

Apoptosis and Aging

Eugenia Wang, Chantal Autexier, and Edwin Chen

I. Introduction

Since its initial discovery by Kerr *et al.* (1972) as a mechanism of cell death distinct from necrosis, the process of "apoptosis" has been the subject of intense scientific interest, and deregulation of apoptosis has been implicated as a fundamental pathogenic mechanism in a myriad of aging-related human diseases. For example, inefficient elimination of malignant or autoreactive cells can result in the development of cancer or autoimmune diseases. On the other hand, excessive apoptotic cell death may result in aberrant cell loss and organ atrophy, pathological events that underlie neurodegenerative diseases, cardiovascular dysfunction, muscle atrophy, intestinal disorders, and kidney disease. Indeed, the numerous and wide-ranging manifestations associated with dysregulation of apoptosis reflect the complexity of apoptotic signaling and the importance of its role in organismic homeostasis.

Unlike necrosis, which usually results from severe trauma to the cell and is manifested by an uncontrolled breakdown of cellular and organelle structure,

cell lysis, and an inflammatory response, apoptosis can be induced by mild signals and occurs through an ATP-dependent, gene-driven, non-inflammatory process (Majno & Joris, 1995). Morphologically, necrosis exhibits cell swelling and loss of membrane integrity, whereas apoptosis is characterized by cell shrinkage, formation of membrane-enclosed bodies, preservation of organelles, and maintenance of membrane integrity. Phagocytosis of the apoptotic bodies by macrophages prevents an inflammatory response. Apoptosis is also characterized by chromatin condensation and margination and by fragmentation of the nuclear DNA into integer multiples of the internucleosomal length (approximately 180 bp) (Wyllie, 1980). Biochemically, apoptosis is accompanied by the *de novo* expression of a spectrum of genes that facilitate the execution of the cellular suicide program and/or the processing of pre-existing gene/protein entities (such as caspase) into functional mode.

Apoptosis is necessary during development when excess cells need to be removed, for example, during organ morphogenesis. However, with age, the

exquisite control of gene expression for apoptotic events may not be finely tuned, resulting in either retaining cells that should be eliminated or losing cells that should be retained. We hypothesize here that cells that inappropriately fail to die (apoptosis-resistant cells) and cells inappropriately prone to apoptotic death (apoptosis-susceptible cells) are dangerous to the tissues in which they reside. The former cells may act as seeds for transformation and neoplastic growth, whereas the absence of the latter may compromise tissue function. The activation or repression of "killer" or "survival" factors that modulate the regularity of apoptotic activity may be crucial to understanding the etiology of some age-dependent debility. Investigation of the molecular mechanisms leading to dysregulated apoptosis may provide tools necessary to the prognosis, diagnosis, and eventual treatment of age-related diseases such as cancer, Alzheimer's disease, and cardiovascular degeneration.

The past decade has seen an explosion of research into the biochemical signaling pathways of apoptosis. In this review, we provide an overview outlining important findings in the molecular mechanisms of apoptosis in general. We further discuss how these pathways may be initiated or circumvented in cells that exhibit apoptotic sensitivity or resistance, respectively, and the relevance of these phenotypes to aging and aging-associated diseases.

II. General Molecular Mechanism of Apoptosis

A. Caspases

A common end event in the execution of apoptotic cell death is the activation of a family of proteases called caspases [reviewed in Cohen (1997)], a family of cysteine proteases that mediate proteolysis of a variety of intracellular proteins at

specific aspartate residues. Among the many caspase substrates that have been identified are poly(ADP-ribose) polymerase (PARP) (Lazebnik *et al.*, 1994), lamin A (Orth *et al.*, 1996), U1 70-kDa small nuclear ribonucleoprotein (Casciola-Rosen *et al.*, 1996), actin (Mashima *et al.*, 1995), fodrin (Martin *et al.*, 1995), and the retinoblastoma tumor suppressor Rb (Janicke *et al.*, 1996). The first mammalian caspase to be described, a protein previously known as interleukin-1 β converting enzyme (ICE, now designated caspase 1), was identified as playing a role in apoptosis by virtue of its homology with the proapoptotic *Caenorhabditis elegans* protein, *ced-3* (Yuan *et al.*, 1993). Ten caspase family members have since been identified; caspases 3 and 7 are the key effector caspases upon which divergent apoptotic signaling pathways appear to converge. Overexpression of caspase 1 or 3 is sufficient to trigger apoptosis (Miura *et al.*, 1993), and caspase 3 knockout mice exhibit an embryonic lethal phenotype due to abnormal brain formation caused by the inability to diminish neuronal numbers during development (Kuida *et al.*, 1996).

Caspases are synthesized as proenzymes with variable prodomains and an enzymatic domain. Activation occurs by posttranslational cleavage within the enzymatic domain at specific aspartate residues, yielding large and small subunits; two large and two small subunits subsequently associate into an active heterotetrameric complex. Removal of the prodomain is not necessary for proteolytic activation, nor is its presence necessary for enzymatic activity (Salvesen & Dixit, 1997; Villa *et al.*, 1997). Because activation requires cleavage at specific aspartate residues, an ability unique to the caspase family, activation can only be mediated through autoactivation or by another caspase.

Once the activation of caspases is effected, cell death appears to be inevitable. Therefore, researchers have turned to studying the upstream mechanisms that initiate the activation of caspases. A plethora of data now suggests that activation of caspases can be mediated through proapoptotic receptor signaling complexes at the cell surface, by mitochondrion-dependent processes within the cytosol, or by p53-dependent processes originating in the nucleus (Fig. 1).

