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Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation☆

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Abstract

We have established an in vitro culture model of cellular aging in which antigen-specific T cells are stimulated repeatedly to divide until they reach the irreversible state of growth arrest known as "replicative senescence." T lymphocytes that reach replicative senescence in culture show complete loss of CD28 expression, shortened telomeres, undetectible telomerase, and reduced ability to produce heat shock proteins. We now document that in response to treatment with apoptotic stimuli, senescent CD8+ T-cell cultures show reduced apoptosis and diminished caspase 3 activity compared with quiescent early passage cultures from the same donor. Our results suggest that the progressive accumulation of T cells showing many of the hallmarks of replicative senescence during aging, chronic infection, and autoimmune disease may, in part, reflect the diminished capacity of such cells to undergo normal programmed cell death.

Keywords: Human; T lymphocytes; Apoptosis; Cellular proliferation; Replicative senescence

1. Introduction

We have analyzed the process of replicative senescence in CD8+ T cells by using a long-term culture system in which T cells from healthy young adults are stimulated repeatedly with antigen and monitored over time for a variety of functional and phenotypic parameters. Our research has documented that after multiple rounds of antigen-

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specific proliferation, CD8+ T-cell cultures enter an irreversible nondividing state known as "replicative senescence" (Effros and Pawelec, 1997). Similar findings have been reported for both clonal and bulk CD4+ T-cell cultures (Adibzadeh et al., 1996; Grubeck–Loebenstein et al., 1994), for autocrine driven CD4+ T-cell proliferation (Weng et al., 1995), and CD4+ T-cell proliferation induced by a combination of CD3 and CD28 antibodies (Levine et al., 1997).

There is accumulating evidence that replicative senescence, thus far characterized exclusively in cell culture, may be occurring in vivo in several clinical contexts. For example, endothelial cells isolated from arterial walls in areas associated with increased hemodynamic stress have shorter telomeres, indicative of increased cell turnover, than arterial endothelial cells from other vascular sites (Chang and Harley, 1995). Epithelial cells and keratinocytes with an unusual form of β-galactosidase associated with fibroblast senescence in culture are increased in frequency in skin samples from aged donors (Dimri et al., 1996). Lymphocytes from centenarians and individuals with Down syndrome share many characteristics with senescent T-cell cultures (Vaziri et al., 1993). The proportion of CD4+ T cells lacking CD28 expression increases over time in patients with rheumatoid arthritis (Schmidt et al., 1996), similar to CD4+ T cells undergoing multiple rounds of cell division in long-term culture. Finally, a dramatic example of the potential effect of replicative senescence in vivo is the recent demonstration that the proportion of CD8+ T cells resembling cells in senescent cultures increases progressively during human immunodeficiency virus disease, comprising >65% of the peripheral T-cell pool (Effros et al., 1996). Thus, the process of replicative senescence may have profound effects on cellular dynamics and disease pathogenesis in a variety of clinical situations.

Replicative senescence can be distinguished from other nonproliferative states such as quiescence and anergy (Beverly et al., 1992) by the irreversible nature of its cell cycle arrest. Senescent T-cell cultures are in a permanent G0/G1 state that cannot be overcome by stimulation with specific antigen, supra-optimal doses of interleukin-2 (IL-2), or PMA plus ionomycin (Perillo et al., 1993). Because activation state and/or entry into cell cycle have been implicated in several forms of apoptosis (Boehme, Lenardo, 1993; Lissy et al., 1998; Radvanyi et al., 1996; Renne et al., 1997; Renno et al., 1995; Tuosto et al., 1994), we hypothesized that replicative senescence in T cells might lead to alterations in the process of programmed cell death that is essential for proper immune function. To test whether replicative senescence affects the ability of T cells to undergo apoptosis, we compared the same population of T cells at different stages of their in vitro replicative lifespan for their response to apoptotic stimuli. In this report, we provide the first documentation that CD8+ T-cell replicative senescence is associated with resistance to apoptosis. Our study suggests that if a similar resistance to apoptosis is true for the putatively senescent T cells that have been identified in vivo, the defect will have a profound effect on the quality and function of the peripheral T-cell pool.

