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Apoptosis and Aging: Role of the Mitochondria

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Apoptosis research is a rapidly developing area, but the role of apoptosis is still unclear and controversial. For example, several studies document a significant loss of cardiac and skeletal myocytes during normal aging, possibly by apoptotic mechanisms. This loss in cells may be directly mediated by mitochondrial dysfunction caused by chronic exposure to oxidants and increased activation of mitochondrial permeability transition pores. This review will discuss apoptosis in the context of normal aging of T cells, cardiac myocytes, skeletal muscle, and brain cortex. Particular attention is paid to the role of the mitochondria, because they have been implicated as a major control center regulating apoptosis. Mitochondrial oxidative stress and a decline in mitochondrial energy production in vitro often leads to activation of apoptotic pathways, but whether this occurs in vivo is unclear.

IN 1951, Gluckmann first described the apoptotic process. In 1972, Wyllie, Kerr, and Currie coined the term *apoptosis* to describe a form of cell death with morphological characteristics that are distinct from necrosis (1). Necrosis is a passive form of cell death that results from acute cellular injury, which causes cells to swell and lyse. In contrast, apoptosis is an active process in which cells die by design and apoptotic bodies are removed without inflammation (Figure 1). These findings were largely ignored in the early 1980s, but since 1987 the number of papers in this field has been growing rapidly. Researchers became interested in apoptosis after it was demonstrated in the nematode *Caenorhabditis elegans*, followed by the identification of homologous death genes in other organisms (2). For example, CED-9, CED-4, and CED-3 are homologous to the mammalian gene products for Bcl-2, Apaf-1, and caspase-9, respectively (1,3,4).

Apoptosis can be divided into three nondistinct phases: an induction phase, an effector phase, and a degradation phase. The induction phase depends on death-inducing signals to stimulate proapoptotic signal transduction cascades. Some of these death-inducing signals include reactive oxygen and nitrogen intermediates, TNF-α, ceramide, overactivation of Ca^{2+} pathways, and Bcl-2 family proteins such as Bax and Bad (5–8). In phase two, the effector phase, the cell becomes committed to die by the action of a key regulator, that is, death domain activation on the cell surface, nuclear activators (such as p53), endoplasmic reticulum pathways, or activation of mitochondrial-induced pathways (release of cytochrome c or apoptosis-inducing factors; Figure 2). The degradation phase involves both cytoplasmic and nuclear events. In the cytoplasm, a complex cascade of protein-cleaving enzymes called *caspases* (cysteine proteases) becomes activated. In the nucleus, the nuclear envelope breaks down; endonucleases are activated, causing DNA fragmentation; and the chromatin condenses. Finally, the cell is fragmented into apoptotic bodies and phagocytosed by surrounding cells or macrophages (1,9).

One classic case of the role of apoptosis in development is the elimination of tissues, transitory organs, and phylogenetic vestiges. For example, the pronephros and mesonephros are eliminated by apoptosis in higher vertebrates. Anuran tails and gills undergo apoptosis as tadpoles change into frogs. Moreover, the roundworm *C. elegans* eliminates exactly 131 of its initial 1090 cells as it changes into its adult form (10). Another classic example of programmed cell death is tissue remodeling. As vertebrate limb buds develop, for example, in chick, duck, and humans, webbing between digits in the hind limbs is removed by apoptosis. This indicates that the ectoderm sends signals to initiate programmed cell death (10). In these cases, aging is under strict genetic control and therefore is truly *programmed* cell death. However, senescent aging—such as found in recent studies—may largely be due to "wear and tear" mechanisms. But it is also possible that a programmed mechanism becomes activated with aging that triggers cell loss; future investigations will have to distinguish between these two possibilities.

