Rac2 and H_2O_2 and inhibition of PLCγ, IP_3-synthesis and Ca^{2+}-release.

(Supported by DFG and American Diabetes Association)

B8-205 ANTIGEN NON-SPECIFIC, FAS INDEPENDENT APOPTOSIS OF IMMATURE THYMOCYTES INDUCED BY A THYMIC EPITHELIAL CELL LINE IS MEDIATED BY STEROIDS. Rina Guy#, Eitan Yefenof#, Eti Oron#, Noga Rosenheimer# and Yael Zilberman*, The Lautenberg Center for General and Tumor Immunology# and The Department of Pharmacology*, The Hebrew University, Hadassah Medical School, PO Box 12272, Jerusalem 91120, Israel.

We have studied antigen non-specific apoptosis of thymocytes induced by a thymic epithelial cell line (TEC). Immature, double positive thymocyes and thymic lymphoma clones were sensitive to TEC-induced apoptosis, while mature, single positive thymocytes and other thymic lymphoma clones were resistant. TEC induced apoptosis in thymocytes from mice of different MHC haplotypes. Thymocytes from Fas defective (MRL Ipr-/-) mice were also sensitive to TEC-induced apoptosis. Studies with [3H]-thymidine pre-labeled TEC-sensitive or TEC-resistant clones revealed that the TEC-resistant clone turned into a sensitive one provided that the unlabeled TEC-sensitive clone was present in the culture, suggesting mediation through a TEC-derived soluble mediator. TEC-induced apoptosis of the sensitive target clone was blocked by the glucocorticoid (GC) synthesis inhibitor aminogluthetimide, and by the GC-receptor antagonist RU38486. Thus, TEC induces apoptosis of double positive thymocytes in an antigen non-specific, MHC non-restricted, Fas independent manner. This TEC-induced apoptotic signal is mediated by steroid hormones.

B8-206 EXPRESSION OF THE CED-3-HOMOLOG NEDD-2, Georg Häcker and David L. Vaux, The Walter and Eliza Hall Institute, PO RMH, Victoria 3050, Australia

Mechanisms of physiological cell death have been conserved from primitive organisms to mammals. Programmed cell death in the nematode C.elegans has been extensively studied, and genes have been found to regulate the death of all 131 cells which die during normal development of the hermaphrodite. Physiological cell death in mammals also is regulated by an internal program and usually exhibits a typical morphological pattern termed apoptosis. The structural similarity of components of this system in different species suggested that a cell death pathway was conserved. This has been proven biochemically by transgenic expression of some of these genes in different species. When transfected into rodent cells both the C. elegans gene ced-3 and its mammalian homolog the cysteine protease interleukin-1-β–converting enzyme (ICE) are able to induce apoptosis. Another mammalian member of this family of proteases, nedd-2, had originally been cloned as a hybrid cDNA lacking the 5' part of the nedd-2 coding regions. We cloned a full length nedd-2 cDNA from a library constructed from a lymphoid cell line. The longest clone consists of a 3.5 kb molecule carrying an open reading frame of 1.35 kb. In vitro translation gives a protein of 50-55 kD. Expression of this clone in RAT fibroblasts leads to the death of the cells, suggesting that nedd-2 is also involved in the implementation of cell death. A second set of clones derived from the same library lacks the 5' part of the coding region but is identical in the 3' part and contains an in frame insertion of 153 nucleotides, possibly as a result of differential splicing. Expression of this variant after complementation of the 5' part by the 5' end of the full length clone does not kill RAT fibroblasts. To investigate its possible role as a modulator of cell death, we are generating cell lines stably expressing this variant. Northern blot analysis shows nedd-2-expression in a variety of normal murine tissues. The level of RNA-expression of neither ICE nor nedd-2 changes during the induction of apoptosis in two cell lines suggesting that the activity of the proteins is unlikely to be regulated at the level of gene expression. We are investigating the differential expression of the two variants in tissues and cell lines.

B8-207 COLORECTAL TUMOUR CELLS EXPRESS BCL-2 AND UNDERGO APOPTOSIS IN RESPONSE TO SHORT CHAIN FATTY ACIDS AND BILE SALT DEOXYCHOLATE, Angela Hague, Morgan Moorghen, Laurie W. Haynes, Douglas J. Elder, Diane J. Hicks and Christos Paraskeva, Department of Pathology and Microbiology, University of Bristol, Bristol BS8 1TD, U.K.

As BCL-2 is a survival factor in several tissues we examined colorectal tumour specimens and cell lines for BCL-2 protein. BCL-2 protein was detected in 19/22 adenocarcinomas, 12/13 adenomas, 6/9 carcinoma cell lines and 7/8 adenoma cell lines. BCL-2 was retained in metastases to regional lymph nodes (3/3 specimens) and in the cell line SW620 derived from a lymph node metastasis. BCL-2 deregulation may be a relatively early event in colorectal carcinogenesis and retention of BCL-2 expression in carcinomas and lymph node metastases may explain the resistance of colorectal tumours to chemotherapy. The incidence of colorectal cancer correlates with a high fat/low fibre diet. The short chain fatty acids acetate, propionate and butyrate are produced when dietary fibre is fermented by the colonic bacteria. We previously showed that in colorectal tumour cells apoptosis was induced by sodium butyrate at physiological concentrations, but not by $TGFß_1$. We extended the study to compare butyrate- induced apoptosis in 7 cell lines (3 adenoma, 4 carcinoma). Two carcinoma lines (PC/JW/FI and S/KS/FI) were more resistant to butyrate-induced apoptosis than the adenoma lines, suggesting that some carcinomas may evolve mechanisms to protect cells from induction of apoptosis. All three short chain fatty acids induced apoptosis in colorectal adenoma and carcinoma cell lines in a p53 independent pathway, but butyrate was most effective. Since these fatty acids result from bacterial fermentation of fibre, this may in part, explain the correlation between a high fibre diet and low colorectal cancer incidence. The bile salt deoxycholate is considered a tumour promoter in large intestine and its levels are reduced by dietary fibre. Significant induction of apoptosis was obtained using 0.5mM deoxycholate, raising the possibility that the tumour promoting action of deoxycholate may be by selecting for cell populations resistant to deoxycholate-induced apoptosis.

B8-208 EXPRESSION OF THE C-REL TRANSCRIPTION FACTOR AND ITS IκB INHIBITORY PROTEINS IN THE CHICK THYMUS. C. Huguet, F. Bouali, D. Stéhelin, B. Vandenbunder and C. Abbadie. Laboratoire d'Oncologie Moléculaire, Institut Pasteur de Lille, 1 rue Calmette, 59019 Lille cedex, France.

The chicken c-rel protooncogene encodes a transcription factor which is predominantly located in the cytoplasm of numerous cell types complexed with three ankyrin-repeat proteins of the IκB family, the p40, p115 and p124.

Various physiological functions have been attributed to cRel from regulation of normal hemopoiesis to induction of programmed cell death. We examined the expression of c-rel and its inhibitory proteins in the chick thymus , where lymphoid cells undergo differenciation or elimination by apoptosis

The patterns of expression of the four genes, evidenced by *in situ* hybridization, are almost similar. All four mRNAs are present both in the cortex and medulla, but more abundant in the medulla. Inside the medulla, the more intense signal is seen on cells exhibiting morphological features of dendritic cells. Thymocytes show only a low but significant level of expression. Some differences in the intensity of the signal appear between c-rel, p115 and p124 : c-rel mRNAs are less abundant than p115 and p124 mRNAs. p40 mRNAs accumulate also in dendritic cells and additionaly in single cells, probably macrophages, scattered in the cortex and medulla.

The nucleocytoplasmic distribution of cRel was studied by immunocytochemistry. The protein is mainly cytoplasmic in dendritic cells. Although c-rel mRNAs are not very abundant in macrophages, the cRel protein is easily detected both in the cytoplasm and in the nucleus of these cells. Therefore, the cRel protein seems to localize in the cytoplasm when the three inhibitors are expressed (dendritic cells) and both in the nucleus and cytoplasm when only p40 is highly expressed (macrophages). The cRel protein is not detected in the thymocytes.

In conclusion, in the chick thymus, cRel and its inhibitors are preferentially expressed in macrophages and dendritic cells. The function of cRel in these antigen presenting cells might be to activate the expression of MHC class II molecules. Indeed, MHC class II was shown to be a target of cRel. Clearly, thymocytes show only discrete expression of c-rel and its inhibitors. The possible activation of cRel when these cells undergo apoptosis is under study.

B8-209 THE COUPLING OF EVENTS IN APOPTOSIS. C.M.Ireland and S.M.Pittman. Children's Leukaemia and Cancer Research Centre, Prince of Wales Children's Hospital, Randwick, NSW, Australia.

In the normal mammalian cell tubulin nucleates from a single microtubule organising centre (MTOC) except during cell division. At S-phase the MTOC duplicates and migrates to the cellular poles where the spindle is formed. γ-tubulin has been shown to be required for nucleation of the MTs at the spindle. We have shown that tubulin reorganisation occurs during apoptosis and have found that the MTs involved nucleate from multiple MTOCs. In order to investigate the time course of events involving the tubulins and centrosomes during apoptosis Northern analysis of mRNAs and FCM and immunocytochemical analysis of proteins were performed using probes and monoclonal antibodies for α, β, and γ-tubulins and a centrosomal protein[1]. Apoptosis was induced in the CCRF-CEM leukaemia cell with methotrexate, dexamethasone and VP-16. During apoptosis Northern analysis has revealed that the earliest detectable change is an increase in γ-tubulin mRNA which occurs concurrently with multiplication of the centrosome and migration to the nuclear region in the cell. At this time β-tubulin protein levels are massively reduced in the treated cells with respect to the controls and immunocytochemical studies show that few cells are recognisably apoptotic. As more cells enter the process it can be seen that the microtubular stuctures which we have identified as occurring in apoptotic cells are formed, and β-tubulin mRNA levels then increase. Concomitantly, FCM analysis reveals increasing staining of the centrosomal protein followed by increasing β-tubulin immunofluorescence. Finally the formation of apoptotic bodies is apparent in the formation of a hypodiploid peak in the DNA histogram. Usually at this stage DNA fragmentation can be detected. Both centrosomal and β-tubulin protein levels are at their highest in the treated cells since the initial decrease. Our analysis suggests that events are coupled in the apoptotic process and that in these cells there is a defined pathway of events that is followed involving the same sequence of events in the same order. This sequence of events is independent of the mode of action of the drug used to induce apoptosis and independent of the stage of the cell cycle at which the cell enters apoptosis.

MAb1la138kindly provided by K.A.O.Ellem, QIMR, Brisbane.

B8-210 ***APPARENT REQUIREMENT FOR EXPRESSION OF THE C-JUN ONCOGENE IN CERAMIDE-INDUCED APOPTOSIS IN MYELOID LEUKEMIA CELLS.*** W. David Jarvis, Michael J. Birrer[1], and Steven Grant. *Medical College of Virginia, Richmond, VA 23298, and [1]National Cancer Institute, Bethesda, MD 20850.*

Generation of the lipid messenger ceramide through the sphingomyelin pathway mediates apoptotic cell death in several mammalian cell lines, including the human myeloid leukemia cell line U937. More recent effort has focused on the participation of the nuclear phosphoprotein c-Jun, the product of the c-*jun* oncogene, in the induction of apoptosis by ceramide. c-Jun is an essential component of the AP-1 transcription-activating complex. Treatment of U937 cells with exogenous sphingomyelinase (SM'ase; 100 mU/ml) for 6-hr induced apoptotic degradation of genomic DNA as indicated by resolution of (*a*) ~50-kbp DNA loop fragments by pulsed-field gel electrophoresis, and of (*b*) ~0.2- to 1.2-kbp oligonucleosomal DNA fragment ladders by static-field gel electrophoresis. Corresponding studies using quantitative spectrofluorophotometry demonstrated that 6-hr exposure to SM'ase (0.002-200 mU/ml) triggered the formation and release of apoptotic double-stranded DNA fragments in a concentration-dependent manner. Ceramide-related DNA damage was closely asociated with the expression of apoptotic cytoarchitecture. Transfection of U937 cells with a deletion mutant of c-*jun* (TAM-67) which lacks the transactivation domain (and thus suppresses c-Jun-dependent transactivation events) markedly altered susceptibility to ceramide. There was no evidence of DNA loop fragments or DNA fragment ladders on agarose gels on agarose gels following exposure of U937/TAM-67 transfectants to SM'ase, while the formation and release of DNA fragments as measured by spectrofluorophotometry was virtually abolished. In addition, SM'ase failed to elicit apoptotic morphology in these cells. These findings demonstrate that the apoptotic capacity of ceramide is substantially impaired in c-Jun dominant-negative transfectants; these studies support a role for c-Jun as an essential down-stream target for ceramide, suggesting that AP-1-mediated transcriptional activation is essential for ceramide-induced apoptosis in U937 cells.

B8-211 KAINIC ACID INDUCED DNA DAMAGE IS ASSOCIATED WITH A UNIQUE IMMEDIATE-EARLY GENE RESPONSE IN c-*fos*-*lac*Z TRANSGENIC RATS, G. M. Kasof, A. Mandelzys, T. Curran, and J. I. Morgan, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07710.

To discriminate between events that are products of seizures elicited by kainic acid (KA) and those that are specifically associated with its excitotoxic properties, we have examined the expression of cellular immediate-early genes (cIEGs) following KA or pentylenetetrazole (PTZ) treatment. While both chemoconvulsants elict seizures, only KA leads to neuronal damage as determined by the TUNEL (terminal transferase biotinylated-UTP nick end labeling) procedure. In order to unambiguously follow the expression of one of the prototypic cIEGs, c-*fos*, transgenic rats were generated that produce a Fos-lacZ fusion protein. Fos-lacZ was induced by either KA or PTZ; however, its expression was more protracted following KA. In addition, within 6 hours following KA treatment, Fos-lacZ expression appeared in the cytoplasm of neurons that were destined to die 1-2 days later, a situation never observed following PTZ. Examination of other basic zipper genes, revealed a unique immediate early response following KA. These features included: the expression of *fra*-1 and *fra*-2 in KA treated animals; protracted and differential expression of several cIEGs; and a biphasic elevation of c-*fos* and *jun*B in which the first peak was correlated with the initial seizure and then the second peak occuring just prior to the onset of cell death. These changes in cIEG expression were correlated with sustained increases in AP-1-like DNA binding activity in the hippocampus of KA treated rats. These features of the KA induced cIEG response suggest that there are unique AP-1 complexes that could have a critical role in leading to, or counteracting, neuronal death.

B8-212 EXPRESSION AND INVOLVEMENT OF C-JUN PROTOONCOGENE IN VP-16 INDUCED APOPTOSIS IN HUMAN LEUKEMIA CELLS, [1]Shiro Kataoka and [2,3]Takashi Tsuruo, [1]KIRIN BREWERY CO.,LTD. Pharmaceutical Research Laboratory. Miyahara-cho, Takasaki-shi Gunma, 370-12. Japan, [2]Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan. [3]Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan.

Human monocytic leukemia U937 cells express c-jun protooncogene when the cells are treated with DNA damaging stimuli such as DNA topoisomerase II inhibitor etoposide (VP-16), antimetabolite 1-β-D-arabinofuranosylcytosine (Ara-C), and uv irradiation. To examine the role of c-jun expression in response to DNA damage, we compared the expression in U937 cells to that in apoptosis-resistant UK711 cells which was previously established from U937 cells by mutagenesis. U937 cells expressed c-jun gene within 1 h of the end of treatment, and then they underwent apoptosis within next 1 h. In contrast, though UK711 cells were induced same level of topoisomerase II dependent DNA damage by VP-16, as were U937 cells, they did not express c-jun gene and did not undergo apoptosis. Therefore, c-jun expression was associated well with apoptosis inducing phenotype. These observations suggest that c-jun expression plays an important role in apoptosis rather than in repair against DNA damage.

B8-213 HTLV-I AND HIV-1 INDUCED LYMPHOCYTE APOPTOSIS: POSSIBLE CORRELATION WITH PATHOGENICITY, Thomas J Kindt, Michel Leno, Florence S. Bowers and R. Mark Simpson, Laboratory of Immunogenetics, NIAID Twinbrook II Facility, Rockville, MD 20852

A study of HTLV-I infected rabbit T cell lines indicated that rabbits inoculated with certain lines developed acutely fatal disease with signs similar to adult T cell leukemia lymphoma(ATLL). However, rabbits inoculated with most other HTLV-I cell lines became chronically infected but displayed no overt signs of disease. Thymuses from mature outbred rabbits inoculated with a lethal HTLV-I infected T cell line (RH/K34) showed morphological and biochemical evidence of apoptosis, whereas thymuses from rabbits inoculated with non-lethal lines showed no signs of apoptosis. In vitro exposure of rabbit or human PBMC to purified virus from the lethal, RH/K34, line caused rapid induction of apoptosis, mainly in the T cell population. By contrast, virus isolated from the non-lethal cell line mediated dose-dependent lymphocyte proliferation. Mechanisms of apoptosis by lethal HTLV-I remain unknown, but cell surface protein acquired by the virus membrane in the process of budding from the cell are suspected to play a role in lymphocyte apoptosis or activation mediated by the viruses. The fact that only certain HTLV-I lines cause disease in rabbits is analogous to human infection where only about 2% of HTLV-I infected individuals develop serious disease. The search for factors contributing to HTLV-I pathogenicity is facilitated by correlation of in vitro apoptosis with ATLL-like disease. Studies of HIV-1 show that exposure to infectious virus causes apoptosis of PBMC from human CD4 transgenic rabbits, but not of normal rabbit lymphocytes. Death of CD4- cells in the transgenic PBMC indicates a bystander effect in the cell killing. HIV-1 induced apoptosis is accompanied by upregulation of cell surface molecules including class II MHC, CD25 and fas.

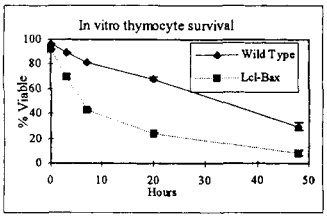
B8-214 SUPPRESSION OF APOPTOSIS BY SIGNALING THROUGH THE IL-3/GM-CSF RECEPTORS, Kinoshita, T.Yokota, T. Arai, K. Miyajima, A

DNAX Research Institute,901 California Ave., Palo Alto,CA94304 Institute of Medical Science, The Universty of Tokyo, Shirokanedai Minato-ku, Tokyo 108, Japan.

Interleukin 3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF) exert their biological functions through the specific receptor which consists of a ligand-specific α subunit and the shared common β subunit. Inhibition by genistein of a subset of IL-3 mediated signals including c-*myc* induction resulted in the abrogation of DNA synthesis; however, IL-3 still protected cells from apoptotic cell death. Conversely, a C-terminal truncated GM-CSF receptor, which is missing a critical cytoplasmic region required for the activation of Ras/Raf-1/MAPK pathway, induced DNA synthesis, but failed to prevent apoptosis in response to GM-CSF. However, expression of activated Ras protein complemented defective signaling through the mutant receptor and supported long-term proliferation in concert with GM-CSF. Interestingly, the activation of Ras signaling pathway resulted in the up-regulation of Bcl-2 expression. These results indicate that IL-3/GM-CSF prevent apoptosis of hematopoietic cells by activating a signaling pathway distinct from the induction of DNA synthesis, and that long-term proliferation requires the activation of both pathways. The Ras signaling pathway appears to play an essential role in the prevention of apoptosis in hematopoietic cells.

B8-215 EFFECT OF BAX ON THYMOCYTE DEVELOPMENT AND CELL DEATH, C. Michael Knudson, Debra T. Chao, and Stanley J. Korsmeyer. Department of Pathology and Medicine, Division of Molecular Oncology, Howard Hughes Medical Institute, Washington University School of Medicine St. Louis, MO 63110.

We have recently identified Bax based on it's association with the Bcl-2 oncogene. Bcl-2 has been shown to suppress cell death in many systems and Bax was able to counter the protective effects of Bcl-2 in a growth factor dependent cell line. In order to study the effect of Bax on thymocyte maturation and death, transgenic mice were derived which are engineered to express Bax in immature thymoctyes. Seven transgenic lines were initially identified. Several low expressing lines showed no obvious phenotype. However 3 lines have been identified in which Bax is highly expressed and the level of expression inversely correlates with the total number of thymocytes. In the highest expressing line, the transgenic mice contain <10% of the thymocytes in comparison to their wildtype littermates. Thus this finding is consistent with the Bcl-2 to Bax ratio being important in determing the "set point" or sensitivity of a cell to apoptosis. However, flow cytometry analysis illustrates that thymocytes from these mice retain their ability to mature to CD4 and CD8 single positive cells and migrate to the periphery. Although some of these mice have a decrease in the number of CD4+ thymocytes relative to their littermates and to the number of CD8 thymocytes. The explanation for this is under study. The figure shows the in vitro survival of thymocytes from wild type and transgenic animals. The data clearly illustrate that the Bax transgenic thymocytes have greatly accelerated cell death. Thus one explanation for the reduction in thymocyte numbers in the transgenic animals is that the normal life span of a developing thymocyte has been reduced. This would result in a shorter window for positivie selection. Studies are underway to test this hypothesis.



B8-216 EXPRESSION OF RECOMBINANT CLUSTERIN, A PROTEIN IMPLICATED IN APOPTOSIS, IN *Saccharomyces cerevisiae* and *Pichia pastoris*. Johnathon Lakins[1,2], Mark Wilson[3], Hailun Tang[1] and Martin Tenniswood[1,2], [1]W. Alton Jones Cell Science Center, Lake Placid, NY 12946, [2]Dept of Biochem., Univ. of Ottawa, Ottawa and [3]Univ. Wollongong, Wollongong, NSW, Australia.

Clusterin is a secreted glycoprotein expressed as a single chain polypeptide and matured by limited proteolysis between N205 and S206 during secretion in the human to generate a disulfide linked dimer of α and β subunits. It has been shown to be upregulated in response to cell injury in general and in particular in several models of active cell death (ACD). Human clusterin has been shown to bind a variety of other macromolecules including ApoA1/HDL, IgG, the cell free form of β amyloid protein, paraoxanase, and components of the terminal attack complex of complement. To study the relation between these binding activities and the role of clusterin in ACD we have expressed the human clusterin in *Saccharomyces cerevisiae* and *Pichia pastoris*. In *S.cerevisiae* the protein has been expressed as a galactose inducible gene in the pYes2 vector (Invitrogen) driven by the Gal 1-10 promoter. Human clusterin is expressed predominately as a secreted N-glycosylated 64 kDa protein. Minor immunoreactive bands between 21 and 31 kDa under reducing conditions indicates that there is limited proteolysis between the two disulfide linked subunits but the majority of the immunoreactivity remains associated with the 64 kDa band demonstrating that most of the protein remains uncleaved. In *P.pastoris* the protein has been expressed as a methanol inducible gene in the pHIL-D2 vector, driven by the Aox1 promoter. Expression is 100 to 1000 fold higher than in *S.cerevisiae* (approximately 10 to 10^2 μg/ml). The *P.pastoris* recombinant protein appears as a series of distinct bands between 45 and 67 kDa under non reducing conditions that are nearly quantitatively cleaved to products between 25 and 37 kDa under reducing conditions. Using N terminal sequencing of PVDF blots we have shown that there are apparently only two sites of processing, of similar primary structure, in the β subunit, the authentic site between N205 and S206 and a second between H241 and S242. Only the N terminus produced by signal peptidase cleavage has been detected for the α subunit, though the existence of low M, polypeptides detected by α subunit specific mAb G7, 78E, and 41D after N-glycanse treatment suggests that processing sites exist in the C terminus of this subunit. Despite these additional sites of cleavage we demonstrate that all secreted recombinant clusterin is a dimer of α and β subunits and that the stability of the α chain to processing events appears to depend upon the choice of cleavage site in the β chain.

(Supported by the Medical Research Council of Canada)

B8-217 **Regulation of the Bcl-2 Gene in Normal Human B Cells** Georgina Lang, Wendy Gombert, Hannah Gould. Randall Institute, Kings College London, 26-29 Drury Lane, London, WC2B 5RL

The Bcl-2 protein plays a major role in apoptosis , rescuing cells from apooptotic cell death by an as yet undetermined mechanism. DNaseI hypersensitive mapping of the 5' region of the Bcl-2 gene (1) has identified a single site, DNaseI hypersensitive site 8, (Dhs8) which is observed in B cells which express Bcl-2 but not in B cells which do not. We have confirmed the mapping of Dhs8 in B cells from human tonsil germinal centres. Footprint analysis of Dhs8 will determine the protein binding site(s) within this region of the Bcl-2 gene . It can then be determined whether protein(s) binding to Dhs8 are known or novel regulatory protein(s). Once the gene(s) for any novel regulatory protein(s) have been cloned, cell transfection studies and the production of transgenic animals will allow the function of such binding protein(s) in relation to Bcl2 gene expression and apoptosis to be analysed.

1 Young, R. L. & Korsmeyer, S.J. (1993). A Negative Regulatory Element in the bcl-2 5' Untranslated Region Inhibits Expression from an Upsteam Promoter. Mol. Cell. Biol., **13**, 3686-3697.

B8-218 **ISOLATION OF MODULATED GENES DURING PROGRAMMED DEATH OF A CLONAL INVERTEBRATE BY DIFFERENTIAL mRNA DISPLAY**, Robert J. Lauzon[1,2] and Wen-Teh Chang[2], [1]Department of Pediatrics, [2]Department of Microbiology, Immunology & Molecular Genetics, Albany Medical College, Albany, NY 12208

Programmed cell death is a fundamental physiological event in which cells actively participate their own destruction. In the lifecycle of a clonally-modular urochordate *Botryllus schlosseri* (Tunicata, Ascidiaceae), individual organisms (zooids) with identical genetic backgrounds die in cyclical fashion principally by apoptosis in a 24 hour period, a process called **takeover**. This animal model provides an exceptional study system of programmed cell and organismal death by providing large quantities of synchronized biological material amenable to molecular characterization. Preliminary evidence indicating that genes are modulated during takeover has been demonstrated by two-dimensional protein gel electrophoresis of *in vitro* translated mRNAs from various developmental stages of the animal. The distinct profiles of translated proteins observed within specific stages strongly suggest that changes in gene expression are associated with the early stages of zooid death in this organism. We have attempted to identify genes whose expression patterns are modulated during takeover using **differential mRNA display**, and have isolated several putative cDNA fragments with defined primer sets. In order to confirm developmentally-regulated expression of these clones, slot and northern blot analysis of ^{32}P-labelled cDNA fragments were carried out using RNA from various developmental stages. Two of the isolated clones (GAP1/790.3 and GAP1/790.4) were selectively down and upregulated respectively throughout takeover. Collectively, these results suggest that zooid regression is accompanied by modulated gene expression. Further characterization of these genes is currently underway in our laboratory.

B8-219 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN(TCDD)-AND DEXAMETHASONE (DEX)-INDUCED APOPTOSIS IN GROWTH FACTOR-DEPENDENT AND RESISTANCE IN AUTONOMOUS RAT Nb2 LYMPHOMA CELLS. MA Leff, H Liang, DJ Buckley, JT McCormack[1], M Friederichs[1], and AR Buckley, Pharmacology & Toxicology and [1]Anatomy & Cell Biology, UNDSM, Grand Forks, ND, USA, 58202.

TCDD and DEX are immunotoxic agents which induce T lymphocyte lysis subsequent to activation of apoptosis. Utilizing a growth factor (prolactin, PRL or interleukin-2, IL-2)-dependent T lymphoma cell line (Nb2-11) and a growth factor-independent subline (Nb2-SFJCD1) as a T cell model system, the mechanisms which underlie TCDD- and DEX-induced apoptosis have been investigated. Treatment of growth-arrested Nb2-11 cells with either TCDD (50 nM) or DEX (100 nM) rapidly induced DNA fragmentation, determined by agarose gel electrophoresis and quantitated using the diphenylamine reagent. Moreover, analysis of fragmented DNA by *in situ* 3´-end labeling revealed that each of these compounds induced morphological characteristics of active cell suicide including the formation of "apoptotic bodies" within 1 hr. Importantly, addition of maximally mitogenic concentrations of PRL (10 ng/ml) or IL-2 (10 U/ml) to Nb2-11 cultures completely abrogated all apoptotic characteristics provoked by TCDD or DEX. In contradistinction, the Nb2-SFJCD1 subline was completely resistant to activation of programmed cell death by either agent even when each was added at suprapharmacological concentrations. Since the protooncogene, bcl-2, has been previously reported to protect against the consequences of apoptosis, its expression was assessed in each of the cell lines treated with TCDD or DEX alone or in combination with PRL. Notably, bcl-2 mRNA was undetectable by northern blot analysis in either of the cultured cell lines, irrespective of the treatment protocol. However, an 8.5 kb bcl-2 species could clearly be distinguished in RNA preparations obtained from lymphocytes from chronic lymphocytic leukemia patients included as a positive control. We conclude that: (1) TCDD and DEX induce apoptosis in growth factor-dependent Nb2-11 lymphoma cells; (2) stimulation of mitogenesis by PRL or IL-2 blocks activated cell death mechanisms; (3) mutations which confer autonomous growth may also alter normal apoptotic pathways; and (4) suppression of apoptosis induced by PRL or IL-2 in Nb2-11 and resistance in Nb2-SFJCD1 cultures is most likely independent of bcl-2 expression. Supported in part by DK44439 from the NIH.

B8-220 ROLE OF PROTEIN KINASE C (PKC) IN REGULATION OF RAT HEART VASCULAR SMOOTH MUSCLE CELL (SMC) PROLIFERATION AND APOPTOSIS, Dariusz Leszczynski* & Marie L. Foegh**, *Department of Research, Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland and **Department of Surgery, Georgetown University Medical Center, Washington, DC 20007, USA.

In a previous study (Am. J. Pathol, in press) we found SMC proliferation and apoptosis to be regulated by PKC. Inhibition of PKC activity caused a decline in SMC proliferation and induced apoptosis. The PKC-regulated apoptosis appeared to be accompanied by a decline in expression of oncoprotein bcl-2 but not by nucleosomal fragmentation of DNA. Also, no change in the expression of proliferating cell nuclear antigen (PCNA) was observed following PKC inhibition, even though decline in cell proliferation was observed.

Here, using Western blot we determined that the rat heart coronary vascular SMC express the following isozymes of PKC: - α, - γ, - δ , - ε but do not express isozymes - βI, - βII, - η, - ζ, and - Θ. PKC- α was shown previously to be involved in regulation of cell cycle of SMC (Sasaguri et al. Exp. Cell Res. 208, 1993, 311) thus we examined whether the antisense tio-oligonucleotide directed against rat PKC- α (AS-PKC- α; GTAAACGTCAGCCAT) will affect SMC proliferation and/or apoptosis. Exposure of SMC to 100μM AS-PKC- α caused 36% decline in cell proliferation during 3-day treatment, this was accompanied by significant decline in the expression of PCNA as assessed by Western blot. In spite of the decline in SMC proliferation, the morphological apoptotic cells were not induced. We did not detect any DNA cleavage in the SMC exposed AS-PKC- α by *in situ* hybridization. Due to its mean of action, the response of cells to AS-PKC- α might be delayed as compared with direct inhibition of PKC activity or PKC- α may regulate cell proliferation but not apoptosis. Longer treatment periods with AS-PKC- α are currently being examined.

B8-221 REGULATION OF SIGNALLING PATHWAYS STIMULATED BY ENGAGEMENT OF THE *FAS* ANTIGEN, by Steven D. Levin, Jennifer L. Mitcham, Robert E. Miller, Teresa W. Tough, Mark R. Alderson, Peter Baum, and David H. Lynch, Immunex Corporation, 51 University Street, Seattle, WA.

The *fas* antigen is expressed on a wide variety of cell types including cells of myeloid and lymphoid origin. *Fas* engagement with monoclonal antibodies has varied effects depending on the cell. Chronically activated T cell clones and most transformed cell lines that express *fas* die by an apoptotic mechanism upon antibody engagement. Primary T cells, on the other hand, do not die in response to anti-*fas* antibody treatment, and in fact when anti-*fas* antibodies are included with sub-optimal concentrations of antibodies to the T cell antigen receptor, resting T cells respond more vigorously than with anti-T cell receptor antibodies alone. Thus, by this criteria, the *fas* antigen is also a co-stimulatory molecule on T cells. While something is known of the biological consequences of *fas* engagement by antibodies or by its cognate ligand, very little is known of the intracellular signalling pathways that produce these events, or how signals originating from one receptor can be alternatively interpreted by different cells of the same lineage. To investigate this, we used the yeast two-hybrid system to look for molecules capable of interacting with the cytoplasmic domain of the *fas* antigen, reasoning that such molecules would be involved in the signalling cascade. This approach has lead to the cloning of two novel, *fas*-interacting proteins, designated fip1 and fip2. The role of these proteins in signalling through the fas antigen is currently under investigation.

B8-222 REGULATION OF CELL GROWTH AND APOPTOSIS IN HUMAN B LYMPHOCYTES BY RAR-, NOT RXR-LIGANDS.

Jon Lømo[1], Erlend B. Smeland[1], Rune Blomhoff[2], Uma Gandhi[1], Marcia I. Dawson[3], and Heidi Kiil Blomhoff[1], [1]Department of Immunology, The Norwegian Radium Hospital, [2]Institute for Nutrition Research, University of Oslo, Oslo, Norway, and [3]Life Science Division, SRI International, Menlo Park, CA 94025.

We have previously shown that retinoids potently inhibit activation of human peripheral blood B lymphocytes. In the present study, the mechanisms of this effect have been explored using ligands specific for the retinoic acid receptor (RAR) and retinoid X receptor (RXR). We found that the RAR-specific ligand TTAB inhibited anti-IgM-induced activation, as measured by a reduction in DNA synthesis and entry into the G1 phase of the cell cycle (7-actinomycin D-staining). Both effects paralleled those of all-trans retinoic acid (RA). In contrast, the RXR-specific lignad SR11217 had no effect on these parameters. We also examined the action of the receptor ligands on apoptosis, which occurs spontaneously in unstimulated cells. Interestingly, TTAB and all-trans RA significantly inhibited cell death, as demonstrated by vital dye staining and DNA end labeling. Again, SR11217 had no effect. 9-cis RA, which is considered to be the natural ligand for RXR, although it also binds to RAR, was only a weak inhibitor of B cell activation, but counteracted apoptosis as efficiently as all-trans RA and TTAB. In conclusion, retinoic acid both inhibits activation and stimulates the survival of peripheral blood B lymphocytes, and these effects appear to be mediated via nuclear RARs, not RXRs.

B8-223 COMBINED TNF-α/IFN-γ CAN INDUCE APOPTOSIS IN WERI-Rb1 BUT NOT Y-79 RETINOBLASTOMA CELLS. Michele C. Madigan, R. Max Conway. Department of Clinical Ophthalomology, University of Sydney. NSW 2006. Australia.

Retinoblastoma (RB) is the most common intraocular malignancy of childhood, with an average incidence of 1:15,000 live births. As the only established neuronal-derived tumor of the retina, RB is an important model for the study of neural malignancy. While TNF-α has been shown to arrest the growth of neuroblastoma cells and possibly promote neural differentiation, the effects of cytokines on cell growth, apoptosis and differentiation in RB have not been fully studied.

Y-79 and WERI-Rb1 cells were incubated in the presence of varying concentrations of TNF-α and IFN-γ alone and combined for up to 120 hours. Apoptotic cell death was quantified at regular intervals in acridine orange/ethidium bromide stained samples, and further assessed with transmission electron microscopy (TEM) and DNA electrophoresis. Cell viability was assessed by trypan blue exclusion. The effects of pre-treatment with either cycloheximide or actinomycin D on TNF-α/IFN-γ -induced apoptotic involution were also assessed.

Treatment of the 2 cell lines with either TNF-α or IFN-γ alone (100U/ml or 1000U/ml) did not induce levels of apoptotic cell death above those observed in control cultures. However, when cells were incubated with combinations of TNF-α and IFN-γ (10U/ml to 10000U/ml), dose-dependent apoptotic involution was induced in the WERI-Rb1 cells accompanied by a significant decrease in cell viability. Increased levels of apoptotic involution were not obvious in Y-79 cells at any of the concentrations used, although cell viability was significantly decreased compared to controls. TEM confirmed the presence of cells with morphological features of apoptosis in treated WERI-Rb1 cells. When WERI-Rb1 cells were pre-treated with cycloheximide or actinomycin D, the incidence of apoptotic cell death was reduced; Y-79 cells did not appear to be affected.

This study suggests differences in the susceptibility of the 2 cell lines to TNF-α and IFN-γ-induced apoptosis, and indicates a role for cytokine interactions in mediating RB tumor growth and differentiation, and perhaps tumor regression via apoptosis.

B8-224 COMPLEMENTATION AND CELL DEATH REGULATION BY BCL-2, P53 AND C-MYC DURING *IN VIVO* LYMPHOMAGENESIS. Maria C. Marin, Brenda Hsu and Timothy J. McDonnell. Department of Molecular Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030

Carcinogenesis is a multistep process resulting from the accumulation of genetic lesions within the cell. These genetic lesions will alter the rate at which the cells proliferate as well as the susceptibility of these cells to undergo apoptosis. The most frequent targets for genetic alterations in human lymphomas are: bcl-2, p53 and c-myc, genes known also as regulators of apoptosis. The bcl-2 gene was characterized by its association with the t(14;18)(q32;q21) of follicular lymphoma. Bcl-2-immunoglobulin transgenic mice initially exhibit a polyclonal hyperplasia due to the overriding of apoptosis by deregulated bcl-2. After a latency period of 15 months, 20% of the animals developed clonal lymphomas. Approximately, 50% of these high grade lymphomas possess chromosomal translocations involving c-myc. In order to assess the complementation of myc and bcl-2 we generated myc/bcl-2 double transgenic mice. Simultaneous bcl-2 and c-myc deregulation resulted in accelerated lymphomagenesis. The basis for this complementation resulted from the ability of bcl-2 to inhibit myc induced apoptosis. A PCR-SSCP analysis of bcl-2-Ig lymphomas revealed that p53 mutations were uncommon in these tumors, however, p53 mutations occur commonly in Eμ-myc lymphomas. To assess the extent of complementation between p53 inactivation and c-myc or bcl-2 gene deregulation, p53 "knockout" mice were bred to either bcl-2-Ig or Eμ-myc mice in order to produce hybrid mice. Our findings suggest that bcl-2 and p53 are regulators of a common cell death pathway and do not genetically complement during lymphomagenesis. Furthermore, deregulated myc expression and p53 inactivation result in extremely rapid tumor onset. The basis of this complementation will be discussed.

B8-225 GRANULOCYTE-TYPE-SPECIFIC MODULATION OF APOPTOSIS BY GLUCOCORTICOIDS

L.C. Meagher, J.R. Seckl* and C. Haslett
Respiratory Unit, Dept. Med., Univ. Edinburgh, EH8 9AG and *Mol. Endocrinology Lab., Dept. Med., Western Gen. Hosp., Edinburgh EH4 2XU, U.K.

Eosinophils (EO) and neutrophils (PMN) are closely related, terminally differentiated cells which undergo constitutive cell death by apoptosis *in vitro*. Preparations of EO (>99% pure) from non-asthmatic donors were 30.5±11.5% (n=11) apoptotic following overnight culture while PMN cultures (>98% pure) were 73.2±9.2% (n=6) apoptotic. Addition of 1μM dexamethasone (Dex) to parallel cultures increased EO apoptosis by 220±53.1% (significant $p<0.001$, paired t-test) but decreased PMN apoptosis by 55.2±12.8% ($p<0.001$). Similar results were obtained with the endogenous glucocorticoid cortisol and apoptosis was not affected by the sex steroid progesterone. The glucocorticoid receptor antagonist RU38486 did not alter constitutive rates of apoptosis in EO or PMN but completely abolished Dex-induced up-regulation of EO apoptosis and Dex-induced inhibition of PMN apoptosis, indicating that Dex is acting to modulate apoptosis via the glucocorticoid receptor. This is the first description of a single agent which promotes apoptosis in EO while inhibiting PMN apoptosis and thus presents a novel opportunity to the study of the control of apoptosis in these closely-related cell types.

This work is supported by the National Asthma Campaign.

B8-226 INVOLVEMENT OF RAF-1 IN THE PROTEIN KINASE C APOPTOTIC SIGNALING PATHWAY IN COLO205 HUMAN COLON CARCINOMA CELLS.

Ana T. Menendez, Michael Hoffmann*, and Robert Kramer. Department of Tumor Biology, Medical Research Division, American Cyanamid Company, Pearl River, NY 10965 and *NY Medical College, Department of Microbiology/ Immunology, Valhalla, NY 10595.

We have previously identified 5 human tumor cell lines that were ~300 fold more sensitive to growth inhibition by phorbol esters (TPA) and other PKC agonists than the additional 21 cell lines tested. Sensitivity correlated with elevated PKC levels and activation rather than down-regulation of PKC. (Menendez, et al., Keystone Symposia on PKC, Taos, NM, March, 1994). The mechanism of TPA sensitivity was studied using TPA sensitive Colo205 (IC50=13 ng/ml) and resistant LS174T (IC50=4,600 ng/ml) colon carcinoma cell lines. Quantitative isotyping of TPA responsive PKC's showed that both cell lines expressed the same PKC isotypes but that Colo205 expressed significantly more α, β1, ε, and γ PKC, whereas LS174T expressed more β2PKC. δPKC and non-TPA responsive ιPKC and ζPKC were equivalent in both cell lines. 15 minutes following TPA exposure, raf-1 activation was detected using a gel shift hyperphosphorylation assay and apoptotic DNA ladders were evident after 24 hours in the sensitive Colo205 cells. Raf-1 hyperphosphorylation and apoptosis did not occur in the resistant LS174T cells. However, treatment with the phosphatase inhibitor okadaic acid resulted in raf-1 activation and apoptosis in both Colo205 and LS174T cells. These results suggest that PKC may induce an apoptotic pathway by directly activating raf-1. This may be dependent upon the intensity of the PKC-raf-1 activation signal.