2. Materials and methods

2.1. Long-term T-cell cultures

Alloantigen-specific T-cell cultures were initiated as described previously (Perillo et al., 1989). Briefly, 10^6 peripheral blood mononuclear cells are mixed with 10^6 allogenieic irradiated (10 000 Rad) EBV-transformed B cells. Cultures are maintained in RPMI with

20% fetal calf serum (Life Technologies, Grand Rapids, NY, USA) containing 20 U/mL recombinant IL-2, and are subcultivated to a density of 2×10^5/mL whenever the cell counts exceeded 8×10^5/mL. In some experiments, CD4+ T cells are removed by negative selection using magnetic bead depletion (Dynal). Purity of the remaining CD8+ T-cell population is verified by flow cytometry and is routinely >95%. Restimulation is performed every 3–4 weeks with the same stimulator cells as in the original activation culture. At each passage, viable cell counts (determined by trypan blue dye exclusion) are recorded and used to calculate the number of population doublings since the previous passage. A senescent culture is defined as one that shows no proliferation in response to antigenic stimulation, monoclonal antibodies (mAbs) to CD3+CD28, or increasing doses of IL-2. No decrease in cell viability is associated with replicative senescence. In our T-cell cultures, replicative senescence is reached after approximately 25 population doublings, but because of variation between donors, results are presented by using "percent total culture lifespan completed" rather than "number of population doublings completed."

2.2. Apoptosis assays

Because senescent cultures are by definition quiescent, to standardize the experimental conditions between early and senescent passage cells, all apoptosis experiments were performed on quiescent cultures. Both early passage and senescent cultures were restimulated with antigen and apoptosis was induced 21 days later, the time-point at which antigen-induced proliferative activity in the early passage cultures had ceased. The following stimuli were used in these studies: dexamethasone (2×10^{-6} M); anti-CD3 mAb (2 μg/mL and coated onto wells of flat-bottomed 24-well plates and placed at 4° overnight); anti-Fas mAb; the protein kinase C (PKC) inhibitor, Staurosporine (SRS; final concentration, 20 nM, Sigma), hyperthermia (a one hour incubation in a 43° water bath), galectin-1 (20 μM, graciously provided by Dr. Linda Baum, UCLA Department of Pathology and Lab Medicine), and IL-2 withdrawal. After apoptosis induction, cells were plated in complete media at 10^6/well in flat-bottom, 24-well plates (Costar, Cambridge, MA, USA), and placed in a 5% CO_2 humidified 37° incubator for periods ranging from 24 to 96 h. Apoptosis was evaluated both microscopically by morphologic criteria, and flow cytometrically using the Oncor Annexin V/propidium iodide assay system, in which live cells are analyzed for membrane expression of phosphatidylerine (PS), one of the earliest features of apoptosis. Flow cytometric evaluation of Annexin V binding was performed using a FACScan flow cytometer. At least 20 000 events were collected for each sample, and data analysis was performed by using a Macintosh computer equipped with FACScan Cell Quest software (BectonDickenson, Palo Alto, CA). Results are shown for the timepoints at which Annexin V staining was highest, which varied Between cultures, but was generally either at 48 or 72 h for all stimuli except dexamethasone, where maximum levels were reached at 96 h.

2.3. Caspase 3 activity

The Pharmacia "Apo-Alert™" assay system was used to detect caspase 3 activity. At time points ranging from 20 min to 6 h after apoptosis induction, $2–4 \times 10^6$ cells were resuspended in 50 μL of lysis buffer, incubated for 10 min at 4°, and centrifuged at 12 000 rev./min. The supernatant was diluted 1:1 with 2× reaction buffer contain-

ing 10 mM dithiothreitol, and was then incubated at 37° for 1 h. Caspase 3 protease activity was detected by spectrophotometry (405 nm). Fold increase in protease activity was determined by comparing the OD (Optical Density) for experimental cells to the OD of the uninduced control cells.

