Fifth Annual Pezcoller Symposium: Apoptosis

In 1972 Kerr, Wyllie and Currie coined the term “apoptosis” to describe a set of cellular phenomena (surface membrane blebbing, contraction in cell size, chromatin condensation, and DNA digestion) associated with physiological death in insect and animal cells. The outcome of the apoptotic process is engulfment by phagocytic cells. The past 20 years has seen an increased interest in cell death as an important mediator of a number of developmental processes, and in the past few years, a role for apoptosis in cancer biology and a potential role of various “oncogenes” in abnormal cell growth phenomena have brought into focus the realization that cell death is an important component of the behavior of all cells. The fifth annual Pezcoller Symposium addressed various genetic and cellular aspects of cell death with an emphasis on cancer biology and immunology.

The first session dealt with genetics of apoptosis as studied in Caenorhabditis elegans and with aspects of the biology of genetically determined retinal degeneration. Robert Horvitz described the known genetic determinants of (programmed) death of specific cells in C. elegans. A number of genes have been identified and can be classified into different categories. In category 1, genes involved in cell death, ced-3 and ced-4 are required for cell death in all cells undergoing apoptotic (programmed cell) death and are presumably expressed in the cells undergoing apoptosis. ced-9 controls cell death, but in contrast to ced-3 and ced-4, it functions to prevent cell death. The DNA sequence of the ced-9 gene indicates that it encodes a protein with properties similar to the mammalian bcl-2 gene, a gene that is involved in cell survival as discussed by other presenters at this symposium (see the remarks of Hockenberg and White, below). In category 2, genes involved in phagocytosis, seven identified genes are involved in engulfment of dying-dead cells but are not involved in cell death per se. However, a major defect in engulfment occurs only when there is a mutation in ced-1, ced-6, ced-7, or ced-8 and another mutation in ced-2, ced-5, or ced-10. Horvitz suggests that there are two, redundant, pathways involved in engulfment by neighboring cells: In category 3, a nuclease gene, a nuc-1 gene controls DNA nuclease activity. Interestingly, mutations in this gene block DNA degradation but not cell death, suggesting that this nuclease is not essential for cell death. Category 4 contains genes affecting death of specific cell types. Whereas the above genes affect all programmed cell deaths, other genes have been identified that affect death of highly specific cell types. ces-1 and ces-2 appear to act in a negative fashion to prevent the death of two specific cells in the pharynx. egl-1 similarly affects the life/death decisions of specific motor neurons. Studies are under way to clone and analyze a number of these genes.

D. Papermaster discussed apoptosis within the context of genetic defects resulting in progressive retinal degeneration as a model for cell death in the nervous system. Two rodent models of degeneration of retinal rod cells were studied. Retinal degeneration occurs in the rd8 mouse following birth. The genetic defect results in an altered form of peripherin, the consequence of which is the insertion of rhodopsin randomly into membranes, rather than insertion into membrane stacks, thereby imparting abnormal morphology to rod cells. Progressively, these cells die by an apoptotic mechanism (chromatin condensation and DNA digestion) and are engulfed by adjacent cells. It remains to be determined why such an altered structure results in cell death. In the RCS rat, whose genetic defect is unknown, the overlying retinal epithelium is incapable of phagocytosing the released outer rod segments, and such segments accumulate extracellularly. Progressively, rod cells undergo cell death, with classical microscopic indications of apoptosis. Papermaster described work from other laboratories, most predominantly that of LaVail, which indicate that retinal degeneration may be reduced by administration of various growth factors, i.e., survival factors. The theme of opposing death and survival genes (ces-3, ces-4 versus ces-9 in C. elegans) or opposing death/survival signals (factors) may be a common theme in determining the fate of cells.