Apoptosis and Mitochondrial Control

Recent evidence has implicated mitochondria as accelerating and contributing to many forms of apoptosis. (However, other forms of apoptosis exist, e.g., cell death brought about through death domain activation on the cell surface.) Mitochondria were not originally assumed to be participants in the effector phase, but recent evidence shows that in some forms of apoptosis, mitochondria undergo major functional and structural changes that regulate and accelerate cell death. First, the mitochondrial inner transmembrane potential ($\Delta\Psi_m$) collapses prior to classical morphological signs of apoptosis. Second, studies using cell-free systems and isolated mitochondria suggest that specific mitochondrial proteins are released (cytochrome c and apoptosis-inducing factor) for the activation of endonucleases and caspases that cleave substrates at Asp-Xxx bonds (i.e., after aspartic acid residues) (11,12). Third, drugs, such as cyclosporin, which stabilize mitochondrial membranes, have

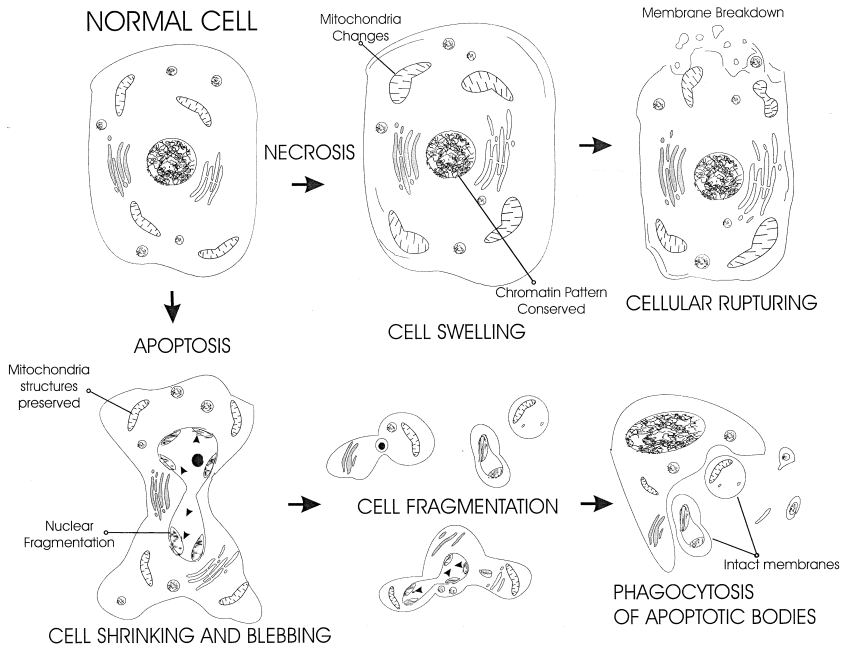

Figure 1. Apoptosis vs necrosis. There are distinctive differences between necrotic and apoptotic cell death that can be observed and measured. Necrosis occurs when a cell suffers lethal injury and is characterized by swelling, rupturing of the cell, and inflammation. Apoptosis, however, is an event characterized by cell shrinkage, membrane blebbing, and chromatin condensation. Additionally, activation of endogenous endonucleases and caspases results in irreversible DNA fragmentation along with fragmentation of the cell into membrane-bound apoptotic bodies. Surrounding cells or macrophages subsequently phagocytose apoptotic bodies.

been shown to inhibit apoptosis (3,13). Fourth, the antiapoptotic protein Bcl-2 blocks the release of the intermembrane mitochondrial protein cytochrome c, thus often blocking apoptosis as well (3,13).

Cytochrome c release from mitochondria is one of the most intensively studied pathways of apoptosis (Figure 3). The mitochondria can serve as key regulators of apoptosis via this cytochrome c-mediated pathway (12). Cytochrome c in its holoform (i.e., with its haem group attached) associates with Apaf-1 (apoptotic protease-activating factor 1), caspase-9, and adenosine triphosphate (ATP) to form a complex called an *apoptosome*. When the apoptosome is formed, it can proteolytically activate caspase-9, which leads to the activation of the caspase cascade (caspase-3, caspase-6) and the degradation phase of apoptosis (3,4). Apaf-1 is a predeominantly cytosolic protein, and in *C. elegans* likely interacts with Bcl-2. However, a recent study using rat embryo fibroblasts found that Bcl-2, Bcl-xL, and Bax do not interact with Apaf-1 in normal and apoptotic cells (14). To further complicate the matter, several endogenous cytosolic caspase inhibitors are present, such as XIAP, c-IAP1, and c-IAP2, which can inhibit activation of the caspase cascade (15,16).