3. Results

Long-term culture alloantigen-specific T cells were used to determine the effect of replicative senescence on the response to apoptosis-inducing stimuli. In this study we compared early passage cultures, which have completed $<$50% of their total replicative lifespan, and late passage or senescent cultures, which have completed $>$75% or 100% of their respective replicative lifespans in the absence of experimental apoptosis induction, no difference in cell death was observed between early passage and senescent cultures. Indeed, the viability of all cultures we have studied exceeds 90%, and senescent cultures can be maintained in a viable, albeit nonproliferative state, for several months.

In preliminary experiments on early passage cultures, we determined that the inducible apoptosis levels in the actively proliferating cultures immediately after antigenic restimulation are significantly greater than those observed in the same cultures immediately before antigenic restimulation, when the cells were in a quiescent state (data not shown). However, because senescent cultures are in a permanent state of quiescence, it was critical to ensure that the early passage cultures were in a quiescent state as well. Thus, the levels of apoptosis presented in this report for young cultures do not represent their maximum potential, but rather the extent of apoptosis inducible during the non-cycling, quiescent state that is reached 21 days after antigenic stimulation. By using propidium iodide staining on semipermeabilized cells, we have previously documented that both early passage quiescent cultures and senescent cultures have comparable levels ($>$93%) of cells with a G_1/G_0 DNA content (Perillo et al., 1993). To ensure that all cultures were treated in identical fashion, the senescent cultures were also restimulated with antigen 21 days before apoptosis induction, although in these cultures antigenic restimulation did not induce proliferation or augment apoptosis. In all experiments, early passage and senescent cultures from the same donor were tested in parallel.

The first series of experiments compared early and late passage T-cell cultures established from two different donors for cell death over a 4-day period after treatment with dexamethasone. Whereas the maximum level of cell death was reached at 72–96 h irrespective of culture age, T cells from early passage cultures showed significantly higher proportions of cells undergoing apoptotic death at all timepoints tested (Fig. 1). These experiments provide the first demonstration that replicative senescence in T cells is associated with a resistance to apoptosis.

The initiation phase of apoptosis can be signaled by a wide variety of stimuli, some involving receptor ligation and others constituting subnecrotic damage. To determine whether the diminished apoptosis observed in late passage cultures is restricted to particular modes of induction, we evaluated apoptosis after a wide range of stimuli. In preliminary experiments (data not shown), we verified that flow cytometric evaluation of Annexin V staining and fluorescence microscopy morphologic analysis of propidium iodide stained cells gave similar results. Fig. 2 (panels a–f) shows the percentage of Annexin V positive cells at the timepoint of maximum response following treatment with (a) anti-Fas; (b) galectin-1, an endogenous lectin that is abundantly present in vivo in

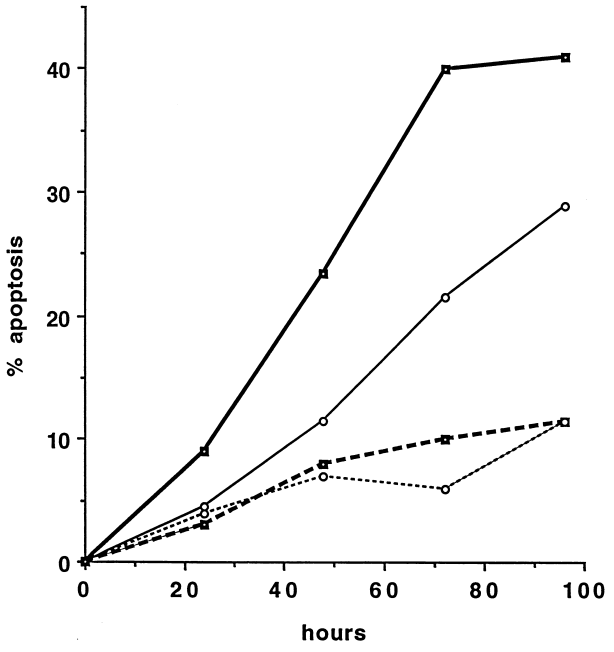

Fig. 1. Time course of apoptosis in early passage and senescent cultures. Apoptosis induced by dexamethasone treatment of quiescent early passage (solid lines) and senescent cultures (broken lines) derived from two different young healthy donors. Apoptosis was evaluated using the Oncor Annexin V kit at 24, 48, 72, and 96 h.