B8-227 PROGRAMMED CELL DEATH IN HIGHER PLANTS,

Ron Mittler and Eric Lam, AgBiotech Center and Department of Plant Sciences, P.O. Box 759, Waksman Institute, Rutgers The State University of New Jersey, Piscataway, NJ 08854, USA.

In plants, programmed cell death (pcd) is thought to occur during the hypersensitive response (HR) to certain avirulent pathogens and in the course of several differentiation processes. We describe two model systems for studying pcd in higher plants. The first is a transgenic model system that mimics the pathogen-triggered activation of HR. In this model system, expression of a bacterial proton-pump in transgenic tobacco plants activates a HR pcd pathway, in the absence of a pathogen. Transgenically triggered HR was coordinated with the activation of multiple defense mechanisms and the induction of systemic resistance against a variety of pathogens. The second system involves the differentiation of vascular tissue in higher plants. In this model system pcd was coordinated with differentiation of primary xylem elements in pea roots. Onset of pcd occurred in elements that contained mature, lignified secondary cell wall structures and preceded elongation of the mature element. We further demonstrate that pcd in higher plants is likely to involve the degradation of nuclear DNA, one of the hallmarks of apoptosis in animal cells.

B8-228 ANTAGONISM OF p53 FUNCTION BY HIV-1 TAT, Cindy A. Morris, Barbara Ensoli*, Ming Lu and Aizen J. Marrogi, Department of Pathology, Tulane University School of Medicine, New Orleans, LA 70112; Laboratory of Tumor Cell Biology*, NCI/NIH, Bethesda, MD 20892

The interaction between viral regulatory proteins and the tumor suppressor protein, p53, and the dysregulation of wild-type p53 function by these viral proteins may be important in regulation of viral replication and in mediation of virus-associated malignancies. The ability of p53, which is mutated or aberrantly regulated in many human cancers, to suppress tumorigenesis by mediating growth arrest and apoptosis appears to correlate with its function as a transcriptional regulator. Preliminary data coupled with recent reports suggest that Tat, an essential HIV-encoded regulatory protein that acts as a potent transactivator of HIV gene expression and replication and as a paracrine growth factor for AIDS-associated Kaposi's sarcoma (AIDS-KS), may have a novel role as a functional antagonist of p53 in AIDS-associated malignancies. Immunohistochemical analyses of macular, papular and nodular stage AIDS-KS lesions obtained as fresh punch biopsies from AIDS patients demonstrate the presence of Tat in spindle cells and infiltrating mononuclear cells within the AIDS-KS lesions. Additionally, spindle cells derived from AIDS-KS lesions express significant cytoplasmic levels of the cellular oncogene, Bcl-2, a known antagonist of p53-mediated apoptosis, as well as nuclear expression of p53. A recent report suggesting Tat-associated induction of Bcl-2 expression provides indirect evidence, along with preliminary data to be presented, that Tat may antagonize p53-mediated apoptosis and/or growth arrest within AIDS-KS cells through upregulation of Bcl-2. Other preliminary data demonstrating that Tat abrogates transcriptional effects of p53 regulation suggest that Tat may antagonize the tumor suppression function of p53 by several mechanisms. The overall objectives of this study are to elucidate the molecular mechanism(s) by which Tat antagonizes wild-type p53 function in AIDS-associated malignancies, in general, and to determine how such mechanisms may be operative in the initiation and/or progression of the AIDS-associated neoplasia, KS.

B8-229 REGULATION OF THE HUMAN PROLIFERATING CELL NUCLEAR ANTIGEN PROMOTER BY THE p53 TUMOR SUPPRESSOR PROTEIN. Gilbert F. Morris, Jin Xu, and Weihong Lei. Department of Pathology and Laboratory Medicine SL-79, 1430 Tulane Avenue, Tulane University Medical Center, New Orleans, LA 70112

In human cancers, frequent mutations in the gene encoding the p53 tumor suppressor protein demonstrate the importance of p53 in control of cell growth and suppression of neoplastic transformation. The preponderance of data indicates that the regulatory activities of p53 are related to its function as a transcription factor. Therefore, relevant target genes for the transcriptional regulatory functions of p53 must be identified to clearly understand how p53 controls cell growth and suppresses neoplastic transformation. The gene encoding the proliferating cell nuclear antigen (PCNA) is a likely candidate for a cellular gene that is regulated by p53. PCNA is a highly conserved protein that performs an essential function in DNA replication and repair as an auxiliary factor for DNA polymerase δ. Consistent with its role in DNA repair, PCNA is induced concomitantly with increasing p53 levels in human skin cells exposed to UV irradiation. In addition, PCNA can be found in the same complex with cyclins, cyclin-dependent kinases (Cdk), and the recently identified p53-regulated inhibitor of Cdks, Pic 1.

Our data indicate that, like Pic 1, the PCNA promoter contains sequences that bind and mediate transcriptional activation by p53 in transient co-expression assays. However, higher levels of p53 expression fail to activate or repress the PCNA promoter. We propose that the differential response of the PCNA promoter to increasing amounts of p53, reflects the functions of p53 as an inducer of DNA repair and programmed cell death. To manipulate levels of p53 expression in a physiologically relevant manner, a rat fibroblast cell line (CREF) is exposed to increasing amounts of γ-radiation. In accord with our hypothesis, a PCNA promoter construct with the p53-binding site intact is activated by irradiation, whereas a similar construct with the p53-binding site deleted is not. Activation of the PCNA promoter by radiation via an intact p53-binding site is consistent with induction of a DNA repair gene by p53. Furthermore, the E1A oncoprotein from adenovirus induces apoptosis and represses the PCNA promoter by a mechanism that appears to be associated with wild-type p53 activity. The long term goal of these experiments is to correlate p53 regulation of the PCNA promoter and the activity of p53 as a regulator of cell growth and cell death.

B8-230 DEFECTIVE EXPRESSION OF HEMATOPOIETIC CELL PROTEIN-TYROSINE PHOSPHATASE (HCP) BLOCKS FAS-MEDIATED APOPTOSIS. John D. Mountz, Tong Zhou, Zheng Wang, PingAr Yang, Richard S. Jope* and Xiao Su, Department of Medicine, The University of Alabama at Birmingham, Division of Clinical Immunology and Rheumatology, *Department of Psychiatry and Behavioral Neurobiology and the Birmingham Veterans Administration Medical Center, 473 LHRB, UAB Station, Birmingham, AL 35294-0007.

Protein tyrosine dephosphorylation after Fas crosslinking occurred in Fas apoptosis sensitive CEM-6 cells but not in Fas apoptosis resistant MOLT-4 cells, and apoptosis in the CEM-6 cells could be inhibited by the protein tyrosine phosphatase inhibitor, pervanadate. The time course and level of dephosphorylation were correlated with increased hematopoietic cell protein-tyrosine phosphatase (HCP) activity, but not with the activity of two other tyrosine phosphatases. The level of expression of HCP was correlated with Fas apoptosis function in 11 human and murine Fas positive lymphoid cell lines. Expression of recombinant HCP in the MOLT-4 cell line converted this Fas apoptosis resistant cell line to Fas apoptosis sensitive. HCP-mutant me^v/me^v mice exhibited increased expression of Fas but decreased Fas-mediated apoptosis function in lymphoid organs after anti-mouse Fas antibody treatment *in vitro* and *in vivo*. Thus, HCP-mediated protein dephosphorylation is involved in the delivery of the Fas apoptosis signal in the immune system.

B8-231 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.

Experiments with p53 and E1A in fibroblasts have shown that p53-dependent apoptosis results when cells are given conflicting signals to arrest and grow. To examine if this is occurring in murine thymocytes, we are taking several approaches to study the cell cycle status of normal and p53-deficient thymocytes before and after treatment with radiation. Flow cytometry experiments have shown modest differences in cell cycle induction. We are also analzying changes in mRNA expression following irradiation and assaying cyclin-dependent-kinase activity in these cells as a possible indicator of cell cycle entry or progression.

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 predict that decreased rearrangement in the Rag-deficient animals will reduce the tumor incidence, whereas the combination of *scid* and p53 mutations will lead to an increase. These mice will be monitored for frequency of thymic tumor development and thymocytes from these mice will be used to study apoptosis as outlined above.

B8-232 TRANSCRIPTION STIMULATION OF THE FAS-ENCODING GENE BY NF-IL6 UPON INFLUENZA VIRUS INFECTION, Y. Nakanishi, N. Wada, M. Matsumura, Y. Ohba, N. Kobayashi# and T. Takizawa†, Fac. Pharm. Sci., Kanazawa Univ., Kanazawa, #Yamaguchi Univ. Sch. Med., Ube, and †Inst. Dev. Res., Aichi Pref. Colony, Kasugai, Japan

We previously found that the amount of Fas, a cell surface receptor for an apoptosis signal, increases at the mRNA level in influenza virus-infected HeLa cells prior to the subsequent cell death by apoptosis. Here we investigated the mechanism of the activation of Fas-encoding gene expression upon influenza virus infection. The 5'-upstream region of the human Fas gene contained the binding site of NF-IL6, also known as C/EBPß, that was repeated five times in a 1.3-kbp DNA region. 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 two to threefold upon influenza virus infection. Gene expression driven by the Fas promoter was activated when a DNA expressing human NF-IL6 was co-transfected. Moreover, the DNA-binding activity of NF-IL6 gained a threefold increase by infection of the virus or the treatment with poly(I)·poly(C), a synthetic double-stranded RNA, whereas the amount of NF-IL6 seemed unchanged by either treatment. All the above results strongly 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.

B8-233 **INDUCTION OF APOPTOSIS BY VITAMIN D IN MCF-7 CELLS IS POTENTIATED BY THE ANTI-PROGESTIN ONAPRISTONE.** CJ Narvaez and JE Welsh, W. Alton Jones Cell Science Center, Lake Placid, NY 12946

We have shown that $1,25(OH)_2D_3$, the active metabolite of vitamin D, induces morphological and biochemical markers of apoptosis (chromatin and nuclear matrix condensation, up-regulation of TRPM-2/clusterin and cathepsin B, and down-regulation of bcl-2) in MCF-7 breast cancer cells. These cells are estrogen dependent and also undergo apoptosis in response to anti-estrogens. Since anti-estrogens down regulate progesterone receptors in MCF-7 cells, induction of apoptosis may involve disruption of progesterone signalling. In these studies, we assessed whether the anti-progestin onapristone (Schering AG) induces apoptosis or augments the effects of $1,25(OH)_2D_3$ in MCF-7 cells. Cells were treated with 100nM $1,25(OH)_2D_3$ ± 1µM onapristone for 48 hours prior to assessment of morphological and biochemical indices of apoptosis. Although onapristone alone does not modulate MCF-7 cell number under these conditions, this anti-progestin potentiates the induction of apoptosis by $1,25(OH)_2D_3$. Cells treated with onapristone and $1,25(OH)_2D_3$ display condensed cytoplasm and pyknotic nuclei typical of apoptosis. In addition, cells treated with onapristone and $1,25(OH)_2D_3$ exhibit higher levels of clusterin/TRPM-2 than cells treated with onapristone alone. These data indicate that anti-progestins may increase the sensitivity of hormone dependent breast cancer cells to activation of the apoptotic pathway by vitamin D compounds. *Supported by AICR and the WAJCSC.*

B8-234 A BCL-2-RELATED PROTEIN OF ASFV ENHANCES APOPTOTIC CELL DEATH, J.G. Neilan, L. Zsak, E. Caler, G.F. Kutish, T. Burrage and D.L. Rock, Plum Island Animal Disease Center, USDA, ARS, Box 848, Greenport, NY 11944

We previously identified an ASFV gene (5-HL) encoding a 21 kDa protein which shared a high degree of similarity to the family of bcl-2-related proteins, members of which both positively and negatively modulate apoptosis (J Virol 67:4391). The 5-HL gene was present and the p21 protein expressed in all ASFV isolates examined, including both low passaged field isolates and highly monkey cell-culture-adapted viruses, the latter which no longer grow in the natural target cell, swine macrophages. The conservation of this gene among diverse ASFV isolates suggests an essential role for p21 in some aspect of ASFV-cell interaction. To examine 5-HL gene function, the gene was cloned and expressed in baculovirus (Bv). Infection of Sf21 insect cells with a recombinant baculovirus expressing p21 (BvHL) resulted in a dramatic and highly significant decrease in cell viability from 24 to 96 h post infection (PI) when compared to cells infected with wild type baculovirus. BvHL infected cell lysates at 48 and 72 h PI showed DNA fragmentation with extensive DNA laddering that was absent in baculovirus infected cells. Additionally, BvHL infected cells at 48 h PI had a 7-fold greater incidence of ultrastructural nuclear changes which included extensive chromatin margination when compared to baculovirus infected cells. These data indicate that p21 accelerates cell death by an apoptotic pathway when expressed in baculovirus.

B8-235 INTERLEUKIN-1β CONVERTING ENZYME (ICE) ACTIVITY IS NOT REQUIRED FOR MACROPHAGE KILLING MEDIATED BY ACTIVATED CYTOLYTIC T CELLS OR BY EXTRACELLULAR ATP. Michelle Nett-Fiordalisi, Kevin Tomaselli, and David D. Chaplin, Department of Internal Medicine and the Howard Hughes Medical Institute, Washington University, St. Louis, MO 63110 and IDUN Pharmaceuticals, San Diego, CA 92121.

Production of mature, active IL-1β, a potent mediator of immune and inflammatory responses, is regulated by the IL-1β converting enzyme (ICE). ICE is a cysteine protease which cleaves inactive, precursor murine IL-1β at Asp^{117}-Val^{118} to generate mature IL-1β. In previous studies we had shown that mature IL-1β is generated and released from LPS-stimulated apoptotic macrophages. Recently, it was reported that ICE showed 23% homology with ced-3, a gene involved in regulating cell death in the nematode *C. elegans*. Overexpression of either ICE or ced-3 resulted in increased cell death of transiently transfected rat-1 fibroblasts. In order to determine whether ICE activity is required for macrophage apoptosis, we have exposed macrophages to apoptotic stimuli in the absence and presence of a peptide ICE inhibitor,YVAD-chloromethylketone (YVAD-cmk). The ablility of the YVAD-cmk peptide to inhibit ICE activity was confirmed by immunoprecipitation of IL-1β. Peritoneal exudate cells (PECs) which were cultured with activated, allogeneic cytolytic T cells (CTL) showed extensive processing of pro-IL-1β. Coculturing of PECs with CTL in the presence of 25 µM YVAD-cmk resulted in a complete inhibition of IL-1β processing. The extent of specific ^{51}Cr release from the target cells was unaffected by the presence of the ICE peptide inhibitor or a control peptide, FPR-cmk. In a second model of apoptosis, PECs which were induced to undergo apoptosis by exposure to extracellular ATP showed approximately 40% specific ^{51}Cr release after a 6h chase. Specific ^{51}Cr release from cells which were treated with YVAD-cmk did not differ significantly from untreated cells or cells treated with FPR-cmk. Cells treated with YVAD-cmk showed an 80% reduction in the amount of mature IL-1β found in the culture supernatant. YVAD-cmk had no effect on the processing and release of IL-1α. We conclude that, in these models of macrophage apoptosis, ICE activity is not required for the induction or the execution of cell death.

B8-236 ANTIOXIDANTS INHIBIT A COMMON PATHWAY OF THYMOCYTE APOPTOSIS, C. Stefan I. Nobel, Andrew F.G. Slater, Emilia Maellaro, Monica Kimland, Juanita Bustamente and Sten Orrenius, Institute of Environmental Medicine, Division of Toxicology, Karolinska Institute, Box 210, S-17177 Stockholm, Sweden.

Oxidative stress has recently been suggested to be a mediator of apoptotic cell death, although evidence that this phenomenon is an essential component of apoptosis is lacking. To elucidate this issue antioxidants were used to inhibit apoptosis in rat thymocytes induced by four different non-oxidant compounds (methylprednisolone, etoposide, thapsigargin and TPEN). The antioxidants tested were the nitroso-containing free radical spin traps 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3,5,5-tetramethyl-1-pyrroline-1-oxide (TMPO), the nitroxide-radical antioxidant 2,2,6,6-tetramethyl-1-piperidinyl-1-oxyl (TEMPO) and the endogenous thiol reductant dihydrolipoic acid. The cell shrinkage as well as DNA fragmentation resulting from the four inducers above was reduced by each of these different antioxidant compounds. To support these findings Percoll density gradients were used to isolate different sub-populations of thymocytes undergoing apoptosis. Glutathione, reduced protein thiols and vitamin E were each depleted in both the pre-apoptotic and fully apoptotic cells, while the peroxide-content of apoptotic cells was significantly increased. These observations demonstrate that an oxidative stress is indeed occurring during the apoptotic process. We conclude that intracellular oxidation is a necessary biochemical event during thymocyte apoptosis, even when initiated by agents that are not in themselves oxidants.

B8-237 CELL SURFACE ACTIVATION OF APOPTOSIS BY MHC CLASS I PROTEINS, Leonard C. Norkin, Nandita S. Dangoria, and Walter C. Breau, Department of Microbiology, University of Massachusetts, Amherst, MA 01003

Monoclonal antibodies specific for MHC class I proteins induce apoptotic cell death in serum-starved CV-1 monkey kidney cell cultures within 3 hours of treatment. Antibody-treated cultures showed much reduced numbers of viable cells. Furthermore, they contained cells showing membrane blebbing and nuclear condensation and fragmentation. Apoptosis did not occur if serum was added to cultures along with the MAbs.

We recently found that anti-class I MAbs activate intracellular signaling pathways that upregulate the cellular primary response genes c-myc, c-jun, c-sis, and JE. Activation of these signaling pathways is blocked by the tyrosine kinase inhibitor, genistein. Cell death was reduced, or did not occur, if genistein was added with the anti-class I antibodies. We also found that anti-MHC class I induces the activation of MAP kinase activity in growth-arrested cells. However, these MAbs do not appear to induce mobilization of Ca^{2+}.

B8-238 AN IMMUNOSUPPRESSIVE FACTOR FROM ESOPHAGEAL CARCINOMA INDUCES APOPTOSIS IN LYMPHOID CELLS BY ELEVATING CYCLIC AMP. Alison O'Mahony, John K. Collins* and Gerald C. O'Sullivan.# Dept. of Biological Chemistry, School of Medicine, UC Davis, California and *Immunology Unit, Depts. of Microbiology & Surgery, University College and Mercy Hospital, Cork, Ireland.

An immunosuppressive factor produced by an esophageal squamous carcinoma cell line mediates profound irreversible suppression of *in vitro* proliferative responses of lymphoid cells. The immune suppressive effects of this factor are not inhibited by anti-TGF-b antibodies which neutralize TGF-β isoforms 1, 2 and 3. ISF potentiates the immune suppressive effects of agents such as forskolin, cholera toxin and isoprotenerol, all of which elevate cAMP synthesis. Forskolin induced an increase in cAMP synthesis in both resting and activated cells, in contrast, ISF induced a time-dependent elevation of cAMP levels only in pre-activated PBL. Indeed the ISF induced a 15-fold increase in cAMP levels as compared to a 12-fold cAMP elevation in forskolin treated cells. Exposure of activated normal PBL to the ISF resulted in the induction of an irreversible anergic state with apoptosis evident in 20% of those cells. Flow cytometric cell cycle analysis of mitogenically stimulated normal lymphocytes exposed to the ISF revealed that, despite exhibiting full activation status (IL-2 production, IL-2R and TfnR expression), PBL were arrested at the G1/S interphase of the cell cycle. Transformed lymphoid cell lines, NSO and JURKAT, displayed morphologies characteristic of apoptosis within 24 hours of exposure to the ISF. Agarose gel electrophoresis resolved a DNA fragmentation pattern characteristic of apoptosis in both the NSO cells and to a lesser extent in the activated PBL exposed to the ISF but not in control cells. Induction of programmed cell death by ISF may account for the *in vivo* immune suppression local to the tumor site in squamous carcinoma of the esophagus.

B8-239 **REOVIRUSES INDUCE APOPTOSIS: ROLE OF THE S1 GENE** **S.M. Oberhaus[1], M.K.T. Squier[2], S.E. Rodgers[3], B. Schneider[1], T.A. Grdina[4], D.G. Willis[4], J.J. Cohen[2], T.S. Dermody[3,5], and K.L. Tyler[1,2,6]; Depts. of [1]Neurology, [2]Immunology, [6]Microbiology & Medicine, Univ. of Colorado Hlth. Sci. Ctr., & [4]Research Service Denver VAMC, Denver, CO 80262 and Depts. [3]Microbiology & Immunology and [5]Pediatrics, Vanderbilt Med.Sch., Nashville, TN 37232**

Reoviruses are non-enveloped viruses whose genome consists of 10 dsRNA gene segments. These viruses infect nearly all mammalian species, including humans. Experimental infection of newborn mice is an important model system for the study of viral pathogenesis. Reoviruses also infect and produce cytopathic effects (CPE) in a variety of cultured cells. The mechanism(s) by which reoviruses produce CPE in vitro and cell death in vivo are unknown. We investigated the potential role of apoptosis in reovirus CPE. We found that reovirus infection of L929 fibroblasts causes apoptosis as determined by: 1)quantitation of ^{3}H-thymidine labeled DNA fragmentation, 2)differential nuclear staining with fluorescent dyes, and 3)detection of nucleosomal ladders by agarose gel electrophoresis and Southern blot hybridization. Both the T1 Lang (T1L) and T3 Dearing (T3D) prototypic reovirus strains induce apoptosis using all 3 assays, but T3D does so to a significantly greater extent than T1L. Apoptosis can be induced in the absence of reovirus replication, but requires viral binding and perhaps subsequent internalization and intra-endosomal proteolytic processing. We used 38 T1L x T3D reassortant viruses to identify the gene(s) responsible for strain-specific differences in apoptosis induction. We found that the S1 gene determined the extent of apoptosis, but the M2 gene may also play a role. The S1 gene encodes the outer capsid protein σ1, and is also the major determinant of differences between T1L and T3D in: 1)tropism in vitro and in vivo, 2)patterns of spread in the infected host, 3)inhibition of host cell DNA synthesis. Studies are currently underway to investigate the relationship between these properties and apoptosis, and to identify molecular mechanisms of reovirus-induced apoptosis.

B8-240 REQUIREMENT OF NUCLEAR FACTOR AP-1 FOR THE INDUCTION OF APOPTOSIS BY CERAMIDE IN HUMAN LEUKEMIA HL-60 CELLS. Toshiro Okazaki[1], Hirofumi Sawai[2], Masaro Tashima[2], Hiroyoshi Sawada[2] and Naochika Domae[1], Department of Medicine, Osaka Dental University[1], Osaka, JapanThe First Division, Department of Medicine, Kyoto University[2], Kyoto, Japan

Ceramide, the product of sphingomyelin hydrolysis, plays an important role as a lipid messenger in cell functions including proliferation, differentiation and apoptosis. To clarify how ceramide transduces the apoptotic signal we investigated the role of nuclear factor AP-1 on the apoptosis induced by ceramide in human myelocytic leukemia HL-60 cells. When the cells treated with 5 μM N-acetylsphingosine (C2-ceramide), the apoptosis confirmed by DNA fragmentation was induced at latest 3 hours after treatment. The levels of c-jun and c-fos mRNAs increased 30 min after treatment. At the same time we found that the amount of c-jun protein and the DNA binding activity of AP-1 increased by ceramide. In terms of the mechanisms to increase c-jun mRNA levels, it was found that the transcription rate increased in a run-on experiment. These suggested that ceramide increased AP-1 activity, at least in part, by increasing the generation of c-jun protein. These changes did not occur by C2-dihydroxyceramide which has two hydroxygens at 4th and 5th carbon of sphingosine and could not induce apoptosis in HL-60 cells. The results showed the specificity of ceramide to increase c-jun levels. When 40 μg/ml antisense DNA of c-jun was added to the cells, the growth inhibition caused by ceramide was restored at about 90-100 %. Adding curucumin which decreased c-jun protein level, the similar recovery of growth inhibition by ceramide occurred. These results suggest that apoptosis induced by a sphingolipid second messenger "ceramide" definitely requires a c-jun-AP-1 system in HL-60 cells.

B8-241 THE FAS ANTIGEN: INTERACTING CYTOPLASMIC PARTNERS AND EARLY SIGNALING EVENTS, Jason R. Orlinick and Moses V. Chao, Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021

The Fas antigen was discovered in 1989 by screening a battery of monoclonal antibodies (mAbs) raised against a human B cell lymphoblast cell line (SKW6.4). Initially, it was believed that a mAb could be found which would specifically result in tumor regression upon injection into *nu/nu* mice harboring such tumors. Subsequent cloning and sequence analysis demonstrated that the Fas antigen is a member of a family of proteins of which the TNF receptors I ($p55^{TNFR}$) and II ($p75^{TNFR}$) and the p75 NGF receptor are notable members. In particular, the Fas antigen shares significant sequence homology with the intracellular domain of the $p55^{TNFR}$ in a region which has been demonstrated to be required for the induction of apoptosis. In order to identify cytoplasmic proteins which interact with the intracellular domain (ICD) of the Fas antigen, several GST fusion proteins containing the ICD have been constructed and expressed in bacteria. These GST fusion proteins have been used to identify proteins which are specifically associated with the intracellular domain of the Fas antigen. As a complementary approach, a Jurkat T cell cDNA expression library is being screened with ^{32}P-labeled GST fusion proteins to identify cellular proteins which specifically associate with the ICD of the Fas antigen. Finally, a mammalian expression vector which encodes a polyhistidine-tagged and an epitope-tagged form of the ICD is being used to identify interacting partners after expression of this construct in a variety of cell lines.

B8-242 THE ROLE OF ORNITHINE DECARBOXYLASE AND REACTIVE OXYGEN SPECIES IN c-MYC-INDUCED APOPTOSIS, Graham Packham and John L. Cleveland, Department of Biochemistry, St. Jude's Children's Research hospital, 332 N. Lauderdale, Memphis, TN 38105.

The c-Myc transcription factor is a key regulator of cell proliferation and differentiation and is activated in human cancer. In addition to its established role as an oncogene, inappropriate c-Myc expression also induces apoptosis. The transcriptional targets of c-Myc and the mechanism of c-Myc-induced apoptosis are unknown. The ornithine decarboxylase (ODC) gene is a direct transcriptional target of c-Myc, and we have tested the role of ODC enzyme activity in c-Myc-induced apoptosis. Enforced ODC expression, like c-Myc, is sufficient to induce apoptosis in IL-3-deprived 32D.3 myeloid cells. Importantly, c-Myc-induced cell death is inhibited by α-difluoromethylornithine, a specific inhibitor of ODC enzyme actvity, demonstrating that ODC is a mediator of c-Myc function, at least in the induction of apoptosis. In contrast to c-Myc, ODC is not suffcient to promote entry into S phase in the absence of growth factor. Therefore, induction of apoptosis by ODC (and ODC-mediated c-Myc-induced apoptosis) is not the result of a default process in response to inappropriate cell cycle progression. Alterations in polyamine metabolism can result in the production of reactive oxygen species (ROS) and we have investigated the role of ROS in c-Myc-induced apoptosis. Low doses of hydrogen peroxide induce apoptosis in myeloid cells, whereas high doses induce rapid cell death, more consistent with necrosis. Importantly, hydrogen peroxide-induced apoptosis was inhibited by growth factors, demonstrating that these ligands suppress apoptosis downstream of ROS production. Finally, c-Myc-induced apoptosis is inhibited by antioxidants. Therefore, low doses of ROS can accelerate apoptosis, and ROS appear to mediate/modulate c-Myc-induced apoptosis.

B8-243 MOLECULAR REGULATORS OF MYC-INDUCED APOPTOSIS, Linda J.Z. Penn, Rich Y. Lu, Wilson W. Marhin and Gihane W. Wasfy, Department of Microbiology, Immunology and Cancer, The Hospital for Sick Children, The University of Toronto, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8

Here we extend our previous work, focused on the genetics of the Myc-induced apoptosis mechanism[1,2], and describe the cloning of Myc-regulated genes whose expression is rapidly elevated in response to Myc-activation leading to apoptosis in Rat-1 fibroblasts. Using the MycER-inducible system with a subtractive hybridization and differential display PCR approach, we have cloned six Myc-induced cDNAs. Two are known genes: cyclin D1, a G1 phase cyclin; and the rat homologue of human heat shock protein 89-alpha. The four remaining Myc-regulated cDNAs encode novel genes whose mRNA expression is of low abundance. We have taken an anti-sense and dominant negative approach to address the functional role of these cDNAs in the apoptotic mechanism induced by Myc as well as other triggers of programmed cell death.

To delineate the points of convergence of the many regulatory pathways controlling apoptosis, we have tested molecular regulators of programmed cell death for their ability to potentiate or inhibit Myc-induced apoptosis. These include bcl-2 and mutants thereof, wild-type and mutant p53, as well as various growth/survival factors. These results will also be presented.

1.Evan et al., Induction of apoptosis in fibroblasts by c-myc protein. Cell, 69:119-128.
2. Facchini et al., Dysfunction of the Myc-induced apoptosis mechanism accompanies c-myc activation in the tumorigenic L929 cell line. Cell Growth & Diff., 5: 637-646.

B8-244 CENTROSOMAL CHANGES OCCUR EARLY IN APOPTOSIS. S.M.Pittman and C.M.Ireland. Children's Leukaemia and Cancer Research Centre, Prince of Wales Children's Hospital, Randwick, NSW, Australia.

Tubulin reorganisation was observed in cells undergoing apoptosis and we hypothesise that microtubules (MTs) may play a role in the morphological changes that occur during this process. In the normal mammalian cell MTs nucleate from the microtubule organizing centre (MTOC) or centrosome. We wished to determine whether the microtubular reorganisation in apoptotic cells was dependent upon the MTOC or whether in this process isolated, independent polymerisation of tubulin occurred. We used flow cytometry (FCM) and immunocytochemistry with monoclonal antibodies which recognise a centrosomal antigen[1] and β-tubulin to investigate apoptosis induced in CCRF-CEM cells following treatment with a variety of drugs which have different modes of action. The centrosome duplicates during cell division and therefore we used drugs which arrest the cells at different stages of the cell cycle in order to discriminate cell cycle from apoptotic events. Cells were treated with dexamethosone (DEX), which caused G1 arrest; methotrexate (MTX), which induces S-phase arrest, and VP-16, which arrests cells at G2. In addition we investigated the apoptotic process using agents which directly interact with tubulin viz. vincristine, taxol and colchicine. Drug doses were chosen to induce maximum apoptosis with minimum necrosis. The time course of cell death varied from 3 hours with VP-16, to 12 hours with tubulin-binding drugs to three days with DEX and MTX. A consistent pattern of change was seen in all experiments. We found that an early change detected by FCM and immunocytochemistry was an increase in the expression of the centrosomal antigen. Cell preparations showed that the centrosomes had multiplied and migrated to the nucleus. An increase in tubulin polymerisation in cell preparations could be seen as cells were recruited into morphologically recognisable apoptosis. This included cells with invagination of the nucleus associated with tubulin bands surrounding the nucleus and single or multiple apoptotic bodies with strong tubulin staining. β-tubulin expression initially decreased followed by a comparative increase which peaked concurrent with the observation of an hypodiploid peak indicative of cell death on the DNA histogram. The ladders which confirm that these cells were apoptotic were detected at this stage.

[1]MAbIIa138kindly provided by K.A.O.Ellem, QIMR, Brisbane.

B8-245 DNA DAMAGE-INDUCED DEATH IN HUMAN PROMYELOCYTIC HL60 CELLS; RECENT OBSERVATIONS WITH TOPOISOMERASE INHIBITORS AND POSSIBLE RELATIONSHIP WITH CELL CYCLE-RELATED KINASES, Yves Pommier, Tsunehiro Shimizu, Patrick O'Connor, and Kurt Kohn, Laboratory of Molecular Pharmacology, National Cancer Institute, NIH, Bethesda, MD 20892.

HL60 are among the most sensitive cells to anticancer drugs. This is probably related to the fact that HL60 cells die by apoptosis within a few hours after exposure to DNA damaging agents. Interestingly, HL60 cells overexpress amplified *c-myc*, are P53 null and express *bcl*-2. We have developed a novel filter elution assay to measure the apoptosis-associated DNA fragmentation and shown that topoisomerase inhibitor-induced DNA fragmentation can be suppressed by chemicals and monocytic differentiation in HL60 cells, as well as *bcl*-2 overexpression. We have also set up a cell-free system and used it to demonstrate that both staurosporine-induced apoptosis and phorbol ester suppression of apoptosis are regulated at the cytoplasmic level. We will report our recent studies on the regulation of cyclins and cdk's during apoptosis. During 4 h treatments with the topoisomerase I inhibitor, camptothecin, cyclin B1/cdc2 kinase activity first increases within the first hour and then decreases below control value while cells undergo apoptosis. By contrast, the cyclin A/cdk2 kinase activity is not increased. The cyclin B/cdc2 kinase activity changes are not due to changes in cyclin B or $p34^{cdc2}$ protein levels. The secondary kinase inactivation is correlated with tyrosine phosphorylation of cdc2. The cyclin B1/cdc2 kinase changes are also observed after treatment with VP-16 and nitrogen mustard, and are even more pronounced in S-phase synchronized cells. The present results suggest that unscheduled activation of cyclin B1/cdc2 kinase is associated with apoptosis induced by DNA damage in HL60 cells.

B8-246 ACTIVATION VERSUS APOPTOSIS IN NEUTROPHILS -DIFFERENTIAL INVOLVEMENT OF PKC ISOENZYMES. Judit Pongracz, Elizabeth M. Deacon and Janet M. Lord, Department of Immunology, University of Birmingham, Birmingham, B15 2TT, U.K.

Neutrophils are end cells which die by apoptosis within 24-48h of entering the circulation. In addition apoptosis of neutrophils, is important in the resolution of inflammation, allowing removal of neutrophils by macrophages, preventing potential tissue damage by released proteases. Little is known about the signalling mechanisms triggering activation or regulating apoptosis in neutrophils, but PKC has been implicated in both processes. The precise PKC isoenzymes involved have not been clearly established. Phorbol esters (TPA, Dopp, Doppa and Sapintoxin A) with different abilities to activate PKC isoenzymes in vitro, were used to activate neutrophils. TPA and Dopp activate all classical and novel PKC's, Sap A activates all except PKC-δ and Doppa is selective for PKC-β. TPA, Dopp and SapA (1-100 nM) strongly activated neutrophils in superoxide production assays, with 3-4 fold activation above the level of fMLP induced superoxide. Morphological studies showed that these agents also caused rapid disintegration of neutrophils, resulting in an almost immediate decline in cell number (60% loss of total cell number within 1 hr). In contrast, Doppa (1-100nM) had no effect on neutrophil activation and did not cause rapid cell loss. However, Doppa did cause a significant increase in the rate of entry of neutrophils into apoptosis (15% ±3.4% above control by 3h, $p<0.05$) . Taking in to account the PKC isoenzyme composition of neutrophils (α,β,δ,η,ζ), we conclude that PKC α is essential to neutrophil activation, PKCδ does not play a major role in this process and that PKCβ is involved in promoting neutrophil apoptosis. Studies of PKC isoenzyme expression in neutrophils, revealed that PKC-β was greatly increased in apoptotic neutrophils. Furthermore, using GM-CSF and bacterial lipopolysaccharide, the rate of neutrophil apoptosis could be reduced. These agents did not activate PKCβ and instead activated PKC-η, highlighting the differential involvement of PKC isoenzymes in the regulation of neutrophil apoptosis and function.

B8-247 THE MOLECULAR ASPECTS OF PROLIFERATION AND APOPTOSIS IN FIBROBLASTS DERIVED FROM CENTENARIANS. Ewa Radziszewska*, Emanuela Grassilli, Paolo Salomoni, Enrica Bellesia, Paolo Negro, Roberta Tiozzo, Antonietta Croce, Umberto Fagiolo #, Ewa Sikora* and Claudio Franceschi. *Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland; Dept.of Biomedical Sciences, Section of General Pathology, via Campi 287, 41100, Modena, Italy; # Inst. Internal Medicine, Univ. of Padua, Italy.

An intriguing relationship appears to exist between apoptosis and cell proliferation. In particular, we have shown that the same set of early proto-oncogenes (*c-fos, c-jun, c-myc*) and transcription factors (AP-1, NFkB) are expressed and activated in dexamethasone-treated and in ConA-stimulated rat thymocytes (Grassilli et al., BBRC,188, 1261-1266, 1992; Sikora et al., BBRC, 192, 1261-1266, 1993; Sikora et al., 197, 709-715, 1993). These data suggest that the same early pathway is involved in both processes. As our labs are interested in the relationship between cell proliferation and cell death in aging, we performed experiments on fibroblast primary cultures derived from biopsies of healthy centenarians and young controls. It has been reported that *in vitro* senescent fibroblasts are unable to express *c-fos*, as well to activate AP-1 after serum stimulation. On the contrary, we showed that fibroblasts from centenarians, at different passages, are fully capable of expressing *c-fos* and AP-1, when starved for 10 days and then stimulated with 10% FCS. As we observed that the propensity to undergo apoptosis of peripheral blood lymphocytes decreases with age from young subjects to centenarians, we are checking if the same phenomenon occurs in fibroblasts, using 2-deoxy-D-ribose to induce apoptosis, as recently described. Moreover, we are also studying if *c-fos* and *c-jun* expression, as well as AP-1 activation, occur in apoptotic fibroblasts from old people and centenarians.

B8-248 INHIBITION OF APOPTOSIS PLAYS A MAJOR ROLE IN *BCR-ABL* INDUCED LEUKEMOGENESIS, Peter T. Rowley, Margaret Thomas, Barbara Kosciolek, and Peter Keng. University of Rochester School of Medicine, Rochester, NY 14642.

Mutations in oncogenes have traditionally been viewed as inducing malignancy by causing excessive cell division. However an additional possible tumorigenic mechanism is inhibition of normally occurring apoptosis. We have studied the mechanism of action of *bcr-abl* in chronic myeloid leukemia (CML) by inhibiting its expression using antisense oligonucleotides. K562 cells, derived from a patient with CML, were incubated with 16 μM 3′,5′-capped *bcr-abl* antisense phosphodiester 18mer targeting the *bcr-abl junctional* sequence. Antisense reduced cell number by day 5 by 30-50% compared to nonsense or no-oligomer controls. To partition this reduction between induction of apoptosis and reduction in DNA synthesis, flow cytometric assays were used. Analysis of DNA distribution following propidium iodide-staining revealed that, at day 3, antisense increased the proportion of cells with less than 2n DNA by 83% (nonsense 3.6%, antisense 6.7%). Because CML cells are resistant to the induction of apoptosis (as judged by DNA laddering on electrophoresis, which requires double-stranded breaks), we also assayed the binding of terminal deoxynucleotidyl transferase (TdT), which requires only single-stranded breaks. Antisense increased TdT binding at day 4 over two-fold (15.5% positive, compared to nonsense or no-oligomer controls, 7.1% positive). In contrast to its effect on apoptosis, antisense changed DNA synthetic rates less than 10% (whether estimated by ^{3}H-thymidine incorporation or by bromodeoxyuridine incorporation). We conclude that, in CML, *bcr-abl* may lead to the accumulation of myeloid cells to a greater extent by inhibiting apoptosis than by increasing cell division. This *bcr-abl* induced inhibition of apoptosis may thwart chemotherapy and foster the accumulation of further mutations leading to the development of the blastic phase of the disease.

B8-249 INCREASED APOPTOSIS OF T-CELLS IN TRANSGENIC MICE OVEREXPRESSING THE ORPHAN RECEPTOR *nur77*/N10, Rolf-Peter Ryseck, Falk Weih, Lihong Chen, Sergio Lira, and Rodrigo Bravo, Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, New Jersey 08543-4000, USA

We are interested in the function of transcription factors encoded by immediate-early genes like Jun, Fos, Rel/NF-κB and zinc finger proteins. One member of the growth-factor induced genes, *nur77*/N10 shows a significant similarity 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 was identified for Nur77/N10.

Recently the *nur77*/N10 gene product was identified to be involved in the apoptosis of T-cell hybridomas (Woronicz et al., Nature 367, 277-281 (1994); Liu et al., Nature 367, 281-284 (1994)). To further characterize the role this gene plays in this event, we established several transgenic mouse lines either expressing a full-length wild-type Nur77/N10 or a dominant negative mutant under the control of the T-cell specific proximal *lck* promoter. The lines expressing the wild-type gene product show a thymus strongly reduced in size. In contrast, the thymus of animals carrying the dominant negative form of Nur77/N10 is enlarged. These lines are currently used to further analyze the function of this gene in apoptosis of T-cells.