In the apoptosis field, there is debate over whether the release of cytochrome c from mitochondria irreversibly commits a cell to death. Although cytochrome c may in some cases trigger the effector phase of apoptosis, in other cases cytochrome c release is a very late event. For example, in apoptosis induced by death receptors, cytochrome c release is a late event that is likely to be the result of caspase activation rather than its cause (17). At the very least, release of cytochrome c interrupts the transfer of electrons between respiratory chain complexes III and IV, resulting in the generation of radical species that could accelerate apoptotic processes (18–20).

Another key protein released from mitochondria (11) is the apoptosis-inducing factor (AIF; Figure 3). Various apoptotic signals, such as radicals, staurosporin, c-Myc, etoposide, or ceramide can lead to mitochondrial release of this proapoptotic protein. AIF is normally confined to mitochondria and colocalizes with heat shock protein 60 (HSP60). On induction of apoptosis, AIF (but not HSP60) translocates to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation (11). Thus, AIF nuclear translocation indicates that this protein is a caspase-independent mitochondrial death effector responsible for partial chromatinolysis. Therefore, this pathway could contribute to apoptosis during normal aging and should be investigated more thoroughly.

The Bcl-2 Family of Proteins

The Bcl-2 family is composed of over a dozen proteins that have been classified into three functional groups. The first member of the family was isolated from a B-cell lymphoma; hence the name bcl (21). The members of the first group are antiapoptotic and have four short, Bcl-2 homology (BH) domains (BH1–BH4). They also have a C-terminal hydrophobic tail that allows them to localize to the outer surface of the mitochondria. This group includes Bcl-2 and Bcl-xL. The members of the second group are proapoptotic and similar in structure to the first group; however, they do not possess the BH4 domain. This group includes Bax and

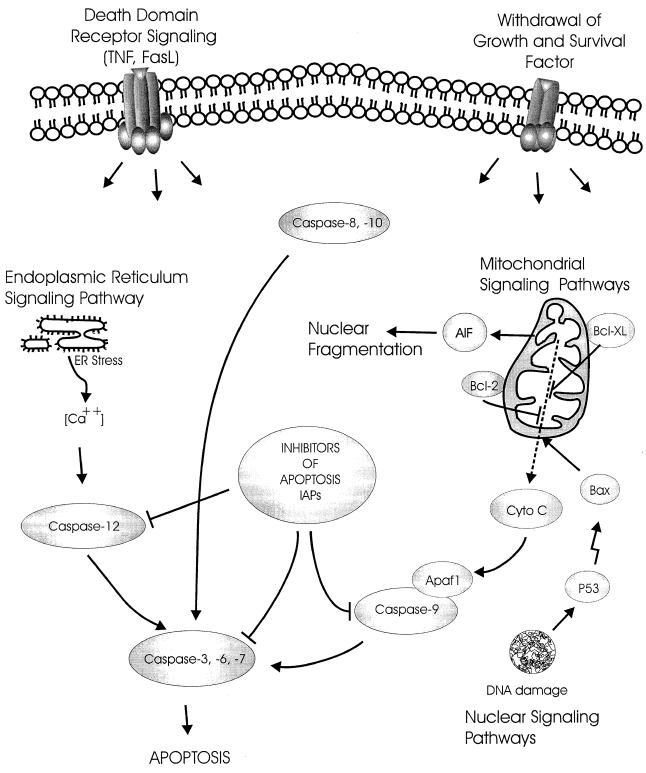

Figure 2. Simplified pathways implicated in apoptotic cell signaling. The mitochondrial-mediated pathway may be stimulated by loss in mitochondrial membrane potential and mitochondrial dysfunction following the release of cytochrome c (Cyto c) or apoptosis-inducing factors (AIF), activating caspases or causing large-scale nuclear fragmentation, respectively. The endoplasmic-mediated pathway may be activated by "ER stress," resulting in increased intracellular calcium content that leads to the activation of caspase-12. Receptor-mediated pathways may be initiated by ligand binding or the withdrawal of growth factors resulting in caspase activation (caspase-8, caspase-10) activation. Nuclear-mediated pathways may provoke apoptosis by means of the signaling of p53 in response to DNA damage. IAPs = inhibitors of apoptosis.

