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Replicative Senescence in the Immune System: Impact of the Hayflick Limit on T-Cell Function in the Elderly

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Gerontologists, lured by the prospect of understanding the cellular events that underlie the gross features of human aging, are focusing increasingly on replicative (or cellular) senescence. The term "replicative senescence" describes the irreversible state of growth arrest experienced by all mitotically competent cells of human origin following a fairly predictable number of cell divisions in culture. First identified in human fetal fibroblasts by Hayflick, replicative senescence, or the so-called Hayflick limit, results from an intrinsic natural barrier to unlimited cell division exhibited by all normal somatic cells.

The characteristics of replicative senescence have been explored in a variety of cell types (reviewed in Smith and Smith 1996; see also Bérubé et al. 1998 [in this issue]), but only recently has this model been applied to the immune system. Ironically, the Hayflick limit may be particularly deleterious for immune cells, since the ability to undergo rapid clonal expansion is absolutely essential to their function. The decline of T-cell immune function during aging suggests that T cells might be an ideal system in which to explore the potential role of replicative senescence during in vivo aging. Well-characterized cell surface markers, signal transduction pathways, and functional traits further enhance the potential utility of T cells as a model system, both to elucidate the process of replicative senescence itself and to assess its physiological consequences.

This review will summarize the results of research on T-cell replicative senescence in cell culture and will demonstrate that cells from elderly people have undergone changes in vivo that are similar to those observed in the cell culture model. I will argue that T-cell replicative senescence contributes to increased morbidity and mortality during aging and that the proportion of replicatively senescent T cells within individual subjects may serve as a measure of "immunological age." The striking interindividual variation among elderly people in health status and immune competence underscores the need to define parameters other than chronological age to identify individuals at risk of immunodeficiency.

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Proliferation Is Essential for Lymphocyte Function

The major cellular components of the immune system are T and B lymphocytes, cells that derive from hematopoietic stem cells within the bone marrow. The intricate genetic mechanism by which lymphocyte antigen receptors are generated allows a limited number of immunoglobulin and T-cell receptor (TCR) gene segments to create an immune system with an enormous range of specificities. Briefly, during a lymphocyte's development, one member of a set of gene segments is joined to other gene segments by an irreversible process of DNA recombination. This random series of juxtapositions forms a sequence of gene segments, thereby yielding a receptor molecule that is unique to that cell. The consequence of this mechanism is that just a few hundred different gene segments can combine in a variety of ways to create thousands of receptor chains. The diversity so generated is further amplified by the pairing of two different chains, each encoded by distinct sets of gene segments, to form a functional antigen receptor. By these mechanisms, a small amount of genetic material is utilized to generate $\geqslant 10^8$ different specificities. Each lymphocyte bears many copies of its antigen receptor, and, once generated, the receptor specificity of a lymphocyte does not change. Thus, only one specificity can be expressed by a single lymphocyte and its progeny (Janeway and Travers 1997).

The process of clonal selection is central to adaptive immunity. When an antigen interacts with receptors expressed on a mature lymphocyte, that lymphocyte becomes activated and starts dividing, giving rise to a clone of identical progeny bearing identical receptors for antigen. Antigen specificity is thereby maintained as the dividing cells continue to proliferate and differentiate into effector cells. Once antigen is cleared, a small num-

ber of memory cells persist, all bearing the same antigen receptor. When the same antigen is encountered again, the process of activation and clonal expansion is repeated.

The exquisite specificity of each lymphocyte results in the ability of the organism to respond to the nearly infinite number of foreign antigens that could be encountered over a lifetime. However, because of the enormous repertoire of responding immune cells, the number of cells that can recognize and respond to any single antigen is extremely small. Thus, to generate a sufficient quantity of specific effector cells to fight an infection, an activated lymphocyte must proliferate extensively before its progeny differentiate into effector cells. For this reason, a limitation on the process of cell division could potentially have devastating consequences on immune function.