B8-250 **LOSS OF FAS/APO-1 RECEPTOR INDUCED APOPTOSIS CAN ACCELERATE T-CELL TUMORIGENESIS IN (LPR,LPR)/MYC TRANSGENIC MICE**

T. Schmidt, M. Zörnig, A. Grzeschiczek and T. Möröy, Institut für Molekularbiologie und Tumorforschung, Philipps Universität Marburg, Emil-Mannkopff-Str. 2, D-35037 Marburg, Germany

The (lpr,lpr) mutant mice carry an endogenous retroviral Etn element that has integrated into the gene for the FAS/Apo-1 receptor and blocks its expression. The Fas/Apo-1 receptor is able to transduce apoptotic signals in T-cells, and its absence in (lpr,lpr) mice leads to a nonmalignant proliferation of abnormal T-cells very probably through the lack of the induction of apoptosis in peripheral mature T-cells. We wished to test the hypothesis that the loss of Fas/Apo-1 receptor and thereby the loss of proper regulation of T-cell proliferation through apoptosis may be a prerequisite for the process of T-cell tumorigenesis. To this end, we used myc transgenic mice that are predisposed to develop T-cell tumors with long latency periods to cross into the (lpr,lpr) background. In a second approach we used infection with Moloney Murine Leukemia Virus (MoMuLV) which is a non acute transforming retrovirus and also provokes the formation of T-cell lymphomas in mice with long latency periods. We find that deregulated expression of an L-myc transgene in T-cells of (lpr,lpr) mice leads to the accelerated formation of T-cell lymphoid tumors when compared to normal myc transgenics, suggesting that the loss of Fas/Apo-1 and the expression of a myc gene can act synergistically in the formation of T-cell tumors. In addition, the infection of normal (lpr,lpr) mice and (lpr,lpr)/myc transgenic mice with MoMuLV accelerated T-cell tumorigenesis further only in the (lpr,lpr)/myc transgenics when compared to noninfected (lpr,lpr)/myc animals. A significant proportion of these tumors contained integrations of the retrovirus in the newly identified tmi-1 gene. We conclude from this study that the loss of Fas/Apo-1 receptor can be indeed be a prerequisite in the generation of T-cell tumors and is able to cooperate with a number of different oncogenes in lymphomagenesis.

B8-251 IDENTIFICATION OF GENES CONTROLLING PROGRAMMED CELL DEATH DURING METAMORPHIC TADPOLE TAIL RESORPTION, Robert A. Schwartzman, Zhou Wang, Donald D. Brown, Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore MD 21210

The metamorphosis of tadpoles into frogs, instigated by the synthesis of thyroid hormone (TH), involves the removal of several larval organs no longer necessary during adult life. The most dramatic of these, and the final metamorphic step, is resorption of the tail. This striking example of developmentally-regulated programmed cell death involves the simultaneous removal of several different cell types (*e.g.*, skin, muscle, nerve, notochord, connective tissue) and occurs in an autonomous fashion, since isolated tails treated with TH undergo complete resorption. To identify the TH-regulated genes that control tail resorption a sensitive PCR-based subtractive hybridization screen developed in this laboratory was performed on cDNA libraries developed from control and TH-treated *Xenopus laevis* tails. The results of the screen indicate that there are approximately 40 genes in the tail whose expression changes following TH treatment (35 up-regulated genes and <10 down-regulated genes), suggesting that the genetic program controlling tail resorption is fairly simple. We currently have cloned and sequenced fragments or entire cDNAs for 24 of these genes. To date 15 genes have been identified by their sequence homology to known genes. These genes include some transcription factors (TH receptor-ß, a zinc finger protein, and two closely related bZIP proteins) which likely mediate secondary cascades of gene expression. Several genes involved with extracellular matrix were also identified, including collagenase-3, stromelysin-3, and fibronectin. To determine which of these genes is involved in programmed cell death the results of *in situ* hybridization experiments of these genes are being correlated to the location of dying cells as determined by Nile blue staining. Additionally, overexpression of these tail genes in several *Xenopus* cell lines is being performed to determine if any of these genes are capable of inducing cell death *in vitro*.

B8-252 INHIBITION OF GROWTH FACTOR-STIMULATED TYROSINE PHOSPHORYLATION INDUCES APOPTOSIS IN SMALL CELL LUNG CANCER CELL LINES.

T Sethi and A Tallett.

Respiratory Medicine Unit (RIE), Rayne Laboratory, University of Edinburgh, Medical School, Teviot Place, Edinburgh EH8 9AG. Scotland

Small cell lung cancer (SCLC) constitutes 25% of all lung cancers and follows an aggressive clinical course. Novel approaches to treatment are needed. It is likely that these will arise from a better understanding of the intracellular molecular events underlying mitogenisis.

SCLC secrete many hormones and neuropeptides. We have previously shown that multiple neuropeptides induce inositol phosphate hydrolysis, mobilisation of intracellular calcium and stimulate growth, suggesting that SCLC growth is mediated by an extensive network of autocrine and paracrine interactions. There is increasing interest in protein tyrosine phosphorylation as a growth promoting signal and we therefore studied the effects of serum, insulin, bombesin and gastrin (all of which are well recognised growth factors) on tyrosine phosphorylation in SCLC. Following stimulation with growth factors, whole cell lysates were analysed by western blotting. We demonstrated that these growth factors were all able to induce protein tyrosine phosphorylation in SCLC in a concentration and time-dependent fashion. An increase in tyrosine phosphorylation was evident at 30 secs increasing to a maximum between 10-20 mins and then declining. Though the pattern of tyrosine phosphorylation was agonist specific, some major substrates like $p125^{FAK}$ and bands Mr p130, p90, p80, p75 and p60 were phosphorylated by all growth factors. Tyrphostin 25, a specific tyrosine kinase inhibitor, inhibits this tyrosine phosphorylation, SCLC growth and stimulate apoptosis in the μM range ($I.C._{50}$ 10 μM). Increased rate of apoptosis compared to controls was evident at between 8 and 12 hours and was maximal at 48-72 hours, demonstrated morphologically and by DNA fragmentation analysis.

Time (hrs)	3	8	12	24	48	72	n=4
Apoptosis (%)	3±2	5±4	7±3	15±5	30±8	35±7	±S.E.M.

This is the first report to identify substrates for neuropeptide-stimulated tyrosine phosphorylation, one of these substrates is a tyrosine kinase hence this is an important mitogenic signal for small cell lung cancer. Inhibition of tyrosine phosphorylation may be an important target for novel therapeutic strategies.

This work has been supported by the British Lung Foundation.

B8-253 TRANSCRIPTION FACTORS SHOWING AFFINITY FOR BOTH GRE AND AP-1 CONSENSUS SEQUENCE ARE INVOLVED IN DEXAMETHASONE-INDUCED APOPTOSIS OF RAT THYMOCYTES, Ewa Sikora *, Gian Paolo Rossini, Emanuela Grassilli, Enrica Bellesia, Paolo Salomoni and Claudio Franceschi, *Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland; Dept. of Biomedical Sciences, via Campi 287, 41100, Modena, Italy.

The early molecular events of glucocorticoid-induced apoptosis have been investigated by studying glucocorticoid receptor levels, as well as binding activities for GRE and AP-1 sequences, using nuclear extracts from dexamethasone-treated rat thymocytes. We found that nuclear accumulation of radioactive glucocorticoid-receptor complexes occurred in the first hour of incubation, and was followed by a progressive decline. This trend was confirmed by immunoblotting of nuclear proteins using a monoclonal anti-glucocorticoid receptor antibody. The kinetics of binding activity to AP-1 and GRE sequences studied by using nuclear extracts and gel shif assay showed the maximum within 1h and 2h, respectively, after dexamethasone treatment, and returned to basal levels in the following hours. Competition studies using nonradioactive sequences showed that retarded bands were inhibited by both unlabelled GRE and AP-1 oligonucleotides in a dose-dependent manner, and that binding to GRE was better competed for by AP-1 sequence than by GRE itself. On the basis of these observations and of the asynchronies we detected among nuclear levels of glucocorticoid receptor complexes and binding activity to AP-1 and GRE sequences, we propose that different AP-1 complexes could be involved in glucocorticoid-induced apoptosis of thymocytes, within an intriguing pattern of interactions with glucocorticoid receptor and its responsive element.

Poster Session 3

B8-300 MOUSE BCL-X BLOCKS ANTIGEN RECEPTOR INDUCED PROGRAMMED CELL DEATH IN IMMATURE B LYMPHOCYTES. Timothy W. Behrens, Wei Fang, James R. Rivard, Daniel Mueller. Department of Medicine -Rheumatology and the Pathobiology Graduate Program, University of Minnesota, Minneapolis, MN 55455

Programmed cell death (PCD) is important in regulating the immune repertoire, and defects in apoptosis can lead to autoimmunity. We have recently cloned the mouse homologues of bcl-x. In addition to bcl-x 'Long' and 'Short', we identified a novel cDNA isoform, bcl-xΔTM, which deletes via alternative splicing the C-terminal transmembrane domain of bcl-x and is a soluble, rather than membrane bound, protein. The ΔTM form of bcl-x was the major species found in a pre-B cDNA library (17 of 24 cDNA clones), and is abundantly expressed in a panel of pre-B cells tested. The ΔTM isoform is functional in delaying apoptosis of an IL-3 dependent cell line following growth factor withdrawal. Bcl-x mRNA is highly inducible following mitogen treatment of B and T lymphocytes, and transfected Long and Short target to mitochondria in Hela cells. Overexpression of mbcl-x or mbcl-2 in the WEHI 231 immature B cell line resulted in resistance to programmed cell death induced by anti-IgM crosslinking, gamma irradiation, serum deprivation and a variety of oxidant stresses. bcl-x appears to be more potent in this system than bcl-2. Strikingly, addition of antioxidants alone to the control cell line abrogated IgM triggered apoptosis. The effects of mbcl-x overexpression and antioxidants were not additive or synergistic, suggesting that bcl-x, like bcl-2, may participate in an intracellular antioxidant pathway. These findings indicate that bcl-x may function to regulate PCD during lymphoid development, and suggest the possibility that negative selection of lymphocytes may be mediated by an increased production of or sensitivity to intracellular oxidants.

B8-301 MUTATIONAL ANALYSIS OF BACULOVIRUS P35 DEFINES TWO CHARGED DOMAINS ESSENTIAL FOR SUPPRESSION OF APOPTOSIS, John Bertin and Paul D. Friesen, Institute for Molecular Virology and the Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706

The baculovirus P35 protein suppresses programmed cell death in diverse organisms including insects, nematodes and mammals. P35 shares no obvious amino acid sequence similarity with other known apoptotic suppressors and may therefore function by a distinct mechanism. Due to apoptotic death of the host cell, replication of baculovirus mutants lacking *p35* is impaired in a cell-line and insect-specific manner. To identify functional domains of P35, we introduced 18 independent two-codon insertion mutations throughout the *p35* gene and determined their effect on *p35*'s ability to complement replication of a *p35* null mutant virus by suppressing apoptosis in cultured insect cells. All insertions within the amino-terminal half interfered with P35 complementation of mutant virus growth. In contrast, most insertions within the carboxy-terminal half had no effect on P35 function, with the exception of those at the extreme carboxy-terminus or in small hydrophobic regions. To identify specific P35 amino acid residues that are involved in anti-apoptotic activity, we performed clustered charged-to-alanine mutagenesis of three charged regions (CR1, CR2 and CR3) that span the amino-terminal half of P35. Charged amino acids in CR1 and several within CR2 that are present in a putative amphipathic alpha-helix were found to be essential for P35 function. Charged-to-alanine mutations in CR3 had no effect on P35 anti-apoptotic activity. We conclude that specific amino acid residues within CR1 and CR2 are essential for suppression of apoptosis by P35. Due to the clustering of charged residues within CR1 and CR2, these domains likely reside on the surface of P35 and thus may function as sites of interaction with other proteins, including P35 itself or other host death regulators.

B8-302 **EXPRESSION OF CELL SURFACE PROTEASES IN NEUTROPHILS UNDERGOING APOPTOSIS**

Simon B Brown and John S Savill, Division of Renal and Inflammatory Disease, University Hospital, University of Nottingham, UK NG7 2UH.

The ageing neutrophil in vitro provides an ideal model for the study of cell surface phenomena following onset of apoptosis. Dransfield *et al*[1] have demonstrated that in such a model, apoptosis is associated with the loss of specific markers (eg CD16), shedding of which is presumed to be due to metalloprotease activity. Studies by Brown et al with HeLa[2,3] have shown a stimulation of HeLa cell surface (ecto-) metallopeptidase activities in response to a variety of agents which correlate with the proportion of cells undergoing apoptosis which remain impermeant to non-vital dyes. We therefore sought to address the role of cell surface peptidase activity in neutrophils isolated from normal donors and aged in culture for up to 24 h. Evidence will be presented that as an increasing proportion of senescing neutrophil populations become apoptotic, as judged by morphology, no significant variation in cell surface aminopeptidase/esterase activity could be detected. To exclude the possibility that decreased activity in aged non-apoptotic cells was masked by increases in apoptotic cells we performed counterflow centrifugation to selectively enrich for the apoptotic cells. Again no increase in activities was seen compared with non-apoptotic or fresh fractions. This is supported by direct linear kinetic analysis for the hydrolysis of Leucine- and/or Alanine- para-Nitroanilide substrates as well as by a nonapeptide substrate incorporating a radio-iodinated tyrosine[4]. Using this latter substrate we did, however, observe decreases in endopeptidase activity with ageing. Counterflow centrifugation again revealed no apparent differences between the sub-populations other than that which could be attributed to cell lysis. The data indicate that in senescing neutrophil populations any changes to cell surface protease activity are unrelated to morphological changes of apoptosis

[1] Dransfield I, A-M Buckle, J Savill, et al (1994); **J. Immunol.**, ***in press***
[2] Brown SB, Krause DR, & Ellem KAO (1993); **J. Cell. Biochem.**, 51:102-115.
[3] Brown SB, Kluck RM, & Ellem KAO (1994); **J. Cell. Biochem.**, 54:320-331.
[4] Brown S, Krause D, Townsend E & Ellem K (1992); **J. Cell. Biochem.**, 48:411-423

B8-303 **MOLECULAR INTERACTION OF BACULOVIRUS P35 AND INTERLEUKIN-1β CONVERTING ENZYME (ICE): PROPOSED MECHANISM FOR THE REGULATION OF AN APOPTOTIC PATHWAY,** Nancy J. Bump, Kenneth Brady, Patrick Chen, Catherine Ferenz, Simon Franklin, Tariq Ghayur, Margaret Hugunin, Ping Li, Peter Licari, Lois K. Miller, and Winnie W. Wong, BASF Bioresearch Corp., Worcester, MA 01605 and Dept. Entomol., U. Georgia, Athens, GA 30602

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 and ICH-1, are thought to play essential roles in apoptosis. Expression of the 32 kD fragment that led to enzymatically active human ICE (20 kD + 10 kD fragments) in Sf-9 cells resulted in apoptosis as evidenced by DNA fragmentaton. Expression of the 20 kD fragment of ICE alone did not have the same effect. We purified the recombinant enzyme via active site affinity chromatography, and found that it was stably associated with the cleavage fragments of the baculovirus anti-apoptotic protein, p35. When the cDNA encoding p35 was cotransfected into COS cells with the cDNA of ICE and pro-IL-1β, the amount of active IL-1β released was inhibited by ≈60%, similar to the results observed upon cotransfection with crmA, a cowpox serpin previously shown to inhibit ICE activity. We propose that p35 may mediate its antiapoptotic effects through interaction with an insect cell homolog of ICE/CED-3 (ICH-1).

B8-304 ELECTROSTATIC REVERSAL OF GRANZYME B SUBSTRATE SPECIFICITY, Antonio Caputo and R. Chris Bleackley, Department of Biochemistry, University of Alberta, Edmonton, AB T6E 2E2, Canada

Granzyme B has been implicated to participate in destruction of target cells by cytolytic T lymphocytes and natural killer cells. The enzyme appears to induce DNA fragmentation in cells which have been subjected to plasma membrane damage by the action of the pore forming protien perforin. Interestingly, granzyme B shares an unusual P1 substrate specificity for aspartyl residues with Interleukin-1β converting enzyme, the mammalian homologue of the *ced-3* cell death gene of *Caenorhabditis elegans*, thus implying common cellular substrates for these two enzymes. In order to determine the structural features of granzyme B which determine this P1 substrate specificity we have developed a COS cell expression system to generate enzymatically active recombinant granzyme B in natural and mutant forms. This system was used to show that arginine-208 of granzyme B, the side chain of which partially fills the specificity pocket, determines the preference of this enzyme for substrates with aspartate at P1. Granzyme B synthesized in COS cells from the expression vector pAX142 was detected as two polypeptide species of apparent molecular weight 35,000 and 32,000. Both these species represent N-linked glycosylation variants of a single polypeptide of apparent molecular weight 30,000 which is produced in cells treated with tunicamycin. Here we show that substitution of arginine-208 with aspartate results in an enzyme which prefers substrates with phenylalanine and arginine at P1, whereas a mutant which substitutes arginine-208 with glutamate has an exclusive preference for a substrate with arginine at P1. These data indicate that the P1 substrate specificty of granzyme B is determined by the steric and electrostatic properties of the side chain of the amino acid residue occupying position-208 of the enzyme.

B8-305 **CHARACTERIZATION OF HUMAN FAS GENE: EXON/INTRON ORGANIZATION AND PROMOTER REGION,** Jianhua Cheng, Changdan Liu, William J. Koopman, and John D. Mountz, Department of Medicine, Division of Clinical Immunology and Rheumatology, University of Alabama at Birmingham and the Birmingham Veterans Administration Medical Center, Birmingham, AL 35294

Ligation of the Fas cell-surface molecule induces apoptosis. Defective Fas mediated apoptosis have been associated with spontaneous autoimmunity in mice. Using human Fas/Apo-1 cDNA as a probe, we have molecularly cloned and characterized the human Fas chromosomal gene. The gene consists of 9 exons and spans more than 26 kb of DNA. The lengths of introns vary from >14 kb at the 5' end of the gene to 152 bp upstream of the exon encoding the transmembrane domain. The domain structure of the human Fas is encoded by an exon or a set of exons. Primer extension analysis revealed three major transcription initiation sites. The promoter region lacked a canonical "TATA" and "CAAT" boxes but was "GC-rich" sequence, and contained consensus sequence for AP-1, GF-1, NY-Y, CP-2, EBP20, and c-myb. These data provide the first characterization of the human Fas gene and insight into its regulatory region.

B8-306 **IN VIVO EXPRESSION OF BHRF1, AN EPSTEIN - BARR VIRUS ENCODED BCL-2 HOMOLOGUE**

Paul Clarke, Clive LeBozer, Tamas Hickish, Francesca Di Stefano, Mark Hill, and David Cunningham, CRC Centre for Cancer Therapeutics, and Section of Medicine, Lymphoma unit, Institute of Cancer Research and Royal Marsden Hospital, Sutton, Surrey, United Kingdom.

BHRF1 has significant amino acid sequence similarity with the proto-oncogene bcl-2, a gene implicated in the development of human B cell follicular lymphoma. Bcl-2 is a regulator of apoptosis that has been proposed to function via its association with the mitochondrial membrane. To investigate the functional similarity between BHRF1 and bcl-2 *in vivo* we have established a colony of transgenic mice that constitutively overexpress BHRF1 from an immunoglobulin heavy chain promoter element. These mice develop without any obvious morphological defects, however, histological examination has demonstrated that they have a similar phenotype to bcl-2 transgenic mice. One prominent feature is a gross follicular hyperplasia in the spleens of these animals. With increasing age bcl-2 transgenic mice develop an excess of high grade lymphomas compared to control littermates. We are now monitoring a large number of BHRF1 mice to assess whether BHRF1 expression has a similar effect. In conclusion we have demonstrated that *in vivo* BHRF1 can function in a manner similar to bcl-2. This implies that there is requirement to block apoptosis at some point in the viral life cycle, perhaps early in infection or during lytic replication of the virus. Overexpression of bcl-2 in follicular lymphoma is thought to be an early event in the pathogenisis of the disease. Whether or not BHRF1 has a similar role in EBV-associated malignancies remains to be established.

B8-307 **INDUCTION OF APOPTOSIS BY SODIUM BUTYRATE IN HUMAN RETINOBLASTOMA.**

RM Conway, MC Madigan, PLPenfold. Department of Clinical Ophthalmology, 1st Floor Sydney Eye Hospital, Sir John Young Cres Woolloomooloo NSW 2011 Australia.

Purpose. To examine the mode of cell death induced in the Y-79 human retinoblastoma cell line by sodium butyrate (SB), a short chain fatty acid with potent inhibitory effects on the growth of many transformed cell lines.

Methods. Y-79 cells were treated with a range of concentrations of sodium butyrate. Cell viability and apoptotic involution were assessed qualitatively and quantitatively over a 72 hr period with light and electron microscopy, and fluorescence microscopy using acridine orange and ethidium bromide. DNA fragmentation was assessed at 48 hrs by agarose gel electrophoresis. The effects of cycloheximide (10μM) and actinomycin D (5μg/ml), inhibitors of RNA and protein synthesis respectively and of aurintricarboxylic acid (200mM) and Zn^{2+} ions (0.001 to 1mM), inhibitors of endonuclease activity, on SB-induced apoptosis were also assessed at 48hrs.

Results. Sodium butyrate (1-4mM) induced apoptotic involution in Y-79 in a dose-dependent fashion after a latent period of 24 hrs. Dying cells displayed the characteristic morphology of apoptosis accompanied by DNA laddering with agarose gel electrophoresis. Extensive cell necrosis was noted with 0.5M SB. Induction of apoptosis and DNA laddering by SB was prevented by putative inhibitors of RNA and protein synthesis.

Conclusions. Sodium butyrate treatment of the Y-79 retinoblastoma cell line induced cell death with morphologic features characteristic of apoptosis. Reduction of apoptotic involution by inhibitors of macromolecular synthesis suggests this process requires de novo expression of mRNA and protein. Additionally, DNA fragmenting endonuclease activity in Y-79 does not appear to be sensitive to the endonuclease inhibitors aurintricarboxylic acid and $ZnSO_4$.

B8-308 APOPTOTIS INDUCED BY PHOTOSENSITIZATION WITH PORPHYRINS (PHOTOFRIN) IN A NORMAL MONKEY KIDNEY CELL LINE (CV-1), Marc Dellinger, Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, INSERM U201, CNRS UA 481, 43 rue Cuvier, 75231 Paris CEDEX 05, France.

Photosensitization using the tumour-localizing porphyrin Photofrin induces cell death both in vitro and in vivo, leading to tumour eradication (photodynamic therapy). Impairment of mitochondrial energetic metabolism and of intracellular calcium regulation are the main early effects of photodynamic treatment. However, the sequence of events leading to cell death is not established. Although cell lysis (necrosis) was the accepted mechanism of cell death, photosensitization induced-apoptosis has been shown for certain carcinoma cell lines (He et al., Photochem. Photobiol. 59, 468-473, 1994). Following photosensitization of CV-1 via Photofrin, we observed a cytostatic effect with a low dose of irradiation, while higher doses led to cell death. All doses induced severe morphological changes: cells condensed in spindle shaped form with redistribution of cytoplasmic organelles. Cytotoxic doses also led to cell rounding up and detachement from the substrate. At a later stage, cells often showed plasmic membrane blebbing and fragmentation into vesicles. Using standard dyes, fluorescence microscopy revealed that intracellular organelles did not swell. However, the mitochondria redistributed around the nucleus and later the endoplasmic reticulum might disappear at the cell periphery, suggesting cytoskeleton movements. Tubulin seemed to be not affected in these morphological alterations which differed from those observed after colcemid treatment. In contrast, actin modifications are supported by the partial inhibition of cell rounding up and fragmentation in the presence of cytochalasin B, and by the rhodamine-phalloidin staining of actin showing redistribution from actin-fibres to peripheric and nucleic condensed filaments. These morphological changes point to apoptotic cell death rather than necrosis. Although signs of necrosis (cell lysis, lysosomal alterations) were observed for a few cells photosensitized with a high light dose, most of the cells underwent typical apoptotic morphological changes. Hoechst 33258 stained nuclei of rounded cells had patches of condensed chromatin, and agarose gel electrophoresis on extracted DNA showed nucleosomal degradation, both are also key features of apoptosis. The present data indicate that Photofrin photosensitization may lead to cell growth arrest, apoptosis or necrosis, depending on the light irradiation dose, and it can be considered as a new inducer of apoptosis in normal cells.

B8-309 CELL DEATH REGULATION IN ENDOTHELIUM BY AN APOPTOSIS INDUCING FACTOR (APIF)

Hannes C.A Drexler, Holger Schulz and Werner Risau, Abteilung Molekulare Zellbiologie, Max-Planck Institut für Klinische und Physiologische Forschung, 61231 Bad Nauheim, Germany.

We are interested in the mechanisms regulating the regression of small blood vessels. This process is important for embryonic development where it can be observed e.g. during limb development and it is an essential aspect of wound healing. It also has implications for the development of a tumor therapy based on the targeted elimination of tumor blood vessels. In a first attempt we wanted to know whether soluble factors are present in the developing chick embryo that could be involved in the regulation of blood vessel regression. By assaying for the inhibition of endothelial cell proliferation we have purified a novel low molecular weight compound from chick embryo extracts which was found to have a cytotoxic effect on endothelial cells and to a lesser degree on vascular smooth muscle cells and NIH 3T3 fibroblasts. The nuclear morphology as revealed by PI staining and the DNA fragmentation pattern into oligonucleosomes suggest an apoptotic mode of cell death for this low molecular weight compound. Cycloheximide did not block APIF induced cell death but rather synergized with APIF in inducing endothelial cell death. Cycloheximide alone was sufficient to induce apoptotic DNA fragmentation in endothelial cells. Hence we conclude that APIF induces in endothelial cells the release of a cell death program most likely through the functional elimination of a cell death suppressing activity.

B8-310 INTERACTION OF CELLULAR PROTEINS WITH ADENOVIRUS E1B 19K, Stuart Farrow, Julia White, Tom Raven, Chris Grinham, Tao Pun and Robin Brown, Department of Molecular Science, Glaxo Research and Development Ltd, Greenford Road, Greenford, Middlesex, UB6 0HE, UK.

The Adenovirus E1B 19K protein is known to provide protection from TNF-α, E1A or anti-FAS induced apoptosis. We have attempted to identify the cellular targets for E1B 19K by using the yeast two-hybrid system.

A number of E1B 19K interacting proteins have been identified. The first of these is a clone for lamin A/C, which supports previous observations that E1B 19K co-localises in cells with the nuclear lamina. The second cDNA is a novel member of the bcl-2 gene family, with extensive sequence homology to proteins such as BAX and bcl-X. The third cDNA has no homology to any known sequences, but intriguingly, in yeast, it dimerises to bcl-X_L, but not with any other members of the family. This binding to bcl-X_L is also shared by the novel bcl-2 like clone.

We are expressing these novel clones in mammalian cells in an attempt to demonstrate either protection from, or induction of apoptosis.

The above results suggest that the E1B 19K protein has multiple cellular targets. We are attempting to determine whether all of these interactions are necessary for E1B 19K to inhibit apoptosis.

B8-311 CHARACTERISATION OF AN APOPTOTIC NUCLEASE FROM MURINE BONE MARROW CELLS, Isla Furlong, Prupti Malde, Rosalia Ascaso*, Javier Olivier*, Abelardo Lopez Rivas* and Mary Collins, Chester Beatty Laboratories, London, UK and *Lopez Neyra, Granada, Spain.

The murine bone marrow-derived cell line, BAF3, depends on IL3 for its growth in culture. Cells undergo death by apoptosis upon cytokine removal, displaying the DNA laddering characteristic of endonuclease activity. We have shown that the ionophores valinomycin and nigericin induce rapid chromatin fragmentation when added to BAF3 cells in the presence of IL3. Both ionophores cause a sustained decrease in intracellular pH under these conditions. Increasing the extracellular pH can not only block ionophore-induced fragmentation but also inhibit the fragmentation observed upon IL3 withdrawal. This suggests that cytoplasmic acidification is necessary for the induction of DNA laddering. We have been able to induce chromatin fragmentation in isolated BAF3 nuclei by either decreasing the pH or by adding μM Ca at neutral pH. Fragmentation was inhibited by 2 mM magnesium, 0.5 mM zinc or potassium ions in excess of 60 mM. A 30 kD protein has been purified from BAF3 nuclear extract that displays nuclease activity under identical conditions to those required for induction of fragmentation in whole nuclei.

B8-312 THE ROLE OF APOPTOTIC AND NECROTIC PROCESSES IN CYTOLYSIS MEDIATED BY LAK CELLS WITH DIFFERENT PHENOTYPES,

Nikolai Gnuchev, Lidia Sashchenko, Olga Kabanova, Tamara Luk'yanova, Daulet Satpaev*, Irina Mirkina*, Oleg Chertov*, Elena Blishchenko*.

Institute of Gene Biology Russian Academy of Sciences, Vavilova 34, Moscow, Russia;

*Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry Russian Academy of Sciences , Miklukho-Maklaya 16/10, GSP-7, Moscow, Russia.

The mechanisms of tumor cell lysis mediated by CD16+ CD8+ CD3- and CD3+ CD8+ CD16- LAK cells were studied at L929 and K562 tumor cells. It was demonstrated that:

1/Cytolytic activity of both subpopulations was due to secreted and membrane-associated proteins,

2/Different subsets of LAK cells lysed target cells usind different effector proteins. Both secreted and membrane-associated proteins characteristic for CD16+ and CD3+ cells were identified and their physico-chemical parametres were determined,

3/Target cell cytolysis mediated by these LAK cell subsets was associated both by necrotic and apoptotic cytolytic processes.

4/The realization of different cytolytic processes depended on effector cell phenotype.

Target cell cytolysis mediated by both secreted and membrane-associated proteins will be also discussed.

B8-313 *bcl-x_L* IS THE MAJOR *bcl-x* mRNA FORM EXPRESSED DURING DEVELOPMENT AND ITS PRODUCT LOCALIZES TO MITOCHONDRIA, Maribel González-García*, Rafael Pérez-Ballestero*, Liyun Ding*, Linda Duan*, Craig B. Thompson§ and Gabriel Núñez*, *Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109. §Howard Hughes Medical Institute, Departments of Medicine, Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637.

Mutant mice with targeted disruption of *bcl-2* appear normal at birth and complete maturation of lymphoid tissues before succumbing to fulminant lymphopenia and polycystic renal disease by 2-5 weeks of age. This suggests that there may be genes other than *bcl-2* that can regulate apoptosis during development. To begin to investigate this possibility, we have cloned and characterized the murine *bcl-x* gene, whose human counterpart displays striking homology to *bcl-2*. Just like Bcl-2, the murine *bcl-x_L* gene product can act as a dominant inhibitor of cell death upon growth factor withdrawal. In addition, the bulk of the Bcl-x_L product localized to the periphery of mitochondria as assessed by a *blc-x_L*-tag expression system, suggesting that both Bcl-2 and Bcl-x_L proteins prevent cell death by a similar mechanism. *bcl-x_L* is the most abundant *bcl-x* mRNA species expressed in embryonic and adult tissues. The levels of *bcl-x_L* mRNA appear higher than those of *bcl-2* during embryonal development and in several adult organs including bone marrow, brain, kidney and thymus. In addition to *bcl-x_L*, we have identified another form of *bcl-x* mRNA, *bcl-x_β* that results from an unspliced *bcl-x* transcript. *bcl-x_β* mRNA is expressed in various embryonic and postnatal tissues. Together, these data suggest that, in addition to *bcl-2*, *bcl-x_L* will have an important role in the regulation of developmental cell death and tissue homeostasis.

B8-314 ENHANCED NUCLEAR, SERINE PROTEASE ACTIVITY CONTRIBUTES TO BILE ACID-INDUCED APOPTOSIS IN HEPATOCYTES, Gregory J. Gores, Paul Y. Kwo, Tushar Patel, Steven S. Bronk, Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, MN 55905

Retention of bile salts by the hepatocyte contributes to liver injury during cholestasis. We have recently demonstrated that the bile salt glycodeoxycholate (GDC) induces apoptosis in hepatocytes suggesting bile salt-induced apoptosis is an important mechanism of liver injury during cholestasis. The mechanisms of GDC-induced apoptosis remain unclear. Because increasing data suggests proteolysis may play an important role in apoptosis, our aims were to directly test the hypothesis that enhanced proteolysis is a mechanism causing GDC-mediated apoptosis. In cultured rat hepatocytes exposed to 50μM GDC for 4 hours, nonlysosomal proteolysis increased by 65% compared to controls. No change in lysosomal proteolysis was observed. The serine protease inhibitor TLCK (100μM) reduced nuclear fragmentation by 75% after 4 hours of treatment with GDC. TLCK also inhibited DNA fragmentation as assessed by agarose gel electrophoresis. As TLCK inhibits serine protease activity, we used a serine protease substrate Boc-Val-Leu-Lys-AMC to determine the compartmentation of serine protease activity. There was a 2-fold increase in nuclear, serine-like protease activity during GDC-induced apoptosis accompanied by a 2.5-fold reduction in nonnuclear serine protease activity. These data suggest translocation of a protease from the cytosol to the nucleus occurs during bile salt-induced apoptosis. To further test this hypothesis we developed a novel fluorogenic probe, Cbz-Val-Leu-Lys-CMAC, to dynamically measure serine protease activity in living cells by digitized fluorescent microscopy. The ratio of nuclear to cytosol serine protease activity doubled during apoptosis. Finally, zinc, an inhibitor of apoptosis, also inhibited nonlysosomal proteolysis and nuclear serine protease activity in a dose-dependent manner. The dose dependence for zinc inhibition of the serine protease activity was virtually identical to that for inhibition of nuclear fragmentation. Our data demonstrate for the first time that: 1) TLCK prevents GDC-induced apoptosis; 2) nuclear serine protease activity is enhanced in apoptosis; and 3) Zn^{2+} blocks GDC-induced apoptosis and nuclear serine protease activity. These novel data suggest nuclear, serine-like protease activity contributes to apoptosis.

B8-315 MODULATION OF APOPTOSIS IN THE DEVELOPING MOUSE OCULAR LENS BY INTERACTIONS BETWEEN HPV-16 ONCOPROTEINS AND CELLULAR TUMOR SUPPRESSOR GENE PRODUCTS. Anne E. Griep and Huichin Pan, Department of Anatomy, University of Wisconsin Medical School, Madison, WI 53706.

During ocular lens development, a specific subset of undifferentiated cells acquire the capacity to divide, migrate and differentiate. During the final stages of their differentiation, these cells lose cellular organelles, including the nucleus, through a process resembling apoptosis. To study the involvement of tumor suppressor gene products in regulating cell cycle control during lens cell differentiation in transgenic mice, we utilized the E6 and E7 oncogenes from human papillomavirus type 16 as *trans*-dominant repressors of tumor suppressor protein function (*Genes & Development* **8**: 1285). Expression of E7 in the lens profoundly affected fiber cell differentiation: fiber cell elongation and the down regulation of cell proliferation were severely compromised and ultimately the death of these abnormal cells occurred through apoptosis. The ability of E7 to affect lens fiber cell differentiation was completely dependent upon its ability to associate with Rb and/or Rb-like proteins, indicating that these proteins play essential roles in cell cycle control in the developing lens. The E7-induced apoptosis has both p53-dependent and p53-independent components, as judged by comparison of the level of apoptosis in E7+/p53-/- and E7+/p53+/+ mice. Transgenic mice expressing E6 showed inhibition of fiber cell denucleation and the inhibition of the apoptotic-like DNA degradation observed in the lenses of nontransgenic embryos. However, E6's ability to inhibit denucleation is likely due to E6's interactions with cellular proteins other than p53 since lenses from p53-/- are unaffected while lenses from E6+/p53-/- are similar to those from E6+p53+/+ mice. In lenses from neonatal E6 x E7 double transgenic mice, the level of apoptosis was partially reduced compared to that seen in lenses from neonatal E7 mice, suggesting, as with E7+/p53-/- lenses, that E7 may induce apoptosis by multiple pathways. In these adult double transgenic mice as well as in adult E7+/p53-/- transgenic mice, lens tumors developed, whereas in E7+, E6+, or p53-/- mice, no lens tumors developed. Thus, apoptosis need only to be partially rescued, via E6 action or the absence of p53 to allow for tumor formation. These results point to specific roles for the Rb gene family in regulating the normal cell division cycle in differentiating lens fiber cells, to additional factors involved in regulating normal fiber cell denucleation, and to the existence of multiple mechanisms to induce apoptosis as a means of removing aberrantly proliferating cells in order to avoid tumorigenesis.

B8-316 SUPPRESSION AND INDUCTION OF APOPTOSIS IN B-CELL NEOPLASIA

J. Gruber[1], R. Sgonc[2], G. Boeck[2], M. Herold[3], R. Greil[1]*

[1] Laboratory of Molecular Cytology, Dept. of Internal Medicine, [2] Institute for General and Experimental Pathology, [3] General Laboratory, Dept. of Internal Medicine, Univ. of Innsbruck, Austria.

Resistance to chemotherapeutic agents often indicates the terminal phase of human malignancies. Further understanding of the mechanism(s) which are involved in this process could ultimately lead to new therapeutic strategies. Since apoptosis is involved in both inducing and blocking effects of chemotherapeutic agents the aim of the present investigation was to identify factors which either enhance or block apoptosis. Cells from chronic lymphocytic leukemia (B-CLL)-patients which are resistant to chemotherapeutic agents in vivo rapidly underwent apoptosis in vitro after treatment with the same agents used in the clinical regimen. Interestingly, the observed apoptosis is dependent on culture conditions. In order to identify serum factors responsible for this process we investigated several cytokines with known impact on apoptosis. We added different candidate cytokines to CLL- cells treated with anticancer drugs. Interleukin (IL)-4 inhibited dexamethasone induced apoptosis. The highest degree of inhibition was seen after IL-10. IL-3 also inhibited - at least in some patients - apoptosis induced by dexamethasone and doxorubicin. Additionally, cytokine receptor expression was studied. Untreated cells showed high expression of IL-2 receptor(IL-2R) (33%), the addition of dexamethasone (10^{-6} M) resulted in downregulation of IL-2R (6%). Concomitant evaluation of serum levels revealed higher level of soluble IL-2R in patient resistant to chemotherapy (12.9 ng/ml versus 1.2). Investigation of other cytokine receptors is being performed.

* Supported by FWF grant P 8947 Med

B8-317 NUCLEASE ASSAY BASED ON THE GENERATION OF LARGE AND SMALL DNA FRAGMENTS

Francis M. Hughes, Jr. and John A. Cidlowski, Department of Physiology, University of North Carolina at Chapel Hill, Chapel Hill, N.C. 27599

Recent reports have suggested that both large (300 Kb and/or 50 Kb) and small (oligonucleosomal) DNA fragments are generated during apoptosis. We have developed an assay to analyze the ability of nucleases to generate both forms of DNA cleavage. Briefly HeLa nuclei are suspended in 50 mM Tris (pH 7.4), 2 mM $MgCl_2$, 1 mM $CaCl_2$ in the presence or absence of purified nucleases or experimental preparations. Following incubation, the nuclei are divided and one portion analyzed for large DNA fragments by encapsulating nuclei in agarose, lysing in situ (100 mM EDTA, 1% sarcosyl), treating with Proteinase K and electrophoresis on a CHEF pulsed field electrophoresis system. Another portion of nuclei were analyzed for small DNA fragments by standard agarose gel electrophoresis. Kinetic analysis in the absence of added nuclease showed that HeLa cells do not spontaneously generate large or small DNA fragments (autodigestion) although some very high molecular weight signal in the pulsed field analysis was lost after 4 h of digestion. Analysis of micrococcal nuclease (MN; 1 ng) (known to cleave chromatin in internucleosomal regions) demonstrated a continuous decrease in the average size of DNA fragments until they reached ≈ 40-50 Kb, after which a signal was no longer apparent (indicating degradation of DNA to ≈ 5 Kb fragments which are lost by diffusion). Degradation of DNA by MN was apparent on a pulsed field gel significantly earlier than oligonucleosomal fragments were seen on standard agarose gels, a result probably indicative of the different sensitivities of detection of different size DNA fragments. Similar results were seen with DNase I (50 ng) except that small DNA fragments were of random size (revealing a smear on standard gels). DNase II at pH 6.3 showed no nuclease activity by either form of analysis. Nuclear extracts from glucocorticoid-treated rat thymocytes revealed a degradation pattern similar to that seen with MN. These results show the versatility of this assay for detecting genomic degradation into large and/or small fragments. Supported by DK32078, DK09150 and HD07315.

B8-318 SEARCH FOR GENES WHOSE PRODUCTS INHIBIT TNF/FAS INDUCED APOPTOSIS IN MCF7 BREAST CARCINOMA CELLS.

Marja Jäättelä, Lasse Foghsgaard and Vishva Dixit*. Dept. of Tumor Cell Biology, Danish Cancer Society Research Center, DK-2100 Copenhagen, Denmark and *Dept of Pathology, Univ. of Michigan, Ann Arbor, MI.

To identify genes that confer resistance to apoptosis induced by tumor necrosis factor (TNF), we used a cDNA expression cloning strategy employing cDNA libraries derived from TNF-resistant HeLa cells and Epstein-Barr virus transformed lymphoblasts. The library DNA was transfected into highly TNF-sensitive subclone of MCF7 breast carcinoma cells. Several rounds of selection with lethal doses of TNF resulted in the enrichment of cDNAs encoding for Bcl2 oncoprotein, a recently characterized closely related protein Bclx and an Epstein-Barr virus homolog of Bcl2, BHRF1. Introduction of these cDNAs individually into MCF-Fas cells (a subclone of MCF7 cells transfected with human Fas antigen) conferred complete resistance to apoptosis mediated by both TNF and anti-Fas. Interestingly, overexpression of these protective proteins in MCF-Fas cells blocked TNF and Fas-induced activation of phosholipase A2 whereas TNF-induced activation of NF-kB-like transcription factors was unaffected. These findings suggest that TNF and Fas activate a common death pathway which involves the activation of phospholipase A2 and that members of Bcl2 family interfere with this pathway at a step prior to the release of archidonic acid.