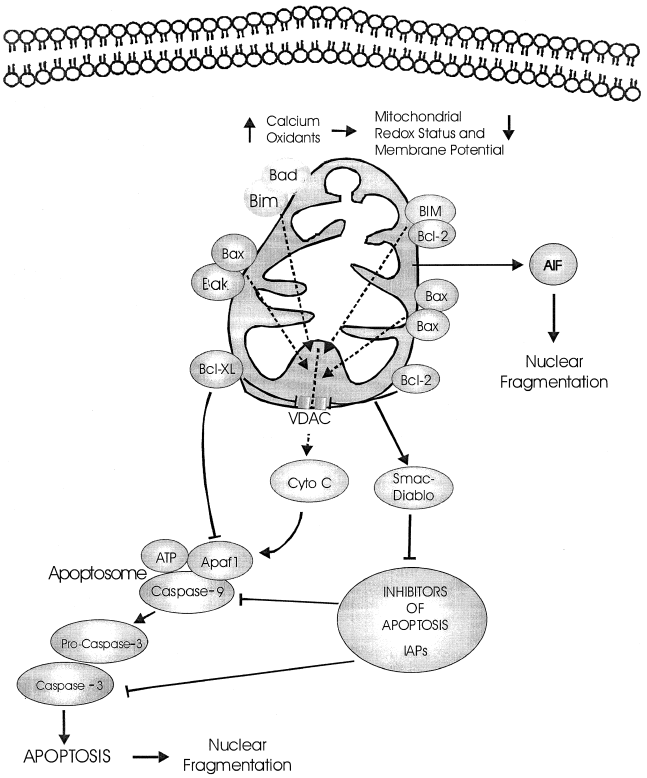

Figure 3. Mitochondrial-mediated pathway. Mitochondrial dysfunction could be caused by factors, such as mitochondrial membrane potential ($\Delta\Psi_m$) collapse, increases in Ca^{++} levels, formation of reactive oxygen and nitrogen species, decline in the redox status (i.e., glutathione, ATP, NADH). Mitochondrial proteins, such as Bcl-2, Bcl-X_L, Bid, and Bax, and their specific ratios ("check point proteins" for cell death) can influence the mitochondrial outer membrane channel, the voltage-dependent anion channel (VDAC), which leads to the release of cytochrome c (Cyto c) from the mitochondria. Cyto c release could lead to the formation of the "apoptosome" (Apaf-1, caspase-9, dATP) resulting in apoptosis. Other proteins released from the mitochondria, such as apoptosis-inducing factor (AIF) located in the mitochondrial intermembrane space are caspase independent and translocate to the nucleus, causing large-scale DNA fragmentation. Smac-Diablo (second mitochondrial activator of caspases) can be released simultaneously with cytochrome c and functions to inhibit inhibitors of apoptosis (IAPs). Signals and proteins responsible for apoptosis may vary remarkably between cell types and may also originate from the extracellular milieu and the nucleus.

Bak. The members of the third group are a heterogeneous collection of proteins that share a BH3 domain; some are divergent homologues of Bcl-2 and Bax, such as Bid, whereas others are likely to possess a BH3 domain through convergent evolution (22). The Bcl-2 family of proteins is able to regulate some forms of apoptosis by controlling the release of cytochrome c. Currently, there are several competing models of how Bcl-2 family members regulate the release of cytochrome c; for a review, see Hengartner (22). The exact alterations in the expression of these proapoptotic or antiapoptotic proteins with age and the effects on cytochrome c release are unclear.

The Role of Free Radicals in Inducing Apoptosis

The overproduction of radicals can induce oxidative stress and cell death (23–26). For example, reactive oxygen and nitrogen species, such as peroxynitrite and hydroxyl radical, are believed to be potent inducers of oxidative damage and necrosis. The role of radicals in inducing apoptosis is supported by the following: (a) reactive intermediates can influence the cellular redox status (27,28) and therefore apoptosis (29), and (b) studies show that antioxidants can attenuate apoptosis (30–33). Aging is characterized by an increased production of radicals in several tissues (29,34–36); this increased production of radicals may promote the induction of apoptosis.