The second session started a series of presentations with the impact of certain oncogenes on aspects of cell death or survival. M. Oren described his studies on the interrelationships between the tumor suppressor gene, p53, and cytokines on apoptosis in myeloid leukemic cell lines. Mouse leukemic M1 cells containing a ts p53 (normal function at 32°C) grow normally at 37°C (where p53 function is defective) but when down-shifted to 32°C arrest predominantly in G1 and undergo rapid apoptosis. When M1 cells are grown in IL-6 at 32°C, cells undergo complete arrest in a cell cycle position more analogous to G0 (small size and low RNA content), and such cells are not subject to apoptosis. This finding suggests that arrest of cells in different cell cycle compartments may affect the signaling pathways that result in apoptosis and are consistent with the concept that an apoptotic program occurs when signals involved in cell cycle progression are conflicting. Oren also described studies in mouse myeloid leukemia cell lines wt for p53 (32D and DA-1) the survival of which is dependent on the continued presence of IL-3. Introduction of vectors expressing antisense p53 mRNA or a dominant-acting mutant p53 protein convert 32D cells to a state where cell survival is not dependent on IL-3. Oren suggested that wt p53 expression results in the need for continual presence of a “survival” factor to prevent apoptosis.

E. White described studies on the role of the oncogenic adenovirus genes, E1A and E1B, on apoptosis and transformation in human cells. E1A gene products are responsible for initiation of a proliferative response but do not transform cells. In cells containing a wt p53, E1A induces p53 accumulation and rapid apoptosis. The addition of E1B prevents apoptosis and allows for generation of the transformed phenotype.

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1 This symposium was held June 9–11, 1993, in Trento, Italy, and was the fifth in a series of annual symposia sponsored by the Pezcoller Foundation. This Foundation was established from the estate of Professor Alessio Pezcoller who spent his life as a surgeon in the Trento Community. This year’s symposium was cochaired by Enrico Mihich (Roswell Park Cancer Institute, Buffalo, NY) and Robert T. Schimke (Stanford University, Stanford, CA). The Program Committee consisted of the co-chairs, Dr. J. M. Bishop (University of California at San Francisco, San Francisco, CA), Dr. A. Levine (Princeton University, Princeton, NJ) and Dr. D. Livingston (Dana Farber Institute, Boston, MA).

Invited participants included: P. Amati, Università di Roma La Sapienza, Rome, Italy; J. Ashwell (NIH, Bethesda, MD); G. Bellomo (University of Pavia, Pavia, Italy); J. Cleveland (St. Jude’s Children’s Research Hospital, Memphis, TN); D. Delia (National Cancer Institute, Milan, Italy); M. D’Incalci (Istituto Mario Negri, Milan, Italy); A. Eastman (Dartmouth, Hanover, NH); G. Evan (ICRF, London, United Kingdom); M. Fried (ICRF, London, United Kingdom); D. Green (La Jolla Institute, La Jolla, CA); T. Goodrich (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); D. Hockenberg (Fred Hutchinson Cancer Research Center, Seattle, WA); R. Horvitz (MIT, Cambridge, MA); J. Isaacs (Johns Hopkins, Baltimore, MD); M. Kastan (Johns Hopkins, Baltimore, MD); P. Kramer (German Cancer Research Center, Heidelberg, Germany); P. Nicotera (Karolinska Institute, Stockholm, Sweden); M. Oren (Weizmann Institute, Rehovot, Israel); D. Papermaster (University of Texas, San Antonio, TX); C. Riccardi (Università degli Studi di Perugia, Perugia, Italy); S. Schlossman (Dana Farber Institute, Boston, MA); S. Sherwood (Stanford University, Stanford, CA); T. Taniguchi (Osaka University, Osaka, Japan); E. White (Rutgers, Piscataway, NJ).

The proceedings of this Symposium will be published in their entirety, including discussions, in the Spring of 1994 by Plenum Publishing Corp., New York [Dr. Mihich and Schimke (eds.)].

The abbreviations used are: IL, interleukin; wt, wild type; IRF, interferon-responsive transcription factor; TCR, T-cell receptor; ts, temperature sensitive.

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notype. EIB codes for two proteins: (a) a M, 55,000 protein which binds p53 and suppresses apoptosis, presumably by inactivation of wt p53; (b) a M, 19,000 protein which also suppresses apoptosis and which bears sequence homology with the human bcl-2 gene. The bcl-2 gene can substitute for EIB in preventing apoptosis and generating cell transformation. A basic conclusion from these studies is that altered function of p53, resulting in a lack of apoptosis upon a stimulus for proliferation, is a common theme in transformation of cells.