To find other cDNAs that protect cells from TNF-mediated apoptosis, we have started a new cloning experiment employing a cDNA library deleted of Bcl2, Bclx and BHRF1 by restriction enzyme digestion. Two rounds of selection with sublethal doses of TNF resulted in the enrichment of several cDNAs. We are presently testing them individually and those cDNAs that confer resitance against TNF and/or Fas will be sequenced and further characterized.

B8-319 THYMIDINE-INDUCED APOPTOSIS IN L929 CELLS IS ASSOCIATED WITH DNA HYDROLYSIS INTO SUB-NUCLEOSOMAL FRAGMENTS,

M.J. Sargus, J.S. Amenta, F. Durante, G. Barbiero, F.M. Baccino, G. Bonelli, University of Pittsburgth and University of Turin, Italy

Mouse L929 cells, when treated with 5 mM thymidine, show a sequence of morphologic changes characteristic of the changes identified with apoptosis in vivo (In Vitro Dev. Biol. 29A:955, 1993). However, no oligonucleosomes were identified at any time, either in the cells or medium. In the current study, we have attempted to ascertain the role of DNA fragmentation, if any, in this model of apoptosis. Difference methods for lysing and purifying the DNA did not account for lack of oligonucleosomes. However, labeled DNA showed extensive fragmentation, with release 50-60% of the total label recovered in the medium by 72h. Flow cytometry studies confirmed that a subpopulation of close to normal sized cells were losing most of their DNA and indicated that DNA fragmentation was associated with a down shift in cell pH. In vitro studies confirmedthat when L929 cells were incubated in a Tis buffer at pH 6.5 - 7.0 containing Triton X100 and EDTA, rapid and extensive DNA hydrolysis occurred, first to oligonucleosomes and then to suboligonucleosomal fragments. We conclude that thymidine-induced apoptosis in L929 cells is associated with a non Ca-dependent endonuclease activated by an increase in cell acidity, which results in an extensive fragmentation of DNA into subnucleonal fragments. We hypothesize that apoptosis is initially associated with chromatin denaturation followed by different degrees of DNA fragmentation, depending upon the specific cells and the different changes induced in cells by various agents inducing apoptosis.

B8-320 UNINTEGRATED HIV DNA SURVIVES IN APOPTOTIC T CELL DEBRIS AND REGENERATES INFECTIOUS VIRIONS AFTER PHAGOCYTOSIS BY MACROPHAGES.

Richard S. Kornbluth and Douglas D. Richman, Dept. of Medicine and Pathology, Univ. Calif. San Diego, and the San Diego V. A. Medical Center, La Jolla, CA 92093-0679.

CEM T cells infected with the lymphotropic strain HIV-LAI (LAI) undergo apoptotic cell death (J. Clin. Invest. 87:1710, 1991). This process generates cellular debris containing degraded cellular DNA but intact unintegrated HIV DNA. Scavenging macrophages avidly take up this debris and we have proposed that they become infected by the viral DNA it contains.

To test this hypothesis, we fed apoptotic debris containing radiolabelled DNA to macrophages and analyzed the DNA that survived intracellular digestion. Whereas almost all of the input DNA apoptotic ladder was destroyed inside of the macrophages, a discrete high m.w. form of radiolabelled DNA remained. This suggests that the DNA in the infected apoptotic debris may follow an intracellular pathway which evades hydrolysis.

The macrophages that take up this debris proceed to release infectious virions. Whereas AZT and soluble CD4 effectively blocked infection by HIV-1 BaL virions in macrophages, these agents were at best only partly effective in macrophages infected with debris containing LAI DNA. Conversely, rabbit polyclonal antibody produced against uninfected CEM cells had little effect on BaL infection but completely prevented infection by HIV-infected CEM debris. Pretreatment of macrophages with TNF-α had no effect on BaL replication but markedly enhanced the production of p24 in debris infected cultures (5-20X). These data suggest a distinct cell biology of a non-virion, DNA-mediated pathway for the infection of macrophages by apoptotic debris derived from HIV-1-infected cells.

B8-321 ANALYSIS OF BCL-2 HOMO-AND HETERO-DIMERIZATION PROPERTIES. Cornelia Kurschner and James I. Morgan. Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

The proto-oncogene product, Bcl-2, has been shown to specifically suppress apoptosis in a number of paradigms, although it cannot protect against all forms of cell death. Bcl-2 belongs to a growing family of Bcl-2-related proteins that share two "Bcl-2 homology domains", BH1 and BH2. Additional family members include Bax, Bcl-X, MCL-1, and A1. We have used the "two-hybrid system" in yeast, a potent method for the investigation of protein-protein interactions, to study the dimerization properties of murine Bcl-2 with other proteins of this family. Using various truncations of Bcl-2 as a "bait" in this system, we could determine different domains of the protein that are necessary for homo- or heterodimerisation. Co-immunoprecipitation studies were performed to confirm the findings. Furthermore, we screened a cDNA library with the two-hybrid method and recovered several clones encoding Bcl-2 binding proteins that are not members of the Bcl-2 or Ras families (R-Ras has been reported to bind to human Bcl-2 in the same system). Studies of the novel Bcl-2 binding proteins may lead to clues to the mechanism(s) by which Bcl-2 exerts its cell survival promoting actions.

B8-322 APOPTOSIS IS INDUCED EARLY DURING BACULOVIRUS INFECTION OF CULTURED INSECT CELLS, Douglas J. LaCount and Paul D. Friesen, Institute for Molecular Virology and the Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706

Baculoviruses are among a diverse group of DNA viruses that induce apoptosis in infected cells. Although apoptosis is important in viral pathogenesis, little is known about the signals that initiate the death program. We have used a genetic approach to define the signals responsible for baculovirus-induced apoptosis. Mutants of the baculovirus AcMNPV lacking the apoptotic suppressor gene *p35* cause *Spodoptera frugiperda* SF21 cells to die by apoptosis. We have constructed a *p35* null mutant (*p35*$^-$) with a temperature sensitive mutation (ts8) in the baculovirus helicase gene to determine if apoptosis is induced early or late during infection. At the nonpermissive temperature, ts8 blocks all late virus events, including DNA synthesis, virion production, and shut-off of host protein synthesis. When infected with the *p35*$^-$/ts8 mutant, SF21 cells underwent apoptosis at both the permissive and non-permissive temperature. Thus, an early gene or an early event in the virus life cycle was sufficient to induce apoptosis. Corroborating evidence was obtained by using a transformed cell line that blocks P35 function during infection. When these cells were infected with a *p35*$^+$/ts8 virus, apoptosis occurred at both temperatures, again suggesting that apoptosis was initiated early. To determine if apoptosis was induced by virus attachment to the cell, SF21 cells were infected with a *p35*$^-$ mutant in the presence of chloroquine, a drug that prevents acidification of endocytotic vesicles and blocks baculovirus infection prior to early gene expression. Since chloroquine prevented apoptosis, virus attachment was not responsible for initiating the death program. Together these experiments indicated that one or more early baculovirus genes were sufficient to induce apoptosis in SF21 cells.

B8-323 ENZYMATIC CHARACTERIZATION OF CYCLOPHILINS: POTENTIAL APOPTOTIC NUCLEASES Jennifer W. Montague[1] and John A. Cidlowski[1,2], Departments of Biochemistry and Biophysics[1], and Physiology[2], UNC-CH, Chapel Hill 27599.

The cleavage of chromosomal DNA into discrete fragments is a biochemical hallmark typifying thymocyte apoptosis. There are two specific patterns of DNA fragmentation: internucleosomal cleavage, which results in oligomers that are integer multiples of approximately 200 bp in length, and the more recently discovered large break cleavage, in which the DNA fragments are approximately 50 kbp in length. We have identified and purified and 18 kD nuclease (NUC18) from apoptotic rat thymocytes. Two peptides resulting from trypsin digestion of NUC18 display remarkable sequence similarity to the cyclophilin family of proteins (Cyps). Cyps and NUC18 also display structural similarity, as evidenced by the cross-reactivity of the antibodies. Based on these similarities, we obtained purified Cyps A, B and C to examine the possibility they may contain nuclease activity. All three forms of Cyp demonstrate the ability to degrade a variety of nucleic acid substrates, including supercoiled and linear plasmid DNA, as well as single-stranded DNA. Interestingly, Cyps can also degrade RNA, suggesting a possibility for a dual role for these proteins during apoptosis. The nuclease activity of Cyps B and C is stimulated by certain divalent cations, with greatest activity observed in the presence of magnesium or manganese, and weaker activation by calcium. Further, Cyps B and C demonstrate strongest activity under basic conditions, with an optimum ranging from pH 8 to pH 10. We are continuing to further characterized Cyp nuclease activity and are exploring the role it may play in apoptosis. This work was supported by NIH Grant DK 32078.

B8-324 THE ROLE OF BCL-2 AND FAS IN CONTROL OF APOPTOSIS IN KERATINOCYTES AND MELANOCYTES. David A. Norris, Marjorie H. Middleton, Kyu Whang, David Davis, and Richard Duke. Dept of Dermatology, Univ. of Colorado, Denver, CO 80262

Apoptosis is a fundamental biologic process for remodelling tissues and removing damaged or transformed cells. We investigated the role of the anti-apoptotic regulatory protein Bcl-2 and the apoptotic receptor Fas in control of apoptosis in keratinocytes and melanocytes.

Early passage cultured keratinocytes and transformed keratinocyte cell lines were susceptible to apoptosis triggered by cytokines, ultraviolet radiation (UVR), ionophores, and anti-Fas. Melanocytes and melanoma cells were generally resistant to induction of apoptosis.

Bcl-2 expression as measured by FACS and immunoblotting was very low in all keratinocyte lines studied. Increasing keratinocyte Bcl-2 expression by retinoids or UVR decreased the apoptotic susceptibility of these cells. Melanocytes and melanoma cell lines had very high constitutive levels of Bcl-2. Keratinocytes and melanocytes expressed high constitutive levels of Fas (measured by FACS). In some keratinocyte cell lines, Fas was further induced by IFN-g.

We propose that Bcl-2 is an important endogenous anti-apoptotic factor in melanocytes and melanoma cells. Fas expression is a constitutive feature of keratinocytes and melanocytes, but Fas-dependent apoptosis can be blocked in cells expressing high levels of Bcl-2. Induction of Bcl-2 expression increases keratinocyte resistance to apoptosis.

B8-325 SPECIFIC CLEAVAGE OF APC AND Rb TUMOUR SUPPRESSOR GENE PRODUCTS IN APOPTOTIC CELLS: POSSIBLE ROLE FOR APC AND Rb IN CELL SURVIVAL. Christos Paraskeva, Ann C. Williams, Angela Hague, Alison J. Butt and Sara J. Browne, University of Bristol, Department of Pathology and Microbiology, Bristol, BS8 1TD, U.K.

Adenomatous polyposis coli (APC) protein is expressed in the normal colonic crypt and believed to be involved in colonic cell maturation. We investigated whether the expression of the APC protein is associated with cell death in colonic epithelial cells. We have previously reported an *in vitro* system to study apoptosis[1]. Briefly, cells attached to the flask have a low frequency of apoptosis (1-3%) whereas cells that detach and float in the medium have a high proportion of apoptotic cells (36-96%). The full-length 300kD or truncated APC protein normally expressed by the attached cells was lost in the floating apoptotic cells in eight/eleven colon tumour cell lines. In addition, the APC antibody FE9 detected a 90kD protein in the floating apoptotic cells of all cell lines investigated which was not present in attached cells. Furthermore, loss of full-length APC and gain of the 90kD protein was observed in the apoptotic cells of two cell lines derived from other tissues: the SV40 transformed fibroblast cell line CMSV40fib and the lymphoblastoid B-cell line BJA-B. Interestingly, the levels of the full length retinoblastoma (Rb) gene product was significantly reduced in the apoptotic cells of most cell lines tested and a novel protein of 30 kD was detected in the apoptotic cells of all eleven colon tumour cell lines tested and in the apoptotic CMSV40 fib and BJA-B cells. In necrotic cells (freeze-thawed), full-length or truncated APC was also lost although a 95kD protein distinct from that in apoptotic cells was observed[2]. Specific loss of full-length or truncated APC (resulting in a 90kD protein in apoptotic cells but a 95kD protein in necrotic cells) is therefore associated with cell death. These findings suggest a possible role for APC and Rb in cell survival and may provide a further link between proteases and apoptosis.

[1]Hague, A. et al. Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: Implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int. J. Cancer* **55,** 498-505 (1993).

[2]Browne, SJ.et al. Loss of APC protein expressed by human colonic epithelial cells and the appearance of a specific low molecular weight form is associated with apoptosis in vitro. *Int. J. Cancer,* (In Press) 1994.

B8-326 PRIMITIVE HUMAN HEMATOPOIETIC PRECURSORS LACK BCL-2 EXPRESSION. Julie R. Park, Irwin D. Bernstein, David M. Hockenbery, Molecular Medicine and Pediatric Oncology Programs, Fred Hutchinson Cancer Research Center, Seattle, WA 98109.

A highly conserved, genetically programmed cell death pathway plays an important role in the regulation of hematopoiesis. Bcl-2 is the prototype of a family of genes which alters programmed cell death. We evaluated the expression of bcl-2 and its family members, bcl-x and bax, in hematopoietic cell populations to better understand their role in the maintenance of hematopoietic homeostasis and ultimately to understand mechanisms of the survival in the pluripotent stem cell population. Two color immunofluorescence cell sorting was utilized to separate adult bone marrow into mature hematopoietic cells lacking CD34 ($CD34^-$), lineage committed hematopoietic precursor cells expressing CD34 ($CD34^+lin^+$), and the more primitive lineage uncommitted precursors expressing CD34 and lacking maturation-linked surface antigens ($CD34^+lin^-$). Using immunohistochemical staining, we demonstrated that both $CD34^+lin^+$ and the majority of the $CD34^+lin^-$ precursor cells stained positive for bcl-2. Notably, the expression of Bcl-2 in the $CD34^+lin^-$ precursor cells was confined to the presumably cycling blast-sized cells while the quiescent small lymphocyte-sized cells lacked bcl-2 expression. Small lymphocyte-sized $CD34^+lin^-$ cells were further enriched for primitive precursors by selecting cells which lacked expression of CD38 ($CD34^+lin^-CD38^-$). These primitive precursor cells also lacked bcl-2 expression but were able to generate colony-forming cells in an in vitro long-term assay, demonstrating their ability to function as stem cells.

We have demonstrated that the primitive population which includes the quiescent hematopoietic stem cell, does not express the bcl-2 protein. We hypothesize that this population utilizes an alternative cell death suppressor protein, or it has not activated a cell death pathway thereby not requiring the expression of a cell death suppressor protein. Utilizing RT-PCR, we have demonstrated the expression of bcl-x and bax in the mature $CD34^-$ cell populations, the $CD34^+lin^+$ cells and the $CD34^+lin^-$ precursor cells. Studies are underway to evaluate the expression of these family members in the more primitive subpopulations of $CD34^+lin^-$ cells.

B8-327 ROLE OF MURINE SCAVENGER RECEPTOR IN THE RECOGNITION OF APOPTOTIC THYMOCYTES BY MACROPHAGES

Nick Platt and Siamon Gordon, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

One of the important phagocytic functions of the macrophage is the specific recognition and engulfment of dying cells. A number of cell surface receptors on the phagocyte, including the vitronectin receptor and thrombospondin, have been implicated in mediating this interaction, particularly in the removal of apoptotic neutrophils (Savill et al; Immun. Today :14,131,1993). Specifically, we have chosen to investigate the role of the endocytic receptor, the macrophage scavenger receptor (MSR) in the phagocytosis of apoptotic thymocytes.

The resident macrophages in the thymus have been identified by immunocytochemistry and found to express a number of markers including the pan-macrophage cell surface molecule F4/80 and class II MHC. In addition, these cells stain strongly with a mAb (2F8) that is specific for the murine scavenger receptor. This mAb was isolated by its ability to inhibit scavenger receptor-mediated macrophage adherence (Fraser et al; Nature:346,343,1993). Isolated thymic macrophages are phenotypically identical to resident thymic macrophages and retain their ability to rosette thymocytes.

We have directly tested the involvement of MSR in the recognition of dying thymocytes by establishing an *in vitro* phagocytosis assay. Elicited peritoneal and thymic macrophages (which are both MSR positive) were overlaid with steroid-treated apoptotic thymocytes which had been labelled with the fluorescent lipophilic dye PHK2. Phagocytosis of the labelled apoptotic cells was quantitated by microscopy and was shown to be specifically inhibited by preincubation of the macrophages with the mAb 2F8, which reduced uptake to 50% of control levels and the MSR ligand polyinosinic acid, which inhibited by almost 90%. An isotype matched mAb and the related polyribonucleotide polycytidytic acid had no effect on phagocytosis.

These data are consistent with the direct involvement of scavenger receptor in the clearance of apoptotic thymocytes by thymic macrophages. We are currently using additional approaches to modulate MSR activity and are examining the contribution of other receptors.

B8-328 BCL-2 PROTECTS AGAINST BOTH OXIDATIVE INJURY AND HYPOXIA-INDUCED PROGRAMMED CELL DEATH. Brent A. Rupnow, Mark S. Gilbert, David A. Ramirez, Kirk D. Trisler and Susan J. Knox, Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305.

The proto-oncogene product, Bcl-2, inhibits programmed cell death (PCD) in a variety of cells and may play a role in tumorigenesis. The mechanism by which Bcl-2 inhibits PCD is unknown. The human B-cell lymphoma cell line, PW, was transfected with Bcl-2 or the same vector for neomycin resistance only. Parental PW cells underwent PCD following γ-irradiation. The Bcl-2 transfected PW cells over-expressed Bcl-2 and were significantly more radioresistant than parental PW cells ($p<0.0005$). The effect of Bcl-2 on reactive oxygen species (ROS) and non-ROS mediated cell death was studied using the bioreductive cytotoxin SR4233. SR4233 generated ROS in the presence of oxygen and a non-ROS SR4233 free radical under hypoxic conditions. The viability of Bcl-2 transfected cells was significantly greater ($p<0.02$) than that of parental PW cells treated for one hour with 100μM SR4233 under aerobic conditions, but there was no significant difference in the viability between the two groups similarly treated with SR4233 under hypoxic conditions ($p=0.2$). Incubation of parental PW cells with N-acetyl cysteine (NAC; 100mM) decreased the cytotoxicity of SR4233 under aerobic but not anaerobic conditions. NAC had no significant effect on the viability of Bcl-2 transfectants, suggesting that Bcl-2 and NAC may be acting in a similar but not additive fashion to inhibit ROS-mediated cytotoxicity. Depletion of cellular glutathione (GSH) with 2mM Buthionine sulfoxamine (BSO) killed nearly 100% of control PW cells, but none of the Bcl-2 transfectants under the same conditions. The TBARS assay for lipid peroxidation byproducts was performed to measure ROS-induced cellular damage. Bcl-2 transfectants had a significantly lower level ($p<0.02$) of lipid peroxidation than parental PW cells following a 24 hour constant exposure to 100μM SR4233 under aerobic conditions. These results provide further evidence that Bcl-2 may act at the level of antioxidant pathway(s) and that Bcl-2-mediated inhibition of PCD may be due, in part, to elimination of ROS. Furthermore, hypoxia alone caused PCD in parental PW cells. Again, Bcl-2 transfectants were significantly more resistant ($p<0.0001$) to hypoxia-induced PCD than parental PW cells. This observation suggests that Bcl-2 may act through multiple pathways and may contribute to both the size of the hypoxic fraction and the relative radioresistance of hypoxic cells in tumors.

B8-329 THE TNF-INDUCED GENE A20 PROTECTS FROM APOPTOSIS, Vidya Sarma, Beth M. Rust, Muneesh Tewari and Vishva M. Dixit, Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109.
Previously, we have reported that the novel zinc-finger protein A20 confers resistance to tumor necrosis factor α (TNF) cytotoxicity. However, since then we have found that A20 expression was also induced by agents that promote cell survival. For example, A20 was found to be induced in B cells following CD40 activation which confers resistance to programmed cell death. Activation of B cells via the CD40 pathway is thought to allow B cells to gain entry into the memory B cell population, an entry normally restricted by apoptosis. A20 is also constitutively expressed in Epstein-Barr virus (EBV) immortalized B cells and further it is the EBV-LMP1 gene that is responsible for the induction of A20. LMP1 protects B cells from apoptosis in part by the induction of *Bcl2*, and we now show in part by the induction of A20 thereby promoting viral persistence.

B8-330 REGULATION OF POLY(ADP-RIBOSE)POLYMERASE ACTIVITY BY THE APPEARANCE OF INTERNUCLEOSOMAL APOPTOTIC DNA FRAGMENTS. - A. I. Scovassi, C. Negri, R. Bernardi, M. Donzelli, E. Prosperi* and F. Guano - IGBE CNR and *Centro per l'Istochimica CNR, 27100 Pavia, Italy.

In HeLa cells treated with the chemotherapeutic agent etoposide (VP-16) we have analyzed different cellular parameters typical of apoptotic cell death. We have shown that nuclear morphology is affected by VP-16, that VP-16 causes complete disruption of nucleoli and that DNA is cleaved at internucleosomal region. In this experimental system, where typical features of apoptosis are visible after VP-16 administration, we investigated whether the ADP-ribosylation process could be regulated by the presence of apoptotic fragments of DNA appearing at early times after administration of 10-100 μM VP-16. By means of specific assays (activity gel, boronate chromatography) we observed that in extracts from cells where fragmentation occurs, the autoribosylated form of poly(ADP-ribose)polymerase is greatly enhanced (1). This observation suggests that the activation of poly(ADP-ribose)polymerase mediated by DNA fragmentation could represent a typical biochemical step in apoptosis. To better understand the sequence of events occurring during etoposide induced apoptosis, we analyzed ADP-ribosylation process in cells treated with Zn, a very well known modulator of small-sized DNA fragmentation. The appearance of characteristic ladder due to the internucleosomal DNA cleavage is affected by the presence of zinc in a dose/time-dependent manner. In cells still apoptotic but in which internucleosomal DNA fragmentation due to the combined VP-16/Zn effect was not visible, poly(ADP-ribose)polymerase was not activated (2,3). Our data indicate that the primary event regulating the poly(ADP-ribose)polymerase activity during apoptosis is the appearance of internucleosomal DNA fragmentation.

1. Negri C. et al. - Carcinogenesis 14: 2559-2564 (1993). **2**. Guano F. et al. - Cell Death and Differentiation 1: 101-107 (1994). **3**. Bernardi R. et al. - Biochimie, in press.

B8-331 NEUTROPHIL APOPTOSIS IS CALPAIN-DEPENDENT. Amy J. Sehnert, Margaret K. T. Squier, and J. John Cohen. Departments of Immunology and Pediatrics, University of Colorado Medical School, Denver CO 80262, USA.

Human peripheral blood neutrophils (PMN) are short-lived leukocytes which are known to die by apoptosis and undergo subsequent ingestion by local macrophages. We have studied PMN survival in vitro using morphological changes as assessed with ethidium bromide/acridine orange staining, and quantitative measurement of DNA fragmentation. The time to 50% apoptosis in untreated normal PMN averages 16-18 hours. Apoptosis can be accelerated by treatment with actinomycin D or cycloheximide, with 50% apoptosis occurring in 3-5 hours. We have previously shown that the calcium-dependent neutral protease calpain is involved in apoptosis of murine thymocytes induced by glucocorticoids. In addition, calpain has been described as playing a role in PMN activation. We investigated the effects of calpain in PMN apoptosis. Inhibitors of calpain blocked both spontaneous and cycloheximide-accelerated apoptosis; lysosomal proteases were not involved in the process. Activation of calpain by inhibiting the synthesis of a specific endogenous calpain inhibitor also induced PMN apoptosis. These studies therefore indicate a central role for calpain in this very important human model of apoptotic death of normal cells (approximately 10^{11} PMN die each day).

B8-332 A NOVEL GENE RELATED TO Bcl-2 IS EXPRESSED IN EARLY HEMOPOIETIC LINEAGES, Hee-Sup Shin, Sunshim Choi, and Youngchul Sung, Department of Life Science, Pohang University of Science and Technology, Pohang, 790-784, Republic of Korea
Bcl-2 was originally isolated by its oncogenic role in B-cell lymphoma. Bcl-2 extends the cell survival by acting against a variety of apoptotic processes. An increasing number of Bcl-2-related proteins have been reported, all of which share two domains of highly conserved aminoacid residues, referred to as Bcl-2 homology 1 and 2 (BH1 and BH2). These two domains are essential for inhibition of apoptosis. A cDNA clone with a homology to Bcl-2 has been isolated from a human fetal liver at 22-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 database to be used for human genome analysis. The initial homology was recognized by its 69 % aminoacid identity to the murine A1 gene, a member of the Bcl-2-related proteins. The homology to the BH1 and BH2 domains of Bcl-2 was especially significant, suggesting that this human cDNA may be a new member of the Bcl-2-related proteins. A northern blot containing total RNAs of a fetal liver, a normal adult liver, several primary liver tumors, a normal bone marrow, and HL60, H9, THP1, Raji, and Molt cell lines were probed with this cDNA. Only the bone marrow, HL60, and Raji RNAs were hybridized with this clone, revealing a transcript slightly smaller than one kb. These results indicate that this novel gene may only be expressed in some of the early hemopoeitic cell lineages. Currently we are carrying out experiments to express this gene in apoptosis-inducible cell lines to examine whether this new Bcl2-related gene has in fact an apoptosis-inhibiting activity.

B8-333 **CELLS DIFFERENTIATING INTO NEUROECTODERM UNDERGO APOPTOSIS IN THE ABSENCE OF FUNCTIONAL RETINOBLASTOMA FAMILY PROTEINS** R.S.Slack, B.Lach#, J.Craig*, K.Jardine*, M.W.McBurney* and F.D.Miller; Montreal Neurological Institute, McGill University, Montreal, Canada; *Dept. Medicine, #Dept.Pathology, University of Ottawa, Ottawa, Canada.

Stable clones of P19 embryonal carcinoma (EC) cells were established in which the activity of the retinoblastoma (Rb) family of proteins has been ablated. We used deletion mutants of the adenovirus early protein, E1A, that were defective in the binding of p300 but could bind and inactivate pRb, p107 and p130. Following induction of neuroectodermal differentiation, cells lacking functional Rb exhibited massive cell death relative to parental cells expressing control plasmid only. Ultrastructural examination revealed chromatin condensation and collapse of the nuclei and cytoplasm into membrane bound vesicles. DNA laddering, indicative of internucleosomal cleavage of DNA, was clearly visible in all clones possessing E1A mutants capable of inactivating pRb, p107 and p130. Stable clones expressing E1A mutants defective in the binding of pRb, p107 and p130, differentiated normally and did not exhibit apoptosis. Clones lacking functional Rb exhibited a dramatic upregulation of c-fos coinciding with the time at which apoptosis was initiated. Our results suggest that in terminally differentiated neurons the Rb family may serve as a molecular link to the signalling cascade leading to apoptosis. We are presently using adenoviral vectors to perturb cell cycle regulatory genes in sympathetic neurons to gain insight as to their function after terminal differentiation.

B8-334 **SIMULTANEOUS ASSESSMENT OF DRUG EFFECTS ON MICROTUBULE STRUCTURE AND APOPTOSIS BY DUAL FLUORESCENCE LABELING.** Charles D. Smith and Anna-Marija Helt, Pharmacology, Fox Chase Cancer Center, 7701 Burholme Ave, Philadelphia, PA 19111.

Drugs which disrupt the dynamic cycling of microtubules, e.g. vinblastine, cryptophycin and taxol, induce DNA fragmentation characteristic of apoptosis, demonstrated by the presence of DNA ladders upon electrophoresis. These ladders become visible at drug concentrations which inhibit cell proliferation by approximately 50%, suggesting that fragmentation is related to cytotoxicity. To overcome uncertainty associated with the analysis of DNA from a population of cells, we have also assessed the effects of these drugs on the ability of terminal deoxynucleotidyl transferase to incorporate digoxigenin-dUTP into DNA using the Apoptag™ kit from Oncor. Using this system, apoptosis was found to be closely related to cytotoxicity for all three drugs, i.e. the percentage of cells incorporating the nucleotide was similar to the percentage of cells killed. Finally, we wished to observe relationships between alterations in microtubule morphology and the induction of apoptosis in individual cells. For these experiments, cells were treated with drug for 48 hr and then fixed. The Apoptag™ assay was performed as usual, except that a mouse anti-β-tubulin antibody was included with the goat anti-digoxigenin antibody. After washing, the microtubules were visualized with a fluorescein-conjugated anti-mouse IgG antibody, while apoptic nuclei were stained with a Texas-Red-conjugated anti-sheep IgG antibody. Using this assay, it is clearly apparent that *these drugs can induce apoptosis without disrupting* cytoplasmic microtubules, indicating that the molecular signal which initiates the apoptic response to antimicrotubule drugs is unrelated to the overall microtubule distribution. This dual staining method can be adapted for the simultaneous staining of apoptic nuclei and any cellular protein of interest, e.g. bcl-2, p53 etc.

B8-335 MOLECULAR CLONING OF PORCINE FAS ANTIGEN AND EXPRESSION IN CELL LINES DERIVED FROM MINATURE SWINE, Timothy P.L. Smith and Craig W. Beattie, Genetics and Breeding Unit, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE 68933

Human and mouse Fas antigens are type II receptors of the NGF family that play a critical role in at least one pathway of apoptosis. We report the sequence of porcine Fas antigen, which has overall identity of 60, 48, and 65% with the proteins from human, mouse, and cow, respectively. The predicted porcine Fas protein is 332 amino acids in length. In comparison to the human protein, the three cysteine-rich domains show 88% identity (70% homology); the signal transduction domain shows 93% identity (65% homology); and the negative regulation domain shows 73% identity (47% homology). One of the five cDNA clones obtained showed evidence of alternative splicing, with one exon in the extracellular domain spliced out. Examiniation of a variety of porcine tissues with primers that amplify this region of the mRNA failed to show expression of this variant form in detectable amounts. Thus far, we have detected Fas mRNA in porcine corpus luteum, lung, ovary, spleen, testicle, and thymus via RT-PCR, but have failed to observe expression in brain, liver, or heart muscle.

We have examined the expression of Fas mRNA in tumors taken from Sinclair miniature swine that display an inherited form of melanoma. In this animal model, tumors on animals that survive to weaning invariably regress spontaneously, a phenomenon that may proceed via apoptosis. *Lesions from all stages of tumor development, from dysplastic nevi to* regression phase, were examined for Fas mRNA expression via RT-PCR. Surprisingly, we detected Fas mRNA in the majority of early stage (rapidly growing) tumors, while only 3 out of 15 tumors in the regression phase tested positive. In contrast, we detected Fas mRNA in all 6 available miniature swine melanoma cell lines that were derived from tumors at various stages of development.

B8-336 HIGH BCL-2 EXPRESSION DOES NOT PREVENT INDUCED APOPTOSIS IN HUMAN MYELOMA CELL LINES, Helena Spets, HelenaWiklund, Patrik Georgi-Hemming and Kenneth Nilsson, Department of Pathology, University Hospital, S-75185 Uppsala, Sweden.

In many cell lines specific growth factors may act as survival factors prevent cell death by apoptosis. In some model systems bcl-2 seems to be involved in the maintenance of survival as an anti-apoptosis gene. To determine the role of IL-6 and bcl-2 in the survival of human multiple myeloma cell lines we analysed the early, IL-6 dependent, and late, IL-6 independent, passages of the human myeloma cell lines U-266 and HL 407 during growth factor deprivation and staurosporine treatment. When the cell lines U-266-1970 and HL 407E, both dependent on IL-6 and 10% FCS for growth ,were cultured for 96 hrs in the presence of 10% FCS but without IL-6 the number of viable cells was almost unaltered. The U-266-1970 cell line could be cultured for a month without IL-6 and not loose more than 20% in viability. When both IL-6 and FCS or FCS alone were absent from culture medium for 96 hrs the number of viable cells decreased with 20-30 % for U-266-1970 and 25-35 % for HL 407E. The viability of the IL-6 independent cell lines U-266-1984 and HL 407 L was also reduced upon FCS depletion. In the absence of FCS the viability declined to 0% in 21-26 days. Apoptosis could be induced in all myeloma *cell lines by inhibition of PKC (staurosporin) and protein* synthesis (cycloheximide). After 4 hrs incubation with either drug the cells showed apoptotic morphology and a DNA-ladder could be visualised on an agarose gel. Cells killed after FCS depletion had the same apoptotic morphology. Bcl-2 is highly expressed in the U-266 cell lines. We show here that Bcl-2 is present in myeloma cells with apoptotic morphology and therefore seems to be unable to prevent apoptosis induced by staurosporine and cycloheximide in these cells.

B8-337 A ROLE FOR CALPAIN IN A COMMON APOPTOSIS PATHWAY, Margaret K.T. Squier, Anita C.K. Miller, Alvin M. Malkinson, and J. John Cohen, Department of Immunology and School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO 80262

Physiological cell death is an active process wherein the cell initiates a sequence of events culminating in chromatin damage, nuclear collapse, and disintegration of the cell into small, membrane bound apoptotic bodies. Examination of this death program in various models has shown common themes, including a rise in calcium, cytoskeletal changes, and redistribution of membrane lipids. The calcium-dependent protease calpain has proposed roles in cytoskeletal and membrane changes in other cellular processes; this led us to test the role of calpain in a well-known model of programmed cell death, that of thymocytes after treatment with dexamethasone. Assays for calcium-dependent proteolysis in thymocyte extracts reveal a rise in activity with a peak at about one hr of incubation with dexamethasone, falling to background at approximately two hr, just prior to development of morphological apoptosis. Western blots indicate autolytic cleavage of the proenzyme precursor to the calpain I isozyme, providing additional evidence for calpain activation. Several "induction" models of thymocyte apoptosis, including that of dexamethasone-treated thymocytes, can be blocked by specific inhibitors of calpain. Apoptosis of bone marrow metamyelocytes incubated with cycloheximide, a "release" model, also shows this calpain dependence. These results suggest a required calpain activation step in a common pathway to apoptotic cell death.

B8-338 SOMATOSTATIN INDUCES APOPTOSIS IN MOUSE PITUITARY TUMOR (AtT-20) CELLS. C.B. Srikant, Fraser Laboratories, McGill University and Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1

Synthetic somatostatin (SST) octapeptide analogs inhibit growth of tumors by decreasing tumor growth rate and, in some instances, induce tumor regression. *In vivo* and *in vitro* studies suggest that SST not only inhibits the mitogenic activity of growth factor receptor kinases (which are dephosphorylated and inactivated by SST sensitive tyrosine phosphatase) but also by inducing apoptosis. Growth factor induced mitogenic signaling for cell growth and proliferation is primarily sensed in G_1 phase of the cell cycle. In order to determine whether SST induces apoptosis in a phosphorylation dependent and cell cycle-specific manner, and to correlate with its inhibitory action on growth factor kinases, AtT-20 mouse pituitary tumor cells were first synchronized in G_1 and then cultured in the presence or absence of octreotide SMS 201-995. Cell cycle progression and induction of apoptosis were monitored by dual label flow cytometric analysis. Correlation of cellular DNA content (propidium iodide staining) and apoptosis (non-radio active terminal transferase mediated end labeling of DNA with digitonin-dUTP and FITC conjugation - Oncor Apoptag kit) revealed that SMS 201-995 induced apoptosis in a dose-dependent manner (10^{-10} - 10^{-7} M) in cells that progressed through the cell cycle and divided. The appearance of apoptotic cells correlated with a reduction in the number of cells in G_1, although the cells undergoing apoptosis appeared to be in S phase. Addition of EGF did not reduce the apoptotic effect of SMS 201-995. No apoptosis was observed in cells arrested in G_1 or G_2. SMS 201-995 was able to induce time dependent apoptosis when added to G_2 synchronized cells, but not in presence of G_2 block (160 nM staurosporine). This effect was found to be both G protein dependent and protein tyrosine dephosphorylation mediated since S01-995 induced apoptosis was blocked by pertussis toxin pretreatment and 10 μM orthovanadate respectively. These findings suggest that SST induced apoptosis is signalled in a cell cycle-specific manner via a G protein dependent activation of protein tyrosine phosphatase.

B8-339 MULTIPLE ETOPOSIDE-INDUCED RESPONSE PATHWAYS IN HUMAN LEUKEMIC CELLS AND THEIR RELATIONSHIP TO 'CLASSIC' APOPTOSIS. Bernard W. Stewart, Robert J. Sleiman & Daniel R. Catchpoole, Children's Leukaemia & Cancer Research Centre, University of New South Wales, Prince of Wales Children's Hospital, Sydney 2031, Australia.

We have described 'classic' apoptosis in a human T-cell lymphoblastoid line, CCRF-CEM (CEM), exposed to etoposide (VP16) at concentrations of 5μM or more; under the same conditions, a similar line, MOLT-4 failed to exhibit 'ladders' or apoptotic bodies (Catchpoole DR, Stewart BW; Cancer Res 53:4287-96,1993). Flow cytometric analysis indicated absence of a G2/M block in either line, although such an effect was evident using lesser concentrations of drug. This 'concentration' effect has now been further studied in relationship to a range of indicators of cell cycle, differentiation & apoptotic pathways. Within 6h of exposure to either cytotoxic (5μM) or cytostatic (0.5μM) VP16, increase (greated than that induced with phorbol ester) in purine nucleotide phosphorylase expression (assayed by RT-PCR) occurred, whilst changes in c-myc expression were less marked. Lesser responses to other indicators of differentiation were observed. In DNA isolated 24 hr after exposure of CEM cells to VP16, 'ladders' were only detected using concentrations of 5μM or greater. Expression of DNase I was barely detectable and not related to drug treatment. Cytospin preparations of VP16-treated CEM cells were used to quantify treatment-related effects on nuclear change and apoptotic body formation; similarly treated cells were employed to assess uptake of trypan blue. Loss of membrane integrity was evident 24h after exposure to 0.2μM VP16 while morphological evidence of apoptosis was not evident except using much higher drug concentrations. The data preclude simple causal relationships between various indicators of apoptosis, and are consistent with multiple response pathways only some of which culminate in cell death.

B8-340 THE ROLE OF ONCOGENES AND TUMOUR SUPPRESSOR GENES IN CELL DEATH REGULTION AND NEOPLASTIC TRANSFORMATION. A. Strasser, A.W. Harris and S. Cory. The Walter and Eliza Hall Institute of Medical Research, PO, Royal Melbourne Hospital, Victoria 3050, Australia.

Apoptosis is a physiological cell death programme responsible for removing obsolete and potentially dangerous cells of multiple lineages and is therefore indispensable for normal development. Oncogenes and tumor suppressor genes play a prominent role in cell death regulation.

bcl-2 is the first example of a new class of oncogenes, which regulates cell survival but does not influence proliferation or differentiation. Enforced *bcl-2* expression prolongs survival of cells after factor deprivation and exposure to various cytotoxic stimuli. Genetic analysis demonstrated that Bcl-2 plays a crucial role in the regulation of cell survival during lymphocyte development and function.

The tumour suppressor gene *p53* is only essential for apoptosis induced by γ-irradiation but not by other cytotoxic stimuli. Loss of function of *p53* and gain of *bcl-2* function do not cooperate in lymphocyte survival in *bcl-2* transgenic *p53* deficient mice. Furthermore *bcl-2* expression directly inhibits wild type *p53* induced apoptosis in mouse erythroleukemia cells. This suggests that *p53* and *bcl-2* are positive and negative regulators in the same apoptosis inducing signal transduction pathway.

Unexpectedly, cycling lymphocytes from $p53^{-/-}$ mice underwent apoptosis after maturation or genotoxic drug treatment. Hence p53 is not the only mediator of DNA damage induced apoptosis. Bcl-2 expressing $p53^{-/-}$ lymphoblasts were subject to growth arrest but resisted apoptosis. Their accumulation in G1 as well as G2 is suggestive of a p53-independent DNA-damage G1 checkpoint. Since Bcl-2 increases the clonogenic survival of irradiated cells, expression of survival genes may pose a greater impediment to genotoxic cancer therapy than loss of p53.

Although Bcl-2 antagonises multiple cell death stimuli it cannot block apoptosis induced by signalling through the TNF or the related Fas/Apo-1 receptor. This indicates either that this death mechanism dominantly inhibits Bcl-2 function or that it constitutes a separate apoptosis inducing pathway. We propose a model to explain the regulatory networks of cell death and survival.

B8-341 *DAD-1*, AN EVOLUTIONARILY CONSERVED CELL DEATH SUPPRESSOR IN VERTEBRATES AND *CAENORHABDITIS ELEGANS*. Asako Sugimoto, Rebecca R. Hozak, Torahiko Nakashima, Takeharu Nishimoto, and Joel H. Rothman, Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706. Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812, JAPAN.

The *DAD1* gene was originally identified in a mutant hamster cell line. These cells undergo apoptosis at a restrictive temperature as the result of a missense mutation in the *DAD1* gene. This suggests that *DAD1* is essential to suppress apoptosis in these cells. *DAD1* homologues cloned from human, hamster, and *Xenopus laevis* encode highly conserved proteins. To understand how *DAD1* participates in programmed cell death, we are characterizing its function using the nematode *Caenorhabditis elegans* as a model system. First, we tested the ability of human *DAD1* to prevent developmentally programmed cell death in *C. elegans*. Human *DAD1* was expressed from a heat-shock promoter. Heat-shocked transgenic worms showed significantly fewer cell corpses than control worms. Moreover, the heat-shocked animals contained extra nuclei corresponding to surviving cells that normally undergo programmed cell death. These results demonstrate that human *DAD1* is sufficient to prevent cell death in *C. elegans*. To initiate genetic analysis of this gene, we have isolated a *C. elegans DAD1* homologue (*Ce-dad-1*). *Ce-dad-1* encodes a protein of 113 amino acids which shares high sequence similarity with vertebrate *DAD1* (63% identical, 83% similar to human/hamster *DAD1*). In addition, the position of the single intron in the coding region is conserved. Similar to human *DAD1*, *Ce-dad-1* expressed from a heat-shock promoter reduced the number of cell corpses and produced extra nuclei, suggesting that *Ce-dad-1* also has cell death suppressor activity. In addition, *Ce-dad-1* rescued the $DAD1^{ts}$ hamster cell line as efficiently as did the human or frog genes. Thus, *DAD1* appears to be an evolutionarily conserved cell death suppressor. In order to further understand the function of *Ce-dad-1*, we are currently isolating loss-of-function mutants and are characterizing the expression pattern of the gene.