anatomical areas associated with high levels of apoptosis (Perillo et al., 1995); (c) IL-2 withdrawal; (d) SRS, a protein kinase c inhibitor; (e) mild heat shock; and (f) anti-CD3. The data in Fig. 2 show that in response to six different stimuli, late passage/senescent cultures show significantly lower levels of apoptosis than quiescent early passage cultures derived from the same donor. Although apoptosis was uniformly reduced at senescence, the differences between early and senescent culture apoptosis showed some variability among donors and according to the mode of induction. The most dramatic reductions in apoptosis in senescent cultures were observed in response to anti-CD3 and staurosproine treatment, where the apoptosis in senescent cultures was reduced by approximately 90% (panels d and f) compared with early passage cultures from the same donor.

As previously reported (Perillo et al., 1993), the particular long-term culture protocol used in these studies results in the progressive increase in the proportion of CD8+ cells, so that at senescence the cultures consist of >90% CD8+ T cells. To determine whether the decrease in apoptosis shown in Figs. 1 and 2 might be attributable to the presence of CD4+ T cells in the early passage culture, we compared apoptosis in cultures of different in vitro ages after magnetic bead depletion of CD4+ T cells. The data in Fig. 3 show that CD8+ T cells from early passage cultures undergo significantly greater apoptosis than CD8+ T cells from senescent cultures, confirming that replicative age is the determining factor in the resistance to apoptosis of senescent cultures.

The differences in apoptosis between senescent and early passage cultures involving stimulation with anti-CD3 (panel f) might be explained by reduced TCR expression and/or signal transduction ability that could be associated with replicative senescence. We believe this to be unlikely, because in earlier studies we showed that both CD3 expression