B. Receptor-Dependent Caspase Activation

Proapoptotic signals can be transmitted from the extracellular milieu by associating extracellular death signal ligands with their respective receptors on the cell surface. Among the best characterized of the cell-surface-initiated apoptotic pathways is that initiated by the tumor necrosis factor receptor family, of which five members have been identified. These receptors include Fas, TNFR1, DR3, DR4,

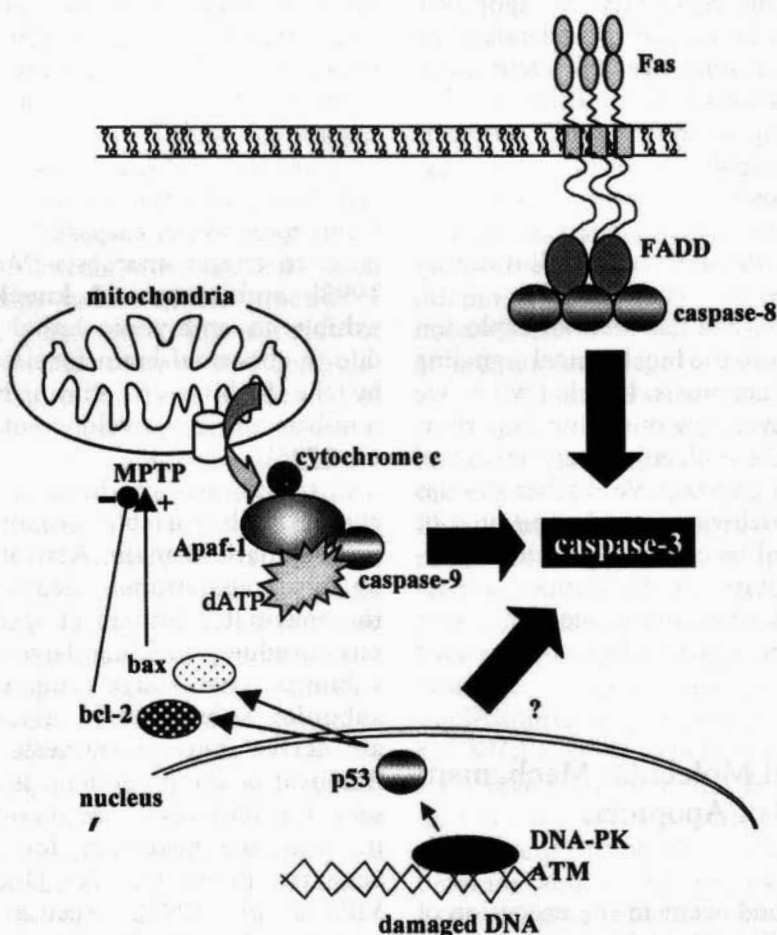


Figure 1. Apoptotic signaling pathways to caspase activation. The machinery involved in the induction of apoptotic signals and the activation of apoptotic effectors, such as caspases, is complex. Three well-studied mechanisms capable of leading to the apoptosis of cells include (i) mitochondrion-dependent pathways, (ii) Fas(receptor)-dependent pathways, and (iii) p53-dependent pathways. The pathways are not completely distinct, but exhibit a limited capacity of crosstalk. See text for details.

and DR5 [reviewed by Haunstetter and Izumo, (1998)]. These death receptors share a motif termed the "death domain," an approximately 80-amino acid stretch that resides in the cytoplasmic domain of these receptors and is necessary for their proapoptotic function. Signal transduction through these receptors requires binding of the appropriate ligand, such as Fas ligand (FasL), which associates with the Fas receptor, TNF- α , which binds to TNFR1, and Apo-3L, which interacts with DR3. The TRAIL ligand has been shown to interact with both DR4 and DR5. Receptor-ligand interaction results in the formation of a homotrimeric complex, facilitating the recruitment to the "death domain" of an intracellular adaptor protein by protein-protein interactions. In the case of TNFR1 and DR3, this adaptor protein is the TNFR-associated death domain protein (TRADD), whereas Fas and DR4 recruit the Fas-associated death domain protein, FADD. The adaptor protein involved in DR5 signaling remains unknown, suggesting the existence of hitherto undiscovered adaptor proteins.

FADD has been shown to interact directly with caspase 8, leading to its autoactivation [Boldin *et al.*, 1996; Muzio *et al.*, 1996], which is followed by the activation of caspase 3 and apoptosis [Fernandes-Alnemri *et al.*, 1996].

C. Mitochondrion-Dependent Caspase Activation

Another potential mechanism by which apoptosis is initiated and caspases are activated is through mitochondrion-dependent pathways. Some apoptotic stimuli evidence a cascade of events that occur at the mitochondria and lead to cell death. These events include (i) opening of the mitochondrial permeability transition pore (MPTP) and (ii) release of cytochrome *c* from the intermi-

tochondrial matrix, which induces (iii) the activation of caspases [Cai *et al.*, 1998; Gross *et al.*, 1999; Halestrap *et al.*, 1998; Zoratti & Szabò, 1995].

The mitochondrial permeability transition pore (MPTP) is a proteinaceous megachannel complex that spans the intermitochondrial matrix. It consists of several inner and outer mitochondrial membrane proteins, including the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), the peripheral benzodiazepine receptor (PBR), cyclophilin D, hexokinase, creatine kinase, and perhaps other unidentified components [Zoratti & Szabò, 1995]. The MPTP functions primarily to control the efflux of mitochondrial matrix solutes with molecular masses <1500 Da from the mitochondria to the cytoplasm, thereby maintaining a mitochondrial matrix environment distinct from that of the cytoplasm [Zamzami *et al.*, 1998]. One of the first steps in the apoptotic process is the opening of the MPTP [Kroemer *et al.*, 1997], followed by mitochondrial depolarization, organelle swelling, and uncoupling of oxidative phosphorylation.

Opening of the MPTP provides a channel for expulsion of key components of the mitochondrial electron transport chain, which is necessary for the activation of several downstream apoptotic executors. In particular, the intermitochondrial matrix protein cytochrome *c* is released from mitochondria following the initiation of apoptosis [Liu *et al.*, 1996] and functions in the formation of an "apoptosome," a complex that also contains apoptosis protease activation factor 1 (Apaf-1) and procaspase 9. This complex subsequently catalyzes the autoactivation of caspase 9 by a dATP- or ATP-dependent mechanism [Li *et al.*, 1997]. Active caspase 9 subsequently activates caspase 3, precipitating cell death.