B8-342 GENOMIC ORGANIZATION AND EXPRESSION OF THE RAT AND HUMAN TRPM-2 (CLUSTERIN) GENES Daniel Taillefer, Ann Clark*, Michael Griswold*, and Martin Tenniswood*, Dept. of Biochemistry, University of Ottawa, Ottawa, Canada, *Washington State University, Pullman, WA, and *W. Alton Jones Cell Science Center, Lake Placid, N.Y.

Many biological systems undergoing active cell death require the induction of specific genes and proteins. We have previously described the cloning of rat and human TRPM-2 (clusterin) cDNA. The gene is induced *de novo* in a subset of anatomically confined, terminally differentiated cells undergoing apoptosis during prostate involution.The mechanisms that control the transcription of genes induced during active cell death have not been elucidated. The rat and human clusterin promoters share more than 80% identity in the first 150 nucleotides upstream of the transcription start site. This homology drops to 34% for the remainder of the upstream sequence. Sequence analysis of the 5'-flanking sequence has revealed a TATAAA promoter element preceding the first exon at position (-30). Two sequences homologous to the CCAATT motif are positioned at (-95) and (-105). Several sequences homologous to binding sites for known transcription factors (AP-1, SP-1 and NFE-2 have been identified.To determine the relative importance of these motifs in controlling the constitutive and inducible expression of the clusterin gene, we have made a series of exo III deletions of the promoter inserted upstream of a luciferase expression cassette (pLuc-Link). The plasmids were transfected into several cell lines, including MCF-7, LNCap, NRK-52E and MSC-1 that express clusterin constitutively. Two distinct regions appear to be required for the regulation of luciferase activity from these constructs. The region spanning -1297 to -1238 appears to act as a repressor element since its presence decreased luciferase activity 2-3 fold. When a deletion mutant lacking a conserved AP-1, but still retaining the canonical TATA element, was transfected into these cells lines, luciferase activity was reduced to that of the promoterless parent plasmid. These data demonstrate that a region from -306 to -48 encompassing a conserved AP-1 domain, previously identified in both rat and human promoters, constitutes the minimal sequence required for efficient transcription of the luciferase reporter gene. Androgen deprivation of LNCaP cell results in an increase in reporter activity, suggesting that one or more addition regions may be important in the de-repression of the expression during active cell death. These results provide *in vivo* evidence for a common trans-acting repressor involved in constitutive expression of the clusterin promoter and suggest that additional cis acting domains are involved in the induction of the gene during ACD. Our future studies are directed at establishing the *in vivo* significance of this interaction and identifying the protein complexes involved.
(Supported by the Medical Research Council of Canada, and the National Cancer Institute of Canada).

B8-343 AGE DEPENDENT REDUCTION OF BCL-2 EXPRESSION IN PERIPHERAL T CELLS OF *lpr* AND *gld* MUTANT MICE, Akiho Tamura and Katsuyuki Yui, Division of Immunology, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Fas, a member of the tumor necrosis factor receptor family, is a cell surface receptor that can induce apoptosis. Mice homozygous for the mutations in the genes encoding Fas (*lpr*) or Fas ligand (*gld*) develop a lupus-like autoimmune syndrome and lymphoproliferative disorder characterized by the accumulation of double negative (DN) T cells [$CD4^-$ $CD8^-TcR\alpha\beta^+$ $CD45R(B220)^+$] with age. These cells are more susceptible to apoptosis *ex vivo* than normal T cells. Since apoptosis of lymphocytes is regulated by multiple gene products, we suspected that the defect in the Fas-mediated apoptosis pathway might influence the expression and function of other apoptosis-related gene products. To this end, we investigated the expression of the Bcl-2 protein in T cells of *lpr* and *gld* mice. The expression of Bcl-2 in peripheral T cells of aged *lpr* and *gld* mice (5-6 months) was significantly reduced when compared with their normal counterparts. This reduction in Bcl-2 expression was most apparent in DN T cells. $CD4^+$ or $CD8^+$ cells showed a broad distribution of Bcl-2 expression, suggesting a transitional reduction in Bcl-2 expression within these populations. This reduction was not observed in young *lpr* mice and progressed with age concomitant with the accumulation of DN T cells in the lymph nodes. In contrast, thymocyte subsets of *lpr* mice expressed Bcl-2 at the level comparable to their normal counterparts, suggesting that the reduction in Bcl-2 expression occurs in the periphery. We next examined whether mature *lpr* T cells expressing the reduced level of Bcl-2 protein are more susceptible to cortisone-induced cell death *in vivo*. The ratio of peripheral T cells recovered after 48 hr treatment with dexamethasone *in vivo* in *lpr* mice was 2 to 3-fold reduced when compared with that in normal mice, while no apparent differences were observed between *lpr* and normal thymocytes.

These results suggest that the alteration in the level of Bcl-2 expression is one of the mechanisms regulating the apoptosis of mature peripheral T cells. This mechanism may work when T cells that are destined to die cannot die due to the defect in the Fas-mediated apoptosis pathway. Alternatively, down-regulation of Bcl-2 may occur independent of Fas-mediated signals, and these T cells may be normally depleted by Fas-mediated apoptosis.

B8-344 THE COWPOX VIRUS *crmA* PROTEIN, AN INHIBITOR OF ICE, BLOCKS TNF- AND Fas-MEDIATED APOPTOSIS, Muneesh Tewari and Vishva M. Dixit, Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Cowpox virus encodes a protein called *crmA* which is an inhibitor of the Interleukin-1β converting enzyme (ICE). ICE, a homologue of the *C. elegans* death gene *ced-3*, has been suggested to play a role in mammalian cell death. We hypothesized that *crmA* might function as an inhibitor of apoptosis induced upon activation of two cytokine receptors: Fas and the TNF receptor. We tested this hypothesis by stably transfecting and expressing *crmA* in cell lines sensitive to either Fas or TNF-induced apoptosis. We report that *crmA* is a powerful inhibitor of cell death, capable of completely abrogating apoptosis induced by both these pathways. The data have two major implications. First, a function of *crmA* may be to inhibit cell death as a means of evading the immune response, since both TNF and Fas (through its role in CTL-mediated killing) are thought to be involved in the elimination of virus-infected cells in-vivo. Second, our results suggest that either ICE or a related protease is a component of the Fas- and TNF- induced death pathway.

B8-345 **INTERLEUKIN-1β (IL-1β) CONVERTING ENZYME IS NOT REQUIRED FOR APOPTOSIS IN AN IL-2-DEPENDENT T-CELL LINE**, John P. Vasilakos, Karen M. Keane, Dave A. Giegel and Brenda D. Shivers, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Co., Ann Arbor, MI 48105, USA.

Clonal T-cells undergo apoptosis (programmed cell death) when cultured without the appropriate cytokines. The cysteine protease, IL-1β converting enzyme (ICE), is implicated in apoptosis based on its similarity to the *C. elegans* CED-3 programmed cell death gene and the ability of ICE to induce apoptosis in transfected fibroblasts. Here we demonstrate that the murine interleukin-2 (IL-2)-dependent CTLL T-cell line expresses ICE mRNA and protein as shown by northern and western blot analysis, respectively. Moreover, this protein is functional as determined by cell extract cleavage of an artificial ICE substrate; however, inhibition of ICE enzymatic activity with two peptide inhibitors modified for cell permeation does not abrogate apoptosis following IL-2 deprivation as measured by DNA fragmentation and viability. Also, overexpression of ICE in transfected CTLL cells does not induce apoptosis in the presence of IL-2 nor increase the rate of apoptosis after withdrawal of IL-2. Our results suggest that ICE is not required for programmed cell death in lymphokine-deprived T-cells.

B8-346 EARLY AND LATE EVENTS INVOLVED IN RADIATION-INDUCED APOPTOSIS, David W. Voehringer, Michael D. Story, Raymond E. Meyn, Department of Experimental Radiotherapy, Univ. of Texas M.D. Anderson Cancer Center, Houston, Tx. 77030

The interrelationship of signal transduction pathways (calcium, protein kinase C, and cyclic AMP dependent protein kinase) and expression of certain genes (p53 and bcl-2) implicated in modulating radiation-induced apoptosis were studied in a B-cell mouse lymphoma from which both apoptosis-sensitive and apoptosis-resistant clones were derived. Modulation of DNA fragmentation was investigated and correlated to clonogenic cell survival. Whereas DNA fragmentation in the sensitive clones could be modulated by activators or inhibitors of signal transduction pathways, clonogenic survival was not necessarily modified. Resistant clones expressed lower levels of DNA fragmentation and enhanced cell survival with respect to ionizing radiation when compared to sensitive clones. Initial investigations into the p53 status in sensitive and resistant clones suggest that the sensitive B-cell clone expressed a functional p53 product and that the radiation resistant clone has a mutation at the p53 gene locus. Taken together, our data suggests early and late components of the cell death program. Events occurring in the early component of apoptosis, when blocked, lead to enhanced clonogenic survival. Inhibiting events in the late component, while blocking some of the characteristics associated with apoptosis, have no effect on eventual cell death. The role of signal transduction pathways, p53 and bcl-2 status will be discussed with respect to multistep carcinogenesis and susceptibility to radiation-induced apoptosis.

B8-347 MYC-MEDIATED APOPTOSIS REQUIRES WILD TYPE p53 IN A MANNER INDEPENDENT OF CELL CYCLE ARREST AND THE ABILITY OF p53 TO INDUCE $p21^{waf1/cip1}$, Andrew J. Wagner[1], John M. Kokontis, and Nissim Hay[2], The Ben May Institute and the Departments of [1]Biochemistry and Molecular Biology and [2]Pharmacological and Physiological Sciences, The University of Chicago, Chicago, IL 60637

The c-*myc* proto-oncogene plays an important and well-defined role in promoting cellular proliferation. In addition to this pivotal role, recent studies have also demonstrated that Myc is a mediator of apoptosis. Deregulation of Myc expression in conjunction with serum deprivation or other means of growth arrest can lead to cell death. The apoptotic pathway is dependent on functional Myc protein and may serve as a mechanism to protect an organism from uncontrolled cell cycles and tumorigenesis. That is, cells in which Myc expression is deregulated may not necessarily develop into tumors and instead can be eliminated by apoptosis unless a second event that blocks the apoptotic pathway occurs. These cells can be rescued from apoptosis by coexpression of "survival genes" such as Bcl-2 that block apoptosis. The product of the tumor supressor gene p53 has also been implicated in apoptosis and has been demonstrated to be an essential component in several apoptotic pathways. Here we present evidence that Myc-mediated apoptosis requires expression of functional p53. Retroviral-mediated expression of Myc in mouse embryo fibroblasts (MEFs) obtained from the p53 knockout mouse did not elicit apoptosis upon serum starvation. Additionally, a conditionally active MycER protein induced cell death in MEFs from wt but not knockout embryos. Furthermore, in a cell line with large deletions of both p53 alleles, Myc induced apoptosis only when a temperature-sensitive p53 was expressed at the permissive temperature, whereas wt p53 expression by itself induced growth arrest. Growth arrest by isoleucine starvation, in a manner independent of p53, was not sufficient for susceptibility to Myc-mediated apoptosis in cells not expressing wt p53. Thus, wt p53 activity is essential and growth arrest per se in the absence of functional p53 is not sufficient to induce apoptosis. Furthermore, apoptosis did not require induction of p53 target proteins including the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. Therefore, the role of p53 in apoptosis may be distinct from its role in cell cycle arrest. Altogether, these data indicate that wt p53 is required for Myc-mediated apoptosis and that overexpression of Bcl-2 or inactivation of p53 may be mechanisms by which Myc deregulation can lead to proliferation instead of programmed cell death.

B8-348 IDENTIFICATION OF CELL DEATH GENES IN *DROSOPHILA*, Kristin White*, John Abrams, Megan Grether, and Hermann Steller, *Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, and HHMI, Departments of Brain and Cognitive Sciences and Biology, M.I.T., Cambridge, MA 02139

Developmentally regulated cell death in *Drosophila* is indistinguishable in most aspects from cell death in higher organisms. Ultrastructurally it resembles apoptosis, exhibiting condensation of the nucleus and cytoplasm, and engulfment by circulating macrophages. Many of the signals which initiate cell death in mammalian systems also function in *Drosophila*, ranging from trophic interactions to X-ray induced damage. The genetic and molecular techniques available in this organism make it an ideal system to investigate the biochemical mechanisms involved in programmed cell death.

By screening for genomic regions which are involved in cell death in the *Drosophila* embryo, we identified a region which is globally required for programmed cell death. Deletions of these region not only blocked all cell death during normal development, but also provided protection against cell death induced by developmental abnormalities and by X-rays. Very high doses of X-rays were capable of inducing apoptosis, indicating that this region does not delete the cellular effectors. We have identified a gene in this region, *reaper* (*rpr*), which is an excellent candidate to be a key regulatory gene in the cell death process. This gene is expressed in cells which are fated to die 1 to 2 hours before they show any signs of degeneration. Expression of *rpr* is increased by treatments which induce cell death. Ectopic expression of *rpr* is sufficient to induce cell death in many types of cells. Eventually we hope that this work will lead us to understanding the entire pathway leading to cell death, from the selection of the doomed cell to the molecular effectors which actually kill the cell.

B8-349 NEUTROPHIL APOPTOSIS AND CLEARANCE BY MACROPHAGES IS ACCELERATED *IN VITRO* BY PROTEIN SYNTHESIS INHIBITION. MKB Whyte, *LC Meagher, JS Savill and *C Haslett. Dept. of Medicine, University Hospital, Nottingham NG7 2UH, and *Dept. of Respiratory Medicine, Royal Infirmary, Edinburgh EH3 9YW, U.K.

Apoptosis of neutrophils (PMN) leads to their phagocytosis and clearance by macrophages (Savill *et al* J. Clin. Invest. 83:865, 1989). PMN undergo apoptosis constitutively but their lifespan is extended, via inhibition of apoptosis, by treatment with inflammatory mediators (Lee *et al* J. Leukoc. Biol.54:283,1993), or by modulation of cytosolic calcium concentrations (Whyte *et al* J. Clin. Invest. 92:446, 1993). This implies that neutrophil apoptosis may normally be inhibited by endogenous survival genes. To further test this hypothesis we examined the effects of protein synthesis inhibition upon PMN apoptosis. Human peripheral blood PMN were cultured in the presence or absence of actinomycin-D (AD) and cycloheximide (CX) (RNA and protein synthesis inhibitors respectively). Apoptosis in PMN populations was determined by morphological criteria and confirmed by DNA electrophoresis. PMN apoptosis was promoted in a concentration-dependent manner by AD and CX. In 5 experiments, after 8h in culture, apoptosis was 8.4±1.9% in control populations, 58.3±4.2% in cells treated with 50μM CX ($p<0.001$) and 72.6±6.7% in cells treated with 1μM AD ($p<0.001$). Promotion of PMN apoptosis was associated with (1) concomitant loss of functional ability, CX and AD-treated cells showed loss of shape-change ability compared with controls and (2) promotion of macrophage recognition and phagocytosis of CX and AD-treated cells ($p<0.001$ in both cases). SDS-PAGE analysis of lysates form these populations showed specific modulation of two polypeptides, of 6 and 86kD. Preliminary sequence of these polypeptides will be presented, which were shown to be DNA-binding and of nuclear origin.

B8-350 ELEVATED LEVELS OF HEME OXYGENASE-1 mRNA DURING RAT PROSTATE REGRESSION. P. Wong (1), R.K. Kutty(1), G. Kutty(1), B. Wiggert(1), G. Chader(1), and M. Tenniswood(2). (1) National Eye Institute, NIH, Bethesda, MD, (2) Alton Jones Cell Science Center Inc, Lake Placid, NY.

Heme oxygenase (HO) consists of two isozymes, HO-1 and HO-2. The induction of HO-1 is thought to be a cellular response to oxidative stress. HO can effectively degrade heme, a prooxidant, to biliverdin, which in turn can be converted by biliverdin reductase into bilrubin, a product which is antioxidant in nature. It has been shown that there is an induction in HO-1 mRNA levels during intense light-induced retinal cell death in rats (Kutty et al., submitted). We were curious if this induction was specific for the retinal damage or if there was a similar induction in other models of cell death. In the present study, we examine HO-1 and HO-2 mRNA levels in the rat prostate one to four days after androgen ablation. In this system, most of the epithelial cell death that occurs, does so between 2 and 4 days after castration. Northern analysis shows that HO-2 mRNA levels remain consistent throughout the time course examined. HO-1 mRNA, however, is absent during the first two days after castration, evident at day 3 and markedly elevated at day 4. In the prostate, this elevation occurs after initial increases in both TRPM-2 and TGF-β mRNA levels have been observed. In situ studies show that the increase in prostate HO-1 mRNA levels after castration is localized to the stromal cells of the prostate. It is possible, therefore, that the elevated HO-1 levels may reflect a response to a general oxidative stress in the prostate during tissue regression.

B8-351 MONOCYTES ARE REQUIRED TO INDUCE APOPTOSIS IN PERIPHERAL BLOOD T CELLS, Mei X. Wu, John F. Daley, Robert A. Rasmussen, and Stuart F. Schlossman, Division of Tumor Immunology, Dana-Faber Cancer Institute; Harvard Medical Shool, Boston, MA 02115

Unlike immature thymocytes which can be readily triggered to undergo programmed cell death (PCD) or apoptosis by treatment with anti-CD3 mAb, ionomycin, or PMA, freshly-isolated, human peripheral blood T (PBT) cells are largely resistant to the apoptotic effects of these reagents. We demonstrate here that PBT cells can be readily induced to undergo apoptosis when cocultured with either autologous or allogeneic monocytes (Mo) in PMA-containing medium. Incubation of PBT cells with Mo at ratios of ≥2:1 for 18 hr resulted in maximal levels (80%) of apoptotic cell death yet apoptotic PBT cells, characterised by showing typical patterns of DNA "step-ladder" fragmentation, could be demonstrated as early as 6 hr after PMA stimulation. The mechanism whereby Mo enable PBT cells to undergo apoptosis in PMA-containing medium appeared to depend upon cell-cell contact or close-proximity between Mo and PBT cells rather than solely via soluble mediators. Supernatant prepared from PMA-stimulated Mo failed to prime PBT cells for apoptosis and no apoptosis was observed in PMA-containing medium if Mo were separated from PBT cells by semipermeable membranes. More importantly, it was demonstrated that Mo acquire the ability to prime PBT cells for apoptosis after treatment with PMA, and that treated Mo maintain this ability even after fixation with formaldehyde. Interestingly, the degree of apoptosis of $CD4^+$ T cells by crosslinking of CD4 molecules via a combination of gp120, anti-gp120 and GAMIg was significantly greater for T cells primed with PMA-treated Mo than for unprimed T cells. Together, these findings reveal an important role for accessory cells in priming resting PBT cells for apoptosis.

B8-352 MOLECULAR CLONING AND EXPRESSION OF THE BOVINE FAS-AG, Jakyoung Yoo, Roger T. Stone, and Craig W. Beattie, USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center (MARC) P.O.Box 166 Clay Center, NE 68933

Fas-Ag, a member of the tumor necrosis factor (TNF) receptor family, is a cell membrane protein which mediates programmed cell death (apoptosis) in certain cell types such as lymphocytes and fibroblasts. A full-length bovine cDNA for Fas-Ag has been isolated and its nucleotide sequence determined. The 2.8 kb cDNA contained 972 bp of coding sequence. The predicted 323 amino acid protein contains a leader peptide, a transmembrane domain, and three cysteine-rich sub-domains in the cytoplasmic region; a characteristic of TNF receptor family. Bovine Fas-Ag shows strong sequence similarity to the human (73%) and mouse (65%) cDNA sequences. Fas-Ag was expressed in peripheral white blood cells, lung, heart, spleen and ovary (corpus luteum) while expression was not detectable in liver, pituitary, cerebral cortex, placenta (coruncle and cotyledon) and skeletal muscle (by Northern blot analysis). Transfection of bovine Fas-Ag cDNA in mouse L929 fibroblasts resulted in positive immunoreactive binding on the outer cell membrane with a monoclonal anti-human FAS antibody. In an effort to study the regulation of Fas-Ag gene expression, its genomic DNA has been cloned. The bovine Fas-Ag gene consists of 9 exons spanning 40 kb. Its promoter region does not contain TATA elements although the sequence is very GC rich and contains several consensus sequences including SP1 recognition sites. The availability of the full-length bovine Fas-Ag cDNA and the genomic DNA enables a better characterization of the physiological roles of Fas-Ag in various bovine tissues including the immune system.

B8-353 FACTORS AFFECTING FLOW CYTOMETRIC DETECTION OF APOPTOTIC NUCLEI, Robert M. Zucker*, David J. Thomas†, and Kenneth H. Elstein*, *ManTech Environmental Technology, Inc., Research Triangle Park, NC 27709 and the †Environmental Toxicology Division, HERL, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711

Apoptotic thymocyte nuclei normally appear on a flow cytometric DNA histogram as a subdiploid peak. We observed that addition of a specific RNase A preparation to the detergent-based lysing buffer increased the fluorescence of toxicant-induced apoptotic nuclei to the level of untreated diploid nuclei. The chelating agent EDTA partially inhibited the RNase effect, suggesting contaminating divalent cations may have been involved. Moreover, spectrofluorometric analysis revealed that addition of RNase or divalent cations decreased the amount of DNA present in the lysate. This suggested that the upscale fluorescence shift was due to a decrease in the ability of the lysing buffer to extract DNA, possibly as a result of cation-induced chromatin condensation, rather than increased accessibility of fluorochrome binding sites due to apoptotic degeneration. Moreover, during a 16 hr culture, we observed a similar, but time-dependent, upscale shift in the fluorescence of thymocytes undergoing apoptosis either spontaneously or as a result of exposure to 1 μM tributyltin methoxide (TBT), 2% ethanol, 2% methanol, or 1 μM dexamethasone phosphate (DEX). This commonality of effect suggests that a similar magnitude of chromatin reorganization occurs in aging apoptotic cells in culture regardless of the method of apoptotic induction. These findings should alert investigators to potential inaccuracies in the flow cytometric quantitation of apoptosis in in vitro systems employing prolonged toxicant exposures or complex lysing cocktails that may contain active contaminants. (This abstract of a proposed presentation does not necessarily reflect EPA policy.)

Poster Session 4

B8-400 GENE EXPRESSION DURING INVOLUTION OF THE MOUSE MAMMARY GLAND, Craig S. Atwood, Mario Ikeda and Barbara K. Vonderhaar, Laboratory of Tumor Immunology and Biology, NCI, NIH, Bethesda, MD 20892

The mammary gland undergoes a reductive remodeling termed involution following the removal of the sucking stimulus at weaning. This process is characterized initially by a rapid decrease in milk synthesis, followed by epithelial cell death and tissue remodeling, resulting in the mammary gland regressing to a state resembling that of the virgin animal. The decrease in milk synthesis and the selective death of the mammary epithelial cells are thought to be a result of the withdrawal of lactogenic hormones. The objective of this project was to determine whether involution of mammary glands in whole organ culture could be used to study the mechanisms involved in the hormonal control of milk synthesis and death of mammary epithelial cells. Following estrogen and progesterone priming *in vivo*, mammary glands from 34 d old mice were cultured for 5-6 d in a serum-free medium supplemented with insulin (I), aldosterone (A), hydrocortisone (H) and prolactin (Prl) in order to induce lobuloalveolar development. Mammary glands were then placed in serum-free media containing only I and removed from culture at 0, 1, 3, 5, 7, 9, 12 and 15 d for assay. As an *in vivo* comparison, mice that had lactated for 8-10 d were weaned and mammary glands removed at 0, 3, 6 and 9 d post-weaning. DNA and RNA were extracted from the mammary glands for the analysis of DNA integrity and gene expression. Following the withdrawal of AHPrl from the culture media, the lobuloalveolar structures of the mammary glands regressed and a characteristic apoptotic DNA fragmentation pattern was observed within 3-4 d. These changes were paralleled by a rapid decrease in the expression of ß-casein and α-lactalbumin and an increase in the expression of TGFß1 and TGFß3. There was no change in the expression of TGFß2. Similar changes in the pattern of DNA fragmentation and expression of ß-casein and TGFß1, ß2 and ß3 were observed *in vivo*. The expression of other genes involved in apoptosis is currently being pursued. These results indicate that the withdrawal of lactogenic hormones from mammary glands *in vitro* results in a rapid decrease in milk protein expression, and an increase in the expression of apoptosis associated genes leading to programmed cell death. In addition, the whole organ culture method is an excellent model for characterizing the events involved in the involution of the mouse mammary gland.

B8-401 **ANDROGEN INDUCES *BCL-2* MEDIATED CHEMOTHERAPY RESISTANCE IN LNCaP ATCC CELLS.**

Berchem, G.J.,Bosseler, M.C., Voeller,J., and Gelmann, E.P. Division of Hematology/Oncology and the Lombardi Cancer Center, Georgetown Univsersity, 3800 Reservoir Road, N.W. Washington D.C. 20007.

Prostate epithelial cells are dependent on androgens and will undergo apoptosis upon androgen withdrawal. Prostate cancer cells initially retain androgen sensitivity, but in the course of the disease become androgen resistant, at a time when cellular *bcl-2* overexpression occurs. Prostate cancer is also widely unresponsive to cytotoxic chemotherapy which often works through apoptosis. We have observed that androgens induce *bcl-2* expression in the androgen-dependent LNCaP prostate cancer cell line. We asked whether androgens would effect chemotherapy cytotoxicity in prostate cancer. Etoposide (VP-16), a topoisomerase II inhibitor, is known to be a potent apoptosis inducer. We show that both growth and survival of LNCaP cells treated with etoposide is enhanced by simultaneous exposure to androgens. This effect is seen within 24 hours of etoposide exposure. We furthermore showed, using *bcl*-2 antisense nucleotides, that Bcl-2 is responsible for the increased number of cells undergoing apoptosis when LNCaPs were androgen withdrawn. LNCaP cells, although sensitive to VP16 induced apoptosis, do not undergo cell death in response to androgen withdrawal. CWR22, does undergo apoptosis in response to androgen withdrawal. We are investigating the block in the apoptosis pathway of LNCaP cells that results in resistance to androgen withdrawal, but not to VP16. Whereas previous therapeutic strategies in disseminated prostate cancer have sought to enhance chemotherapy cytotoxicity by androgen stimulation, our data imply that simultaneous androgen ablation and cytotoxic chemotherapy should be considered.

B8-402 EXPRESSION OF THE PROTO-ONCOGENE *BCL-2* BY CELLS OF OSTEOBLAST LINEAGE *IN VIVO*, Jon N. Beresford, Susan Walsh and Nighat Arnott, Bone Research Group, Bath Institute for Rheumatic Diseases & The University of Bath, Bath BA1 1HD, U.K.

In adult humans the maintenance of skeletal homeostasis is a function of the remodelling cycle. This process involves the removal (resorption) of a quantum of bone, referred to as a bone structural unit, by osteoclasts and its subsequent replacement by a team of osteoblasts. Under normal circumstances there is a precise balance between the amount of bone removed and that replaced. Thus, remodelling serves to prevent the accumulation of microdamage and maintain the mechanical integrity of the skeleton without affecting its mass.

Osteoporosis is the most common metabolic bone disease and it results from an imbalance between the activities of osteoclasts and osteoblasts during the remodelling cycle. Decreased bone formation is a consistent finding in all forms of osteoporosis and there is accumulating evidence that this results from a reduction in the *number* of osteoblasts. The mechanism for this reduction is currently unknown, although it has been widely assumed that it results from a defect in the recruitment and or proliferation of osteoblast precursors. An alternative hypothesis is that the decrease in osteoblast numbers results from aberrant programmed cell death (PCD), a process which has been implicated in the regulation of cellular homeostasis in a variety of adult tissues. To explore this possibility we sought to localise the expression of the product of the *bcl-2* prot-oncogene in an adult human bone.

Undecalcified, frozen sections of adult human osteophyte tissue were incubated with monoclonal anti-human Bcl-2 antibodies (BCl-2 124 & 100), or isotype-matched control antibodies. Sites of immunoreactivity were visualised using the APAAP technique. Bcl-2 was localised reproducibly to osteoblasts actively engaged in bone formation and expressing high levels of the osteogenic marker protein alkaline phosphatase. In contrast, osteoblast precursors and the majority of lining cells and osteocytes, which are both derived from the terminally differentiated, post-synthetic osteoblast population, were negative. This is represents the first demonstration that the product of a gene involved in the regulation PCD is expressed in adult human 0bone and strongly suggests that apoptosis is an important mechanism for regulating the size and ultimate fate of the osteoblast population. Future studies will focus on the localisation in bone of the expression domains of other members of the Bcl-2-related gene family and their expression by cultured adult-human bone-derived cells.

B8-403 **MABS AGAINST THE E2 MOLECULE (CD99) INDUCE APOPTOSIS OF HUMAN THYMOCYTES SUB-POPULATION**

G.Bernard, A.Taheri, J. C. Lambry, J.P.Breittmayer, C.Aussel, J.L. Martin and A.Bernard.

INSERM U 343 Hopital de l'archet BP 79 06202 Nice cedex 3

We described E2 as a 32 kD transmembrane glycoprotein involved in T cells adhesion since anti-E2 mAbs reacting with peculiar epitopes block spontaneous T cell rosettes. E2 displays isomorphism on T cells and mAbs against pan T epitopes induce on CD4 + CD8+ (DP) thymocytes a serie of puzzeling events. They induce DP human thymocytes to a strong homotypic aggregation involving most probably, an unknown integrin; they induce DP thymocytes to apoptosis within 12 hours; they induce exposure of phosphatidylserine (Pdtser) at the outer leaflet of the plasma membrane (flip-flop event). The pdtser flip-flop on platelet has been related to homotypic aggregation; however, on E2 induced thymocyte aggregation, anti pdtser mAbs have no blocking effect. The event was also related to removal of apoptotic cells by macrophages, a possibility we are investigating on thymocytes. Molecular modeling of both E2 and the pan T epitopes through which these event can be triggered revealed a well defined conformation with a proeminent Phe. It remain to explain why only DP thymocytes undergo these events, and their place in T cell differentiation.

B8-404 SUPPRESSION OF APOPTOSIS IN CULTURED OVARIAN GRANULOSA CELLS BY cGMP-MEDIATED MECHANISM Håkan Billig, Bodil Assarsson, David Björnheden. Department of Physiology, Göteborg University, Göteborg, Sweden.

Apoptotic cell death has been suggested to be the underlying mechanism of ovarian follicular atresia. *In vivo*, ovarian apoptosis is induced by androgens and GnRH and is prevented by FSH and estrogens. Apoptosis is confined to the granulosa cells. In isolated non-apoptotic granulosa cells, apoptosis occurs spontaneously *in vitro* in a time-dependent manner suggesting a loss of apoptosis-inhibiting factors present in the ovarian environment. *In vitro* apoptosis can be inhibited by EGF, TGFβ, bFGF and is tyrosine-kinase dependent, while FSH and other cAMP enhancing agents do not affect apoptosis. To study the regulation of apoptosis in granulosa cells by other intracellular second messenger systems, we have analyzed internucleosomal DNA fragmentation in granulosa cells isolated from 25 day old, PMSG (10 IU) treated rats and incubated for 24 h with sodium nitro prusside (SNP 0.01-100 μM). SNP increases cGMP levels by nitric oxide (NO) release which in turn stimulates soluble guanylate cyclase (GC). The extent of apoptotic DNA fragmentation was analyzed by autoradiography of size-fractionated DNA, labeled at 3'-ends by ^{32}P-ddATP, or by quantitation of low molecular DNA in the supernatant after Triton-X treatment and centrifugation at 15.000xG of the cells. SNP (1-100 μM) inhibited DNA fragmentation dose-dependently. When cellular NO was increased by nitroglycerin, DNA fragmentation was also inhibited. Because NO could have other cellular effects than stimulating GC, the cells were treated with 8-Br-cGMP and dibutyryl-cGMP (0.3-3 mM). These analogs inhibited apoptosis at 0.3 mM. The specificity of the cGMP analogs were demonstrated by the lack of effect of corresponding cAMP analogs. In addition, DNA fragmentation was also inhibited by β-phenyl-1, N^2-ethenoguanosine-3',5'-cyclic monophosphate (PET-cGMP, 100 μM), a potent membrane permeant activator of cGMP-dependent protein kinase type 1β. Furthermore, an increased viability, measured as MTT-formazan formation, after SNP treatment was demonstrated. These data suggest that ovarian granulosa cell apoptosis could be regulated by a cGMP-dependent mechanism.

B8-405 **Multiple activation pathways originate at the Fas/Apo-1 (CD95) receptor.**

Cifone, M.G., P.Roncaioli, A.Santoni and R. Testi.

Departments of Experimental Medicine, University of L'Aquila and Rome "La Sapienza", and Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata".

Fas/Apo-1 (CD95) is a widely expressed surface receptor whose crosslinking triggers cell death by apoptosis in a variety of normal and trasformed cell types, and which has been involved in mediated susceptibility of tumor cell to killing by cytotoxic T lymphocytes. Defining the biochemical pathways which leads signals from the membrane to cytosolic or nuclear targets responsible for cellular apoptosis is crucial to understand strategies adopted by neoplastic cell to avoid CD95-mediated cell death. We have recently shown that CD95 activates an acidic sphingomyelinase (aSMase), with ceramide production (Cifone et al., J. Exp.Med.1994). Here we show that in the lymphoid tumor cell line Hut78 aSMase activation is dependent upon activation of a phosphatidylcholine-specific phospholipase C (PC-PLC) through diacylglycerol (DAG) release. PC-PLC activation and DAG release are required for aSMase activation as D-609, an inhibitor of PC-PLC, blocks DAG release from PC vesicles treated with CD95-stimulated cell extract in vitro, and SM hydrolysis after CD95 crosslinking in vivo. Moreover, CD95 crosslinking activates a phospholipase A2 (PLA2), as detected by both arachidonic acid production in vivo and lyso-PC release from PC vesicles in vitro.Arachidonic acid release could be blocked by aristolochic acid, an inhibitor of PLA2, but not by D-609, suggesting parallel activation of both PLA2 and PC-PLC following CD95 crosslinking. Finally, nitric oxide production could be detected by CD95 crosslinking by both nitrite measurement and cGMP generation. These results indicate that multiple signal transduction pathways are generated from the CD95 receptor. Their possible involvement in the regulation of the apoptotic pathways is currently being investigated.

B8-406 ROLE OF APOPTOTIC CELL DEATH IN SALIVARY GLAND INJURY AFTER X-IRRADIATION, Rob P. Coppes, Birgit Peter, Lie J.W. Zeilstra, Arjan Vissink, Julian D. Down, Antonius W.T. Konings, Department of Radiobiology, University of Groningen, 9713 BZ Groningen, The Netherlands.

Mature salivary glands are composed of highly differentiated epithelial cells of slow cell renewal. As such, the expression of radiation is expected to be delayed but this does not appear to be the case in this tissue. Local X-irradiation of rat parotid and submandibular glands with a single dose of 15 Gy induced very morphological changes. Special attention was given to nuclear aberrations and cell lysis. During the first day after irradiation, the most obvious changes were degranulation of serous cells and induction of nuclear aberrations in both the secretory (serous as well as mucous) and intercalated duct compartment. Progressive lysis subsequently occured in secretory units but not in intercalated and striated ducts. Recovery of tissue integrity was observed from day 6. In order to examine if the presence of granules during the irradiation were of importance, isoproterenol induced degranulation of acinar cells before the irradiation was performed. As a consequence, the recovery from radiation damage seemed to occur earlier in the parotid but not in the submandibular gland. Therefore it appears that the radiosensitivity of serous salivary acini is not dependent on the presence of secretory granules at the time of irradiation. The nuclear changes that we have observed is suggestive of cell death by apoptosis. To further investigate this possibility the incidence of apoptosis in irradiated rat salivary gland is being assessed from in situ end-labeling of new 3'-OH double- or single stranded DNA ends generated by intranucleosomal cleavage with a view to identifying the critical radiation lesion and cell population responsible for acute gland dysfunction.

B8-407 THE ROLE OF PROGRAMMED CELL DEATH IN PRE-GASTRULATION DEVELOPMENT IN THE MOUSE

Electra C. Coucouvanis and Gail R. Martin, Dept. of Anatomy, University of California, San Francisco, San Francisco, CA, 94143

Peri-implantation development of the mouse embryo is characterized by morphogenic events that prepare the embryo for gastrulation. During the formation of the proamniotic cavity, the inner cell mass changes from a solid to a hollow ball of cells lined on the inner surface with a columnar epithelium and surrounded by a single layer of endoderm. The mechanism by which the proamniotic cavity forms is unclear. Embryos are especially inaccessible during the implantation period, making developmental processes occurring at this time difficult to study. We have therefore used the teratocarcinoma stem cell line PSA1 as a model system in which to study pre-gastrulation development. Aggregates of PSA1 cells differentiate in vitro in a manner very similar to the early postimplantation embryo, changing from a solid sphere of cells into a hollow sphere lined with a columnar epithelium and an outer layer of endoderm, structures referred to as embryoid bodies (EBs).

Using the PSA1 system, we have shown that cavity formation occurs by the mechanism of programmed cell death (PCD). Cavitating EBs stained with a DNA binding dye clearly contain many cells displaying apoptotic morphology, including condensed and fragmented nuclei. Cycloheximide prevents this cell death (and thus cavitation), providing evidence that the cell death is an active process. Finally, electrophoretic detection of a characteristic ladder pattern in DNA of cavitating EBs reflects the activity of an endonuclease, which is typical of apoptotic cells. We have also gained insight into the way cavitation in EBs is initiated and progresses. In sections of cavitating EBs, we observe that cells 1-2 cell-widths beneath the outer endodermal layer are the first to die, raising the possiblity that cavitation may be intiated by a signal from the endoderm.

We have confirmed that PCD is the mechanism of proamniotic cavity formation in vivo by sectioning cavitating mouse and rat embryos.

In order to better define the role played by PCD in early mouse development, we are currently using overexpression of BCL2 to prevent PCD in embryoid bodies. Specifically, we hope to determine whether PCD-produced cavity formation is necessary for further differentiation, such as the formation of the columnar epithelium, to occur. We hypothesize that the cavity could provide positional information to the remaining cells that instructs or permits further development. We are also investigating the nature of the molecular signal(s) controlling the cavitation process.

B8-408 CHROMATIN INSTABILITY and DNase ACTIVITY in DIFFERENTIATING LENS CELLS. Marie-France Counis, Elisabeth Chaudun, Françoise Chany, Yves Courtois, Jean Claude Jeanny, Alicia Torriglia and Cristina Arruti°. U 118 INSERM, Association Claude Bernard, 29 rue Wilhem, 75016, Paris, France. °Laboratorio de Cultivo de Tejidos, Medical University, Montevideo, Uruguay.

The lens is an attractive system in which to study cellular differentiation. Lens fiber cells exhibit a physiological nuclear degeneration following the classical pathway of chromatin cleavage observed in many other examples of apoptosis (1).

The lens has an elipsoid shape and is composed of a layer of epithelial cells and a mass of fiber cells. In the equatorial region, epithelial cells differentiate into elongated fiber cells. Lens cells that have same origin, but quite different metabolism. Embryonic epithelial cells divide and are transcriptionally active, while fiber cells are post-mitotic, in terminal differentiation, in which the genome is cleaved and finally disappears. Notwithstanding these profound changes, the anucleated cells remain within the lens during the whole life span of the individual.

In the final stages of this nuclear degeneration, fiber chromatin appears condensed and the chromatin is cleaved between nucleosomes. The chromatin cleavage is not due to an accumulation of single strand breaks with 3'OH ends, though the presence of such a nuclease activity is observed (2). Different nucleases of different types are observed in nucleated lens cells as well as in cells that have undergone complete nuclear degradation, as long as one and a half year previously (3). The lens DNases will be compared with the known DNases and those possibly implicated in apoptosis in other systems.

(1) Counis et al., (1989) Int. J. Biochem., **21**, 235-242
(2) Chaudun et al., (1994) J. Cell Physiol., **158**, 354-364
(3) Arruti et. al, Submitted

B8-409 ADENOSINE AND ELEVATION OF cAMP INHIBIT NEUTROPHIL APOPTOSIS *IN VITRO*.

David C. Crossman and *Moira K.B. Whyte.
Dept. of Cardiology, University of Sheffield, Northern General Hospital, Sheffield and *Dept. of Medicine, University Hospital, Nottingham, U.K.