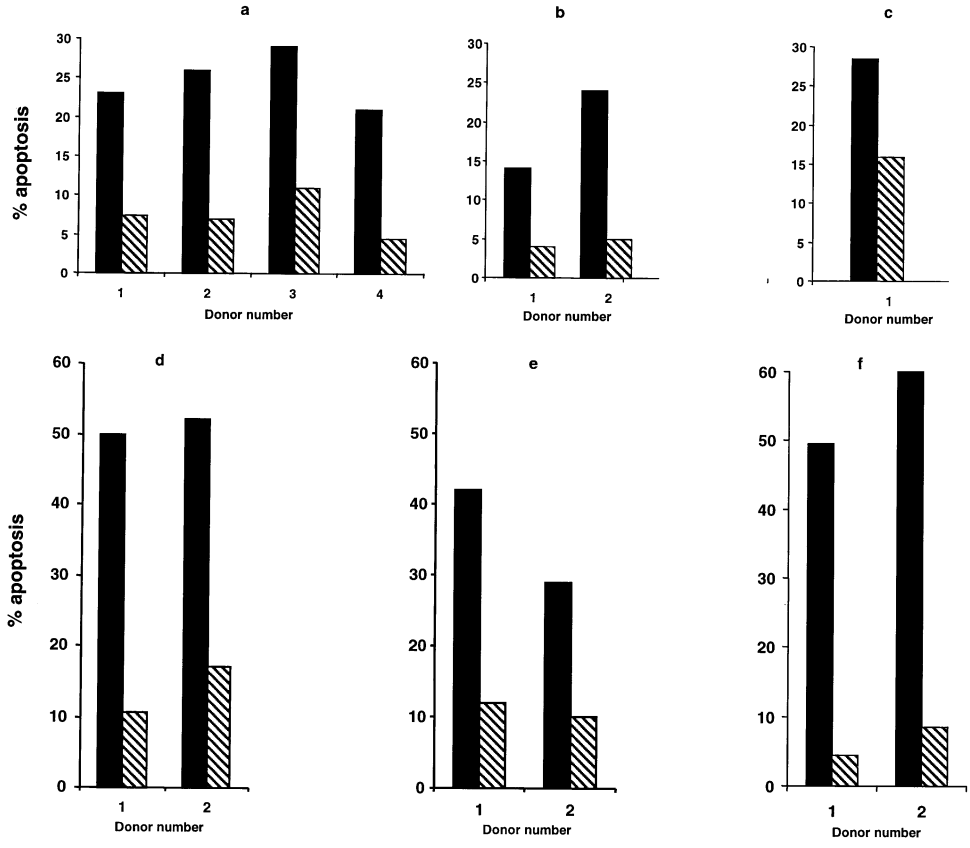

Fig. 2. Apoptosis levels in early passage and senescent T-cell cultures. Maximum values of apoptosis of early passage and senescent T-cell cultures (either 48 h or 72 h after apoptosis induction) in response to: (a) anti-Fas; (b) galectin-1, an endogenous lectin that is abundantly present in vivo in anatomical areas associated with high levels of apoptosis; (c) IL-2 withdrawal; (d) SRS, a protein kinase c inhibitor; (e) mild heat shock: (f) anti-CD3. Apoptosis is expressed as the percent Annexin V positive cells (vertical axis) in early passage (shaded bars) and senescent (hatched bars) cultures.

and antigen-specific cytotoxicity are identical between early passage and senescent cultures (Perillo et al., 1993). Thus, it is specifically the apoptotic pathway, and not all TCR-mediated (T-cell receptor-mediated) functions, that is affected by senescence. The reduced apoptosis after treatment with anti-Fas antibody is also unlikely to be attributable to altered surface expression of Fas, because by flow cytometric evaluation, 100% of the cells in both early passage and senescent cultures express Fas, with no difference in the intensity of fluorescence (unpublished data). Thus, for at least two of the apoptosis-inducing stimuli, receptor density and/signal transduction are probably not the causes of the diminished apoptosis. Indeed, the observation of diminished levels of apoptosis in senescent T cells irrespective of the inducing stimulus (Figs. 1–3) implicates a global apoptosis-regulatory change associated with senescence. This notion is consistent with the large body of data showing that replicative senescence is not merely a cessation of cell division, but involves multiple changes in gene expression and function (Campisi et al., 1996; Smith and Pereira–Smith, 1996).

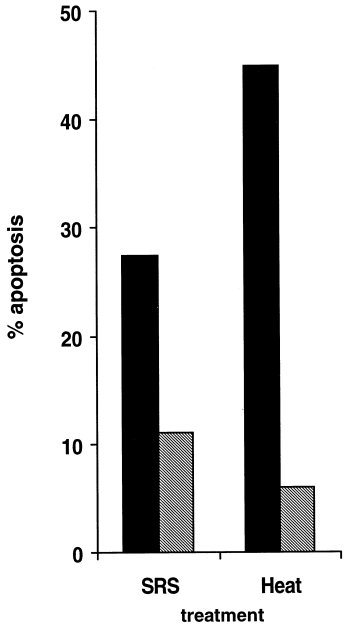

Fig. 3. Apoptosis levels at (24 h) of purified CD8+ T cells from early passage and senescent cultures treated either with SRS or mild heat shock .

Whereas cell death can be induced by a diverse set of signals, it is generally accepted that only one or two centralized mechanisms are operative in the execution of apoptosis. The caspase family of cytosolic serine proteases has been implicated in a variety of apoptotic pathways (Hirata et al., 1998). A key protease that is activated during the early stages of apoptosis is caspase-3 (Zapata et al., 1998; Zhou et al., 1998). The active form of caspase-3, which is found only in cells undergoing apoptosis, proteolytically cleaves and activates other caspases as well as targets in the cytoplasm and nucleus, such as PARP. We observed that the activity of caspase-3 in T-cell cultures after induction of apoptosis diminished with progressive number of population doublings completed. Similar results were obtained using anti-Fas treatment (Fig. 4), actinomycin D and C2-ceramide to induce apoptosis. It should be emphasized that in response to apoptosis induction, the caspase levels of quiescent T cells are all considerably reduced compared with the levels obtained in Jurkat tumor cell line or in even in actively proliferating normal T cells. For example, in a typical experiment, Jurkat cells show an increase in the OD values for CPP32 from 0.22 (control) to 0.99 (anti-Fas treated), whereas in quiescent cultures of normal T cells, the OD levels in the early versus late passage increase to only 0.65 and 0.33, respectively. Nevertheless, the data show that there is a progressive decline with population doublings completed in the activity of this protease after apoptosis induction.