D. p53-Dependent Caspase Activation

Tumor suppressor p53 is a key "guardian of the genome," maintaining genomic stability after DNA damage [reviewed by Yonish-Rouach (1996)]. p53 is a nuclear-localized, sequence-specific transcriptional activator. The amino terminus of p53 functions as the transactivation domain, and the DNA binding domain interacts directly with DNA containing the sequence 5'-PuPuPuC(A/T) (T/A)GPyPyPy-3' (El-Deiry *et al.*, 1992; Kern *et al.*, 1991). The carboxy terminus is responsible for recognizing damaged DNA. Ordinarily, p53 exhibits a short half-life *in vivo*; in the presence of damaged DNA, however, p53 protein levels are increased by the stabilization of preexisting p53 protein and up-regulation of p53 transcription (Kastan *et al.*, 1991).

The first functional role to be ascribed to p53 was the induction of growth arrest at a restriction point in the G₁ phase of the cell cycle to allow the cell sufficient time to repair DNA damage (Diller *et al.*, 1990). Induction of cell cycle arrest involves the activation of transcription of several growth-arrest-associated proteins, such as p21, Gadd45, and cyclin G (El-Deiry *et al.*, 1993; Fornace *et al.*, 1989; Xiong *et al.*, 1993). Alternatively, in the presence of irreparable DNA damage, p53 functions as a proapoptotic factor and induces programmed cell death. Guillof and co-workers (1995) demonstrated that the introduction of p53 into p53-deficient cells is sufficient to induce apoptosis and that p53 also activates growth arrest genes, suggesting that apoptosis may result from a conflict between signals stimulating and arresting cell growth. This hypothesis was later corroborated by the ability of p21-null cells to undergo p53-dependent apoptosis (Deng *et al.*, 1995), suggesting a distinction between the growth arrest and proapoptotic functions of p53.

The mechanism of p53-induced caspase activation and apoptosis remains poorly understood. Conflicting data have been reported regarding the dependence upon p53-mediated transcriptional activation in the execution of apoptosis. Cells can undergo apoptosis in the absence of p53-induced transcriptional transactivation (Caelles *et al.*, 1994). Indeed, Haupt and co-workers (1995) reported that p53 lacking the transactivation domain induces apoptosis in HeLa cells. However, other groups have reported the ability of p53 to activate the transcription of key proapoptotic genes. Miyashita and Reed (1995) demonstrated that p53 functions to up-regulate levels of Bax, a proapoptotic Bcl-2 homologue. Moreover, p53 down-regulates levels of the antiapoptotic protein, Bcl-2 (Hadler *et al.*, 1994). Bcl-2 has been shown to interact directly with components of the MPTP, specifically VDAC and ANT, in order to regulate the release of cytochrome *c* (Marzo *et al.*, 1998; Shimizu *et al.*, 1996). Overexpression of Bcl-2 is associated with decreased cytochrome *c* efflux, whereas increased Bax expression is associated with increased cytochrome *c* efflux. Given the opposing actions of Bax and Bcl-2 in caspase activation, it has been suggested that initiation of apoptosis by p53 may be controlled by its ability to alter the Bax-Bcl-2 equilibrium (Miyashita *et al.*, 1994).

III. Apoptotic Susceptibility in Aging

Untimely loss of irreplaceable cells in certain tissues is one mechanism by which deregulated apoptosis can exert a detrimental effect during the aging of an organism. One of the classical examples of an age-related phenomenon associated with an undesired diminution in cell number is cardiovascular dysfunction. Most cardiovascular

dysfunction-associated fatalities are caused by a process referred to as "ischemic heart disease." The pathology of ischemic heart disease is initiated by thrombosis of a coronary artery, creating an occlusion of blood flow to the myocardium. Coronary blockage results in reduced oxygen supply to the heart, which, if sufficiently prolonged and severe, can induce membrane damage, necrosis, and cell loss. Paradoxically, whereas rapid reoxygenation and reestablishment of blood flow to the ischemic myocardium is critical in preventing excessive hypoxia-induced damage, the reperfusion event itself can exacerbate the damage to the myocardium. The additional injury inflicted upon the myocardium, independent of the ischemic event, is referred to as "reperfusion injury" (Hearse, 1977).

Reperfusion-associated cell death in the myocardium has long been associated with necrosis, because the rapid and early membrane damage and diminished ATP pool associated with hypoxia often leads to a generalized breakdown of cellular structure. For this reason, it was believed for many years that necrosis was the principal mechanism by which cardiac cell death was achieved (Katz, 1992). However, in a landmark study, Gottlieb and co-workers demonstrated that cardiomyocytes undergo cell death via two distinct mechanisms, depending on the insult: ischemia alone induces death in rabbit cardiomyocytes by necrosis, whereas reperfusion initiates cell death by apoptosis (Gottlieb *et al.*, 1994). Kajstura *et al.* (1995) also revealed that post-reperfusion rat hearts contain both apoptotic and necrotic cells, but the abundance of the latter is sixfold greater than that of the former. Several independent studies have confirmed these findings, although some report the presence of apoptotic as well as necrotic cells with hypoxia. The death of irreplaceable, long-lived, terminally differentiated

cardiomyocytes results in fibroblast infiltration into the infarct zone, creating scar tissue; accumulation of scars ultimately results in heart failure.