G. Evan described his studies on the effect of aberrant c-myc expression in enhancing apoptosis. In condescending human and fibroblasts cells deprived of growth factors (serum), c-myc is absent, and upon mitogenic stimulation, mRNA (and protein) levels increase transiently (3 h) and then are maintained at low levels as cells subsequently progress through a cell cycle. When c-myc is placed under high, constitutive expression, rodent fibroblasts progress into S phase at serum levels that would arrest cells where c-myc is under normal control. Under such conditions, the cells undergo apoptosis. Evan showed that the region of the c-myc protein required for allowing cell cycle progression in low serum was also that required for activating apoptosis.

Thus, c-myc appears to promote two conflicting genetic programs, i.e., growth and cell death. Evan proposes two possible mechanisms for initiating an apoptotic program: (a) c-myc expression propels cells into a cell cycle, but in the absence of additional (serum) growth factors, the “signals” for cell cycle completion are “conflicting,” thereby evoking a cell death, i.e., the “conflict model.” (b) Alternatively, c-myc propels a cell into a cell cycle and initiates two possible outcomes, i.e., cell cycle progression and production of viable daughter cells, or apoptosis. By this latter model, successful transit through a cell cycle is dependent on secondary “survival” factors not provided in low serum (dual signal model). Evan favors the latter model, based on his findings that certain growth factors (insulin-like growth factor 1, epidermal growth factor 2, insulin, and platelet-derived growth factor AB), but not others (platelet-derived growth factor, basic fibroblastic growth factor, and bombesin) have no effect on preventing apoptosis. He suggests that such “survival” factors function by a canceling out of the apoptotic “program” which is initiated as a part of commitment to a cell cycle.

J. Cleveland described studies on the role of c-myc expression in the IL-3 growth-survival-dependent mouse myelogenous leukemia cell line D32. Expression of endogenous c-myc is an immediate early response to IL-3, and its transcription is down-regulated upon withdrawal of IL-3, the consequence of which is accumulation of cells in G1. Such cells eventually undergo apoptosis. When myc is expressed constitutively in D32 cells following IL-3 withdrawal, cells continue to enter S and undergo extremely rapid apoptosis. Additionally, constitutive expression of c-myc results in rapid apoptosis even in the presence of either IL-3 or erythropoietin, both of which suppress IL-3 withdrawal-mediated apoptosis in cells displaying normal (down-regulated) c-myc expression. Cleveland also described studies of the effect of activated oncogene raf on apoptosis in D32 cells overexpressing c-myc, raf is a cytosolic serine/threonine protein kinase responsible to growth factors, including IL-3 and erythropoietin. Introduction of v-raf (the activated form of raf), but not c-raf resulted in accumulation of D32 cells in G1 following IL-3 withdrawal with a delayed onset of apoptosis. Simultaneous overexpression of c-myc and v-raf results in tumorigenic alterations. Thus v-raf would appear to constitute a function to delay apoptosis, as do altered states of p53 and bel-2 expression, and participate in transformation/tumorigenesis by reducing cell death resulting from overactive proliferative signals, which, if unopposed, result in apoptosis.

M. Kastan summarized studies on the role of p53 in the cellular response to radiation exposure. In mammalian cells low to moderate radiation exposure results in both G1 and G2 arrest (delay). The G1 arrest is dependent on expression of the wt p53 gene. Human cell lines with mutant forms of p53 progress into S phase without arrest when irradiated, and introduction of wt p53 restores the G1 arrest property. Radiation induces expression of p53 and the G1 arrest requires protein synthesis. In contrast, the G2 radiation arrest does not require protein synthesis. Kastan described studies indicating that p53 expression results in transcriptional activation of a growth arrest and DNA damage-inducible gene, GADD45, thus suggesting a possible transcription cascade in which p53 participates as a transcription factor. Interestingly, cells that overproduce the mtk2 oncogene product, a protein that binds p53, are defective in G1 radiation arrest and in GADD45 induction by radiation. The lack of a radiation-induced arrest occurs in the human genetic disease ataxia-telangiectasia. Kastan indicated that ataxia-telangiectasia complementation groups A and C are defective in the induction of p53 by radiation, suggesting that this genetic defect involves events prior to p53 induction. Radiation sensitivity of cells with wt or mutant p53 are similar, indicating that p53 is not involved in the radiation-induced G1 arrest properties and suggesting that the predominant arrest state involved in radiation survival is a G2 arrest.