To analyze the proliferation systematically, human T cells derived from peripheral blood of healthy young adult donors are propagated in cell culture by repeated stimulation with antigen (Abidzadeh et al. 1996) or with appropriate activating antibodies (Levine et al. 1997) and continuous exposure to the T cell–specific growth factor, interleukin-2 (IL-2). Because lymphocytes grow in suspension culture, it is possible to obtain accurate cell counts and to determine the precise number of population doublings that occur before replicative senescence is reached. The mean number of cumulative population doublings falls consistently between 25 and 40. This limited range has been reported by several different investigators who studied either bulk cultures or clonal populations, and it applies equally to the two subtypes of T cells (CD4+/helper and CD8+/cytotoxic; Grubeck-Loebenstein et al. 1994; Abidzadeh et al. 1996). Descriptions of extremely long-lived T-cell cultures are rare, and in no case has there ever been a report of normal T cells *not* undergoing replicative senescence in culture. The few studies that analyzed cell populations in putatively "immortal" cultures derived from normal T cells invariably found karyotypic abnormalities (Engers et al. 1980). Thus, as had been previously demonstrated in fibroblast studies (Hayflick 1965; Campisi et al. 1995; Smith and Pereira-Smith 1996; Bérubé et al. 1998), replicative senescence is a stringent characteristic of all normal human T cells (reviewed in Effros and Pawelec 1997).

Genetic Alterations in Senescent T Cells

T cells express a large number of well-characterized cell surface molecules. These include antigen receptors; markers whose expression signifies activation; markers that distinguish naive and memory cells; integrins, extracellular matrix–receptor molecules that function in adhesion and migration; and receptors associated with helper versus cytotoxic function. Using monoclonal antibodies in combination with flow cytometry, the expression of a variety of these markers has been shown to be identical between early-passage cultured T cells and cultures that had completed their "programmed" number of cell divisions and had reached senescence (reviewed in Effros and Pawelec 1997).

In light of the stable expression of virtually all T-cell markers tested over the entire proliferative "lifespan" in culture, it is particularly noteworthy that there is one cell surface molecule—CD28—whose expression is dramatically affected by senescence. It has been shown that, with increasing number of population doublings in culture, there is a progressive decrease in the proportion of T cells expressing CD28, so that in senescent cultures, >95% of the cells are CD28− (Effros et al. 1994*a*).

CD28 is a 44-kD disulfide-linked homodimer expressed constitutively on the majority of mature T cells whose signaling is essential for full T-cell activation. Ligation of the T-cell antigen receptor without costimulation by CD28 ligands, such as the "B7" proteins on the surface of antigen-processing cells, results in the inability to proliferate. CD28 signal transduction results in IL-2 gene transcription, expression of the IL-2 receptor, and the stabilization of a variety of cytokine messenger RNAs (June et al. 1994). CD28 has additional biological functions, which include mediation of protective effects against septic shock in vivo, influencing the class of antibodies produced by B cells, and enhancing T-cell migration and homing (Shimizu et al. 1992).

One of the newly identified roles of CD28 is its putative involvement in augmenting the induction of the enzyme telomerase (Weng et al. 1996; see also Bérubé et al. 1998). Telomerase is a specialized reverse transcriptase that functions to extend telomere sequences at the ends of linear chromosomes. In the absence of telomerase, telomeres, the repetitive DNA sequences on the ends of chromosomes, shorten with each cell division (Harley et al. 1990). Telomerase activity has been detected in >90% of human tumors and is believed to enable such cells to divide indefinitely (Shay and Wright 1996). Conversely, telomerase is absent from most normal somatic cells, leading to the hypothesis that telomere shortening is causally related to replicative senescence. Indeed, a landmark event in the field of replicative senescence was the recent validation of the telomere hypothesis of cellular aging. In an elegant series of experiments, Bodnar et al. (1998) demonstrated that gene transfer of a component of telomerase into normal fibroblasts allowed the cells to continue cell division indefinitely and avoid telomere shortening. The transfected cells appear normal and show no signs of transformation, reinforcing the idea that tumor development is a

multistage process in which telomerase induction is a necessary, but not sufficient, component.

The relationship of telomeres , telomerase, and senescence is more complex in T lymphocytes than in fibroblasts. Lymphocytes differ from fibroblasts and most other normal somatic cells in that, under certain circumstances, they exhibit telomerase activity levels similar to those observed in tumor cells. For example, high levels of telomerase activity, further enhanced by CD28 signaling, are observed on T-cell activation (Bodnar et al. 1996; Weng et al. 1996). In addition, telomerase activity has been documented in developing T cells within the thymus and in lymphoid organs (June et al. 1995). Nevertheless, despite initially high levels of telomerase activity, T cells eventually do senesce in culture, reaching the same 5–7-kb terminal restriction fragment (TRF) length (Vaziri et al. 1993) that is characteristic of fibroblasts, a cell type that never expresses telomerase. These findings suggest that T-cell senescence might be forestalled or even reversed if telomerase could be manipulated properly.