The precise mechanism by which cell loss is initiated during reperfusion remains controversial. The favored hypothesis is the "free radical theory of reperfusion injury," which claims that a rapid burst of toxic reactive oxygen species (ROS) mediates cytotoxicity. Indeed, ROS are implicated in all three well-characterized pathways to caspase activation described earlier. In the case of receptor-dependent processes, incubation with ROS, such as H_2O_2 or the semi-quinone menadione, is associated with increased expression of the Fas receptor and up-regulation of Fas ligand mRNA levels (Caricchio *et al.*, 1999). At the mitochondria, ROS promote opening of the MPTP by causing thiol modification of the MPTP component, ANT, enhancing its ability to undergo a calcium-dependent conformational change and resulting in the formation a channel (Halestrap *et al.*, 1998). Moreover, Stridh *et al.*, (1998) and Kluck *et al.*, (1997) demonstrated that H_2O_2 is capable of inducing translocation of cytochrome *c* from the mitochondria to the cytoplasm following 2 hr of oxidative stress in Jurkat and CEM T-lymphoblastoid cells. In both studies, cytochrome *c* release was followed by caspase 3 activation. Direct administration of prooxidants to cytoplasmic extracts that do not contain mitochondria, however, does not result in caspase activation, suggesting that the activation of caspases and the consequent induction of apoptosis induced by free radicals occurs through mitochondria-dependent (and, most likely, cytochrome *c*-dependent) mechanisms (Hampton *et al.*, 1998). Finally, given that ROS are potent inducers of DNA damage and can cause single-stranded DNA breaks, chromosome deletions, dicentrics, and sister chromatid exchanges, it is not surprising

that some investigators have demonstrated that p53-null fibroblasts are more resistant to apoptosis induced by ROS (Yin *et al.*, 1998).

What is puzzling, however, is why these irreplaceable postmitotic cells retain the ability to undergo apoptosis following oxidant damage at all. At first glance, it would seem advantageous for such cells to be impaired in their ability to execute apoptosis. After all, in the presence of overwhelming injury, cell number would be diminished by necrosis. Why would cardiomyocytes respond to a less-than-overwhelming insult, as is often the case with reperfusion, by programmed cell death? Heintz suggests that apoptotic pathways may persist in irreplaceable cells in order to guard against reentry into the cell cycle, which may lead to malignant transformation (Heintz, 1993). Given that one of the primary intracellular targets of oxidants is DNA, in the event of DNA-damage-induced oncogene activation or tumor suppressor inactivation, the survival of an organism may be better served if the potentially dangerous cell is deleted, despite the irreplaceability of the cell. In support of this hypothesis, ectopic expression of oncogenes in terminally differentiated cell types leads to cell death rather than cell proliferation. This "better dead than sick" principle may be the fundamental explanation for the existence of apoptotic mechanisms in cardiomyocytes and other irreplaceable cells (Barr & Tomei, 1994). In the end, the finding that apoptosis also occurs in the myocardium during heart disease will fuel the development of therapies for cardiovascular dysfunction geared toward preventing apoptosis.

IV. Apoptotic Resistance in Aging

One of the best studied models for human aging is the replicative senescence of

human diploid fibroblasts. Hayflick (1965) first showed that fibroblasts possess a limited life span in *in vitro* cell culture, ultimately culminating in a loss of replicative capacity and the development of a flattened, enlarged morphology. Senescent cells are not dead, but rather, if provided regular replenishment of medium and serum, can exist in culture indefinitely (Campisi, 1996). The presence of senescent cells has also been detected *in vivo* in the skin (Dimri *et al.*, 1995). Moreover, senescence may be enhanced by mild oxidative stress. Two teams (Chen & Ames, 1994; de Haan *et al.*, 1995) have shown that administration of H_2O_2 to early passage human diploid fibroblast cultures induces a premature senescence phenotype.

Senescent cells are resistant to serum-deprivation-induced apoptosis, a phenotype that is, in part, attributed to an inability to down-regulate levels of the antiapoptotic Bcl-2 protein (Wang, 1995). In addition, heightened resistance may be due to the inability of the cells to traverse the cell cycle. In this regard, failure to replicate and failure to die may be regulated by the same molecular mechanism, because the activation of apoptosis in quiescent cells requires many of the same G_1 events as replication, and the triage point between life and death may reside at or near the G_1/S boundary. Thus, failure to express key G_1 genes, such as *c-fos* repression, seems to be a double-edged sword, blocking senescent human fibroblasts from both death and replication. Furthermore, this death resistance also may be due to an inability to respond to apoptosis signals.

Our studies have shown that, subsequent to the G_1/S checkpoint, serum-deprived cells are committed to eventual death. They cannot be rescued from death by returning them to serum-containing medium; this commitment point is marked by the proteolysis of a protein, terminin, from a precursor form, Tp90,

to the final product form, Tp30. Therefore, Tp30 can be used as a biochemical marker for the irreversible commitment to apoptotic death. The cleavage of Tp90 to Tp30 closely resembles the noted apoptosis-dependent proteolysis of pro-ICE to ICE and, therefore, suggests key proteolytic steps as the half-way point directing the final apoptotic death.

As described earlier, immediately preceding the commitment to apoptosis marked by the proteolytic production of Tp30, an entire repertoire of immediate early genes is expressed. We and others have noted that there is even an attempt at low level, but significant DNA synthesis in some cells. Ultimately, upon prolonged serum deprivation, cells follow the path leading to DNA replication, though abortive in nature. Perhaps the signals for apoptosis also trigger the machinery preparing for DNA replication; however, in the milieu of a negative environment such as the absence of a functional "anti-death" factor, the enterprise for replicating DNA is aborted, which then sends the alternate signal to commit to death.

Thus, the antiapoptotic state in replicatively senescent human fibroblasts is a unique status marked by the lack of (1) down-regulation of survival factors, such as Bcl-2, (2) key proteolysis of terminin protein to Tp30, its death-specific form, and (3) DNA synthesis activity. This last feature distinguishes senescent fibroblasts from other apoptosis-resistant states, such as rapidly growing cancer cells, that escape the apoptotic fate by continuous proliferation.