Neutrophil granulocytes (PMN) undergo apoptosis, characterised by internucleosomal DNA fragmentation and leading to their recognition and clearance by macrophages (Savill *et al* J.Clin.Invest 83:85). The rate of neutrophil apoptosis is delayed *in vitro* by inflammatory mediators, such as C5a and lipopolysaccharide (Lee *et al* J. Leukoc. Biol. 54:283), and elevations of cytosolic calcium (Whyte *et al* J. Clin. Invest. 92:446). PMNs are known to possess adenosine receptors and tissue adenosine levels are elevated in tissue inflammation and ischemia. This study examines the effect of adenosine receptor stimulation upon PMN apoptosis. PMN were separated from peripheral blood by plasma-Percoll gradient centrifugation. Apoptosis was assessed by morphological criteria and PMN cell surface changes associated with apoptosis by macrophage ingestion of PMNs. Cells were incubated at 37°C for 20h in the presence or absence of adenosine or the selective A_1 and A_2 receptor agonists N^6 cyclopentyladenosine (CPA) and 5'N-ethylcarboxamido-adenosine (NECA) respectively. Adenosine (100μM) significantly reduced apoptosis : control 49.7±4.5 (mean±SE) %, adenosine 28.7±4.6% ($p<0.01$, n=4). Further additions of adenosine at 1 and 3 h had no significant additional effect. CPA (0.01-1nM) had no effect upon PMN apoptosis (n=3). NECA (100μM) significantly apoptosis : control 70.3±6.0%, NECA 33.5±7.8% ($p<0.01$, n=3). Both adenosine and NECA caused dose-dependent inhibition of apoptosis, which was associated with significant reduction in the percentage of macrophages ingesting PMNs. Further studies suggested the effects may be mediated by elevations of intracellular cAMP and activation of protein kinase A, since a synthetic cAMP analogue, dibutyryl cAMP, caused inhibition of apoptosis ($p<0.001$) while a PKA antagonist, KT5720, was without significant effect alone but abolished the inhibitory effect of adenosine on PMN apoptosis ($p<0.005$). Thus adenosine, via A_2 receptors, delays apoptosis in PMNs with functional consequences. This represents a potentially pro-inflammatory effect of adenosine at concentrations which may be relevant to sites of tissue perturbation *in vivo*.

B8-410 AT LEAST TWO MECHANISMS ARE INVOLVED IN THE DEATH OF RETINAL GANGLION CELLS AFTER ABLATION OF THE SUPERIOR COLLICULUS IN NEONATAL RATS, Qi Cui and Alan R. Harvey, Department of Anatomy and Human Biology, The University of Western Australia, Nedlands 6009, Australia

The time-course and extent of retinal ganglion cell (RGC) death following ablation of the superior colliculus (SC) in neonatal Wistar rats was recently described (1). Nuclei of normal and pyknotic RGCs were visualized using the fluorescent dye diamidino yellow (DY). DY was injected into the SC at P2, two days prior to tectal removal. Rats were perfused at various time postlesion (PL). There was an early increase in RGC death (4-8hr PL) followed by a later massive increase in pyknosis about 24hr PL. Injection of cycloheximide (CHX) into the vitreous dramatically reduced the amount of RGC death 24hr PL (2). The present study examined (i) whether lesion-induced RGC death could be reduced by intraocular application of the neurotrophic factors NGF, BDNF, NT-3 or NT-4/5, or by the glutamate receptor antagonists MK-801 and DNQX and (ii) whether these agents had similar effects on RGC viability 6 and 24hr PL. The results show that BDNF and NT-4/5 significantly reduced RGC death 6 and 24hr PL. NT-4/5 was just as effective when its first application was delayed until 20hr PL, suggesting that the neurotrophin can act on RGCs at the post-translational level. NT-3, MK-801 and DNQX were only protective at 6hr PL; they had no influence on RGC death 24hr PL. NGF had no influence on pyknotic rates at either time-point. Contrary to its effect at 24hr, CHX did not reduce RGC death 6hr PL. The different effects of CHX, MK-801 and DNQX on RGC viability at 6 and 24hr PL show that there are at least two distinct mechanisms involved in RGC death after SC ablation. The early increase in RGC death appears to be related to excitotoxic effects mediated by glutamate receptors. In contrast, the late wave of RGC death is an active process requiring protein synthesis and, presumably, new gene expression. It is intriguing that while BDNF and NT-4/5 were both effective 6 and 24hr PL, NT-3 reduced RGC pyknosis only during the early phase of death.

1. Harvey, A.R. & Robertson, D. (1992). J. Comp. Neurol. 325, 83-94.
2. Harvey, A.R. et al., (1994). Eur. J. Neurosci. 6, 550-557.

B8-411 NAK-1, THE HUMAN *NUR77* HOMOLOGUE, AND THE CD95 ANTIGEN (APO-1/FAS) ARE SEQUENTIALLY INVOLVED IN HUMAN T CELL APOPTOSIS

Peter T. Daniel [1,2], Rüdiger W. Pankow [1], Markus Y. Mapara [1,2], Kurt Bommert [1], Ralf Bargou [1,2], and Bernd Dörken [2]
[1] Max Delbrueck Center, Berlin, Germany, and [2] Robert Roessle Klinik, Free University of Berlin, Germany

Activation induced apoptosis can be triggered through the TCR-CD3 complex in normal or malignant T cells. In addition, these cells can be induced to undergo apoptosis by agonistic antibodies against the CD95 (APO-1/fas) antigen, a member of the TNF/NGF receptor family. The latter, like in the case of TNF, requires crosslinking, e.g. trimerization for effective signal transduction. Thus, we previously showed that $F(ab')_2$ fragments of the original anti-APO-1 IgG3 mab bind to the CD95 antigen but do not deliver an apoptotic signal. We therefore used these $F(ab')_2$ fragments to block CD95 mediated signals. Addition of anti-APO-1 $F(ab')_2$ to anti-CD3 stimulated Jurkat T cells almost completely blocked activation induced cell death suggesting that CD3 triggered death is mediated by the CD95 ligand. Using PCR and Northern blot analysis we indeed could show that CD3 triggering upregulates CD95 ligand expression. Since earlier reports demonstrated the involvement of the orphan steroid receptor Nur77 in T cell apoptosis we were prompted to study the relationship between the CD95 pathway and Nur77 in this system. Northern blots and protein analysis by electrophoretic mobility shift assay revealed that CD3 but not anti-APO-1 mab induces a rapid upregulation of NAK-1, the human Nur77 homologue, which preceeds expression of the CD95 ligand. No NAK-1 upregulation was seen in an anti-CD3/anti-APO-1 resistant Jurkat subclone. The addition of NAK-1 antisense oligonucleotides was able to block anti-CD3 but not anti-APO-1 mediated apoptosis. In parallel, anti-APO-1 $F(ab')_2$ fragments were not able to inhibit the CD3 mediated upregulation of NAK-1. These data suggest a potential role for NAK-1 in the regulation of the CD95 ligand mediated apoptosis upon CD3 stimulation.

B8-412 CRITICAL ROLE OF CELL POLYAMINE CONTENT FOR THYMOCYTE APOPTOSIS, M.A. Desiderio*, E. Grassilli, E. Bellesia, D. Limonta*, P. Salomoni and C. Franceschi, *Institute of General Pathology and CNR Center for Research on Cell Pathology, University of Milano; Department of Biomedical Sciences, Section of General Pathology, University of Modena- Italy

Apoptosis is associated with the expression of a number of genes that mediate transition from quiescence to proliferative growth. Activation of apoptotic pathway is supposed to be determined by factors that occur downstream the early gene expression, and that prevent normal cell cycle regulation. Polyamines, acting at various steps of the cell cycle, are modifyed by extracellular signals and affect proto-oncogene expression. Thus, we studied the role of polyamine metabolic enzymes and of polyamine levels in the activation of the apoptotic program. Apoptosis was induced in isolated thymocytes by 10^{-7} M dexamethasone (Dex). Early and transient induction of ornithine decarboxylase (ODC), rate-limiting enzyme of polyamine biosynthesis, was observed. Both ODC mRNA (30 min-2 h) and activity (1-2 h) increased. Therefore, the pattern of ODC expression was similar to that previously reported by us for growth-controlled genes, such as c-*fos*, c-*jun* and c-*myc*, in the same experimental model (Grassilli et al., BBRC *188*, 1261, 1992). However, polyamine acetyltransferases localized in the cytosol (spermidine/spermine N^1-acetyl-transferase, cSAT) and in the nucleus (spermidine N^8-acetyltransferase, nSAT) showed a late increase and no changes of the activities, respectively. Polyamines remained at basal levels within the first 4-8 h after Dex treatment, while markedly decresased thereafter. Thymocytes resulted depleted of spermine at 12 h. The results suggest that in apoptotic thymocytes, at variance with proliferating thymocytes, the induction of ODC was not sufficient to elevate the levels of putrescine and of higher polyamines. Moreover, the absence of nSAT activity modifications were probably related to conformational changes of chromatin also due to loss of polyamines. To examine whether the levels of polyamines are critical for apoptosis, Dex-treated thymocytes were added spermine (1 mM). Apoptosis was prevented for 18 h, as evaluated by cytofluorimetric analysis. However, inhibition of ODC activity by pretreatment (30 min) of Dex-treated thymocytes with 5 mM DFMO, a specific inhibitor of ODC activity, did not prevent apoptosis. Our study emphasize that, besides the expression of early genes including ODC, the thymocytes require further signals, such as polyamine depletion, to undertake the apoptotic program.

B8-413 ROLE OF APOPTOSIS IN FUNCTIONAL LUTEOLYSIS IN THE PREGNANT RABBIT CORPUS LUTEUM:EVIDENCE OF A ROLE FOR PLACENTAL DERIVED FACTORS IN PROMOTING LUTEAL CELL SURVIVAL, A.M. Dharmarajan, K. Kugu and S.B. Goodman, Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, Baltimore, MD 21287

The objective this study is to determine the relationship between luteolysis and apoptosis using the pregnant rabbit CL model. Female rabbits were mated with a sexually mature male rabbit and the day of mating was designated as day 0 of pregnancy (term is day 31 or 32). CL were isolated from one ovary on days 4, 8, 16 (peak luteal function), 28 (functional regression) and 30 of pregnancy and processed for biochemical analysis of DNA integrity. The contralateral ovary was processed for in situ DNA labelling to localize apoptotic cells. Analysis of DNA integrity revealed the presence of oligonucleosomal fragments in day 28 and day 30 CL but not in day 16 CL. The extent of low molecular weight (<15 kb) DNA labelling was 6.6 ± 0.84 fold higher in day 30 as compared to day 11 CL (mean$\pm$SEM, n=4, $P<0.01$). In situ analysis of DNA breakdown confirmed that luteal cells were the primary cell type exhibiting increased DNA breakdown in day 30 CL. There was no indication of DNA breakdown on day 11 CL. In the second series of experiments, healthy CL collected from day 16 pregnant rabbits (time of peak luteal function) were incubated for 2h, 4h, and 8h in the absence or presence of placental extract (PE;250ug/ml). Consistent with our recent data, analysis of DNA integrity revealed that extensive apoptosis occurred in CL incubated in medium alone. PE significantly suppressed apoptosis in the CL incubated for 2 h and 4 h but not 8 h. In the third series of experiments, Northern blot analysis was carried out from total RNA isolated from CL treated without and with PE following 2 h incubation using cDNA probes for Bcl-x and BAX developed in our laboratory by RT-PCR. Treatment with PE increased Bcl-x mRNA levels. By contrast, CL incubated with medium alone expressed higher BAX and lower Bcl-x. These data suggest that functional luteolysis is correlated with the occurrence of apoptosis in the pregnant rabbit CL. Moreover, the data also suggest that a PE derived factor may be responsible for the prolongation of CL life span during pregnancy. (Rockefeller Foundation).

B8-414 IN VIVO DETECTION OF APOPTOSIS IN PATIENTS WITH ACUTE MYELOID LEUKEMIA, Eric Feldman, Xun Li, Frank Traganos, Karen Seiter, Zbigniew Darzynkiewicz. Cancer Research Institute and Division of Oncology, New York Medical College, Valhalla, NY 10595

Apoptosis or programmed cell death is a particular mode of cell death characterized by unique morphological changes in both cell nucleus and cytoplasm. Recent evidence indicates that the effectiveness of anti-tumor drugs depends on the inherent propensity of the target tumor cells to respond by apoptosis. This propensity in turn correlates with the proliferative status of the cell as well with the expression of several oncogenes or tumor suppressive genes such as BCL2, c-myc, RB or P53. Recently, we have described a new flow cytometric method to detect DNA strand breaks associated with apoptosis by in situ labelling of the 3' OH termini with biotin or digoxyginin conjugated DUTP in reaction catalyzed by the enzyme terminal deoxynucleotidyl transferase. This approach offers the possibility to distinguish and quantify the DNA replicating and apoptotic cells in a single measurement and it may have an application to simultaneously assess self proliferation and programmed cell death as markers of tumor prognosis. Analysis of apoptotic cells from peripheral blood and/or bone marrow of 23 patients with acute myelogenous leukemia undergoing induction chemotherapy revealed a wide range of responses. Responses could be seen as early as 8-12 hours after drug administration. The data suggest that as a result of treatment with DNA topoisomerase 2 inhibitors and cytosine arabinoside leukemic cells are still able to divide but their progeny enter the apoptotic pathway. The prognostic value of such analysis with respect to clinical outcome is currently under investigation.

B8-415 LOW PROPENSITY TO APOPTOSIS IN PBL FROM HIV+ LONG TERM NON PROGRESSORS. Franceschi C., Boschini A.**, Portolani M. #^, De Rienzo B. *, Castellani G.**, Smacchia C.**, Monti D., Franceschini M.G., Roncaglia R. ##, Pietrosemoli P. ^, Meacci M. ^, Pecorari M. ^, Sabbatini A. ^, Cossarizza A., Dip. Scienze Biomediche, Sez. di Patologia Generale e # Sez. di Igiene, Univ. di Modena, via Campi 287, 41100 Modena; * Ist. Malattie Infettive e Tropicali, Univ. di Modena; ## Centro Trasfusionale e ^ Serv. Virologia, USL 16, Modena; ** Comunità di San Patrignano (Rimini)

Recent data present in the literature indicate that the gradual depletion of peripheral blood CD4+ T lymphocytes (and also CD8+) during the progression of HIV infection may be due, at least in part, to apoptosis. The molecular and cellular mechanisms of this phenomenon are still poorly understood. In particular, it has been reported that, when cultured *in vitro*, peripheral blood lymphocytes from HIV positive subjects in different stages of the infection undergo apoptosis either spontaneously or following stimulation with superantigens. Such a phenomenon, which is present at a very low level also in peripheral blood lymphocytes (PBL) from normal subjects, is dramatically increased in PBL from HIV positive patients. We have studied 10 young subjects infected by HIV since at least 7 years but who do not present clinical or immunological progression of the infection, the so called long term non progressors (LTNP), in comparison with 10 HIV+ patients and 10 HIV- controls. The data indicate that the percentage of cells undergoing spontanous or induced (by superantigens, PWM, etc.) apoptosis is statistically different between HIV- and HIV+ subjects, but not between HIV- and LTNP. The propensity to apoptosis appears to be inversely correlated to the percentage of CD4+ cells. This data suggest that the propensity to apoptosis can play a major role in the progression of the disease.
Sponsored by *VII Progetto di Ricerche sull'AIDS*, grant 9204/55.

B8-416 EVIDENCE OF A ROLE FOR MAMMALIAN ENDO-EXONUCLEASE IN APOPTOSIS. Murray J. Fraser, Stephen J. Tynan, Arthur Papaioannou, Christine M. Ireland and Sally M. Pittman. Children's Leukaemia and Cancer Research Centre, University of New South Wales, Sydney, Australia 2031.

Mammalian endo-exonuclease (EE) has Mg^{++} (Mn^{++})- dependent, Ca^{++}-stimulated endonuclease activity with single-strand DNA and RNA and 5'-3' exonuclease activity with linear double-strand DNA. Nuclei of human leukaemic CEM cells (CEMs) contain chromatin-bound active and inactive EE. Inactive EE, putative precursor (PRE), is detected by *in vitro* trypsin activation. Full activation requires either the presence of a divalent metal ion (Mg^{++}, Ca^{++} or Mn^{++}) or 120-160nM cyclosporin A (which induces apoptosis in CEMs) and has revealed the presence of up to 10-fold more PRE than active EE in nuclear extracts. Extracts of nuclei of CEMs pre-treated with 100µg/ml cycloheximide for 3h contained 2-fold more PRE indicating rapid turnover of EE in nuclei of untreated CEMs. When CEMs were treated to induce apoptosis with either 17µM VP-16, 6h or 1µM dexamethasone, 48h, total nuclear EE was depleted to about 40% of control levels. PRE is also present in CEM cytosol. Both nuclear and extra-nuclear fractions, when examined by immunoblotting using antibody raised to *Neurospora* EE, were found to contain immunoreactive polypeptides, some with the same sizes as the PRE (93kDa) and active EEs (76, 66, 43, 37 kDa) of *Neurospora*. Depletion of the cytosolic polypeptides was observed in CEMs treated with 17µM VP-16 for 6h, but not in MOLT-4 cells which do not undergo apoptosis with this treatment. Pre-treatment of CEMs with 10µM podophyllotoxin over 24h also resulted in depletion of cytosolic EE activity and immunoreactive polypeptides in 12-24h and correlated with the time course of chromatin DNA fragmentation. MOLT-4 cells treated in the same way did not show such responses. When 3,4-dichloroisocoumarin was omitted from the protease inhibitor cocktail used to make these extracts, smaller immunoreactive polypeptides (18-24 kDa) were observed indicating the sensitivity of human EE to proteolysis as observed previously for *Neurospora* EE. The latter EE showed preferential loss of exonuclease activity during limited proteolysis. Taken together, the present results strongly implicate EE in apoptosis of human leukaemic cells and suggest a role for proteolysis in activation and turnover of this nuclease. (Supported by NH&MRC, Australia).

B8-417 IDENTIFICATION OF AN EPITHELIAL CELL-DERIVED APOPTOSIS-INDUCING FACTOR

Keiko Funa, Akira Ishizaki, Ulf Hellman, Christer Wernstedt, Carl-Henrik Heldin and Mitsuyasu Kato. Ludwig Institute for Cancer Research, Biomedical Center, S-751 24 Uppsala, Sweden

The mechanism behind continuous cell renewal and cell death exhibited by normal skin and squamous epithelial cells is still unclear. We have isolated a molecule secreted by HaCat, a human non-transformed keratinocyte cell line, as well as HSC4, a human oral squamous tumor cell line, which inhibits the growth of a mouse normal keratinocyte cell line, Balb/ MK, as well as a mink lung epithelial cell line, CCL64. This molecule was isolated from conditioned media of cultured HSC4 cells by QAE ion-exchange chromatography followed by Superose 12 gel permeation chromatography. The active fractions exhibited DNA fragmentation on lambda phage DNA and induced apoptosis of several epithelial cell lines confirmed by the TUNNEL method, *in situ* labeling of nicked DNA. The active fraction contained a 16 kDa hydrophobic acidic molecule which is unstable at neutral pH.

We suggest that this molecule might be responsible for the programmed cell death of normal epithelial cells. Further characterization of this molecule will be presented.

B8-418 PHAGOCYTIC CLEARANCE OF APOPTOTIC CELLS: NOVEL PATHWAYS IDENTIFIED BY MONOCLONAL ANTIBODIES TO CELL SURFACE COMPONENTS

Christopher D. Gregory, Pauline K. Flora, Hardeep S. Bhogal and Walter De Smet*, Department of Immunology, University of Birmingham Medical School, Birmingham B15 2TT, UK and *Innogenetics, B-9052 Zwijnaarde, Belgium.

Cells undergoing apoptosis are marked for rapid recognition and phagocytosis. This critically important characteristic, which appears to be lacking in necrotic cells, ensures that swift removal of apoptotic cells by both 'professional' and 'non-professional' phagocytes occurs prior to the loss of plasma membrane integrity and the potentially disastrous consequences of leakage of intracellular contents. To date, macrophage surface molecules that have been implicated in clearance of apoptotic cells are: (1) a putative lectin-like receptor, (2) a phosphatidyl serine receptor, and (3) the $\alpha_V\beta_3$ integrin together with CD36. Our studies indicate that a previously uncharacterised macrophage surface molecule, identified with the monoclonal antibody 61D3, plays an important role in the recognition and phagocytosis of apoptotic cells. Thus, 61D3 effectively inhibited uptake of apoptotic (but not necrotic) lymphocytes and neutrophils by human monocyte-derived macrophages. The 61D3 antigen did not appear to interact with phosphatidyl serine and was discriminated from $\alpha_V\beta_3$. A further monoclonal antibody, 3A9, also blocked recognition of apoptotic leukocytes by macrophages. This antibody specifies ICAM-3 and inhibited the recognition process as a result of its binding to the surface of the apoptotic cell rather than to the macrophage. LFA-1, the only reported ligand for ICAM-3, was not involved in this recognition pathway. Furthermore, we have no evidence as yet that the 61D3 antigen plays any role in this pathway. These results provide further evidence that phagocytic clearance of apoptotic cells is a complex process involving multiple ligand-receptor interactions.

B8-419 DIVERSITY OF APOPTOTIC PHENOTYPE INDUCED BY SUSPENSION CULTURE AMONG HUMAN PAPILLOMAVIRUS OR SV40 LARGE T ANTIGEN-CONTAINING EPITHELIAL CELL LINES,

Takeshi Haga[1], Kentaro Matsumoto[2] and Joel Palefsky[1, 2] [1]Department of Laboratory Medicine, [2]Department of Stomatology, University of California, San Francisco CA 94143

Suspension culture has previously been reported to be sufficient to induce apoptosis of certain cell types including MDCK, HaCat, human umbilical vein endothelial cells and human gut epithelial cells. The human papillomavirus (HPV) and SV40 DNA tumor viruses contribute to immortalization by interacting with p53 and Rb which play an important role in apoptosis. Because acquisition of anchorage independence of transformed cells may be due to the blocking of this suspension culture-dependent apoptotic pathway, and because HPV-transformed cells typically exhibit anchorage-independent growth, we sought to determine if epithelial cells transformed by HPV and SV40 exhibit, or are resistant to, this type of apoptosis.

We studied several different cell lines, including the CaSki and SiHa cervical cancer cell lines containing HPV 16, the HeLa cervical cancer and CCL17 oral cancer cell lines containing HPV 18, Ca922 and HSC3 HPV-negative oral cancer cells with a p53 mutation, primary foreskin keratinocytes (PFK), PFK transfected with HPV16 and with SV40TAg, COS 7, and MDCK cells. Each line was studied in attached culture, and in suspension culture using tissue culture dishes coated with poly-HEMA. DNA was extracted from the cells by proteinase K digestion and subjected to agarose gel electrophoresis.

DNA from suspension culture from all the cell lines except SiHa, HeLa and CCL17 showed DNA degradation, with the latter three lines showing a single band suggesting resistance to apoptosis. These studies show that most primary, SV40- and HPV-transformed epithelial cell lines undergo apoptosis in suspension culture, but some HPV-transformed cell lines exhibit resistance. Studies using other methods to confirm suspension culture-mediated apoptosis, as well as mechanisms of resistance are currently underway.

B8-420 INHIBITION OF LIPID PEROXIDATION ATTENUATES AXOTOMY-INDUCED APOPTOTIC DEGENERATION OF FACIAL MOTOR NEURONS IN NEONATAL RATS,

Edward D. Hall, Sarah L. Smith and Jo A. Oostveen, CNS Disease Research, The Upjohn Company, Kalamazoo, MI 49001

Recent reports suggest that the motor neuronal degeneration in familial amyotrophic lateral sclerosis (ALS) may result from an increased sensitivity to the damaging effects of oxygen radicals due to a genetic defect in the antioxidant enzyme Cu/Zn superoxide dismutase (Rosen et al., Nature 362:59,1993). Similarly, axotomy-induced apoptotic degeneration of facial motor neurons in the neonatal rat appears to involve an oxidative mechanism. For instance, transgenic mice that over-express the BCl-2 oncoprotein, which is believed to have antioxidant properties, show less motor neuron loss after axotomy than non-transgenic mice (Dubois-Dauphin et al., PNAS 91:3309, 1994). A likely mechanism by which free radicals damage motor neurons is through membrane degradation via lipid peroxidation. The purpose of the present study was to test the ability of the lipid peroxidation inhibitor tirilazad mesylate (U-74006F; Hall et al., Adv. Pharmacol. 28:221, 1994) to protect motor neurons in the facial nucleus following axotomy in 14-day-old rat pups. On Day 1, the right facial nerve of each rat was transected at its point of exit from the stylomastoid foramen. Pups were pretreated orally with either 10 or 30 mg/kg U-74006F or cyclodextrin vehicle, 10 minutes before axotomy and post-treated once a day from Day 2-7, and then once every other day from Day 8-21. The rats were sacrificed three weeks post-transection and the surviving motor neurons, identified through choline acetyltransferase immunocytochemistry, were counted. In vehicle-treated rats, 55.1% of the motor neurons in the ipsilateral facial nucleus survived 21 days following facial nerve axotomy in comparison to the non-axotomized contralateral nucleus ($p<.0001$). Pre- and post-treatment with 10 and 30 mg/kg U-74006F significantly enhanced motor neuron survival, with survival rates of 71.0 and 72.3%, respectively ($ps<.0001$ vs axotomized vehicle controls). The fact that the lipid peroxidation inhibitor U-74006F protected facial motor neurons from post-axotomy degeneration suggests that lipid peroxidation may play a mechanistic role in apoptotic neuronal death.

B8-421 THE ROLE OF C-JUN IN PROGRAMMED NEURONAL CELL DEATH,

Jonathan Ham, Carol Babij, Curt Pfarr*, Moshe Yaniv* and Lee L. Rubin, Eisai London Research Laboratories, Bernard Katz Building, University College London, Gower Street, London WC1E 6BT, UK and *Unité des Virus Oncogènes, Département des Biotechnologies, Institut Pasteur, 25 rue du Dr. Roux, Paris 75724, France.

Programmed cell death plays an important role in the development of the mammalian nervous system and is regulated by neurotrophic factors such as Nerve Growth Factor (NGF). We are studying the mechanism by which NGF promotes neuronal survival using primary sympathetic neurons from the rat superior cervical ganglion (SCG) as a model system. When SCG neurons are deprived of NGF they die by apoptosis and in this case, cell death can be blocked by the addition of inhibitors of transcription or translation, suggesting that ongoing transcription or activation of a set of genes is necessary for death to occur. We are currently trying to identify the transcription factors that promote the death of SCG neurons. As a first step we have characterized the pattern of expression of members of the Jun and Fos family in dying SCG neurons, using antibodies that specifically recognize each member of the family. Purified SCG neurons cultured *in vitro* for 7 days in the presence of NGF do not express measurable c-Fos but do express low levels of c-Jun, Jun B, Jun D and Fra 2. After NGF withdrawal, the levels of Jun B, Jun D and Fra 2 fall slightly. c-Fos is induced, but slowly and only in a few cells. This induction cannot be detected by western blotting but can be seen in immunofluorescence experiments. For example, at 24 hours after NGF withdrawal a few dying neurons (less than 1%) express high levels of c-Fos. These cells invariably have slightly deformed nuclei. In contrast c-Jun protein can be readily detected by western blotting and increases several fold in level and becomes more phosphorylated. These changes start about 4 hours after NGF withdrawal and persist for 20 hours or more. In immunofluorescence experiments c-Jun protein can be seen to be strongly induced in the nuclei of all the SCG neurons after NGF withdrawal. Induction of c-Jun clearly precedes any changes in nuclear morphology. To determine what role c-Jun plays in neuronal cell death we have constructed CMV expression vectors that express either full-length c-Jun, antisense c-Jun or a c-Jun deletion mutant that functions as a dominant negative and blocks AP1 activity. We are currently microinjecting these constructs into SCG neurons to determine their effect on neuronal cell death.

B8-422 POSSIBLE ROLE OF FREE RADICALS IN CELL DEATH AND LIMB MORPHOGENESIS. Sid Hunter and Kelly Might, Developmental Toxicology Division, U.S. EPA, and NIEHS, NIH, Research Triangle Park, NC 27711

Interdigital cell death is necessary for normal digit formation, but signals responsible for this apoptosis are not defined. Therefore, we tested the hypothesis that reactive oxygen species (ROS) are involved in normal digit development. Day 12 mouse forelimbs were removed from the embryo, placed into a defined medium and grown in culture for 24-72 hours. During this period, limb bud paddles exhibited digital condensation, formation of bone cartilage models, and loss of interdigital tissues. Limb buds exposed to aurintricarboxylate, an endonuclease inhibitor, exhibited poor paddle development; chondrogenesis appeared to occur in the digital rays without concomitant loss of interdigital tissues. Tempol (4-OH-2,2,6,6-tetramethylpiperdine-N-oxyl) is a broad spectrum antioxidant capable of superoxide and peroxide degradation. Tempol treated limbs exhibited good growth but poor morphogenesis, e.g. little digital development and no loss of interdigital tissue was apparent following treatment. DNA laddering was decreased and 3'-end labeling in limb sections showed a diffuse pattern of staining following Tempol treatment compared to interdigital staining in controls. To determine if other antioxidants were capable of altering limb development, limb buds were exposed to SOD or catalase. These antioxidants were not as effective as Tempol at altering digit formation and required high concentrations to produce dysmorphology. These studies suggest that antioxidants can alter normal limb morphogenesis and that ROS may be involved in the process of programmed cell death.

B8-423 INDUCTION OF APOPTOSIS IN MURINE F9 CELLS AND PROTEIN KINASE CK2/p53 STABILITY,

Olaf-Georg Issinger, Manfred Küpper, Veronika Ziegler and Michael Köster, FR Humangenetik, Universität des Saarlandes, D-66424 Homburg/Saar, Germany.

We have chosen the mouse teratocarcinoma cell line F9 because it can be induced by cisplatin and other chemotherapeutic agents to produce stable p53 and concomitantly undergo apoptosis. Thus, it would allow to investigate the role and interaction of protein kinase CK2 (the active enzyme is a tetramer, $\alpha_2\beta_2$) and tumor suppressor gene product p53 in normal growing cells (in the absence of cisplatin) and in apoptotic cells (after treatment with cisplatin). p53 was detected in cisplatin-treated cells as early as 3 hours after the addition of the drug. The typical DNA ladder was induced by cisplatin concentrations between 1.25 µg up to 20 µg/ml. After treatment of the cells with 10 µg/ml cisplatin the DNA ladder was visible as early as 6 h after the addition. Maximum intensity was reached after 12-15 h. After 24 h the DNA ladder bands became fuzzy. F9 cells were routineously treated for 12 hours and with a cisplatin concentration of 10 µg/ml. A crude cellular extract was prepared and subjected to anion exchange chromatography on MA7Q. Gradient elution was from 0- 1 M NaCl. The individual fractions were tested for CK2 activity, subunit distribution and the presence of p53. A comparison showed, that CK2 activity was reduced by 50% in cisplatin-treated cells. However, the amount of CK2 subunits was the same in both ciplatin-treated and untreated cells which could be due to an endogenous inhibition either by a specific inhibitor, by polymerization or lack of the free subunits to form active tetrameric holoenzyme. Although most of the p53 eluted at low salt, in front of the major CK2 peak, some of the p53 co-eluted with the CK2 holoenzyme supporting the notion of stable complex formation between protein kinase CK2 and p53 as it has been described to occur in Baculovirus-infected SF9 cells.

B8-424 REARRANGEMENT OF CYTOSKELETON DURING CELL DEATH IN *DROSOPHILA* SALIVARY GLANDS. Jana Jochová[1], Zahra Zakeri[2], and Richard A. Lockshin[1] [1]Department of Biol. Sci., St. John's University, Jamaica NY 11439 and [2]Department of Biology, Queens College and Graduate Center of CUNY, Flushing, NY 11367

Programmed cell death (PCD) is an active process by which selected cells at a predetermined time commit suicide. Salivary glands of *D. melanogaster* degrade during the larva-to-pupa metamorphosis, thus providing a convenient model for study of PCD.

Cytoskeleton has been implicated in many fundamental intracellular processes such as cell division, locomotion and intracellular trafficking (i.e. secretion). We were interested how changes in microtubule and microfilament organization correlate with progression of PCD.

Actin filaments were visualized by labeling with Rh-phalloidin. The observed pattern consisted of actin filament bundles and rods located near the plasma membrane. In the mid-3rd instar feeding larvae (F) and wandering larvae (W) the bundles were elongated with prevailing base-to-apex orientation. Beginning at the postfeeding stage (L1) the bundles shrank into irregular patches. The patches became more condensed as the gland degradation proceeded through L2 → white puparium (P1) → brown puparium (P2). Simultaneously, actin filaments along lateral cell boundaries concentrated into a thick irregular layer.

Microtubules were observed by immunofluorescence. In the F-stage larvae the gland cells contained a lace-like microtubular network concentrated more under the cell surface and around nuclei. In the course of W → L1 → L2 the network beneath the plasma membrane became denser and the baskets around nuclei were more apparent. The epithelial wall became thinner and nuclei often moved toward the basal surface of the cells. During P1 and P2 the network disintegrated, microtubular fragments accumulated near the cell surface and scattered patches of intensive fluorescence appeared.

B8-425 PROGRAMMED CELL DEATH DURING HUMAN PREIMPLANTATION EMBRYO DEVELOPMENT.

Andrea Jurisicova*‡, Sue Varmuza‡ and Robert F. Casper*, *Division of Reproductive Sciences, The Toronto Hospital Research Institute, and ‡Department of Zoology, University of Toronto, 200 Elizabeth St, Toronto , Ontario, Canada, M5G 2C4.

One of the most important factors influencing the In Vitro Fertilization (IVF) pregnancy outcome is appropriate selection and assessment of embryo viability. The quality of obtained embryos is variable, which is reflected in their variable developmental potential. In comparison with other mammalian species, human preimplantation development is surprisingly impaired. Less than 50% of human embryos cleave regularly to form equal sized blastomeres without fragmentation. Other embryos contain unequal sized blastomeres with the presence of multiple cellular fragments and often are arrested in development and completely degenerate in vitro. Upon transfer, embryos with many fragments seldom result in pregnancy. Unfortunately, our knowledge about the causes of such impaired development is extremely limited. Previous cytologic and cytogenetic studies confirmed the presence of a wide spectrum of nuclear anomalies eg. multinucleated or anucleated blastomeres, floculent or fragmented nuclei and a wide range of chromosomal abnormalities. Using combined TUNEL and DAPI labelling on arrested and degenerated human embryos obtained through IVF and cultured in vitro, we were able to detect extensive condensation and degradation of chromatin. Moreover, TEM and SEM analysis of such embryos confirmed the presence of typical morphological features of apoptosis (condensation of chromatin and cytoplasm as well as the presence of apoptotic bodies). These results suggest the involvement of programmed cell death in very early human embryo demise. Our present research is concentrated on exploring underlying mechanisms and potential triggers involved in this very complex process.

B8-426 THE ALZHEIMER'S Aß PEPTIDE INDUCES NEURODEGENERATION AND APOPTOTIC CELL DEATH IN TRANSGENIC MICE, Frank M. LaFerla, Brad T. Tinkle, Charles J. Bieberich and Gilbert Jay, Department of Virology, Jerome H. Holland Laboratory, American Red Cross, Rockville, MD 20855

To test the hypothesis that the Alzheimer's ß-amyloid peptide is toxic to neuronal cells, a transgene was introduced into mice to direct expression of this peptide to neurons. *In situ* hybridization and immunohistochemical analyses reveal that the transgene is expressed in regions of the brain that are severely affected in Alzheimer's disease. In these areas, extensive degeneration of neuronal cells is observed. Morphological and biochemical evidence indicates that the eventual death of these degenerating cells occurs by apoptosis. Cells undergoing apoptosis are frequently observed in the cerebral cortex, hippocampus, amygdala, and thalamus; these areas also show a profound loss of cells in the brains of patients with Alzheimer's disease. Coincident with the cell degeneration and cell death, is the ,presence of a striking reactive gliosis. Moreover, greater than 50% of the transgenic mice die by 12 months of age, half the normal life span of control mice. These data show that ß-amyloid is neurotoxic *in vivo* and suggest that apoptosis may be responsible for the accompanying neuronal loss, which is the principal underlying cellular feature of Alzheimer's disease. These transgenic mice will serve as a useful model system to dissect the molecular mechanism responsible for the programmed death of these neuronal cells.

B8-427 INDUCTION AND DETECTION OF APOPTOSIS WITHIN HUMAN MONOCYTIC CELL LINES AND MONOCYTE-DERIVED MACROPHAGES. David A. Lammas[1], Carmel Stober[1], Rodger J.A.Grand[2], and Dinakantha.S. Kumararatne[1] Departments of Immunology[1] & Cancer studies[2], University of Birmingham Medical School, Birmingham B15 2TT, UK.

Programmed cell death(PCD) as a response to intracellular infection has now been observed for a wide range of pathogens and host organisms. Studies were undertaken to develop methods of detecting apoptosis within both transformed monocytic cell lines and cultured human monocyte-derived macrophages to look at cellular responses to infection with mycobacteria. Cells were treated with either ionomycin or cycloheximide, previously shown to induce apoptosis in other cell types, or activated with either TNFα or IFN-γ+/-LPS. The degree of PCD was then assessed by standard morphological criteria following staining with acradine orange or Jenner-Giemsa stain. Such criteria however were found to be very subjective when applied to macrophages, and two novel methods, Comet-Tail analysis and Western blotting, using an anti-45KDa apoptosis-specific protein(asp) antibody were also employed to confirm PCD in treated cells. Marked apoptosis was readily detected within human macrophages and monocyte lines, following incubation with all the above agents, by both methods. Preliminary results however suggest that mycobacterial infection *per se* does not induce apoptosis within human macrophages or monocyte lines. Further studies are being performed to look at possible enhancement or inhibition of apoptosis in response to activation signals by infection with mycobacteria.

B8-428 **ESTROGEN INDUCES APOPTOSIS BY INDUCTION OF TGF-β1 IN PROSTATIC CARCINOMA.** Landström Marene[1]and Funa Keiko. [1],Dep. of Pathology, University of Umeå, Ludwig Institute for Cancer Research, BMC, Uppsala, Sweden.

Rats transplanted with the androgen-sensitive R3327 PAP rat prostatic adenocarcinoma were castrated (C) and either estrogen (E) or vehicle treated for 6 weeks. Tumor growth (TGR%) was retarded significantly by C and even more in the E group. The mitotic (MI) and apoptotic index (AI) was counted. C±E reduced the MI of the tumor epithelial cells. The immunohistochemical expression (IHC) of TGF-β1, -2, -3 and the latent TGF-β binding protein (LTBP), TGF-β type I and type II receptors (TGF-βRI, TGF-βRII) was examined. The epithelium in intact tumors showed no staining for any of the TGF-β isoforms. C induced a weak and diffuse TGF-β-1 IHC in the tumor epithelium, which was stronger in the stroma. E induced an intense staining for TGF-β1 in the basal epithelial tumor cell layer. The number of TGF-β1 expressing apoptotic cells was higher in the E group compared to the C group (1.14 % and 0.15 % respectively, $p<0.05$). Colocalization of TGF-β1 IHC in the apoptotic cells in the E group was further demonstrated by the χ^2 tests ($p<<0.00001$), suggesting that the estrogen-induced apoptosis in the tumor epithelium, might be mediated by TGF-β1. The number of TGF-β1 positive apoptotic cells showed a strong negative correlation to TGR% in the whole material ($RS=-0.83$; $p<0.0001$, $N=15$). TGF-β2 was also induced by C±E but with a weaker intensity. A strong staining of TGF-β3 was found in scattered stromal cells in the C tumors, which disappeared by E treatment. LTBP was present *only* in the tumor stroma. TGF-βRI and RII were observed only in the stroma and increased strongly after endocrine treatment. The inhibitory effect of C on TGR% are linked to an increased expression of TGF-β1 and the receptors. C decreased the AI, when compared to the intact tumors. E increased the amount of TGF-β1 positive apoptotic cells significantly, indicating that the estrogen induction of apoptosis might be exerted by TGF-β1.

B8-429 **APOPTOSIS ASSOCIATED WITH RETINAL DEGENERATION IN OVINE NEURONAL CEROID LIPOFUSCINOSIS,** **Steven C. Lane, R. D. Jolly, and Rose-Mary Boustany, Department of Pediatrics, Division of Pediatric-Neurology, Duke University Medical Center, Durham, NC 27710 and Department of Veterinary Pathology and Public Health, Massey University, Palmerston North, New Zealand.**

Batten disease, or neuronal ceroid lipofuscinosis (NCL), is the most common genetic neurodegenerative disorder of childhood. The pathologic hallmarks of NCL are neuronal degeneration and retinal degeneration. Our work has examined the cause of retinal degeneration, which presents clinically as retinitis pigmentosa, in an ovine model of Batten disease. Since previous studies have indicated that apoptosis is the cause of retinal degeneration in both human and rodent forms of retinitis pigmentosa, we have investigated whether apoptosis might also be the cause of retinal degeneration in Batten disease. Using a TUNEL staining method, our initial results indicate numerous cells scattered throughout the retinal outer nuclear layer of a 14 month old NCL sheep which stain positively for apoptosis. A corresponding age-matched control stains negatively. To further confirm the presence of apoptosis in ovine NCL retinas, we are currently extracting DNA from retinal tissue to examine for the presence of DNA laddering, and we are also applying flow cytometry to paraffin block sections to identify apoptotic cells.

B8-430 HEPATIC STIMULATOR SUBSTANCE INDUCES APOPTOSIS IN NORMAL AND MALIGNANT HEMOPOIETIC CELLS, Karen Li, K.S. Tsang, C.H. Kan and Patrick P.M. Yuen. Department of Pediatrics, The Chinese University of Hong Kong, Shatin, N.T. Hong Kong.