One important source of reactive intermediates is mitochondria (37–39). Although the main function of mitochondria is energy production, isolated mitochondria generate reactive oxygen radicals during oxidative phosphorylation. Studies show that the mitochondrial electron transport chain is imperfect because it generates $O_2^{\cdot -}$ from the one-electron reduction of O_2 (38,39). Enzymatic dismutation of $O_2^{\cdot -}$ then produces H_2O_2, another important biological oxidant. Release of such intermediates accounts for an estimated 1% to

5% of the oxygen consumed during respiration, depending on the substrate and respiration state. However, these studies used isolated mitochondria, and the flux of oxidants was often estimated indirectly (38, 39). Interestingly, recent evidence suggests that complex I is a major site for the production of reactive intermediates in mitochondria. Because seven polypeptides of complex I are encoded by mitochondrial DNA, this complex may be more prone to errors in its structure, resulting in decreased efficiency and increased production of radical species (40,41).

Other sources are also potent mediators of apoptosis, for example, fatty acid metabolites derived from arachidonic acid by the lipoxygenase and cyclooxygenase pathways (42–44). It is unclear whether these products increase the production of reactive intermediates directly in various subcellular compartments or act independently of oxidant production to induce apoptosis. Recent studies have found that overexpressing p53 (45) or treating cells with ceramide, a sphingolipid, will also generate reactive intermediates (46).

The reactive intermediate nitric oxide ($NO^{\bullet}$), a free radical gas, is important in apoptosis induction. Nitric oxide is known to be an important regulator of mitochondrial function, cell signaling, and gene expression, and it functions as the enthothelial-derived relaxing factor (47). For example, nitric oxide, from the $NO^{\bullet}$ donor sodium nitroprusside, resulted in hepatocyte apoptosis and hepatocellular enzyme release, which indicates cell damage (48). Also, exogenous release of $NO^{\bullet}$ from various $NO^{\bullet}$ donors has been shown to trigger apoptosis of rat renal mesangial cells. Researchers believe the mechanism may involve an upregulation of ceramide levels by activating sphingomyelinases while concomitantly inhibiting ceramidases (49,50).

In contrast, at physiological levels, $NO^{\bullet}$ prevents apoptosis and interferes with the activation of the caspase cascade. In one experiment, proinflammatory cytokines were used to activate inducible nitric oxide synthase (iNOS), resulting in full protection for endothelial cells undergoing UV-A radiation. The mechanism involves $NO^{\bullet}$-mediated increases in Bcl-2 expression with a concomitant decrease in the expression of Bax protein (51). Furthermore, in vitro and in vivo experiments indicated that $NO^{\bullet}$ inhibits caspase-3 by S-nitrosation of the enzyme (52). Along with inhibiting caspase 3, $NO^{\bullet}$ has been found to suppress the self-amplification feed-forward loop of apoptosis by inhibiting Bcl-2 cleavage and cytochrome c release (53).

Another reactive nitrogen species with apoptotic effects is peroxynitrite. Peroxynitrite is an anion oxidant generated by the reaction of nitric oxide with superoxide. In one experiment, it was shown that treating HL-60 leukemia cells with increasing concentrations of peroxynitrite induced apoptosis in a time- and concentration-dependent manner (54).

In summary, high levels of oxidants can indirectly induce apoptosis by changing cellular redox potentials, depleting reduced glutathione, reducing ATP levels, and decreasing reducing equivalents, such as reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (55–58). These changes can facilitate the formation of permeability transition pores, leading to the subsequent release of cytochrome c. Interestingly, these pores possess several redox-sensitive sites, including one in equilibrium with mitochondrial matrix glutathione, and one directly activated by oxidants (58).

Apoptosis and Aging

The molecular mechanisms of aging likely involve both programmed changes in gene expression and "wear and tear" from reactive oxygen and nitrogen intermediates, lipid peroxidation products, and advanced glycation end (AGE) products. Both of these intertwined processes may contribute to apoptosis. For example, AGE products have been found to induce apoptosis (59–61). They are produced from glycoxidation reactions, and the formation of several AGE products can be influenced by the presence of reactive oxygen intermediates. Such wear-and-tear processes may result in nonfunctional or abnormal cells, which may later be eliminated by apoptosis (5,7,62).