Apoptosis-resistant cells, as described previously, provide many tissues with sufficient cellular building blocks. However, the accumulation of these death-refractile cells may be deleterious to the well-being of their host tissue; when dysfunctional cells fail to be elimi-

nated, they may become a "hot-bed" for further insults and, thus, a platform leading to dysplasia. The presence of damaged neurons in the brain, or functionally impaired cardiomyocytes in the heart, may become weak spots that compromise organ function. Dysfunctional cells may either cause further damage when additional insults occur or by themselves prevent the whole tissue from functioning to its maximal capacity. Therefore, the accumulation of senescent cells during aging may contribute to increased loss of the function of organs, and the inability of an organism to delete these deleterious cells by apoptotic mechanisms may exacerbate this condition (Warner *et al.*, 1995). This is consistent with a theory suggested by Franceschi *et al.*, 1992), who assert that apoptosis can serve as an important cellular defense mechanism by deleting genetically unstable cells and that the oldest-old centenarian populations have attained such long life spans by their body's ability to effect apoptosis more efficiently. Also consistent with this theory are reports that calorically restricted rodents (which exhibit greater life spans than littermates fed *ad libitum*) display increased apoptotic incidence, which functions to remove potentially neoplastic cells (Grasl-Kraupp *et al.*, 1994; James & Muskhelishvili, 1994). Warner *et al.* (1997) have proposed that the increased apoptosis in calorically restricted mice may also function to counteract the accumulation of otherwise apoptosis-resistant senescent cells, as another mechanism for extending life span. Thus, caloric restriction appears to increase the apoptotic capabilities of organisms to combat the age-dependent accumulation of defective apoptosis-resistant cells. How then to reactivate the apoptosis program to get rid of these dysfunctional cells is an avenue of future work, possibly employing gene therapy.

V. Telomerase, Telomeres, and Apoptosis

A. The Telomere Hypothesis

Telomeres, the ends of linear eukaryotic chromosomes, consist of short repeats and specialized proteins (Pardue & DeBaryshe, 1999; Wellinger & Sen, 1997). Telomeres and their associated proteins are essential for maintaining chromosomal stability (Price, 1999). Without telomeres, eukaryotic chromosomes undergo end-to-end fusions and rearrangements. The action of telomerase is one mechanism that organisms use to maintain telomeres (Nugent & Lundblad, 1998).

Human telomerase activity was first identified in 1989 in HeLa cells (Morin, 1989). Subsequent studies surveying telomerase activity in various human cell types found that telomerase activity is absent from most primary human cells, but is active in germ line cells and 85% of immortalized and tumor cells (Autexier & Greider, 1996; Shay & Bacchetti, 1997). In most human primary somatic cells, telomeres shorten with each round of cellular division due to the incomplete replication of linear chromosomes, as well as the absence of a compensatory telomere maintenance mechanism (Greider, 1996; Colgin & Reddel, 1999; Pardue & DeBaryshe, 1999). By contrast, in germ line cells and telomerase-positive immortal and tumor cells, telomere length is maintained. The correlation between telomere maintenance and telomerase activity serves as a foundation for the telomere hypothesis, first proposed by Calvin Harley (Autexier & Greider, 1996; Harley, 1991). This model postulates that, in cells lacking a telomere length maintenance mechanism, telomeres act as a "mitotic clock." After a certain number of doublings, when the telomeres become "critically short," a signal is transmitted, perhaps via a DNA

damage checkpoint mechanism, and the cell exits the cell cycle and enters replicative senescence (Vaziri & Benchimol, 1996). The telomere hypothesis also proposes that telomerase activation may be one step in immortalization and/or tumorigenesis, making telomerase an attractive target for anticancer therapy.

B. Telomerase, Telomere Maintenance, Cell Growth, and Cancer

Human telomerase is a ribonucleoprotein consisting minimally of a catalytic reverse transcriptase protein subunit (hTERT) and an integral RNA component (hTR) that contains a short template region complementary to the telomeric d(TTAGGG)_n repeats at the ends of vertebrate chromosomes (Nugent & Lundblad, 1998; Weinrich *et al.*, 1997; Beattie *et al.*, 1998). Telomerase activity correlates best with the expression of hTERT, whereas hTR is commonly expressed in both telomerase-positive and telomerase-negative cell lines, tumors, and tissues (Autexier, 1999; Price, 1999).

The identification of the hTERT gene permitted direct testing of the prediction that telomere length acts as a "mitotic clock." The first experimental evidence establishing a causal relationship between telomere shortening and replicative senescence came from the overexpression of hTERT in certain telomerase-negative primary cells (Bodnar *et al.*, 1998; Counter *et al.*, 1998; Vaziri & Benchimol, 1998). hTERT-transfected cells become telomerase-positive, exhibit increased or stabilized telomere lengths, and are able to divide beyond their "Hayflick limit" number of population doublings. Such extended life span cells do not display a transformed phenotype, as indicated by their inability to form foci in soft agar, normal cell cycle checkpoints and karyotypic stability, and inability to generate tumors *in vivo* (Jiang *et al.*, 1999; Morales *et al.*, 1999;

Vaziri *et al.*, 1999). The cells in these studies are defined as immortal because they more than double their maximum replicative life spans in culture. However, certain primary cells are not immortalized by the overexpression of hTERT alone, but also require the inactivation of the Rb/p16 pathway (Kiyono *et al.*, 1998).