J. Isaacs reviewed his studies on apoptosis in the rat prostate and the potential for increasing rates of cell death as a means of facilitating treatment of prostate cancer. In rat prostate 1.7% of glandular cells die (apoptosis) daily and a comparable number are generated. Upon castration, androgen levels are reduced to virtually undetectable levels in 24 h; apoptosis commences several days later; and 7–10 days post-castration, 80% of glandular cells are eliminated by engulfment by macrophages or adjacent epithelial cells. During this time period, there are changes in expression of a number of genes as well as increases in intracellular calcium. Androgen withdrawal-induced apoptosis does not require cells to progress into S phase (DNA content by flow cytometry) and Isaacs concludes that the cells are dying from a resting cell cycle position. Such a conclusion may suggest that an androgen-dependent “survival” factor is required to maintain viability of resting (G0) cells. Following castration bromoexoyuridine incorporation increases in cells (up to 10-fold over control) by 4 days. Although Isaacs interprets the finding to represent “unscheduled DNA synthesis/repair,” an alternative possibility is that androgen withdrawal results in cell cycle movement to a G1-S position (not detected as cells in S phase) from which they cannot progress through a productive cell cycle, resulting in apoptosis. Interestingly, castration is associated with elevated c-myc expression, the aberrant expression of which in other cell types (see the previous presentation of Evan) leads to apoptosis. Isaacs also described preliminary exploration of the use of thapsigargin, a plant sesquiterpene lactone that inhibits a Ca2+-ATPase and causes progressive intracellular accumulation of free Ca2+ to facilitate apoptosis and serve as an adjuvant chemotherapeutic agent in prostate.

The immune system has been a major focus of studies on apoptosis for several reasons: (a) the bel-2 gene was discovered as the breakpoint of a chromosomal translocation in follicular B-cell lymphomas; and (b) cell death plays a critical role in the negative selection against the existence of B- and T-cells capable of reacting with self-antigens. Thus the Symposium featured a number of presentations dealing with the immune system.

D. Hockenbery reviewed aspects of the biological properties of bel-2 expression. bel-2 is normally expressed in a limited number of B-cells in germinal centers of lymph nodes and in the medullary thymocytes of the thymus (T-cells), suggesting possible roles for bel-2 expression in B- and T-cell selection. bel-2 is also expressed in intestinal crypt cells (mitotic cells). More definitive results indicating the effects of bel-2 expression come from studies with transgenic mice.
constitutively expressing bel-2. In mice expressing bel-2 in B-cells, B-lymphocyte hyperplasia occurs, suggesting that bel-2 circumvents death at multiple stages of B-cell differentiation. Immunological tolerance (peristence of memory B-cells) as studied by B-cell transfer into naive mice is prolonged in cells expressing bel-2. Although bel-2 expression prevents the rapid thymocyte apoptosis classically induced by glucocorticoids or γ-radiation (thymic involution), there is no compelling evidence that bel-2 expression is involved in the negative selection of T-cells during development. Expression of the bel-2 gene, either as studied in transgenic mice or as studied in cell lines transformed with a bel-2 expression vector, renders cells resistant to any number of killing agents, including growth factors, heat shock, glucocorticoids, γ-radiation, azide, tumor necrosis factor, and various cancer chemotherapeutic agents with differing mechanisms of action. It remains to be determined how this protein modifies or delays the events of apoptosis.