The free radical theory of aging proposed by Harman links senescence to damage inflicted by superoxide-derived radicals and other reactive intermediates generated primarily in mitochondrial respiration (63–65). Another related theory of aging, the mitochondrial theory of aging, proposes that aging is the result of accumulated free radical damage to mitochondrial DNA (mtDNA). The accumulation of errors in mtDNA results in errors in the polypeptides encoded by mtDNA, leading to dysfunctional proteins in mitochondrial respiratory chain complexes. If a complex is defective, then more radicals are produced, thereby leading to a vicious cycle of increasing mtDNA damage, radical generation, and possibly apoptosis (24,26,29,66).

Mitochondria from the brain and liver of aging mice exhibit enhanced permeability transition pore (PTP) activation. This results in a lower threshold for the release of apoptogenic proteins into the cytosol. These studies showed that hydrogen peroxide and Ca^{2+} have additive effects on PTP formation in vitro (67) and may, in part, play a role in the increased susceptibility of PTP activation with age. Aged cells have been associated with increased mitochondrial oxidant production (36) and elevated intracellular Ca^{2+} levels (68,69). These events lead to a favorable intracellular environment for PTP formation and the release of apoptogenic factors.

Aging and Apoptosis in T Cells

Apoptosis in T cells is well characterized, and recent studies indicate that with advancing age, defects in T-cell apoptosis may correlate with increased autoimmune disorders and susceptibility to infections in young as well as older individuals. Apoptosis is a fundamental part of normal T-lymphocyte maturation and selection. While the T cells are maturing in the thymus, any T cells that bind to self-antigens or make nonfunctional receptors undergo apoptosis (10). In several studies, aging was found to increase CD8 T-cell apoptosis by hyperstimulation through the T-cell receptor, thus leading to decreased levels of CD8 T cells (70). Furthermore, lymphocytes from elderly subjects overexpressed the apoptosis molecule CD95/Fas antigen, which induces T-cell apoptosis in the presence of Fas ligand (70). Thus, the percentage of apoptotic cells collected from the blood was increased versus young individuals, and cells were more "vulnerable" to apoptosis. Also, mononuclear cells

from elderly subjects underwent more apoptosis in response to mitogen or anti-CD3 than mononucleocytes from young controls. Moreover, experiments using interleukin-2 (IL-2) to rescue lymphocytes indicated that far fewer of the cells from old donors were rescued versus cells from young controls, and this effect was independent of differences in Fas expression (70).

Some data suggest that the CD95 pathway is independent of cytochrome c-mediated apoptosis. For example, the clonal deletion of autoreactive T cells that recognized endogenous antigens was not prevented by a *Bcl-2* transgene (71). However, the CD95/Fas pathway does interact with the mitochondria through Bid. Studies so far indicate that CD95-mediated apoptosis proceeds as follows: CD95 ligation activates the adaptor protein FADD (Fas-associated protein with a death domain). FADD then directly activates caspase-8, which acts directly on caspase-3 (72). However, caspase-8 cleavage of Bid results in translocation of Bid to the mitochondria, with subsequent cytochrome c release leading to caspase-9 activation (73). Bcl-2 proteins may also be involved in the regulation of CD95-mediated apoptosis through a signaling protein named Daxx. Daxx can bind to the Fas death domain and is sensitive to Bcl-2. Moreover, its Fas-binding domain is an inhibitor of Fas-induced apoptosis (74).

Aging, Apoptosis, and Caloric Restriction

Caloric restriction, which retards both aging and tumor formation (75), increased the rate of apoptosis of T lymphocytes in a mice model (76). Furthermore, other studies show that caloric restriction decreases the incidence of tumor formation and cancer (77,78). An imbalance between proliferation and apoptosis may result in neoplasia or tumor formation. Therefore, a mechanism by which caloric restriction prevents cancer and tumor formation may be an increased rate of apoptosis. This would clear damaged or preneoplastic cells from the body.