4. Discussion

This study demonstrates that replicative senescence in CD+8 T-cell cultures is associated with significant resistance to apoptosis. Previous studies have documented

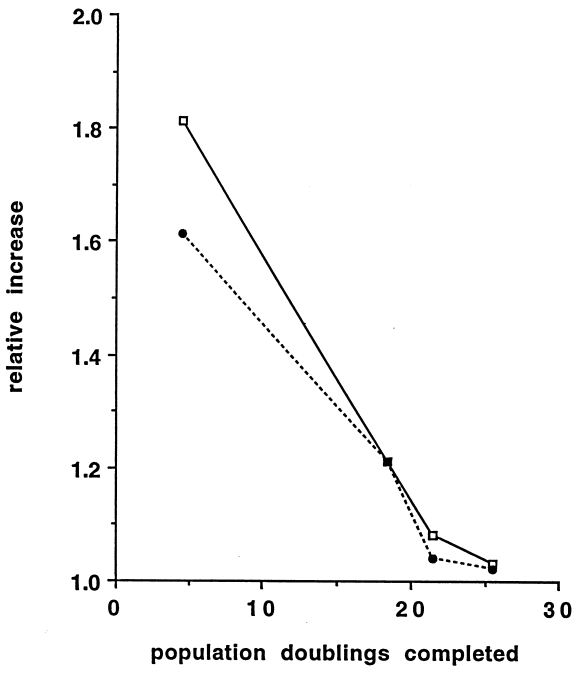

Fig. 4. Reduced caspase 3 activity with increasing numbers of population doublings. Six hours after anti-Fas antibody treatment, cells were lysed and caspase 3 activity measured. Results are expressed as the fold-increase in OD over the caspase 3 levels in untreated cultures (vertical axis) as a function of population doublings completed (horizontal axis) in long-term cultures initiated from two separate donors.

that replicative senescence in human T cells is associated with an irreversible block in cell division (Adibzadeh et al., 1996; Grubeck–Loebenstein et al., 1994; Levine et al., 1997; Weng et al., 1995), reduced hsp 70 production in response to stress (Effros et al., 1994), progressive telomere shortening from an initial 10–11 kb length to a 5–7 kb size at senescence (Vaziri et al., 1993), absence of telomerase activity (Bodnar et al., 1996), and loss of CD28 expression (Adibzadeh et al., 1996; Effros, 1997). The present study adds an important new characteristic to this list, namely, resistance to apoptosis. As was previously demonstrated for the reduced hsp70 gene transcription, the resistance to apoptosis is unlikely to be attributable to cell-cycle related differences at the time of apoptosis induction between early passage and senescent cultures, because all cultures tested were allowed to reach a noncycling, quiescent state by waiting 21 days after antigenic restimulation. Clearly, because apoptosis is linked to proliferative status, the inability to enter cell cycle is a factor in the reduced apoptosis observed in senescent cultures. However, our observation that noncycling early passage cultures from the same donor undergo higher levels of apoptosis suggests that additional control mechanisms related to replicative senescence itself may be involved. Moreover, because reduced ability to undergo apoptosis is observed in response to a variety of stimuli, several of which have not been linked to cycling, it may be that the state of replicative senescence itself may involve a central block in apoptosis signaling pathways. In this regard, it is highly relevant that replicative senescence in fibroblasts is associated with increased Bcl-2 expression and resistance to apoptosis (Warner et al., 1997), consistent with the notion that the senescence

"program" may influence apoptosis. We have preliminary evidence that T-cell replicative senescence is also associated with increased Bcl-2 expression (unpublished data), which may contribute to the prolonged survival of senescent CD8+ T cells in culture.