Another prediction of the telomere hypothesis is that telomerase activation may be an essential step in tumorigenesis. Experiments to test this prediction indicate that the expression of the catalytic subunit of telomerase, in conjunction with alterations in p53, Rb, and *ras*, converts some, but not all, primary cells into tumorigenic cells (Hahn *et al.*, 1999a; Morales *et al.*, 1999). In one study, transfection of human fibroblasts with hTERT and *ras* (H-rasV12) combined with the human papillomavirus-16 E6/E7 viral oncoproteins (to inactivate Rb and p53) failed to stimulate transformation (Morales *et al.*, 1999). In a second study, transformation of fibroblasts and epithelial cells is possible by transfecting first SV40 large T-protein (to inactivate Rb and p53) and subsequently hTERT and *ras* (H-rasV12) (Hahn *et al.*, 1999a). The order in which the genes are introduced, and/or whether SV-40 large-T perturbs additional cellular functions, may explain the different results (Weitzman & Yaniv, 1999). These data indicate that telomerase activation may be one step toward immortalization and tumorigenesis for certain cell types. However, 15% of immortal and tumor cells utilize an alternative mechanism for telomere maintenance (ALT), suggesting that telomerase itself is not essential for immortalization and tumorigenesis. Furthermore, ALT cells invariably have long heterogeneous telomeres, indicating that telomere length maintenance clearly is essential for unlimited cell proliferation (Colgin & Reddel, 1999).

C. Telomerase, Telomere Maintenance, and Cellular Resistance to Apoptosis

The association of chromosomes through their telomeres (end-to-end fusions) is an early manifestation of programmed cell death, both *in vivo* and *in vitro*, and can be induced in normal fibroblasts and some tumor cell lines (Pathak *et al.*, 1994a,b). Penclomedine, a synthetic compound under evaluation in clinical trials as an anticancer agent, induces chromosome associations in HeLa cells and normal human fibroblasts (Pandita *et al.*, 1997). Penclomedine-induced chromosome end associations are significantly higher in telomerase-positive cells, suggesting that the effects of penclomedine on telomere maintenance may be telomerase-mediated. Indeed, penclomedine treatment elicits a decrease in the level of telomerase activity in HeLa cells (Pandita *et al.*, 1997). Cells with mutant p53 are more sensitive to penclomedine than cells with wild-type p53, as measured by cell viability assays, indicating that the absence of a p53 DNA-damage checkpoint and growth arrest may play a role in penclomedine-mediated cell death (Pandita *et al.*, 1997). In another study, the p53-negative non-small-cell lung cancer cell line H1299 shows reduced telomeric signals (shortened telomeres) and increased telomeric associations upon p53-mediated apoptosis; this occurs prior to nuclear fragmentation, indicating a role for p53 in telomere shortening (Mukhopadhyay *et al.*, 1998). Treatment of HeLa cells with the apoptosis-inducing anticancer drug cisplatin results in markedly shortened telomeres prior to the onset of apoptosis (Ishibashi & Lippard, 1998). These studies indicate that the induction of telomeric associations as a consequence of telomere shortening appears to be a common step preceding apoptosis in different cell types.

Chromosome end (telomeric) associations may also occur as a result of

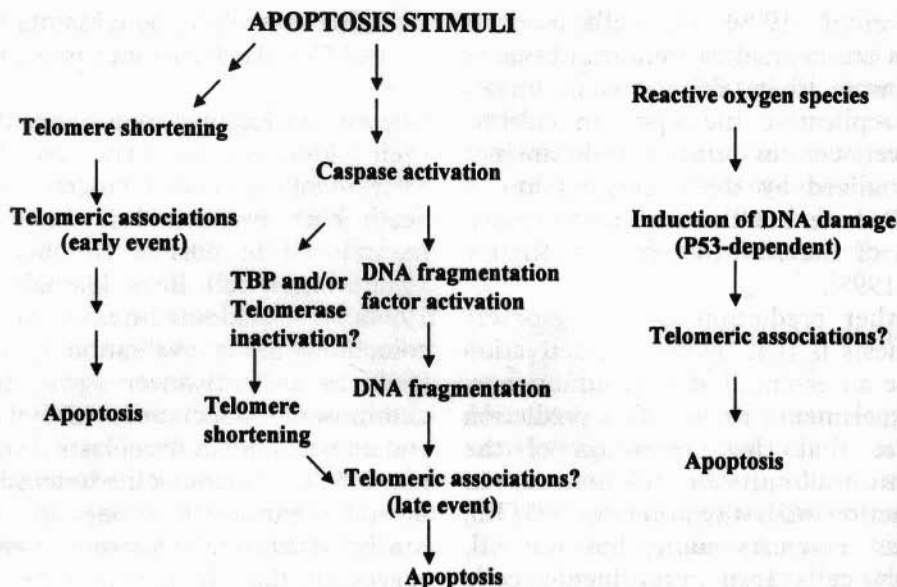


Figure 2. Role of telomeres in apoptosis: a model. Chromosome end (telomeric) associations resulting from telomere shortening appear to be an early event preceding apoptosis in different cell types. Telomeric associations may also occur as a result of DNA fragmentation and/or inactivation of telomere-binding proteins (TBP) and/or telomerase by caspase cleavage. Such associations presumably are late events. Telomeric associations may also result from the induction of DNA damage by reactive oxygen species.

telomere loss due to DNA fragmentation (Fig. 2). The DNA fragmentation factor (DFF), which mediates DNA fragmentation during apoptosis, is activated by caspase 3 (Liu *et al.*, 1997). The potential loss of telomeres during DNA fragmentation may result in telomeric associations; however, in this case, telomeric associations would be a late event in apoptosis. p53-dependent telomeric associations may also result from DNA damage caused by ROS; however, this hypothesis remains to be tested. Telomerase or telomere-binding proteins that regulate telomere length also may be specific substrates for cleavage by caspases (Holt *et al.*, 1999). Potential caspase sites are present in the human telomerase reverse transcriptase component (Holt *et al.*, 1999; Nakamura *et al.*, 1997). The inactivation of telomere-binding proteins, such as TRF2 (telomere repeat binding factor 2) (Karlseder *et al.*, 1999) and/or telomerase

(Hahn *et al.*, 1999b; Zhang *et al.*, 1999), results in telomere loss and apoptosis.