J. Ashwell discussed aspects of immune cell selection and the role of apoptosis in "negative" selection of T-cells during thymic development. Cells expressing TCRs of low to moderate "self-avidity" do not die, thus resulting in the destruction of T-cells that can react strongly with self antigens or no antigen. In addition to TCR occupancy, glucocorticoids are potent inducers of apoptosis in immature T-cells, but not in mature, peripheral T-cells. Simultaneous TCR occupancy and glucocorticoid prevent cell killing in vitro; i.e., the two treatments are mutually exclusive. Ashwell proposes that glucocorticoids play a role in thymic (central) T-cell-negative selection. He presented data consistent with glucocorticoid synthesis in certain thymic epithelial cells during early development. When fetal thymic organ cultures were treated with agents that inhibit the glucocorticoid-biosynthetic pathway, T-cell killing was reduced significantly. These findings are consistent with a role for locally generated glucocorticoid in negative selection for T-cells. Retinoic acid, and more specifically its 9-cis isomer, is a potent inhibitor of TCR-mediated cell killing as studied in vitro. Ashwell reported that in vitamin A-deficient mice there was a significant decrease in CD4+ /CD8+ cells in the thymic population, suggesting that vitamin A may play a physiological role in T-cell selection. The links between TCR occupancy and presence of glucocorticoids and retinoic acid isomers and apoptotic events remains to be elucidated.

D. Green described studies on the role of c-myc and fas/apol genes on negative selection that occurs in the thymus (central selection) as well as that which occurs in more mature circulating T-cells (peripheral selection). In thymoma cell lines, TCR activation-induced apoptosis requires c-myc expression, independent of effects on cell cycle position, whereas transforming growth factor B down-regulates c-myc expression and prevents this apoptosis. Green notes that in other cell types transforming growth factor-β actually induces apoptosis, thus indicating that effects of various growth factors/effectors on apoptosis may depend on cell type-specific signal transduction pathways. Green also described studies using T-cells obtained from wild type mice and those homozygous recessive for the fas/apol gene, a gene implicated in negative selection. T-cells recessive for fas/apol undergo activation-induced apoptosis in a fashion similar to that of normal cells, and Green suggests that the fas/apol gene is not involved in central (thymic) negative selection. He presented results with circulating specific T-cells consistent with the conclusion that fas/apol is involved with peripheral T-cell negative selection. Thus, although the killing event appears to be apoptosis in both central and peripheral deletion phenomena, the signaling events differ, depending on the differentiation state of the T-cells.

P. Krammer described studies from his laboratory on the isolation and characterization of apol as detected as an antibody reacting with surface membranes of a human lymphoblast cell line, which, upon binding to cells, resulted in apoptosis. This gene is the same as that isolated by Itoh et al. and called fas. In the mouse the gene maps to chromosome 19 at the position of the lpr locus, a defect associated with accumulation of aberrant T-cells in peripheral sites (lymph nodes) and by autoimmunity. apol, as studied by immunochemoical methods, is present in various epithelial cells, including immature layers of squamous epithelia in the biliary tract and intestine. In the immune system, apol is expressed among histicioid cells but is restricted to minor subpopulations of peripheral T- and B-cells. The apo-1/fas gene is a member of the nerve growth factor receptor superfamily, suggesting that the biological function of this gene is mediated by a growth factor/cytokine-induced death phenomenon.

T. Taniguchi described his studies on the interactive relationships of the interferon-responsive transcription factors IRF-1 and IRF-2 on cell growth. These factors are transcriptional regulators for certain interferon-responsive genes involved in cytokine-induced cellular responses. IRF-1 is induced as an early response to interferon, followed by induction of IRF-2. The IRF-1 protein is highly unstable, relative to IRF-2, and thus the IRF-1/IRF-2 ratio may be critical in cell responses. In serum-starved mouse NIH 3T3 cells, IRF-1 is induced during growth arrest and declines following addition of serum, again increasing as cells progress towards S. In contrast IRF-2 expression is constant through the cell cycle. Cells overexpressing IRF-2 (transfection-mediated overexpression) grew to a higher density and grew in methylcellulose gels. Additionally cell populations expressing high IRF-2 levels had enhanced tumorigenicity. This transformation phenotype of high IRF-2 expression is reversed by overexpression of IRF-1. These studies indicate that the ratio between transcriptional factors expression is important rather than absolute levels and that IRF-1 can constitute a tumor suppressor gene. Interestingly the IRF-1 gene in humans maps to 5q31, a site which is deleted in cases of myelodysplasia and certain leukemias.