T-cell senescence in culture is associated with additional changes in gene expression. For example, in response to a mild heat shock, production of the major mammalian stress protein, *Hsp70*, is significantly reduced in senescent T cells compared to quiescent early-passage cells. The lower protein levels are caused by reduced transcription of the *Hsp70* gene (Effros et al. 1994*b*). This change represents a potentially important defect in view of the involvement of the *Hsp70* family of cytoplasmic proteins in the response to oxidative stress, viral infection, and the pathways of antigen processing and presentation. Replicative senescence in T cells also affects the precisely orchestrated series of steps involved in programmed cell death. Mild heat stress, low-dose gamma irradiation, and antibodies to the T-cell receptor and to the Fas antigen can all initiate apoptosis in early-passage T cells, but this response is dramatically blunted in senescent cultures, where higher levels of the apoptosis protective protein, Bcl2, accumulate (R. B. Effros, unpublished data). A final change in gene expression documented for senescent T-cell cultures is the reexpression of the CD4 marker on mature CD8+ T cells. T cells with this "double positive" (CD4+ CD8+) phenotype have also been observed to increase with age in vivo (R. B. Effros, unpublished data).

T-Cell Replicative Senescence Occurs In Vivo

The Holy Grail of replicative senescence studies has been to demonstrate that aging in vivo is accompanied by the accumulation of cells, showing all the characteristics of replicative senescence identified in cell culture. Information of this type would provide strong evidence that the complex process of biological aging is somehow reflected in the in vitro model of cellular aging. Our identification of loss of CD28 gene expression as a marker of T-cell senescence in vitro therefore provided an unparalleled opportunity to test whether similar cells accumulate during aging in vivo.

We measured the proportion of peripheral blood T cells expressing the CD28 costimulatory molecule in donors of different ages. Previous studies had documented that 99% of all T cells in neonates express CD28 (Azuma et al. 1993). Our own studies demonstrated that CD28 expression on peripheral blood T cells decreases progressively with age, reaching levels as low as 45% CD28− T cells in some centenarians (Effros et al. 1994*a*). The possibility that T-cell replicative senescence occurs in vivo was also addressed in telomere studies. Telomere length measurements of peripheral blood lymphocytes of subjects ranging from newborn to age 100 years show a progressive shortening to the same 5–7-kb TRF length observed in T cells that had reached senescence in culture (Vaziri et al. 1993). Moreover, by flow-cytometric cell sorting of T cells derived from peripheral blood samples into CD28+ and CD28− fractions, we and others have shown that CD28− T cells have shorter telomeres than do CD28+ T cells from the same donor, indicating a distinct replicative history for the two cell populations (Effros et al. 1996; Monteiro et al. 1996). To determine the proliferative potential of the putatively senescent lymphocytes, sorted populations of CD28+ and CD28− T cells were stimulated with mitogens. Experiments of this sort have repeatedly shown that CD28− T cells have minimal proliferative capacity, even when cultured with stimuli that do not require binding to the TCR or CD28. Thus, in vivo aging is associated with a progressive increase in a population of T cells with the key characteristics of senescent T-cell cultures—namely, a loss of CD28 cell surface expression, shortened telomeres, and an inability to proliferate.

Increased proportions of CD28− T cells have been documented in other clinical situations in addition to aging. For example, in HIV-infected persons, $\leqslant 50\%$ of the peripheral blood T cells lack CD28 expression, and this value exceeds 65% within the CD8+ T-cell subset. Telomere measurements of sorted CD8+ CD28− T cells showed such cells to have the 5–7-kb TRF size observed in centenarians. Moreover, such T cells increase over time in individual donors and correlate directly with disease progression. The accelerated accumulation of T cells with characteristics of replicative senescence in HIV-infected individuals suggests that immune exhaustion, a hallmark of AIDS, may be caused in part by the Hayflick limit.