An increased rate of apoptosis has been reported in mitotic tissues such as liver and T cells of caloric-restricted animals compared with ad libitum counterparts (77–80). These studies reported the change in the rate of apoptosis but did not evaluate alterations in apoptotic proteins or signaling pathways that may have been involved. Warner (5,6,62) hypothesized that retardation of aging could be due to upregulation of apoptosis in certain cells. This would prevent an accumulation of damaged and potentially less functional cells. It therefore remains possible that the remaining cells are more functional; however, this hypothesis has not been tested.

Aging and Apoptosis in Cardiac Myocytes

The human heart loses a significant number of myocytes during aging as a result of apoptotic and necrotic cell death. In fact, the initial ventricular myocyte population may decline by as much as 30% as the heart ages; however, it appears that apoptosis is more prevalent during the late stages of aging (81). In one study of the aging heart in which Fischer 344 rats were used, both necrotic and apoptotic cell death occurred (81). These rats were injected with myosin monoclonal antibody to localize and quantify necrotic myocyte cell death. Apoptotic myocytes were quantified by using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling) assay followed by DNA laddering to confirm DNA strand breaks and thus apoptotic cell death. The TUNEL assay is known to give false positives as necrotic cells will sometimes stain positive, and immune cells "trapped" in tissues may also stain positive. Thus, the use of DNA laddering gels provides a useful check to ensure apoptotic death has occurred. During apoptosis, DNA is cleaved at sites between nucleosomal units, thus generating DNA mononucleosomal and oligonucleosomal fragments (180-bp multimers). These fragments can then be visualized on agarose gels and should form a ladder-like pattern.

The study revealed that myocyte apoptosis increased with age and was restricted to the left ventricular free wall (81). This study also showed that apoptosis in the left ventricles increased by more than 200% in the 24-month-old animals compared with the 16-month old rats, with no change in necrotic cell death. These data strongly suggest that apoptosis may be more prevalent than necrosis in very old rats, and interventions to attenuate this loss could benefit cardiac health.

The underlying mechanism for apoptosis in the heart has not been investigated, but reactive intermediates from nitrogen and oxygen may contribute to this process. In support of this hypothesis, it should be noted that superoxide and hydrogen peroxide production increased with age in isolated mitochondria and submitochondrial particles from the hearts of Mongolian gerbils (34). Therefore, increased oxidative stress may play an important role in cardiovascular abnormalities, perhaps through the loss of viable cardiac myocytes (82).

In addition, this hypothesis is supported by studies of drugs used to treat heart failure. Carvedilol, a beta-blocker drug used to treat hypertension, angina, and heart failure, has significant antioxidant properties. In two studies, apoptosis was attenuated by carvedilol, suggesting a link between oxidative stress and apoptosis (83,84). Furthermore, long-term therapy with the drug enalapril was found to attenuate cardiac myocyte apoptosis in dogs with moderate heart failure (85). Similarly, chronic administration of enalapril in aging mice was found to decrease apoptosis in cardiac myocytes (86). The antiapoptotic mechanisms of these compounds are unclear and further investigation is required.

Research in our laboratory was conducted on the hearts of male Fischer 344 rats. Various assays were performed to measure markers of apoptosis and oxidative stress to determine if there was a relationship between these factors. We found that cytosolic cytochrome c was significantly elevated in the 16- and 24-month-old animals compared with the 6-month-old animals and could render myocytes more vulnerable to apoptosis (87). Also, Bcl-2 levels were decreased with age whereas no alterations in Bax proteins were observed. Because Bcl-2 can block cytochrome c release, this may provide one mechanism for the increase in apoptosis in the aged heart. Nevertheless, no significant differences in caspase activity were found among the three age groups, possibly suggesting the upregulation of caspase inhibitors with age. Oxidative stress was apparent, because

we did find increases in the activity of several key mitochondrial antioxidant enzymes, which indicates an adaptation to age-associated increases in reactive oxygen species (87). Despite the adaptations of antioxidant defenses, oxidative stress was still increased as measured by lipid peroxidation. Others have shown increases in mtDNA damage in the hearts of old animals, which may suggest a link between mitochondrial deterioration and apoptosis (29,66).