P. Nicotera provided a review of the role of calcium in apoptosis. That increases in free, intracellular Ca 2§ can be correlated with apoptosis has been well documented in the literature. He reviewed the role of Ca2 § in the context of two possible roles in generating DNA degradation: (a) activation of endonuclease(s). In many but not all cell types, a Ca2 §-dependent endonuclease can be identified, but there are other endonucleases that could equally well participate in the end stage formation of apoptotic DNA ladders. More recent studies have indicated that DNA is first degraded into fragments of approximately 50 kilobases, and it remains to be determined what types of nucleases may be involved in this first type of cleavage.

P. Nicotera raised interesting questions concerning a presumed prior event in progression to overt apoptotic bodies, i.e., the rapid and abnormal nuclear condensation that precedes the appearance of apoptotic bodies, and suggests that Ca2 § may play a role in alteration in chromatin structure that allows DNA to be subject to endonucleolytic attack. What events in nuclear structure (DNA-protein interactions) are involved and how such structures are altered are major unknowns in understanding the biochemical basis of apoptosis. High levels of polyamines can stabilize chromatin in a compacted state and protect cells from apoptosis as measured by DNA ladder formation. A question that has not been addressed adequately, given the fact that the initial events of apoptosis involve cell surface activation and membrane blebbing, is whether a cell is actually "dead" prior to any effects on chromatin structure (condensation or degradation). Thus, although nuclear events are dramatic components of apoptosis and readily identified, are they secondary events occurring in dying cells?

A. Eastman reviewed the role of endonucleases in apoptosis. There are a number of nucleases present in cells, including a Ca2 §/Mg2 §-dependent endonuclease, a Mg2 §-dependent nuclease, as well as DNases I and II. He concludes that there is no compelling evidence that...
any of the identified nucleases are necessarily required for the DNA digestion that occurs in apoptosis. Eastman purified the major DNase from Chinese hamster ovary cells undergoing apoptosis and found that it is independent of divalent cations but is active at low pH values, *i.e.*, a DNase II. Using flow cytometry he provided data indicating that onset of apoptosis in HL-60 cells resulting from etoposide treatment and in IL-2-dependent T-lymphocyte (CTLL-2) cell lines is associated with a reduction in intracellular pH. This finding suggests to Eastman that a prior event resulting in progression to apoptosis involves alterations by protein kinase activation of the activity of Na⁺/H⁺ transporters, resulting in reduction in cellular pH and a subsequent activation of DNase II. According to this model, growth factors, *p53*, *c-myc*, etc. impinge on apoptosis by virtue of complex interrelationships in controlling intracellular pH. Eastman also described cell cycle perturbation events that occur in cells following cisplatin treatment. Following exposure of cells to moderate concentrations of cisplatin, they arrest/delay in G₂ and subsequently progress through aberrant mitosis with aberrant chromosome segregation as detected by flow cytometry. Such cells undergo apoptosis in the next G₁, presumably because they lack necessary gene expression components to allow for subsequent cell viability.

S. Sherwood described studies with HeLa cells on the induction of apoptosis within the context of cell cycle progression, using Colcemid as an agent that arrests cells in metaphase and aphidicolin, an inhibitor of DNA synthesis. HeLaS3 cells undergo apoptosis when arrested in metaphase, but only after approximately 18 h (one cell cycle time) of arrest. If Colcemid is removed prior to the onset of apoptosis or if concentrations of Colcemid are used which result in defective assembly of the mitotic apparatus, cells undergo multipolar mitoses with the generation of microcells (cells with less than G₁ DNA content by flow cytometry). Such cells are viable but continue to reside in a “pseudo-G₁” state and subsequently undergo apoptosis approximately 18 h after microcell generation. HeLa cells treated with concentrations of aphidicolin that completely block DNA synthesis undergo apoptosis from a G₁-S position, but again, only after some 30-36 h of arrest. When lower concentrations of aphidicolin are used (partial inhibition of DNA synthesis), the time from initiation of treatment to apoptosis is delayed, suggesting that cells can “sense” the rate of progression through S phase. At even lower aphidicolin concentrations, cells can progress through mitosis, but such mitoses are multipolar and generate nonviable cells, which, like the microcells generated by Colcemid, commence apoptosis only some 18 h after their birth. Sherwood suggests that an “apoptotic clock” starts at the G₁ commitment point and is cancelled out when cells accomplish transit through mitosis, irrespective of whether it is normal or aberrant with respect to chromosome segregation.

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