The hepatic stimulator substance (HSS) is a liver-specific peptide substance extracted from weanling pigs. In China, HSS has been used in the clinical trials of several human liver diseases (e.g. fulminant viral hepatitis, chronic active hepatitis and liver cirrhosis) with significant success. In this project, effects of HSS on several human hemopoietic cells were studied: the myeloid cell line K562, one normal bone marrow (BM), one peripheral blood (PB) after granulocyte-colony stimulating factor (G-CSF) mobilization and one leucopheresed product from a patient with T-cell acute lymphoblastic leukemia (ALL). HSS significantly inhibited proliferation of K562 in culture by depleting cells at G_0/G_1 phase and blocking them at S and G_2/M. In liquid cultures of BM, PB and ALL, the induction of apoptosis by HSS has been demonstrated by flow cytometry and electron microscopy. The addition of concanavalin A failed to reverse this apoptotic phenomenon. HSS also inhibited colony formation of BM, PB and ALL stems cells on semi-solid cultures in the presence of erythropoietin, stem cell factor, G-CSF and granulocyte macrophage-colony stimulating factor. The apoptotic-inducing activity of HSS would have significant implications for its potential use as an agent for treating liver diseases or malignancy.

B8-431 ROLE OF APOPTOSIS IN OXIDATIVE GLUTAMATE TOXICITY, Pamela Maher* and John Davis#, *Department of Cell Biology, The Scripps Research Institute and The Salk Institute, La Jolla, CA 92037

Glutamate kills neuronal cells either by a receptor-mediated pathway or by the inhibition of cystine uptake, the "oxidative pathway". In order to investigate the mechanism of cell death via the oxidative pathway we utilized an immortalized mouse hippocampal cell line, HT-22 cells, which were particularly sensitive to glutamate. We previously showed that protein kinase C (PKC) activation could inhibit glutamate-induced cell death in the HT-22 cells. Since PKC activation has been associated with the inhibition of apoptosis in other cell types, we asked if glutamate toxicity had any characteristics consistent with apoptosis. Morphologically, glutamate-treated cells were seen to undergo plasma membrane blebbing and nuclear compaction prior to death. In addition, macromolecular synthesis inhibitors protected the cells from glutamate toxicity. Since glutamate treatment of cells depletes glutathione, the protection from cell death afforded by these agents could be due to an increase in the availability of cysteine for glutathione synthesis. However, similar concentrations of protein and RNA synthesis inhibitors protected the HT22 cells from cell death brought about by treatment with the glutathione synthetase inhibitor, buthionine sulfoximine, suggesting that this was not the case. In order to characterize the temporal sequence of events in the oxidative pathway, a series of time course experiments were carried out to determine the latest time after glutamate addition that the macromolecular synthesis inhibitors could be added and still protect the cells from death. Actinomycin D protection decreased sharply between 2 and 4 hr after the addition of glutamate whereas protection by cycloheximide disappeared between 4 and 6 hr after the addition of glutamate. Thus, oxidative stress brought about by treatment of cells with glutamate activates a pathway of cell death with many of the characteristics of apoptosis.

B8-432 APOPTOSIS IN MURINE AUTOSOMAL RECESSIVE POLYCYSTIC KIDNEY DISEASE, Ruth A. McDonald, James B. Miller, William E. Sweeney, Harriet S. R. Coles*, and Ellis D. Avner, Department of Pediatric Nephrology, University of Washington, Children's Hospital, Seattle, WA 98105; *University College, London, UK

Bcl-2 is a proto-oncogene that plays a key role in protecting cells against apoptosis or programmed cell death. Veis *et al.* (Cell 75:229, 1993), reported that bcl-2 -/- "knockout" mice developed polycystic kidney disease (PKD) soon after birth. We have studied developmental expression of Bcl-2 protein and apoptosis in the C57BL/6J cpk/cpk (CPK) murine model of autosomal recessive PKD.

Normal C57 control and CPK cystic kidney tissue was sampled at 0, 7, 14, and 21 days after birth. A 2-3 fold decrease in the amount of Bcl-2 protein was detected by Western Immunoblot analysis in each of the CPK samples in comparison to the age matched control C57 tissue. In both normal C57 and cystic CPK renal tissue, the amount of Bcl-2 protein increased to maximum expression at postnatal day 7 and subsequently decreased by postnatal day 21.

To identify apoptotic cells, kidneys from 7 day old affected CPK animals and normal littermates were stained with propidium iodide to label nuclei and sections were examined by florescence microscopy. The percent of pyknotic nuclei in the kidneys of the affected animals was approximately 2-3 times greater than that seen in the age matched controls.

The biochemical hallmark of apoptosis is extensive cleavage of the cell's DNA into oligonucleosome sized fragments. Paraffin imbedded kidney sections from 7, 14, and 21 day old CPK and C57 mice were pretreated with protease and then biotinylated nucleotides were in situ incorporated into DNA breaks by terminal deoxytransferase. The sections were subsequently stained with DAB via peroxidase-conjugated avidin allowing morphologic discrimination between apoptotic and normal nuclei. There was a significant increase in the number of apoptotic cells in the polycystic CPK kidneys relative to the age matched C57 control kidneys at all developmental stages.

We have found both a decrease in the amount of Bcl-2 protein and an increase in the degree of apoptosis in the polycystic kidneys of CPK mice. Abnormal regulation of apoptosis may mediate an altered developmental profile and hyperplasia in PKD.

B8-433 NEUTROPHILS INDUCE DNA FRAGMENTATION IN BRONCHIAL EPITHELIAL CELLS, Ruth J. McDonald, Jodie Usachenko, Eva Lew, Dallas M. Hyde. Depts of Pediatrics, Medicine & Vet Anatomy & Cell Bio, CRPRC, University of California, Davis, Davis CA 95616.

The human respiratory tract is often exposed to oxidants or neutrophils in pulmonary disease states such as bronchitis or following exposure to ozone. We have previously found cells of the bronchial epithelium to be resistant to H_2O_2 induced damage when measured by release of ^{51}Cr, but very sensitive to DNA damage measured by alkaline elution. To further investigate this process, we incubated cells from the human bronchial derived cell line, BEAS 2B R1.4 with H_2O_2 or quiescent human neutrophils (PMN) and measured DNA release into cell supernatants or following lysis in BrdU labelled cells using a cellular DNA fragmentation ELISA. BEAS cells incubated in media alone demonstrated no release of BrdU labelled DNA. Incubation with 1mM H_2O_2 for 24 hr did not produce release of DNA into supernatants but did result in increased DNA from cell lysates (OD 450=0.12 control vs OD=1.1 H_2O_2). Incubation for shorter time periods at lower concentrations of H_2O_2 produced correspondingly less cell associated DNA fragmentation. When PMN were added to cultures of BEAS cells, no DNA was present in cell supernatants but dose dependent increases were seen (OD= 0.32 $4x10^4$ PMN, OD=0.76 $4x10^5$ PMN) in cell lysates. We conclude that incubation of BEAS cells with H_2O_2 or PMN produces DNA fragmentation consistent with apoptosis and speculate that PMN may induce apoptosis in the respiratory epithelium.

B8-434 BACTERIA-INDUCED CELL DEATH IN EPITHELIAL TISSUES DURING MORPHOGENESIS OF THE SYMBIOTIC ORGAN OF THE HOST SQUID *Euprymna scolopes*. Margaret J. McFall-Ngai, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-0371.
The cooperative association between the sepiolid squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* is an emerging, experimental model system for the study of the influence of bacteria on development in animals. The host species has a complex, bilobed organ in the center of the mantle cavity, the core of which is epithelial tissue containing a culture of luminous *V. fischeri*. The association is established anew each generation, similar to the establishment of intestinal flora in mammals; the host squid acquires the specific symbiont from the free-living population of *V. fischeri* in the surrounding water within hours of emerging from the egg. At hatching, the juvenile host bears two ciliated, microvillous epithelial fields on each lateral face of the incipient symbiotic organ. These fields serve to entrain bacteria-ladened water toward a series of pores on the surface of the organ. These pores lead to epithelia-lined crypts that are the colonization site of the bacterial partner. The bacteria induce a 4 to 5 day program of morphogenesis in the host in which the symbiotic organ loses the ciliated, microvillous field through massive cell death; morphogenesis does not occur in the absence of the bacterial symbiont. Experiments in which the organ was "cured" of *V. fischeri* with chloramphenicol (with chloramphenicol-resistant mutants of *V. fischeri* as controls) revealed that within 12 hours post-colonization of the organ, the bacteria send a irreversible signal to the host tissue such that the entire morphogenetic program ensues. Concurrently, at 12 hours, a burst of cell death occurs at the edges of the ciliated field, several cell layers away from the bacterial culture. The wave of cell death continues over the 4-5 day period of morphogenesis until the entire field is regressed. Present studies are defining the mechanisms by which bacteria induce cell death events. This work is funded by the Developmental Mechanisms Program of NSF, Grant #IBN9220482 and by the Office of Naval Research, Grant #N00014-91-J-1357.

B8-435 H-Ras ONCOGENE EXPRESSION IN IMMORTALIZED INTESTINAL EPITHELIUM CONFERS RESISTANCE TO PROGRAMMED CELL DEATH INDUCED BY THREE-DIMENSIONAL GROWTH CONDITIONS. Yoshihiro Mitsuhashi, Janusz W. Rak, Victor Erdos, Jorge Filmus, Hassan Jamal and Robert S. Kerbel, Cancer Research Division, Sunnybrook Health Science Centre, University of Toronto, Toronto, Ontario, M4N 3M5, Canada.
Degregulation of molecular pathways controlling cell survival is thought to be an important mechanism of malignant transformation and tumor progression. The function of oncogenes and tumor supressor genes in regulation of programmed cell death is likely to depend on cell type and expression of other genes (cellular context) as well as interactions of the cell with its surroundings (extracellular context). We found that immortalized non-transformed rat intestinal epithelial cells (IEC-18) that grow readily in monolayer culture undergo massive cell death (within 48-72 hours) when cultured under three-dimensional conditions as multicellular spheroids. During this process the IEC-18 cells display features of programmed cell death including: chromatin condensation (Hoechst 33258 staining), apoptotic morphology, DNA degradation and loss of colony forming ability in the absence of apparent membrane damage. This cellular death could be prevented by overexpression of H-*ras* oncogene in three independently stably transfected cell lines (ras 3, ras 4 and ras 7) as well as upon addition of dexamethasone to IEC-18 cells carrying *ras* under control of a glucocorticoid inducible promoter (clone 25). The expression of *ras* was also associated with enhanced cell aggregation and spheroid formation capacity. The latter property, but not resistance to cell death, could be abrogated in ras7 cells by expression of antisense CD44 mRNA, implying that although CD44 is an important mediator of three-dimensional organization of IEC-18 cells, the presence of oncogenic *ras* itself is necessary and sufficient to confer cell survival under these conditions. Similar resistance to cell death was observed in IEC-18 cells transfected with *src* oncogene (src2, src3, src4 cell lines). These observations suggest that intestinal epithelial cells may be programmed to activate the apoptotic pathway upon detachment from physiological two-dimensional (monolayer) configuration. Activation of *ras* (or *src*) oncogene may be a necessary requirement for cell survival and three dimensional tumor growth during intestinal or colonic neoplasia.

B8-436 **AGE-RELATED CHANGES IN THE INTRINSIC RATE OF APOPTOSIS IN LIVERS OF DIET-RESTRICTED AND AD LIBITUM-FED B6C3F1 MICE,** Muskhelishvili,L. and James S.J. FDA-National Center for Toxicological Research, Jefferson AR 72079
It has been proposed that apoptosis may provide a protective mechanism in multicellular organisms whereby DNA-damaged or potentially neoplastic cells are selectively eliminated. Thus, interventions that increase cellular sensitivity to apoptotic cell death would tend to protect genotypic and phenotypic stability with age; on the other hand, the *failure* to initiate appropriate signals for apoptosis would tend to accelerate the accumulation of age-associated genetic lesions and neoplasia. Dietary restriction (DR) is a non-invasive manipulation that retards both physiologic aging and tumor incidence. As such, it provides a powerful model to study common mechanistic processes. In a recent study, we established that chronic DR induces an increase in the spontaneous rate of apoptotic cell death in hepatocytes of 12 month old B6C3F1 mice and was associated with a reduction in subsequent hepatoma development. The purpose of the present study was to extend and confirm these original observations by determining whether the increased rate of apoptosis with DR is maintained throughout the life span in this strain. We quantified the spontaneous apoptotic rate in liver sections from DR and ad libitum-fed (AL) B6C3F1 mice at 12, 18, 24, and 30 months of age. Apoptotic bodies (ABs) were enumerated in non-tumor bearing mice by scoring 50,000 random hepatocytes per liver utilizing in situ end-labelling (ISEL) immunohisto-chemistry and expressed as the mean percent incidence per 100 cells. The rate of apoptotic cell death was found to be elevated with aging in both diet groups; however, the rate of apoptosis was consistently higher in the DR, relative to the AL mice, within each age group. The increase in apoptotic rate with age in the surviving tumor-free AL-fed mice may be a biased reflection of a "surviving cohort" of mice with enhanced resistance to tumor development. These results suggest that variations in the intrinsic sensitivity to apoptotic cell death may contribute, in part, to tumor incidence and degree of "successful" aging.

B8-437 THE MODULATION OF THE IFNγR EXPRESSION CONTROLS HUMAN T LYMPHOCYTE APOPTOSIS. F. Novelli*, A. Allione*, P. Bernabei*, G. Garotta+, L. Mallucci*, G.Trinchieri, and G. Forni*,* Dept. of Clinical and Biological Sciences, Univ. Turin, Italy, +Hoffmann-La Roche Ldt., Basel, Switzerland, *King's College, London, UK, Wistar Inst., Philadelphia, PA.
IFNγR expression is subject to contrasting modulation on human T cells. IFNγR constitutive expression is low on malignant T cells growing in medium supplemented with serum. The addition of IFNγ down modulates IFNγR expression and increases both proliferation and MHC class I Ag expression. By contrast, when malignant T cells are cultured in medium without serum, IFNγR dramatically increases and the cells undergo a slow apoptotic death. The addition of IFNγ enhances apoptosis and inhibits cell rescue in serum-supplemented medium. This opposite ability of IFNγ to stimulate malignant T cell proliferation or death correlate with the intensity of IFNγR cell expression, high expression being a marker for cell apoptosis. These data indicated that environmental stimuli regulate T cell expression of IFNγR. In function of such expression, IFNγ itself promotes either proliferation or rapid apoptotic death. Recently, new findings showed that treatment of malignant T cells with a newly discovered citokine (βGBP, β galactoside binding protein) which exerts negative control of cell proliferation (Wells and Mallucci, *Cell* 64:91, 1991) up-regulates IFNγR expression, down-modulates those of bcl-2 and favours the apoptosis induced by IFNγ. Moreover, in addition to the p90 chain of IFγR, it has been recently cloned a second component (p35) of the human IFNγR, which is responsible for the signal transduction in to the nucleus following the interaction with IFNγ. To better define the signals influencing the apoptosis of T cells we studied the regulation of p90 and p35 expression chains of the IFNγR by other cytokines. We observed that during the differentiation of functional activated T lymphocytes, the expanding Th1 lymphocytes obtained by the priming in the presence of IL12 express low levels of the p35 and p90 IFNγR chains when compared with T lymphoblasts primed in the absence of IL12. The low expression of IFNγR correlates with a refractoriness of T lymphoblasts primed in the presence of IL12 to activation-induced programmed cell death when compared to the lymphoblasts primed in the absence of IL12.

B8-438 *BCL-X* IS EXPRESSED IN EMBRYONIC AND ADULT NEURONAL TISSUES AND ITS EXPRESSION PREVENTS NEURONAL CELL DEATH, Gabriel Nuñez, Maribel González-García, Liyun Ding and Irene García, Department of Pathology, The University of Michigan Medical School, Ann Arbor, MI 48109 and Department of Pathology, Centre Medical Universitaire, 1211 Geneva, Switzerland.

Previous studies have implicated the *bcl-2* proto-oncogene as a potential regulator of neuronal survival. However, while the Bcl-2 protein is retained in neurons of the peripheral nervous system, Bcl-2 levels are greatly reduced or undetectable in the majority of post-mitotic neurons of the central nervous system (CNS) of adult mice, rhesus monkeys and humans. Furthermore, adult mice in which the *bcl-2* gene has been disrupted by homologous recombination, exhibited normal development and maintenance of the CNS. Since *bcl-2* appears dispensable for neuronal development and survival, we have examined the expression and function of *bcl-x*, a new member of the *bcl-2* family of death regulatory genes. We report here that in contrast to *bcl-2*, *bcl-x* is highly expressed in CNS neurons of embryos and adult mice. Two different forms of *bcl-x* mRNA and their corresponding products, Bcl-x_L and Bcl-x_b, were expressed in neurons of the CNS. Microinjection of *bcl-x_L* or *bcl-x_b* cDNAs into primary sympathetic neurons inhibited their death induced by nerve growth factor withdrawal. Furthermore, co-expression of *bcl-x_L* and *bcl-x_b* had an enhanced effect on neuronal survival. Thus, Bcl-x proteins appear to play an important role in the regulation of neuronal survival in the adult CNS.

B8-439 DISRUPTION OF THE MICRO-ENVIRONMENT INDUCES APOPTOSIS IN HUMAN SEMINOMA CELLS, R.A. Olie, M.C. Dekker, J.W. Oosterhuis, T. Boersma, K. Nooter and L.H.J. Looijenga. Laboratory of Experimental Patho-Oncology, Dr. Daniel Den Hoed Cancer Center, Rotterdam, The Netherlands.

Human seminoma (SE) cells and their benign counterparts (primordial germ cells, PGC), are sensitive for DNA damage induced by irradiation and chemotherapy. SE and (murine) PGC are hard to culture *in vitro*, probably because of the induction of apoptosis upon cell isolation. Using an *in situ* end labeling procedure, fresh SE were studied for the presence of single strand DNA breaks. In addition, the presence of double strand DNA breaks was confirmed by DNA ladder formation on electrophoresis gels, before and after mechanical dissociation. This approach revealed that DNA breaks are rapidly induced in SE cells upon dissociation. Less than 5% apoptotic tumor cells were present in most SE before dissociation, while this number increased up to 90% in the single cell suspension derived from the same tumor. DNA ladders could only be obtained from cell suspensions or from SE with high numbers of apoptotic cells in the intact tissue. The presence of cycloheximide during mechanical dissociation decreased the number of apoptotic SE cells in the final cell suspension. Currently, the possible abrogation of apoptosis using stem cell factor, among others, is studied. Cells from 17 SE were brought into *in vitro* culture. Only cells from three SE with a mutation in the *N-* or *KRAS* gene showed enhanced survival or initial proliferation. This is in agreement with recent publications on the abrogation by a mutant *RAS* gene of apoptosis induced by disruption of cell-matrix interactions (Arends, Br J Cancer 68, 1993). However, in these tumors DNA breaks could be detected. The relation between DNA breaks, *in vitro* behavior and mutant *RAS* is under investigation. Our results suggest that an intact (and defined) micro-environment is critical for the prevention of apoptotic death of SE cells, which may relate to the relatively low metastatic potential of SE.

B8-440 BAX APPEARS CENTRAL IN THE REGULATION OF BCL-2 TYPE CELL DEATH CHECKPOINTS, Zoltán N. Oltvai*, Thomas W. Sedlak*, Elizabeth Yang*, Kun Wang*, Lawrence H. Boise#, Craig B. Thompson# and Stanley J. Korsmeyer*, *Division of Molecular Oncology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110, # Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637

A cascade of intracellular checkpoints define a cell's response to an apoptotic stimulus, that includes the prototype checkpoint of Bcl-2/Bax. To repress apoptosis, Bcl-2 heterodimerizes with Bax through their highly conserved BH1 and BH2 domains. A family of Bcl-2 related proteins share BH1 and BH2, including Bcl-x_L, a functional homolog of Bcl-2. We report here that Bcl-x_L, similarly to Bcl-2, also formed heterodimer with Bax. A gly159ala substitution in BH1 of Bcl-x_L disrupted its hetero-dimerization with Bax and abrogated its inhibition of apoptosis in mammalian cells. A yeast-two-hybrid assay accurately reproduced these intracellular interactions and defined a selectivity and hierarchy of further dimerizations. Bax also heterodimerized with Mcl-1 and A1. This suggests that the susceptibility to apoptosis is partly determined by multiple, competing dimerizations among Bcl-2 family members in which Bax plays a central role.

B8-441 FAS ANTIGEN IS INVOLVED IN CD4-CROSSLINKING MEDIATED APOPTOSIS IN PERIPHERAL BLOOD LYMPHOCYTES.
Savita Pahwa, Thomas McCloskey, Soe Than, Rong Hu, and Naoki Oyaizu. North Shore University Hospital- Cornell University Medical College, Manhasset, New York 11030, USA.

Lymphocytes from patients with HIV-infection have been shown to undergo accelerated spontaneous apoptosis. Binding of CD4 molecules by HIV envelope protein gp120 and anti gp120 antibodies can lead to crosslinking of CD4 molecules (CD4XL) in-vitro and conceivably, in-vivo. We have recently shown that CD4XL in-vitro, when performed in unfractioned peripheral blood mononuclear cells (PBMC) of normal HIV seronegative donors, is by itself sufficient to induce T cell apoptosis. In this study, we induced CD4XL in PBMC obtained from normal donors. either by anti-CD4 mAb Leu3a or by HIV-1 envelope protein gp160. PBMC subpopulations were examined for Fas antigen expression and for apoptosis induction by flow cytometry. CD4XL was found to result in increased Fas Ag expression as well as Fas mRNA in lymphocytes and the up-regulated Fas Ag was closely correlated with apoptotic cell death. Addition of the tyrosine protein kinase (TPK) inhibitor genistein or of the immunosuppressive agent cyclosporin A (CsA) abrogated these effects. CD4XL in PBMC also resulted in induction of the cytokines INF-γ and TNF-α in the absence of IL-2 and IL-4 secretion. Both induced cytokines contributed to Fas Ag upregulation and antibodies to these cytokines abrogated CD4XL-induced Fas Ag upregulation and apoptosis in lymphocytes. Patients with advanced HIV disease manifested increased Fas Ag expression in peripheral blood lymphocytes. These findings suggest that CD4XL occurring in-vivo might play an important role in inducing an aberrant cytokine profile (which has been observed in HIV infected individuals) and also in the triggering of T cell apoptosis.

B8-442 CELL DEATH IN ABSENCE OF HORMONAL TARGET SIGNALS: PITUITARY-REGULATING NEURONS IN HYPOPITUITARY MICE, Carol J. Phelps, Mario I. Romero and David L. Hurley, Departments of Anatomy and Cell & Molecular Biology, Tulane University, New Orleans, LA 70112

Hypophysiotropic (pituitary-regulating) neurons in genetically hypopituitary dwarf mice exhibit abnormalities that reflect specifically both the identity and nature of absent target hormone feedback. These animals fail to produce prolactin (PRL) as well as growth hormone (GH). Dopamine (DA), produced by neurons of the hypothalamic arcuate nucleus, is a tonic inhibitor of PRL secretion, and normal PRL feedback stimulates DA production. In dwarf mice, hypophysiotropic DA is severely reduced, as assessed by both biochemical measurement and by immunostaining for tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis; other DA neurons in dwarf mouse brain are unaffected. Developmental assessment has shown that the number of these TH-immunoreactive neurons actually regresses between 21 and 60 days of age postnatally in dwarfs, from 2708±437 cells to 1001±94 cells ($p<0.01$). The phenomenon may be an exaggeration of a tendency for cell loss which also occurs in normal mice (3306±572 to 2159±127 cells) at the same age interval, but not to a statistically significant degree. Treatment of dwarfs with PRL, prior to this period in development, maintains DA expression and TH cell number, but is without effect in adults. Whether PRL treatment is preventing cell death or loss of phenotype among these TH/DA neurons is not known. However, in the same hypothalamic nucleus, neurons which stimulate GH secretion by production of GH-releasing hormone (GHRH) are present in excessive numbers in dwarfs, compared with normal littermate mice (977±123 *vs.* 342±54; $p<0.01$), reflecting the absence of negative feedback by GH. Developmental studies indicate that GHRH mRNA increases in dwarfs postnatally. Studies are in progress to determine whether PRL replacement during development in dwarfs will affect GHRH expression and neuron number. In order to test directly whether such a change in phenotype accounts for a portion of the DA cell loss, a methodology is needed for deducing whether a neuron that was originally TH/DA-producing later exhibits a GHRH phenotype. In addition, we would like to examine the developmental loss of TH/DA neurons using a method for identifying cell death *in situ*.

Supported by PHS grant NS25987 (to CJP).

B8-443 APOPTOSIS: A BASIC HISTOLOGICAL PROCESS DURING THE GENESIS OF THE GIZZARD GLANDS OF Gallus gallus (Linnaeus,1758) Nadja Lima Pinheiro (1) and Luiz Ludovico George. (2). (1) Instituto de Biologia., D.B.A., Universidade Federal Rural do Rio de Janeiro,Km 47. Antiga Rio-São Paulo, Seropédica ,RJ, Brasil CEP 23851-970. (2) Universidade de São Paulo.

An ultrastructural study, in the 10th day embryos, shows us the cells of the basal portion of the gizzard epithelium initiating a process of proliferation and growth, resulting in compact epithelial buds that invade the underlying connective tissue. The glands are formed as ingrowths of the epithelium into the underlying mesenchyme. By the 15th day, those epithelial glands buds show some signs of gland lumen,which occur either through a process of apoptosis- in consequence of cellular fragmentation, or by an accumulation of secretion in the intercellular spaces. The cellular fragmentation, by coalescence of secretory granules, results in ultrastructurally well-preserved fragments and is responsable for focal cell deletion. There still is an epithelial portion obstructing the opening of the tubular glands. Together with the formation of the tubular glands, in the mid-zone of the epithelium, it was detected, by the 14th day, an accumulation of secretion, a product of the epithelial cells, discharged in the intercellular space. These cells, also pertaining to the epithelial portion to be discarded, continue in a process of exocytosis, incrementing the intercellular and intraepithelial secretion and, paradoxically, augmenting the possibilities of a faster rejection of that portion. The accumulation of intraepithelial secretion displaced the upper part of the superficial epithelium, splitting it. It must be emphasized,as well,the presence of citoplasmatic projections in the apical cell, already detected in the 9th day of the embryonic development, as it represents the first step to the focal elimination of cells, in the apical portion of the epithelium. In consequence of constrictions in its basis,those projections detach themselves from the cellular surface, resulting in cellular fragmentation followed by death.

B8-444 OVEREXPRESSION OF BCL-2 IS ASSOCIATED WITH APOPTOSIS IN NEURONAL DEATH. Kasturi L. Puranam, Steven C. Lane, Wei-Hua Qian and Rose-Mary Boustany, Department of Pediatrics, Division of Pediatric-Neurology, Duke University Medical Center, Durham, NC 27710.

Batten disease is a common genetic neurodegenerative disorder of childhood, its pathological manifestations being retinal and neural degeneration that ultimately cause death. Based on recent studies that show the involvement of apoptosis in retinal degeneration, we have investigated whether apoptosis may also be responsible for retinal and neural death in Batten disease. We detected the presence of apoptotic neuronal cells in brain tissue from Batten disease patients by TUNEL staining method that specifically labels the endolytically fragmented nuclear DNA. In order to understand apoptosis in post mitotic neurons, we have been looking at the role of proto-oncogenes like Bcl-2 in terminally differentiated neurons. We have been able to detect an increase in the level of protein expression of Bcl-2 in Batten disease patient brain tissue by immunocytochemical and Western blot methods. We present this as an example of overexpression of a proto-oncogene leading to cell death in terminally differentiated neural cells.

B8-445 COMPARISON OF BCL-2 EXPRESSION IN THE NEURODEGENERATIVE DISEASE: BATTEN DISEASE, NIEMANN-PICK TYPE C AND ALZHEIMER DISEASE. Wei-Hua Qian and Rose-Mary Boustany, Department of Pediatrics, Division of Pediatric-Neurology, Duke University Medical Center, Durham, NC 27710.

The hallmarks of Batten disease, a neurodegenerative disease are neuronal cell death and photoreceptor degeneration. We have evidence of apoptosis being the mechanism of cell death in the juvenile and late infantile forms of this disease. The mechanism of neuronal death is by overexpression of the bcl-2 proto-oncogene which in nondividing, postmitotic neuronal cells causes cell death as opposed to proliferation. We compare expression of bcl-2 by immunocytochemistry in three different neurodegenerative diseases: Alzheimer disease, Niemann-Pick, type C and the late infantile (LINCL) and juvenile (JNCL) forms of Batten disease. We found intense staining for bcl-2 suggesting overexpression in temporal cortex, occipital cortex and midbrain from an LINCL case and moderate staining in occipital cortex, cerebellum and midbrain from a JNCL case. There was no staining for bcl-2 in any of the brain regions in Alzheimer's disease nor in cortex from a Niemann-pick, type C case. Although apoptosis may be a mechanism common to many neurodegenerative diseases, there is specificity with regards to the exact mechanism by which it occurs.

B8-446 PROGRAMMED CELL DEATH, AUTOPHAGIC VACUOLATION AND PRION INFECTIVITY ARE MODIFIED BY NERVE GROWTH FACTOR IN A NEURONAL CELL LINE. H. M. Schätzl, L. Laszlo, D. M. Holtzman, R. I. Weiner, W. C. Mobley and S. B. Prusiner, University of California, San Francisco, CA 94143-0518

Prions cause spongiform degeneration, astrocytic gliosis and sometimes PrP amyloid deposition in the CNS of humans and other mammals. While vacuolation of neurons is a hallmark of prion diseases, it is not a feature in scrapie-infected cell lines currently used to study the cell biology of prions. We report on a prion-infected neuronal hypothalamic cell line which secretes GnRH synchronously and was transfected with the trk-A gene encoding the nerve growth factor (NGF) receptor[1]. A subclone designated ScGT1-1-trk9 was infected with mouse RML prions and was passaged more than 40 times without any decrease in PrP^{Sc} production over the past year. These infected cells show extensive autophagic vacuolation and the characteristics of programmed cell death (PCD). Electrophoresis of soluble DNA extracted with Triton X 100 revealed the "DNA-ladder" fragmentation pattern typical for apoptosis in ScGT1-1-trk9 cells, but not in uninfected control cells. The viability, proliferation rate and survival of ScGT1-1-trk9 cells in serum-free medium was reduced compared to those of uninfected controls. Treatment with NGF reduced the morphological and biochemical signs of PCD in ScGT1-1-trk9 cells. Although NGF also diminished the level of PrP^{Sc}, PrPmRNA and PrP^{C} levels remained unchanged. The decrease in PrP^{Sc} was selective since NGF elevated the level of neuronal nitric oxide synthetase (NOS). Of note, NGF administered intraventrically to neonatal mice elevated PrPmRNA levels[2]. The intense autophagic vacuolation and programmed neuronal cell death of ScGT1-1-trk9 cells may provide a useful model for the studies on the molecular pathogenesis of neurodegeneration. Whether NGF treatment of animals will slow the course of scrapie in animals as it seems to do in ScGT1-1-trk9 cells remains to be established.

1. J. Zhou, D. M. Holtzman, R. I. Weiner, W. C. Mobley, *Proc. Natl. Acad. Sci. USA* **91**, 3824-3828 (1994).
2. W. C. Mobley, R. L. Neve, S. B. Prusiner, M. P. McKinley, *Proc. Natl. Acad. Sci. USA* **85**, 9811-9815 (1988).

B8-447 **A PEAK OF PROGRAMMED CELL DEATH APPEARS DURING POSTNATAL LUNG DEVELOPMENT,**

Schittny, J.C. and **Burri, P.H.** Institute of Anatomy, University of Bern, CH-3000 Bern 9, Switzerland

During the first three postnatal weeks the rat lung undergoes the last two developmental stages, the phase of alveolarization followed by the phase of maturation of the parenchymal microvascular network. The latter involves a merging of the two capillary layers to a single one and a decrease of the connective tissue mass in the alveolar septa.

Speculating that programmed cell death may play a role during postnatal lung remodeling, we searched for the presence of apoptotic cells in rat lungs between days 5 and 24. Lung cryo-, or paraffin-sections, respectively, were treated with 3'-terminal transferase, digoxigenin-dUTP, and anti-digoxigenin-fluorescein-F(ab)-fragments, and the number of fluorescent nuclei was compared between sections at different days. While the number of apoptotic cells was low until the end of the second week and at day 24, we observed an about eight fold increase of fluorescent nuclei towards the end of the third week. We conclude that programmed cell death is involved in the structural maturation of the lung. Furthermore, preliminary results indicate that type II epithelial cells represent one kind of the dying cells.

B8-448 IMPAIRED T-CELL APOPTOSIS IN ANIMAL MODELS WITH SPONTANEOUS AUTOIMMUNITY

Sgonc, R., Gruber, J., Dietrich, H., and Wick, G.
Institute for General and Experimental Pathology, University of Innsbruck, Medical School, Fritz-Pregl-Str. 3, 6020 Innsbruck, Austria

Since autoimmune diseases are characterized by a hyperreactive immune system, we are interested to investigate the deletion of T-cells during thymic maturation in order to clarify if a possible defect during negative selection may be responsible for the development of autoimmunity. Our attention was focussed on the possible alterations of glucocorticoid-induced apoptosis of thymocytes. Chickens of the Obese strain (OS), which develop a spontaneous hereditary autoimmune thyroiditis closely resembling human Hashimoto thyroiditis, University of California at Davis (UCD) line 200 chickens, which develop a hereditary scleroderma - like connective tissue disease, and normal healthy control White Leghorn (NWL) chickens were injected i.p. with 1 - 50 mg dexamethasone per kg body weight. Thymic tissue was removed after 8 hours and analyzed for apoptosis by agarose gel electrophoresis of the DNA, or on frozen tissue sections by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL). The results reveal that OS thymocytes are relatively resistant against glucocorticoid-induced apoptosis. This phenomenon may be involved in the inefficient thymic negative selection process in this strain, leading to deficient deletion of autoreactive clones. UCD 200 chickens show normal thymocyte apoptosis after injection of dexamethasone, but on thymus sections of untreated UCD 200 chickens only very few apoptotic cells are found compared to untreated NWL. This could possibly indicate a disturbed antigen-induced apoptosis during T cell maturation in this animal model.

Supported by the Austrian Research Council (project No. 8809) and the Dr. Kolassa-Stiftung zur Rheumaforschung.

B8-449 Comparative Analysis of the Effects of RB or p53 Transfection on the Regulation of Cell Growth and Apoptosis in Human Cancer Cells. Takahashi, R.[1], Tamura, T.[2], Hashimoto-Shima, T.[3], Liang, P.[1] Aoyama, N.. [1]Dept. of Pathol., Faculty of Med., Kyoto Univ., [2]Dept. of Int. Med., Kobe Univ. School of Med., [3]Dept. of Genet., Hyogo College of Med., Japan.

To examine the effects of growth-suppressing genes on specific cell properties, including the growth factor-dependent regulation of cell cycle and induction of apoptosis, stable RB or p53 transfectection was performed using human cancer cell lines. Although growth rates of RB-transfected bladder carcinoma cells were reduced compared to those of the control cells in a serum dependent manner, p53 transfection caused severe growth inhibition even in 10% serum-containing media. HTB9 cells lost responsiveness to apoptosis-inducing stimuli after aquiring normal RB expression. In case of the colon cancer cell line, WiDr, the effects of mut-p53 were overridden by smaller amounts of exogenous wt-p53 resulting in slower growth rates and low viability. After being transfected with the wt-p53 gene, squamous cell carcinoma cells showed marked keratinization including cancer pearl formation in nude mice xenograft, These findings suggest that RB and p53 share the common feature of inhibiting cell cycle, but have separate functions which correlate with apoptosis and on TGFβ-dependent growth properties.

B8-450 Fas-Mediated Apoptosis Causes Wild-Type p53 Gene Overexpression under the Control of Heterologous HCMV Promoter in Human Colon Carcinoma Cells, Takao Tamura[1,2], Rei Takahashi[1], Nobuo Aoyama[2], Hironori Haga[1], and Taketoshi Sugiyama[1]. [1]Department of Tumor Biology and Pathology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, JAPAN; [2]2nd Department of Internal Medicine, Kobe University School of Medicine, Kusunoki-cho, Chuo-ku, Kobe 650, JAPAN.

To investigate the effects of wild-type p53 gene transfection in human colon carcinoma (WiDr) cells, a wild-type p53 expression plasmid under the control of human cytomegalovirus (HCMV) promoter was stably transfected into WiDr cells carrying a mutation at codon 273 of the p53 gene. Exogenous wild-type p53 transcripts expressed at various expression levels in 8 of 117 isolated clones. The growth rates of the wild-type p53+ clones in culture were not significantly changed. However, the efficiency of colony formation in soft agar was drastically suppressed in four wild-type p53+ clones. Marked cytolysis of the wild-type p53+ cells was induced by incubating the cells with anti-Fas IgM whereas most parental p53- cells did not show significant cell death. Overexpression of the WAF1 and wild-type p53 genes was also induced in the wild-type p53+ cells when they started apoptotic process. The link between Fas and p53 in these p53-transfected cells may be created by heterologous recombination of the wild-type p53 gene and the cytomegalovirus promoter. This model, however, should provide a clue to analyze the mechanism and signal pathways of apoptosis in colon carcinoma cells.

B8-451 APOPTOSIS IN NORMAL HUMAN RENAL DEVELOPMENT AND POLYCTSIC KIDNEY DISEASE. Patricia D. Wilson and Christopher R. Burrow, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

Mammalian renal development is an example of embryonic induction of mesoderm to epithelium. Undifferentiated metanephric blastemal cells (nephroblasts) are induced by the branching ureteric bud to undergo a series of molecular and morphological events leading to their conversion to the several epithelial cell types characteristic of the nephron. It has recently become appreciated that apoptosis is a normal process that occurs at the time of induction and epithelial conversion, and that prevention of apoptosis in bcl-2 deficient mice leads to the generation of malformed cystic kidneys. We have carried out morphological and peroxidase labelling analysis of renal apoptosis in situ during normal fetal development (12-24 weeks gestation), normal adult aging (newborn - 63 yr) and in genetically determined polycystic kidneys of autosomal dominant (ADPKD) and autosomal recessive (ARPKD) origin. Many apoptotic nuclei were seen in fetal and polycystic kidneys, but few were seen in normal adult kidneys. In fetal kidneys apoptosis was most prevalent in blastemal cells surrounding the induced condensing mesenchyme, S-bodies and tubules. In ADPKD and ARPKD, the apoptotic nuclei were predominantly in the epithelial cells lining cysts and appeared to increase with the degree of cystic involvement during progression of the disease. Ultrastructural analysis and immunolocalization of membrane proteins failed to show necrotic changes in these kidneys, suggesting that apoptosis was associated with normal renal development and to be a specific alteration in polycystic kidney disease. To examine the mechanisms of induction, we have developed a novel cell culture system in which human nephroblast progenitor cells can be grown indefinitely and remain differentiation-competent in response to specific growth factors and matrix proteins. Nephroblast cell death is inhibited by addition of NB-GF, a soluble factor secreted by the G401 Wilms' tumor cell line and allows indefinite proliferation of uninduced cells. This culture system should provide insights into the regulation of apoptosis in normal and diseased kidneys.

B8-452 CHOLINE DEFICIENCY INDUCES APOPTOSIS IN SV40-IMMORTALIZED CWSV-1 RAT HEPATOCYTES IN CULTURE. S.H. Zeisel and C.D. Albright. Department of Nutrition, University of North Carolina, Chapel Hill, NC 27599.

Rats fed a diet deficient in choline developed hepatocarcinoma spontaneously. Within weeks of eating this diet, the rate of liver cell death increased dramatically. We reasoned that this might be due to the induction of apoptosis and undertook studies in rats aind in cells in culture (CWSV-1 rat hepatocytes). DNA fragmentation, a hallmark for apoptosis, was detected by measuring the addition of digoxigenin-11-dUTP to free 3'-OH ends of DNA. In rats fed a choline deficient diet for 6 weeks, there was a large increase in apoptotic heaptocytes (from 1% in control to 10% in deficient cells). CWSV-1 cells were plated in 70 μM choline sufficient serum-free medium for 4 days and then cultured in CS or choline deficient (CD) (5 μM or 0 μM choline) medium for 3 days. We demonstrated that fragmentation of genomic DNA increased over time in cells treated with CD medium. Cells undergoing DNA fragmentation retained the ability to exclude trypan blue, and showed an increased ability to convert a tetrazolium salt (MTT) to an insoluble formazan product. This was followed by the accumulation of end-stage apoptotic cells exhibiting nuclear chromatin condensation, pyknosis, and fragmentation of the nucleus found within intact cytoplasm. These apoptotic cells contained intact cell organelles. Proliferating cell nuclear antigen (PCNA) was detected in 79.9%, 62.8% and 26.0% of the cells treated for 72 hrs. with 70 μM, 5 μM, and 0 μM CD medium, respectively. However, the mitotic index (MI) in these same cultures was 2.6%, 0.7%, and 0.0%, indicating that CD medium not only reduced the growth fraction of CWSV-1 cells, but also inhibited the ability of cells to transit the cell cycle. Cells treated with CD medium for 72 hrs. detached from the substrate in high numbers (1.2 x 105 cells/culture). These detached cells exhibited a high incidence of apoptosis (80-90% of cells), an absence of mitosis and PCNA expression, a retention of trypan blue (50-55% of cells), and a decreased ability to reduce MTT to formazan.

Supported by a grant from the American Institute for Cancer Research.

Late Abstracts

GENES INDUCED BY GLUCOCORTICOIDS IN MURINE T-CELLS DURING APOPTOSIS, Gail Baughman and Suzanne Bourgeois, Department of Regulatory Biology, The Salk Institute for Biological Studies, La Jolla, CA 92138.