Aging and Apoptosis in Skeletal Muscle

To our knowledge, only one report exists that documents an age-dependent loss of human skeletal muscle cells by apoptosis (88). An age-dependent increase in apoptosis detected by TUNEL staining of the striated muscle fibers of the rhabdosphincter led to a dramatic decrease in the number of striated muscle cells. A direct linear correlation between the age of the specimens and decrease in volume densities of the striated muscle cells was evident. This study concluded that apoptosis represents the morphological basis for the high incidence of stress incontinence.

Studies in our laboratory have been directed to determine how apoptosis influences age-related muscle loss. Recent results obtained by using locomotor skeletal muscle (gastrocnemius) from eight 6-month-old and 24-month-old male Fischer 344 rats (89) showed a significant increase in mononucleosomes and oligonucleosomes in the skeletal muscle of old rats compared with that of young animals, which is indicative of apoptosis and/or loss of nuclei. Because skeletal muscle is multinucleated, further studies are required to determine what significance a loss of nuclei would have. No differences were observed in the levels of cytosolic cytochrome c, caspase-3 activity, Apaf-1, and Bcl-2 with age. Although aged skeletal muscle shows increases in DNA fragmentation, these studies do not support a cytochrome c-dependent mechanism of apoptosis. However, it remains possible that specific inhibitors for caspases that prevent the loss of irreplaceable cells in skeletal muscle are increased with age. Also, the differences seen in heart and skeletal muscle in regulating apoptosis may stem from the chronic oxidative stress in the heart mitochondria of aging rats.

Apoptosis and Normal Aging of the Brain

Apoptosis seems to play an important role in neurological diseases (90), but is unlikely to play a major role in the cognitive functional decline observed during normal aging of the brain. Previous studies on neuronal loss with aging reported disparate results, but as a group they reported that most neocortical areas and certain hippocampal subfields lose 25% to 50% of their neurons with age. However, these studies shared a similar design flaw: they measured neuron density in a given structure instead of total neuron number (91). More recent research—based on new stereological techniques to estimate neuron number—has revealed that normal aging does not result in a significant loss of hippocampal or neocortical neurons. However, some age-related loss does occur in the hilus of the dentate gyrus and the subiculum (92).

In fact, research in our laboratory confirms these recent findings. Our studies focused on detecting apoptosis and apoptotic markers in brain cortex from young (6-month-old) and old (24-month-old) male Fischer 344 rats. We showed an increased superoxide production in isolated mitochondria from the brain cortices of the old rats, but no changes in the levels of cytochrome c in the cytosol or in caspase-3 activity. In addition, there were no differences in apoptosis determined by cytosolic mononucleosome- and oligonucleosome-bound DNA using monoclonal antibodies directed against DNA-associated histones (93).

Therefore, apoptotic cell loss is unlikely to be a major factor in the cognitive functional decline that occurs with age. Functional declines, such as normal age-associated memory loss, are more likely a result of subtle molecular changes. Such changes include impaired synaptic transmission, defects in long-term potentiation, decreased number of synapses, and the like (92). The molecular biology behind these functional and morphological changes is still poorly understood, but some of the alterations may involve deleterious changes in mitochondrial function. For example, a recent investigation of nigral dopaminergic neurons from humans undergoing normal aging found that only 2% of the neurons studied showed morphological characteristics of apoptosis. However, a majority of the neurons showed signs of oxidative stress, vacuolation of mitochondria, or shrunken mitochondria (94). Furthermore, defects in cytochrome c oxidase (complex IV of the mitochondrial respiratory chain) were detected in the substantia nigra of 36 normally aged human brains (95).

Conclusions

In summary, the role of apoptosis in aging will continue to be an exciting but complex area of research. Future investigations will have to examine the molecular mechanisms behind aging interventions and their effects on the rate of apoptosis and relevant apoptotic pathways. For example, caloric restriction has been reported to increase the rate of apoptosis in liver and T cells of caloric-restricted animals compared with ad-libitum-fed controls (77–80). However, no studies were done to evaluate the apoptotic pathways involved. In contrast, caloric restriction may prevent apoptosis in postmitotic tissues, and this could be an attractive area of future investigation. In vivo studies in aging animals will be challenging, and therefore the use of innovative models may provide more conclusive results; some examples of these are the use of transgenic mice (Bcl-2 overexpression), senescence accelerated mice, or *klotho* mutants (96,97).

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