The induction of apoptosis in immortal human cells may or may not be mediated by the down-regulation of telomerase activity. Several studies have indicated decreases in telomerase activity upon the treatment of cells with apoptosis-inducing agents. In some reports, telomerase activity is down-regulated prior to apoptosis, whereas in one report, telomerase activity appears to be down-regulated as a consequence of apoptosis. In pheochromocytoma (PC12) cells, a reduction in telomerase activity occurs prior to the detection of apoptotic nuclei following treatment with staurosporine, amyloid β -peptide, or Fe^{2+} (Fu *et al.*, 1999). Similarly, in the cytotoxic T-cell line, CTLL-2, down-regulation of telomerase activity is detected 8 hr after IL-2 deprivation before G_0/G_1 cell cycle arrest and/or apoptosis become evident at 24 hr (Mandal &

Kumar, 1997). However, exposure of leukemic U937 cells to etoposide reveals that decreases in telomerase activity occur only in late stage apoptotic or necrotic cells; these findings indicate that telomerase down-regulation is a consequence of apoptosis or necrosis (Akiyama *et al.*, 1999). There also have been a few reports indicating no change in telomerase activity upon the treatment of cancer cells with apoptosis-inducing agents. Treatment of a metastatic murine melanoma cell line with paclitaxel induces apoptosis and telomeric associations, but no decrease in telomerase activity (Multani *et al.*, 1999). Similarly, telomerase activity in the immortalized endothelial cell line ECV-304 is unaffected by the overexpression of p53 and apoptosis (Maxwell *et al.*, 1997), indicating possible cell-type-specific differences in the interactions between cell cycle regulators, apoptotic signals, and telomerase regulation.

Inactivation of human telomerase and the subsequent induction of apoptosis may be an effective cancer treatment strategy (Autexier, 1999). In this approach, the potential role of telomere length and telomerase activity in providing resistance to apoptosis is an important experimental parameter. However, the apoptosis resistance and potential risk of cancer that may be conferred by telomerase could be a concern in the use of telomerase in cell replacement therapy (Bodnar *et al.*, 1998). Fu *et al.* (1999) demonstrated that PC12 cells overexpressing Bcl-2 have increased levels of telomerase activity and increased resistance to apoptosis. Moreover, upon differentiation of PC12 cells, levels of telomerase activity decrease and the cells exhibit increased sensitivity to apoptosis (Fu *et al.*, 1999). Decreasing telomerase activity in PC12 cells through the use of telomerase inhibitors is also associated with increased vulnerability to apoptotic stimuli (Fu *et al.*, 1999).

In addition, telomerase-negative normal human cells that have short telomeres are more susceptible to apoptosis than telomerase-negative normal human cells with longer telomeres (Holt *et al.*, 1999). Telomerase-negative foreskin fibroblasts transfected with hTERT and with telomere lengths of 8.1–14 kb are more resistant to apoptosis than untransfected cells with telomere lengths of 6.6 kb (7.5–13% apoptosis versus 20%) (Holt *et al.*, 1999). Moreover, telomerase-positive immortal SW39 cells exhibit greater survival and resistance to apoptosis than their telomerase-negative counterparts, SW13 and SW26 (Holt *et al.*, 1999). Experimental elongation of the telomeres in telomerase-positive cells such as IDH4 (a line of T-antigen-immortalized human lung fibroblasts) or DU145 (a colon adenocarcinoma cell line) results in resistance to apoptosis and greater survival than in isogenic parental cells with shorter telomeres (Wright *et al.*, 1996; Holt *et al.*, 1999). The increased resistance of these cells to apoptosis appears to be associated with defects in two major apoptosis execution mechanisms: the induction of nuclear calcium-dependent endonucleases and the activation of caspases (Holt *et al.*, 1999). These results clearly suggest a link between telomerase activity, telomere maintenance, and cellular resistance to apoptosis.

D. Roles of Bcl-2 and c-myc in Telomerase Regulation

The regulation of apoptosis involves a number of gene products, including the antiapoptotic factor Bcl-2. Interestingly, overexpression of Bcl-2 in HeLa cells is accompanied by increased levels of telomerase activity; moreover, down-regulation of Bcl-2 in the cytotoxic T-cell line, CTLL-2, inhibits telomerase activity with no detectable apoptosis (Mandal & Kumar, 1997). Similarly, in PC12 cells overexpressing Bcl-2, levels of telomerase

activity are increased by twofold (Fu *et al.*, 1999). However, in Jurkat T-cells, Bcl-2 has no effect on telomerase activity or telomere length (Johnson *et al.*, 1999). These apparently contradictory results indicate that it will be necessary to evaluate the link between Bcl-2 and telomerase regulation along with the regulation of other cell cycle and apoptosis factors in different human cancer cells.

c-myc is a key regulator of both cell proliferation and apoptosis in normal and cancer cells. Overexpression of c-myc in normal human mammary epithelial cells lacking telomerase activity results in the induction of hTERT mRNA and the stimulation of telomerase activity (Wang *et al.*, 1998). This stimulation of hTERT expression is due to transcriptional activation of the hTERT gene, whose promoter contains a number of conserved MYC-binding sequences (Cong *et al.*, 1999; Greenberg *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wu *et al.*, 1999). It is tempting to speculate that the elevated expression of MYC in numerous tumors may be responsible for the telomerase activity in these tumors (Greider, 1999). The link between MYC and telomerase is supported by studies in which the inhibition of MYC expression in leukemic cell lines inhibits telomerase activity (Fujimoto & Takahashi, 1997). However, the role of MYC in cellular immortalization is not simply a consequence of its effect on telomerase activation. In culture, rat embryonic fibroblasts are transformed efficiently by the cooperation of immortalizing proteins such as MYC and activated RAS (H-RAS^{G12V}). The substitution of MYC by mTERT (mouse TERT) in such a cooperation assay is not sufficient to immortalize primary rat cells, indicating that TERT is not equivalent to MYC in cellular immortalization (Greenberg *et al.*, 1999).