Interestingly, in both aging and HIV infection, the majority of CD28− T cells are in the CD8+ subset, the cell type mainly responsible for the immune control over viral infections and tumor progression. CD8+ T-cell se-

nescence may therefore partially account for the clinical observations of increased susceptibility to viral infections and cancer in both of these cohorts. Interestingly, similar clinical findings suggestive of diminished T-cell function have been reported in individuals with Down Syndrome (DS). DS, a congenital condition of complete or partial trisomy 21, is characterized by multiple signs of early senescence, and individuals with DS have increased proportions of CD8+ T cells and greatly enhanced susceptibility to viral and bacterial infections and acute leukemia (Cuadrado and Barrena 1996). Consistent with these clinical observations, accelerated telomere shortening has been documented in peripheral blood lymphocytes from DS versus age-matched controls (Vaziri et al. 1993).

The occurrence of replicative senescence in vivo may offer an explanation for many of the observations regarding immunosenescence. Numerous investigators have demonstrated that one of the major immune changes associated with aging is a decline in T-cell proliferation (Murasko et al. 1990). A second feature of the aging immune system is an increase in the ratio of memory to naive T cells, with the greatest proliferative deficit present in the memory cells (Miller 1994). It is also within the memory population that the most dramatic decline in calcium signaling occurs. Clearly, the cells that have reached replicative senescence in long-term culture are memory cells. They have been repeatedly stimulated to divide both with specific antigen as well as exogenous IL-2. Furthermore, >99% of the cells in a senescent culture express the CD45RO antigen, the marker for memory cells. This suggests that the memory T cells in an aged individual that show reduced proliferative potential may arise in vivo by the same mechanism as senescent cells emerging in long-term cultures. If senescent cells that arise in vivo are resistant to apoptosis, like their in vitro counterparts, their presence might compromise homeostatic control over the peripheral T-cell pool (Rocha et al. 1989). By remaining in the circulation instead of undergoing programmed cell death, senescent T cells potentially prevent renewal of the T-cell pool with more functional T cells.

In both aging and chronic HIV infection, replicative senescence has been observed mainly within the cytotoxic (CD8+) versus helper (CD4+) T-cell subset, possibly as a result of excessive stimulation during acute or chronic viral infections. CD8+ T cells are "programmed" to recognize peptides derived from *endogenously* processed proteins, such as the proteins synthesized in virally infected cells. The viral peptides are processed and presented on the cell surface of an infected cell in conjunction with class I major histocompatability complex molecules. The presence of high proportions of circulating CD8+ T cells showing characteristics of replicative senescence suggests that virus-specific T cells may be periodically restimulated in vivo to undergo proliferation. Examples of antigens that can provoke repeated or chronic stimulation of memory T cells are latent viruses (Epstein-Barr virus), pathogens with cross-reactive T-cell epitopes (e.g., influenza A; Effros et al. 1977), or immunogenic self-antigens. The result of this repeated stimulation would be that the progeny of certain clones of memory T cells eventually reach a state of replicative senescence. Consistent with this possibility, oligoclonal expansions of memory CD8+ T cells have been documented in elderly donors. The expanded T cells are reminiscent of T cells that reach senescence in culture, in that they are in an activated state, they lack CD28 expression, and they are unable to undergo proliferation (Posnett et al. 1994). In addition to the activation of CD8+ T cells in an antigen-specific manner, increased CD8+ T-cell turnover during acute viral infections has also been documented for T cells that are not involved in the specific antiviral response (Tripp et al. 1994). This so-called bystander stimulation, which preferentially affects CD8+ T cells, can be mimicked experimentally in mice by injection of certain cytokines whose levels normally increase during actual infections (Tough et al. 1996).

Whatever the underlying mechanism leading to the presence of high proportions of senescent CD8+ T cells in some elderly persons, the ultimate outcome is that fewer functional CD8+ T cells are available to combat viral infections and cancer. Thus, T cell replicative senescence may indeed affect lifespan by contributing to the increased mortality from certain diseases in the elderly. A major unanswered question that requires additional research is, are there *intrinsic* factors that may account for the large variability in the rate of human aging? One might speculate that telomerase induction patterns may vary between individuals, or that levels of *Hsp70* or Bcl2 proteins are subject to regulatory mechanisms, or even that the expression levels of CD28 or its ligands may contribute to variability in the aging of the immune system. These and other possibilities present exciting challenges for future investigation into the immunology and immunogenetics of aging.

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