Glucocorticoid treatment induces programmed cell death (apoptosis) in susceptible T-lymphocytes. We have isolated and characterized cDNAs corresponding to thirteen different genes induced by agents (glucocorticoids and cAMP) that cause apoptosis in the murine thymoma cell line WEHI-7TG and in thymocytes. Several of these cDNA clones correspond to known genes whose regulation by glucocorticoids had not been previously reported (mitochondrial phosphate carrier protein, calmodulin, mouse retroelement VL30, chrondroitin sulfate proteoglycan core protein, mouse early transposon, ETn, and the Ig-related glycoprotein GP-70). Several genes, however, are of particular interest to our laboratory as they are observed to be predominantly or exclusively expressed in murine thymus. These genes include a new orphan G-protein coupled receptor (termed GIR), the p70 subunit of the Ku autoantigen involved in transcriptional activation, a new gene Tcl-30 that is expressed only is immature, HSA^+, glucocorticoid-sensitive thymocytes, a previously undescribed immunophilin of the FK506 binding protein (FKBP) subclassification, and an unknown gene designated clone 59. Sequence analysis of a full length cDNA corresponding to the new immunophilin indicates that this gene shares significant sequence similarity with, but is not identical to, the 52-59 kDa immunophilin that binds the immunosuppressive drugs FK506 and rapamycin and exists as part of the untransformed, non-DNA binding form of the glucocorticoid receptor. Data on the characterization of this new FKBP will be presented. (Supported by NIH grant CA-36146 to S.B. and a grant from the Children's Leukemia Research Association to G.B)

IDENTIFICATION AND CHARACTERIZATION OF CELL DEATH ASSOCIATED GENES. Wolfgang Bielke, Guo Ke, Susan Saurer, Lawrence M. Schwartz and Robert R. Friis, Laboratory for Clinical and Experimental Research, University of Bern, Switzerland and University of Massachusetts at Amherst, Biology Department, USA

We have employed several different strategies to identify Cell Death genes in various biological systems. One of these strategies involved the comparison between the apoptotic death of secretory epithelia cells during the involution of the mammary gland after weaning and apoptotic events during the regression of the ventral prostate after androgen depletion. The application of a modified Differential Display Analysis (Liang and Pardee, 1992) allowed the semiquantitative detection of several gene fragments regulated coordinately in both organs. For our approach, PCR fragments from RNAs of consecutive involution stages of the rat mammary gland and the rat ventral prostate were displayed in parallel on a polyacrylamide gel. Fragments which showed similar regulation in both tissues were chosen for further analysis. The detection of similar expression patterns of genes in the two glands implies their probable association with the apoptotic process itself, since the expression patterns of many characteristic and abundant genes involved in the tissue remodelling of the mammary gland differed remarkably from the ventral prostate, whereas this is not the case for several known apoptosis markers tested. Beside several, yet undescribed gene sequences, we could identify fragments displaying high homologies with the growth arrest specific gene *gas1* (Del Sal et al., 1992) and the integrin associated protein *IAP* (Lindberg et al., 1993) which RNA levels are strongly induced during early timepoints of apoptosis in both organs.

Another approach to identify genes associated with cell death in mammals was to clone homologs of presumptive Cell Death genes for the moth *Manduca sexta*. One of these genes, 18-44a, displays 55% amino acid identity between human and moth. The human gene is expressed in the hippocampus and further testing is currently underway.

ISOLATION AND CHARACTERIZATION OF GLUCOCORTICOID-INDUCED GENES FROM APOPTOTIC THYMOCYTES

Mark S. Chapman and Roger L Miesfeld, Department of Biochemistry, University of Arizona, Tucson, AZ 85724

Steroid-regulated apoptotic cell death of thymocytes is thought to require transcriptional regulation of a small set of genes. We have used differential display reverse transcription PCR (DD RT PCR) to identify and isolate numerous differentially expressed sequence tags (DESTs) corresponding to glucocorticoid induced genes. Since bcl-2 seems to block apoptosis at a step late in the pathway, we have taken advantage of Hb12, a Wehi 7.2 (murine T cell line) derived cell line which has been stably transfected with an expression vector for human bcl-2. Since Hb12 cells do not die, we have been able to distinguish between primary GR target genes and non-specific 'death-associated' changes in transcription. We have targeted genes which are induced by glucocorticoids in both Wehi 7.2 cells and Hb12 cells in hopes of identifying genes which are involved in the early commitment steps of thymocyte apoptosis. We have isolated a DEST we call GIG10 which is rapidly induced in Wehi 7.2 cells and Hb12 cells within 2 hours after dex treatment (maximal induction of 18-fold at 16 hours). The corresponding GIG10 transcript is 6 kb and sequence analysis of the 300 bp GIG10 tag revealed no significant homologies to GENBANK sequences. We are in the process of characterizing a number of other glucocorticoid-induced DESTs identified in the same way as GIG10. The potential role in thymocyte apoptosis of genes identified by these methods will be tested in several ways. First we will determine if they are induced by other agents which trigger apoptosis in Wehi7.2 cells (cAMP and γ-irradiation). We will also analyze their expression in a unique panel of glucocorticoid resistant (but GR positive) mutant Wehi7.2 cell lines isolated in our lab (J. Cell Biol., 1994, in press). This should allow us to both evaluate the genes role in thymocyte apoptosis and to further characterize the mutant cell lines. Finally, we will use antisense experiments to 'knockdown' expression of candidate genes as a way to directly test their involvement in thymocyte apoptosis.

BOTH IG-α AND IG-β ARE REQUIRED TO INDUCE APOPTOSIS IN WEHI-231 CELLS.

Marcus R Clark, Jeannie Tseng, Young Jae Lee, Shara Kabak, and Bartholomew J Eisfelder. Departments of Medicine and Pathology. University of Chicago, Chicago,IL 60637.

Stimulation of the B-cell antigen receptor complex in immature B-cells induces apoptosis. Although a great deal has been learned about the mediators of this process, little is known concerning which specific component(s) of the antigen receptor complex initiate the apoptotic signal. To address this question we developed a system in which we could examine the ability of Ig-α and Ig-β, either expressed individually or together, to induce apoptosis. Receptor chimeras were made by fusing the extracellular and transmembrane domains of the platelet derived growth factor receptor (PDGFR) alpha or beta chains to the cytoplasmic tail of the Ig-α or Ig-β chain (PDGFRβ/Ig-α, PDGFRβ/Ig-β, PDGFRα/Ig-β). We stably expressed these constructs individually and in combination in the immature B-cell line WEHI-231. To stimulate the chimeras we first added PDGFBB ligand which formed homodimers in single-chain transfected cells and homo- and heterodimers in the double-chain transfected cells. We then added antibodies specific for each receptor to achieve higher orders of aggregation. While cross-linking of Ig-α or Ig-β homodimers induced tyrosine phosphorylation of multiple cellular substrates, neither chain individually induced apoptosis. In contrast, aggregation of chimeric receptor heterodimers, which contain both Ig-α and Ig-β, induce an apoptotic signal indistinguishable from that induced via the endogenous antigen receptor. Thus, we have shown that both Ig-α and Ig-β are necessary and sufficient to induce apoptosis in WEHI-231 cells. It is not clear from this data if Ig-α and Ig-β generate independent signals or if the two chains combine to activate a single apoptotic pathway. However, it is clear that Ig-α and Ig-β contribute non-redundant signalling capacities to the B-cell antigen receptor complex.

C-*KIT* ANTISENSE (AS) OLIGONUCLEOTIDE (ODN) INDUCES APOPTOSIS IN NEUROBLASTOMA (NB) CELL LINES. **Cohen PS[1], Chan J[1], Sickafuse S[2], Carnahan J[3], Thiele CJ[2]. [1]Harbor-UCLA Medical Center, Torrance, CA, [2]Pediatric Branch, NCI, NIH, Bethesda, MD, [3]Amgen, Thousand Oaks,CA.** We have previously shown that stem cell factor (SCF) and c-*kit* are coexpressed in human NB cell lines and tumors, suggesting the presence of an autocrine growth loop involving SCF/c-*kit* in this disease (*Blood*, in press). In this study, we investigated the capacity of an unmodified c-*kit* antisense oligonucleotide to inhibit the growth of the NB cell line SK-N-BE(2). Cells grown in AS-ODN for up to 9 days demonstrated a dose-dependent inhibition of ^{3}H-thymidine (3HTdR) incorporation when compared to cells similarly grown in sense (S) ODN. Maximal inhibition of 3HTdR occured at 100 ug/ml of AS-ODN (mean reduction of 82%), comparable with the 75% reduction seen after cells were grown in the presence of SR-1, a human anti-c-*kit*-blocking antibody. When analyzed by RT-PCR, synthesis of c-*kit* mRNA was completely inhibited after 48 hours in culture with AS-ODN. Analysis of propidium iodide-stained nuclei by flow cytometry after ODN treatment revealed a three fold-increase of apoptosis (up to 29%) in cells treated with AS-ODN for 7 days, when compared to those treated with S-ODN. These data indicate that (1)c-*kit* AS-ODN inhibits 3HTdR incorporation of SK-N-BE(2) cells and (2)inhibition of 3HTdR incorporation of cells treated with AS-ODN occurs in part via induction of apoptosis, and suggests that SCF and c-*kit* expression supports NB proliferation and survival by blocking apoptosis.

INTERLEUKIN-1β CONVERTING ENZYME IS NOT A GRANZYME B SUBSTRATE, Alison J. Darmon, Nancy Ehrman, Antonio Caputo, Jocelyne Fujinaga and R. Chris Bleackley, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

Murine granzyme B (Cytotoxic Cell Proteinase-1/CCP1) is a member of a family of seven serine proteases found in cytoplasmic granules of cytotoxic T lymphocytes (CTLs). Evidence has suggested that it is involved in target cell DNA fragmentation during CTL-mediated cytotoxicity, although intracellular substrates for granzyme B have not yet been identified. The substrate specificity of granzyme B, requiring an aspartic acid residue at site P1, is unique among eukaryotic serine proteases and is shared with only one other known eukaryotic protease, Interleukin-1β Converting Enzyme (ICE). ICE is responsible for processing pro-IL-1β to produce biologically active IL-1β and is itself synthesized as an inactive precursor. Recent evidence has suggested a role for ICE in programmed cell death which led to a model for CTL-mediated cytotoxicity. In this proposal granzyme B activates ICE in the target cell by proteolytically processing the ICE precursor, resulting in active ICE heterodimer which induces apoptosis in the target cell. We have isolated the cDNA encoding murine ICE and generated *in vitro* translated ICE precursor. Using lysates from COS cells expressing granzyme B we show that ICE precursor is not a substrate for granzyme B and propose an alternate mechanism for CTL-mediated cytotoxicity.

(Supported by the Natural Sciences and Engineering Research Council, Alberta Heritage Foundation for Medical Research, the Medical Research Council and the National Cancer Institute of Canada.)

INDUCTION OF APOPTOSIS IN RESTING B CELLS BY ANTIGEN-SPECIFIC T CELLS. John H. Freed, Elizabeth D. Ward, Jennifer VanderWall, Richard C. Duke, Avi Kupfer, Brian L. Kotzin and M. Karen Newell. National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206

Class II MHC molecules expressed on the surface of B lymphocytes function as restriction elements in the antigen specific activation of T lymphocytes. The interaction between T cells and *primed* B cells ultimately leads to activation and differentiation of both cell types. However, the engagement of class II on a *resting* B cell inhibits subsequent B cell growth and activation. We have recently demonstrated that treating high density, resting B cells with anti-class II mAb induces apoptosis in these cells. Using a combination of microscopy and flow cytometry, we have have extended these observations to the interaction between resting B cells and antigen-specific, MHC-restricted T cells. Small, high-density, Percoll gradient-purified (resting) B cells were combined with antigen-specific, MHC-restricted T cell hybridomas or clones. B cells were loaded with antigen non-specifically to avoid involvement of their antigen specific receptors (*i.e.*, surface Ig), an interaction that would prime them. When combined with T cells, the resting B cells were induced to undergo apoptosis only under conditions in which their class II molecules were engaged by the TcR of the T cells. Preactivated T cells induced apoptosis in resting B cells only in an antigen-dependent and MHC-restricted manner suggesting that ligation of the class II molecule rather than the activation of the T cell was the determining factor for the induction of apoptosis. Pre-activated and fixed T cells also could induce apoptosis ruling out a mandatory role for cytokines in the process. Freshly *ex vivo* activated B cells, in contrast, were not induced to undergo apoptosis by their interaction with T cells. In fact, the activated (but not the resting) B cells produced readily detectable amounts of immunoglobulin after 5 days of culture with a Th2 helper clone and the appropriate antigen.

(NZB x NZW)F_1 and (NZB x SWR)F_1 animals develop severe autoimmune disease clinically similar to systemic lupus erythematosis (SLE) and characterized, in the early stages of disease, by polyclonal hypergammaglobulinemia. We reasoned that the breakdown of class II-mediated apoptosis in resting B cells of these animals might underlie this condition. In accordance with our prediction, we found that resting B cells from the autoimmune animals were refractory to anti-class II mAb-induced apoptosis.

INVOLUTION/APOPTOSIS ENHANCED TRANSCRIPTION FACTOR BINDING TO THE TRPM 2/CLUSTERIN PROMOTER, Eileen Furlong, Fiona Thornton, Niamh Keon and Finian Martin, Department of Pharmacology and Biotechnology Centre, University College Dublin, Dublin 4, Ireland.

TRPM-2/clusterin gene expression is triggered when apoptosis is initiated in the epithelial component of the rat ventral prostate by androgen deprivation. We have carried out DNase I footprinting analysis on a 693 bp region of the TRPM-2/clusterin gene promoter immediately 5'- of the start site for transcription. The promoter was footprinted with extracts from normal rat prostate and prostate from rats 2- and 3- days after castration. In the promoter of the TRPM-2 gene we detected two changes in the DNase I footprinting pattern that are involution/apoptosis induced: Firstly, we observed a significant enhancement of DNase I hypersensitivity at position -93 (start site for transcription, +1) on the sense strand of this promoter; an accompanying hypersensitivity site was found on the anti-sense strand (at -72). These hypersensitivity sites encompass an AP-1 binding site (-84 to -78). As our previous studies (Marti et al, 1994) had shown that transient increases in nuclear AP-1 DNA binding activity accompany the onset of apoptosis in the prostate, it has been of interest to identify a potential target for this AP-1, in the TRPM-2 promoter. Transient transfection studies show the activity of the TRPM-2 promoter to be significantly AP-1 dependent.

Secondly, we have identified an element within the TRPM-2 promoter over which the formation of an expanded footprint is enhanced in nuclear extracts from apoptotic tissue, when compared to normal tissue. This element extends from nucleotides -353 to -310 in the TRPM-2 promoter and encompasses two NF-1 binding elements. It would appear that the onset of apoptosis results in the recruitment of NF-1 to occupy the second of the two sites while the other site is occupied under all conditions of study.

Future studies will address the relationship of these changes in transcription factor binding activity to intracellular signals which are triggered at the onset of epithelial programmed cell death.

DELAY OF VACCINIA VIRUS-INDUCED APOPTOSIS IN NONPERMISSIVE CHINESE HAMSTER OVARY CELLS BY THE COWPOX VIRUS *CHOhr* AND ADENOVIRUS *E1B 19K* GENES. Barbara S. Ink, Christopher S. Gilbert, and Gerard I. Evan, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom. The infection of vaccinia virus in chinese hamster ovary cells produces a rapid shut down in protein synthesis and the infection is abortive (Drillien, R. R., D. Spehner, and A. Kirn, Virology 111:488-499, 1978; Hruby, D. E., D. L. Lynn, R. Condit, and J. R. Kates, J. Gen. Virol. 47:485-488, 1980). Cowpox virus, which can productively infect CHO cells, had previously been shown to contain a host range gene, *CHOhr*, which confers on vaccinia virus the ability to replicate in CHO cells (Spehner, D., S. Gillard, R. Drillien, and A. Kirn, J. Virol. 62:1297-1304, 1988). We found CHO cells underwent apoptosis when infected with vaccinia virus. The expression of the *CHOhr* gene in vaccinia virus allowed for the expression of late virus genes. *CHOhr* also delayed or prevented vaccinia virus-induced apoptosis in CHO cells such that there was sufficient time for replication of the virus before the cell died. The *E1B 19K* gene from adenovirus also delayed vaccinia virus-induced apoptosis, however, there was no detectable expression of late virus genes. Furthermore, *E1B 19K* also delayed cell death in CHO cells which had been productively infected with vaccinia virus. This study identifies a new anti-apoptotic gene from cowpox virus, *CHOhr*, for which the protein contains an ankyrin-like repeat and shows no significant homology to other proteins. This work also indicates that an anti-apoptotic gene from one virus family can delay cell death in an infection of a virus from a different family.

EARLY EVENTS DURING APOPTOSIS IN THE MOUSE MAMMARY GLAND, Birgit Jehn, Andreas Marti, Feng Zhiwei, Guo Ke and Rolf Jaggi, Laboratory for Clinical and Experimental Research, University of Bern, Tiefenaustrasse 120, 3004 Bern, Switzerland.

At weaning the mammary gland undergoes a remodelling process (involution) which is associated with the apoptotic cell death of the milk-producing epithelial cells. Elevated nuclear PKA activity was observed from one day postlactation, paralleled by increased *c-fos*, *junB* and *junD* gene expression. Furthermore AP-1 (cFos/JunD) DNA binding activity was transiently induced. Comparison of nuclear extracts derived from normal glands and from glands which had been cleared of all epithelial cells revealed that the measured effects depend on the presence of the epithelial compartment of the mammary gland. In addition, induction of PKA and AP-1 was reversible within the first day(s) of involution, implying that their induction preceeds apoptosis in the mouse mammary gland.

Early G1-phase markers such as *c-myc*, p53 and different D-type cyclins are transcriptionally induced in the involuting mammary gland, indicating that epithelial cells enter G1-phase. ^{3}H-thymidine incorporation experiments revealed that there is no entry into S-phase at any stage of involution, suggesting that committment to apoptosis might occur in G1-phase of the cell cycle.

Implantation experiments of slow-release pellets containing different combinations of lactogenic hormones were used to analyze their role during the induction of apoptosis in the mammary gland. Implantation of pellets containing dexamethasone alone or in combination with prolactin resulted in the inhibition of both involution and apoptosis after weaning. Although apoptosis was blocked under these conditions the expression of *c-fos*, *junB* and *junD* genes and also AP-1 DNA binding activity were still induced. We conclude from these data, that increased expression of *c-fos*, *junB* and *junD* and the induction of AP-1 DNA binding activity preceed programmed cell death events in the mouse mammary gland, but are not sufficient for the induction of apoptosis.

HTLV-I TAX PREVENTS DELETION OF PERIPHERAL MATURE T CELLS BY APOPTOTIC CELL DEATH VIA FAS ANTIGEN, Shuji Kishi[1], Shinobu Saijyo[2], Yoshiko Nishimura[1], Sigeru Karasawa[3], Susumu Ueda[3], Yoichiro Iwakura[2], Shin Yonehara[4], [1]Pharmaceutical Basic Reseach Laboratories JT Inc., [2]Institute of Medical Science, University of Tokyo, [3]Nippon Institute of Biological Science, [4]Institute for Virus Research, Kyoto University

Human T cell leukemia virus type-I (HTLV-I) is a pathogenic agent of adult T cell leukemia (ATL) and has also been suggestted to be involved in several autoimmune diseases. Mice transgenic for HTLV-I pX gene have been reported to develope inflammatory arthropaty. We found here that peripheral T cells from pX transgenic mice are refractory to apoptosis mediated by Fas antigen (Fas). In normal mice, thymocytes and long-term anti-CD3 antibody (anti-CD3)-stimulated mature T cells died by apoptosis in the presense of anti-Fas antibody (anti-Fas), and pX transgene protected mature T cells but not thymocytes from anti-Fas-induced apoptosis. In addition, clonaly expanded Vβ8 positive peripheral mature T cells by in vivo infusion of Staphylococcus aureus enterotoxin B (SEB) are eliminated by in vitro anti-Fas-treatment in normal mice but not in pX transgenic mice developed chronic arthritis. Furthermore, human Jurkat T cells transfected with tax gene of pX clearly acqired the resistancy to anti-Fas. These results suggest that Tax-mediated suppressive regulation of death signal via Fas is involved in pathogenesis of HTLV-I-related diseases.

DIFFERENT SUSCEPTIBILITY TO BCL-2 MEDIATED CELL SURVIVAL IN THE MYELOID LINEAGE. Eric Lagasse and Irving L. Weissman, Department of Developmental Biology, Stanford University School of Medicine, Stanford CA 94305.

Hematopoietic cells have a finite life span and must be constantly renewed. In bone marrow, the majority of proliferating cells are myeloid cells. They age as blood neutrophils and monocytes. Neutrophils have the shortest half-life among blood leukocytes. They are rapidly programmed to die and engulfed by macrophages of the spleen and liver. This early death fate is also supposed to play a critical role in the generation of an effective inflammatory reaction. Monocytes are longer-lived cells, giving rise to tissue macrophages. However in the absence of serum, monocytes progressively lose viability via an apoptotic cell death mechanism. To investigate the importance of apoptosis in myeloid cells, we created transgenic mice expressing human bcl-2 in mature neutrophils and monocytes. bcl-2 protected neutrophils and monocytes from programmed cell death. Despite this, neutrophil homeostasis is essentially unaffected. In fact, macrophage uptake of neutrophils expressing bcl-2 still occurred, indicating that the mechanism which trigger phagocytosis of aging neutrophils represents a bcl-2 independent pathway unrelated to apoptosis. In contrast, the monocyte compartment expanded with abnormal cellular infiltrates suggesting that the bcl-2 transgene confers a survival advantage to this population. This transgenic model indicates that neutrophils and monocytes showed different susceptibility to bcl-2 mediated cell survival.

APOPTOSIS DURING B CELL DEVELOPMENT AND ACTIVATION. C. Martinez-A. A.M. Genaro, A. Gonzalez, E. Baixeras and L. Bosca, Centro Nacional de Biotecnologia, CSIC, Universidad Autonoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain.

Apoptosis or programmed cell death (PCD), is an important physiological mechanism for elimination of B and T cell populations at various stages during their development. The hallmarks morphological changes associated with apoptosis include membrane blebbing, cytoplasmic and nuclear condensation, disruption of the cytoskelton, and DNA fragmentation. For apoptosis to occur, it is accepted that an extracellular ligand is required to initiate a cascade of signaling in the cell that couples to gene regulatory changes that ultimately lead to cell death. The complex regulation of apoptosis and cell survival involves particular combination of signals and therefore, a number of extracellular stimuli, artificial inducers and inhibitors of signal transduction and metabolism, may enhance or diminish apoptosis in lymphocytes

Cell death plays a pivotal role in sorting of B lymphocytes during cell maturation. The developmental program is tightly regulated since it has to promote the generation of functional B lymphocytes but at the same time, the demise of non-functional as well as self-reactive B cells. By using either B cell lines at different stages of development, Pre-B cells and immature B cells, or mature peripheral B cells, we have characterized the role of Bcl-2 and Egr-1 in preventing the apoptosis induced under different experimental conditions. Furthermore, since B cell differentiation is also associated with differential expression of cell surface receptors, we have analyzed the role of some of those molecules in preventing apoptosis, with special emphasis in the role that CD2 might have, by signaling B lymphocytes.

EFFECTS OF ADMINISTRATION OF THE ANTI-FAS ANTIBODY IN MICE, Yoshiko Nishimura [1], Ai Ishii [2], Yoko Hirabayashi [3], Tohru Inoue [4], and Shin Yonehara [5], [1] Pharmaceutical Basic Research. Lab., JT Inc., Yokohama 236, Japan, [2] Department of Cell Biology, The Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan. [3] Department of Pathology, Yokohama City University School of Medicine 236, Japan. [4] Division of Physiology and Pathology, National Institute of Radiological Science, Chiba, Japan. [5] Institute for Virus Research, Kyoto University 606-01, Japan.

Fas antigen, which is a cell surface protein belonging to the tumor necrosis factor/nerve growth factor receptor family, mediates apoptosis. We have prepared both anti-human and anti-mouse Fas mAbs with associated apoptosis-inducing activity. And we have reported the involvement of Fas antigen in negative selection/clonal deletion of superantigen-reactive human thymocytes and mouse peripheral T cells. To analyze the function of Fas antigen *in vivo*, we studied the effects of administration of the anti-Fas antibody into mice. Ogasawara et al. reported that intraperitoneal administration of hamster anti-mouse Fas mAb into mice caused severe damage of the liver and killed the mice within 3-6 h (Nature, 364: 806, 1993). On the contrary, administration of our prepared hamster anti-mouse Fas mAb, RK-8, which shows strong apoptosis-inducing activity both *in vitro* and *in vivo*, never kill adult mice. Histrogical analyses indicated severe damage of the thymus, moderate damage of the spleen and liver, and weak damage of the heart. Flow cytometric analyses revealed that CD4, CD8-double positive thymocytes and CD4-single positive splenocytes were significantly decreased. Administration of RK-8 into new-born mice caused severe damage of the liver and thymus. Growth of the mice was inhibited and jaundice was induced. Finally, the mice died within 5-6 days. These approaches must clalify the unknown function of Fas antigen *in vivo* and more detailed analyses are now in progress.

APOPTOSIS AND NECROSIS IN A PIGLET MODEL OF CEREBRAL HYPOXIA-ISCHAEMIA: THE EFFECT OF MILD POST-INSULT HYPOTHERMIA. H Mehmet, X Yue, *MV Squier and AD Edwards. *Department of Paediatrics and Neonatal Medicine, Royal Postgraduate Medical School, London, UK and *Department of Neuropathology, Radcliffe Infirmary, Oxford, UK.*

Transient hypoxia-ischaemia to the newborn piglet brain leads to both apoptotic and necrotic cell death that is proportional to the severity of high energy phosphate depletion during the insult. This study investigated the effect of cooling the animal for 12 hours, following resuscitation, on cell death in the cingulate sulcus.

Twenty piglets, all less than 24 hours old, were anaesthetised and ventilated while continuous ^{31}P magnetic resonance spectra were collected. Fourteen piglets were subjected to bilateral carotid artery occlusion and a reduced inspired oxygen fraction until cerebral nucleotide triphosphate concentration (which reflects cerebral [ATP]) fell to less than 30% of baseline. The animals were then resuscitated: 8 animals were maintained at normothermia throughout, while in 6 subjects (the hypothermia group) rectal temperature was reduced to 35°C for 12 hours following resuscitation after which normothermia was resumed . As a control, six further animals underwent sham surgery without hypoxia-ischaemia at normal temperature. Normothermic piglets were followed for 48 h, and hypothermic animals for 68-72 h.

Following sacrifice brains were perfusion-fixed using 1% paraformaldehyde and the cingulate sulcus was examined after staining with haematoxylin and eosin by workers unaware of the experimental conditions. Cells were classified as apoptotic if they showed intense uniform nuclear basophilia, chromatin condensation or fragmentation, or the presence of apoptotic bodies. Cells were counted as necrotic if they showed cytoplasmic eosinophilia combined with dispersed chromatin.

Apoptosis and necrosis occurred in 0% and 4% of the cells respectively in control animals and increased to 14% and 8% following transient hypoxia-ischaemia under normothermic conditions. In the 12 hour hypothermia group apoptotic cell death was reduced to 1% although the number of necrotic cells was not significantly different (7%). These results suggest that moderate hypothermia after hypoxia-ischaemia ameliorate cell loss by reducing the degree of apoptotic, but not necrotic cell death. If confirmed, this would have implications for neuronal rescue therapies following cerebral hypoxia-ischaemia in newborn infants.

GRANZYME B BINDS A NUCLEAR PROTEIN OF 80kDa AND A CYTOPLASMIC PROTEIN OF 94kDa IN EL4 TARGET CELLS. Michael J. Pinkoski and R. Chris Bleackley. Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

Granzyme B (CCP1) is a cytotoxic T lymphocyte (CTL) specific serine protease that has been implicated in a number of processes leading to CTL induced apoptosis of target cells. Potential substrates of granzyme B remain elusive, but the technological breakthrough producing active enzyme by removal of an activation dipeptide has facilitated the search for granzyme B substrates.

Using enzymatically active granzyme B expressed in COS M5 cells coupled with a monoclonal antibody raised against a conserved octapeptide in the CCP family of serine proteases, we have observed granzyme B interactions with fixed EL4 target cells. *In situ* protease-linked immunohistochemical techniques with analysis by confocal microscopy of the CTL target cell line EL4 suggest that granzyme B binds to a nuclear substrate which is located surrounding, but excluded from, the nucleolus. Cytoplasmic staining exclusive of the endoplasmic reticulum is also observed. Far-western blotting data demonstrates a putative nuclear substrate of M_r = 80kDa and a putative cytoplasmic substrate of M_r = 94kDa. No binding is observed in the S-100 membrane associated fraction. Attempts to derive protein sequence from immunoprecipitated substrates have shown these two proteins to be N-terminally blocked.

(Supported by the Alberta Heritage Foundation for Medical Research, the Medical Research Council and the National Cancer Institute of Canada).

APOPTOSIS IN CEREBELLAR GRANULE CELLS IS BLOCKED BY HIGH KCl, FORSKOLIN AND IGF-1 THROUGH DISTINCT MECHANISMS OF ACTION: THE INVOLVEMENT OF INTRACELLULAR CALCIUM AND RNA-SYSNTHESIS. Gennaro Schettini, Olimpia Meucci, Antonella Scorziello, *Thomas Werge, *Pietro Calissano and *Cinzia Galli, Dipartimento di Neuroscienze Sezione di Farmacologia Universita' di Napoli Federico II, 80131 Napoli, *Instituto di Neurobiologia, Consiglio Nazionale delle Ricerche, 00137 Roma, Italy.

Cerebellar granule cells deprived of depolarizing concentration of extracellular potassium, $[K^+]_o$, undergo apoptosis. We report that this apoptotic process is associated with an immediate and permanent decrese in the levels of free intracellular calcium $[Ca^{2+}]_i$. Althought forskolin and IGF-1 are both able to prevent apoptosis, only forskolin is able to counteract the instantaneous decrease of $[Ca^{2+}]_i$. However, the early effect of forskolin on $[Ca^{2+}]_i$ is lost after longer incubation in low $[K^+]_o$. The calcium antagonist nifedipine is able to interfere with the survival effect of high $[K^+]_o$, while it does not affect forskolin and IGF-1 promoted survival, as assessed by viability and genomic DNA analysis. Accordingly, the L-type calcium channels agonist Bay K 8644 significantly enhanced the survival of low KCl treated neurons.

We determined also the time course of the rescue capacity of high $[K^+]_o$, forskolin, IGF-1 and actinomycin D, in order to characterize the signal trasduction events and the essential trascriptional step in cerebellar granule cells apoaptosis. Addition of high KCl, forskolin or IGF-1, 6 hr after the initial KCl deprivation saves 50% of cells. Remarkably, 50% of neurons loss the potential to be rescued by actinomycin D after only 1 hr in low [K+]o. Finally we show that the survival promoting activities of high $[K^+]_o$, forskolin and IGF-1 do not require RNA synthesis.We conclude that $[Ca^{2+}]_i$ is involved in the survival promoting activity exerted by high $[K^+]_o$ but not in those of forskolin and IGF-1, and that all three agents, although rescuing neurons from apoptosis through distinct mechanisms of action, do not necessitate RNA trascription.

(CNR T.P. on Aging 1994 to G.S.)

TRANSGLUTAMINASE ACTIVATION DURING IN VIVO APOPTOSIS OF MOUSE THYMOCYTES INDUCED VIA THREE DIFFERENT SIGNAL TRANSDUCTION PATHWAYS IS DUE TO A SHIFT TOWARDS THE EXPRESSION OF A HIGHER MOLECULAR WEIGHT TRANSGLUTAMINASE, Zsuzsa Szondy, Michael Boyiadzis, Zoltán Nemes, András Mádi, Edit Susán and László Fésüs, Department of Biochemistry, University Medical School of Debrecen, Debrecen, H-4012, Hungary

Apoptosis is a programmed mechanism of cell death. In thymus it functions to prevent autoimmunity and to dispose T cells expressing low affinity T cell receptors (TCRs). In thymocytes at least 3 signals (TCR activation, glucocorticosteroid administration or DNA damage) can induce cell death. Though these forms of apoptosis are anatomically indistiguishable, recent research has shown that the 3 signals act via separate signalling transduction pathways and activate a different set of genes. Previous work in our department has suggested that in some forms of apoptosis tissue transglutaminase may be activated. In the present study it was investigated in both an *in vitro* (thymocyte culture) and *in vivo* (dexamethasone, antiCD3s ab treated or irradiated mice) system whether transglutaminase activation is related to any form of thymocyte cell death. In the *in vitro* system we could not detect any change in the transglutaminase activity though the concentration of its crosslinked product (Glu-Lys) increased with time. Most of the transglutaminase was present in these cells in a 64 KD protein form. In each *in vivo* system transglutaminase activity significantly increased after apoptosis induction with a concomittant disappearance of the 64 kD and appearance of a 78 kD transglutaminase protein. The expression of the 78 kD protein was localised in the cells of the cortical layer of the thymus, which contains the bcl-2 negative, immature thymocytes. Concomittantly with the involution of the thymus the Glu-Lys content within the blood significantly increased. It is suggested that transglutaminase activation is part of all the 3 forms of apoptosis in thymocytes. However, the complete program is initiated only within the thymic environment. The activation of the enzyme is related to a regulated shift towards the expression of a higher molecular weight protein.

EFFECTS OF ACTIVATION ON THE REGULATION OF APOPTOSIS IN LYMPH NODE T CELLS, Elma Z. Tchilian, Graham Anderson, Nel C. Moore, John J. Owen and Eric J. Jenkinson, Center for Clinical Research in Immunology and Signalling, Medical School, University of Birmingham, Birmingham B15 2TT, UK

In order to study activation induced apoptosis in mature T cells we have developed an *in vitro* system, in which SEB activated murine lymph node T cells undergo apoptosis following a rechallenge with SEB. We show that SEB can induce cell death, following the second stimulation, only in the presence of MHC class II transfected L cells, but not when is presented in a soluble form. We also show that thymic epithelium, which lacks B7 molecule is as efficient as MHC class II transfected L cells in mediating apoptosis, suggesting that CD28/B7 interactions are not required for this process. This model has been further used to study how the sensitivity to apoptosis is regulated in the resting and activated T cells. The difference in gene expression of various cytokines, bcl-2, fas and myc in the cells following the SEB induced activation (first challenge) and SEB induced apoptosis (second rechallenge) will be discussed. In addition we will evaluate whether CD4 crosslinking induces T cell elimination in this system since previous reports suggest that prior engagement of CD4 predisposes towards programmed cell death.

APOPTOSIS DURING REMODELING OF ENDOTOXIN-INDUCED TYPE II CELL HYPERPLASIA IN RAT LUNG, Tesfaigzi, J., Johnson, N. F., Lechner J. F. Inhalation Toxicology Research Institute, Albuquerque, NM 87185

The purpose of this study was to delineate mechanisms involved in tissue remodeling after endotoxin-induced Type II cell hyperplasia. F344/N rats were intratracheally instilled with endotoxin and sacrificed every 24 h for up to 8 d. Endotoxin instillation caused transient proliferation of Type II cells. An influx of polymorphonuclear cells, which returned to background levels 2 d post-instillation was also observed. The number of macrophages in the bronchoalveolar lavage fluid increased 24 h post-instillation and stayed elevated during the 8 d. Rats were injected 2 d after the endotoxin instillation with BrdU to trace the fate of the proliferating Type II cells during the remodeling process. Hypertrophy of Type II cells was located primarily in alveoli close to conducting airways, and was evident 2 d after endotoxin instillation. Type II cell hyperplasia was observed and nuclear BrdU incorporation was maximum 3 and 4 days after instillation. The numbers of hyperplastic Type II cells decreased dramatically in rats sacrificed 5 d post-instillation. At this time cells with BrdU-labeled nuclei showed a flattened morphology which suggested a transition to Type I cells. However 8 d post-instillation, very few nuclei with BrdU stain could be found. Since it has not been reported that dead Type I and Type II cells are shed and cleared through the conducting airways, it is likely that the hyperplastic Type II cells undergo a controlled cell death. Preliminary experiments with lung sections from rats 4 d after endotoxin instillation indicated an increased number of cells immunostaining for transglutaminase and showing incorporation of biotinylated ATP into the nuclei by terminal deoxynucleotidyl transferase, an assay for detecting fragmented DNA. These results suggest that lung tissue after endotoxin-induced Type II cell hyperplasia is remodeled within 24 h, and 3 d after hyperplasia the newly formed hyperplastic cells are removed by apoptosis-like mechanism.

(Supported by OHER, DOE DE-AC04-76EV01013).

ANALYSIS OF TRANSGENIC MICE OVER-EXPRESSING FAS ANTIGEN ON T-LYMPHOCYTES UNDER THE CONTROL OF *LCK* PROXIMAL PROMOTER, Yoshiki Yamasaki [1], Yoshiko Nishimura [1], Yoichiro Iwakura [2] and Shin Yonehara [3], [1] Pharmaceutical Basic Research Laboratories, JT Inc. Yokohama 236, Japan, [2] Institute for Medical Science, Tokyo University, Japan. [3] Institute for Virus Research, Kyoto University, Japan.

To assess the role of apoptosis mediated by Fas antigen in the development of T-lymphocytes, we generated two lines of transgenic mice which carry a mouse Fas cDNA under the control of *lck* proximal promoter as transgene. Flow cytometric analysis revealed that both line over-expressed transgenic Fas antigen only on T cells in thymus and peripheral tissues. One line (hi-Tg) expressed Fas antigen in both thymus and spleen with higher level than the other line (low-Tg). Hi-Tg mice had significantly decreased numbers of double-positive and CD4 single positive cells in thymus. In hi-Tg mice, however, the numbers of double-negative and CD8 single positive thymocytes were same as those of normal mice. In spleen of hi-Tg mice, the number of mature CD4 positive T cells specifically decreased, although the number of CD8 positive T cells were same as that of normal mice. These results indicate that Fas antigen may play an important role in T-lymphocyte development in thymus, especially at the differentiation stage from double-positive to CD4 single positive T cells. Interestingly, the transgenic thymocytes from both hi- and low-Tg mice underwent very rapid apoptosis during *in vitro* culture without any additional stimulation. The reason for this rapid apoptosis *in vitro* , however, is not known yet. On the contrary, thymocytes of hi- and low-Tg mice did not undergo rapid apoptosis *in vivo* . Thus, thymic environments may possess some mechanisms which can protect thymocytes from apoptosis. This mechanism may regulate the function of Fas antigen on thymic development described above.

IN SITU DEMONSTRATION OF T-CELL ELIMINATION BY APOPTOSIS IN DIFFERENT MODELS OF EXPERIMENTAL AUTOIMMUNE NEURITIS, Uwe K. Zettl, Ralf Gold, Graham K. Harvey, Eilhard Mix*, Hans-Peter Hartung, Klaus V. Toyka, Departments of Neurology, Julius-Maximilians-Universität Würzburg and *Universität Rostock, Germany

Experimental autoimmune neuritis (EAN) in Lewis rats serves as a model of the human disease Guillain-Barré-Syndrome. An important point in understanding the pathogenesis of this T-cell mediated neuropathy is the elucidation of how and where T-cells are eliminated. We investigated this issue in Lewis rat EAN induced by immunization with peripheral myelin (active EAN), adoptive transfer of P_2-specific T-cells (AT-EAN), or ovalbumin specific T-cells in rats intraneurally injected with ovalbumin (OVA-EAN).

T-cell infiltrates in sciatic nerve were detected by immunohistochemistry. Apoptosis - a physiological mode of the cell death - was assessed using morphological criteria and was confirmed by molecular labeling techniques (in situ translation, in situ tailing).

Clinical disease peaked at day 17 in active EAN, at day 7 in AT-EAN and at day 4 in OVA-EAN. In all EAN models studied, apoptotic T-cells were already found in sciatic nerves at the onset of disease and plateaued thereafter: in active EAN 19% apoptotic T-cells were detected on day 35, in AT-EAN 17% on day 14 and in OVA-EAN 25% on day 7. Apoptotic cell death of T-cells was observed throughout the subsequent recovery periods.

These data obtained on different models of EAN show that apoptosis is a general mechanism of elimination for infiltrating T-cells in the peripheral nervous system. Identification of the molecular signals inducing apoptosis may have therapeutic implications.

INDUCTION OF APOPTOSIS BY TRANSIENTLY TRANSFECTED WILD TYPE p53 IN TRANSFORMED CELL LINES: STRUCTURE-FUNCTION ANALYSIS. Elisheva Yonish-Rouach[1], Zohar Mishal[2], Janine Bordé[1], Tatiana Zaitchouk[1], Valérie Deguin[1], John Jenkins[3] and Evelyne May[1]. Laboratoire d'Oncologie Moléculaire[1] and Laboratoire de Cytométrie[2], IRSC, CNRS, 7 rue Guy-Môquet, 94801 Villejuif Cedex, France; [3]Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 OTL, UK.

The p53 tumor suppressor gene is a frequent target of genetic alterations in human cancer. The protein was shown to function as a transcrition factor: it can up-regulate certain genes by interacting with specific DNA elements, or down-regulate some other genes by other mechanisms. The levels of p53 protein increase in response to DNA damage, resulting in growth arrest in G1, presumabely 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 only the population expressing wt, but not mutant, p53 had a significant amount of cell debris. In the present study we use this model system to analyse 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 these proteins to induce apoptosis was analyzed. All the functional domains were found to be necessary for the induction of apoptosis. We therefore conclude that p53 acts, at least in this cell system, as a transcription factor in the induction of apoptosis.