The regulation of apoptosis is complex and involves a number of survival genes,

like Bcl-2, as well as killer genes, such as interleukin-1 β -converting enzyme (ICE) and other key cellular proteases, the caspases (Adams & Cory, 1998; Thornberry & Lazebnik, 1998; Wang, 1995). In addition, genes such as p53, Rb, p21, and c-myc, which control cell cycle progression and checkpoints, often are up-regulated in cells undergoing apoptosis (Duttaroy *et al.*, 1997; Evan & Littlewood, 1998; Pandey & Wang, 1995; Wang, 1997). It will be important to delineate the role of telomerase and telomere maintenance in cellular resistance to apoptosis in various cell types. The next step will be to evaluate the molecular mechanisms of resistance, through analysis of the expression and activity of Bcl-2, ICE and other caspases, p53, Rb, p21, c-myc, and other genes.

VI. Potential Use of Telomerase Inhibition and Induction of Apoptosis as an Effective Anticancer Therapy

Studies with immortal human cells indicate that the expression of antisense telomerase RNA leads to telomere shortening and cell death (Feng *et al.*, 1995). A study reveals that antisense telomerase treatment of U251-MG human glioma cells induces two distinct responses: apoptosis and differentiation (Kondo *et al.*, 1998b). The U251-MG transfectants that apoptose express a high level of ICE. In a similar report, an antisense directed against telomerase RNA inhibits telomerase activity and induces apoptosis in human glioma cells in culture and *in vivo* in nude mice (Kondo *et al.*, 1998a). In further support of a link between telomerase activity and apoptosis, treatment of the cisplatin-resistant U251-MG human glioblastoma cell line with an antisense telomerase expression vector decreases telomerase activity

and increases susceptibility to cisplatin-induced apoptotic cell death (Kondo *et al.*, Kondo, Tanaka, Haqqi, Barna, & Cowell, 1998c). These studies indicate that telomere integrity is compromised during apoptosis and that inhibition of telomerase may sensitize cells resistant to anticancer agents such as cisplatin. In addition, late generation mice in which telomerase RNA is inactivated genetically exhibit defective spermatogenesis, with increased programmed cell death (Lee *et al.*, 1998). These studies indicate that cells defective in telomerase, due to germ line targeting of the telomerase RNA component, can die by apoptosis.

Telomerase inhibitors that target hTERT have been reported (Hahn *et al.*, 1999b; Zhang *et al.*, 1999). The effects of inhibiting hTERT in various human cancer cell lines by the expression of dominant negative mutants include inhibition of telomerase activity, telomere loss, chromosome damage, apoptosis, and cell death. Apoptosis of cells with initially shorter telomeres (2–3 versus 3–5 kb) occurs more rapidly (Hahn *et al.*, 1999b; Zhang *et al.*, 1999). Although telomerase inhibition in cells with long telomeres (10–12 kb) results in telomere shortening, no apoptosis occurs (Zhang *et al.*, 1999). When the telomere length in these cells approaches 4 kb, the dominant-negative protein is lost, with the subsequent reactivation of telomerase activity and maintenance of telomere length. Interestingly, all the cell lines examined in these studies are p53-defective, indicating that the apoptosis that occurs is p53-independent. These studies convincingly indicate that p53-deficient cells in which telomerase is inhibited can die by apoptosis and suggest that tumors with short telomeres may be effectively and rapidly killed by inhibitors of telomerase. Moreover, there are a number of issues to consider in the development of telomerase inhibitors (Autexier,

1999). For instance, induction of the ALT pathway for telomere maintenance in cancer cells undergoing antitelomerase therapy could lead to resistance to telomerase inhibitors.

The evidence indicating a role for telomeres and telomerase in cellular resistance to apoptosis, as well as data showing that inhibition of telomerase can result in apoptosis, suggests an intimate link between telomeres, telomerase, and apoptosis. However, studies indicate a complex relationship between cell cycle, apoptosis, and telomerase regulation. The challenge of future experimentation will be to address and understand this complexity. Only then will it be possible to evaluate the consequences of antitelomerase therapies in various cell types.

VII. Concluding Remarks

The notion that a gene-directed program has evolved to remove extra cells during development, in order to optimize the pattern and shape of each organ, may seem counter-intuitive. However, this process of creating more cells than necessary at the start and then getting rid of them later offers precise control. Now, over a quarter century since the initial discovery of apoptosis, a plethora of information from our own and other laboratories suggests that apoptosis is regulated by the interplay of two opposing "Yin-Yang" families of genes. Members of families that function to tilt the balance toward killing are termed "death" genes, whereas those that promote survival are termed "antideath" genes. The final cell demise may be the result of up-regulation of "death" gene expression and/or down-regulation of "anti-death" gene expression. In certain cases, a lack of key proapoptotic gene expression results in resistance to apoptosis (as seen in senescent fibroblasts). Cells

with defective cell cycle checkpoints and/or resistance to apoptosis can undergo unchecked proliferation. The accumulation of dysfunctional or potentially neoplastic cells within a tissue ultimately can compromise organ function or result in oncogenesis. On the other hand, excessive apoptosis is undesirable as well; examples include massive cell depletion of neurons, cardiomyocytes, or skeletal myotubes during neurodegenerative diseases, cardiac abnormalities, or muscle atrophy.

In this respect, then, it is important to understand that apoptosis itself is neither "good" nor "bad" for the aging individual, but that it must be regulated and balanced. The health of tissues, and indeed of organisms, thus depends upon the balance between controlled expression of both "death" and "anti-death" genes. This balance must be integrated with the many other signals that operate to accomplish normal cellular function. The balance of signaling pathways for cellular maintenance, replication, and apoptosis will be the focus of future research, which must also explain why, during aging, the apoptotic program is dysregulated and how this dysregulation precipitates the disability and degeneration associated with the aging process.

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