Impaired T-cell apoptosis in vivo has been reported during both normal aging and autoimmune disease. Based on the findings of the present study, it is possible that some of the diminished apoptosis capacity may reflect an increased proportion of putatively senescent T cells. For example, in centenarians, peripheral blood mononuclear cells show marked resistance to 2-deoxy-D-ribose-induced apoptosis compared to cells from young donors (Cossarizza et al., 1997). Because centenarians have T cells with two hallmarks of replicative senescence, namely an increased proportion of CD28− T cells and lymphocytes with 5–7-kb telomere lengths, the diminished apoptosis may be due to the dilution of functional T cells by apoptosis-resisitant, senescent T cells. Memory (CD45RO+) T cells have also been documented to show markedly reduced Fas-induced apoptosis (Herndon et al., 1997). The proportion of memory T cells is significantly increased both in elderly persons (Miller, 1990) and with progression to replicative senescence in culture (Perillo et al., 1993). In elderly persons, expanded clonal populations of T cells have been documented in two separate studies, and impaired apoptosis was suggested as one likely cause of the long-term persistance of these T cells (Posnett et al., 1994; Schwab et al., 1997). In the context of rheumatoid arthritis, clonally expanded populations of CD4+CD28− T cells present in the synovial fluid show increased Bcl-2 expression and resistance to apoptosis induced by IL-2 withdrawal, characteristics similar to cultured senescent CD8+CD28− T cells (Schirmer et al., 1998). It is unclear, however, whether all modes of apoptosis are impaired in senescent CD4+ T cells; anti-CD3 treatment of cultured senescent CD4+ T-cell clones actually results in augmented apoptosis compared (Pawelec et al., 1996). Studies in aged mice document reduced T-cell apoptosis in response to Fas (Zhou et al., 1995), mild hyperthermia, anti CD3, γ irradiation, and staurosporine (Spaulding et al., 1996). Interestingly, old mice subjected to the regimen of dietary caloric restriction retain youthful levels of T-cell apoptosis compared to their ad lib-fed littermates (Spaulding et al., 1996). This increase in T-cell apoptosis is associated with improved viral immunity (Effros et al., 1991), reduced tumor incidence, diminished autoantibodies and dramatic lifespan extension (Weindruch and Walford, 1982; Weindruch et al., 1986), suggesting that appropriate T-cell apoptosis contributes to the increased health and longevity of these mice.

Replicative senescence in T cells is in many ways at the opposite end of the spectrum from T-cell development in the thymus. Early T-cell development in the thymus is accompanied by robust cell division and massive apoptotic cell death, whereas replicative senescence constitutes a late stage of T-cell differentiation and is associated with the absence of proliferation and diminished apoptosis. These two features of replicative senescence may prominently influence the composition of the peripheral T-cell pool, which is critically dependent on the combined effects of clonal expansion and T-cell apoptosis. Furthermore, because the size of the T-cell pool, and specifically, the proportion of virgin and activated CD8+ T-cell populations, has been demonstrated to be rigidly controlled by homeostatic mechanisms (Freitas et al., 1996; Rocha et al., 1989; Tanchot, Rocha, 1995), the absence of appropriately timed apoptosis in a significant proportion of memory T cells will undoubtedly influence the

overall T-cell repertoire. An example of a repertoire effect that may be the outcome of replicative senescence is the expanded oligoclonal populations of CD8+ T cells seen in many elderly persons (Posnett et al., 1994). The expanded cells described in that study resemble senescent T-cell cultures in that they are poorly proliferative and lack CD28 expression (Effros, Pawelec, 1997). Moreover, the documented reduction in the telomere length of CD28− versus CD28+ T cells (Effros et al., 1996; Monteiro et al., 1996) further reinforces the notion that the CD28− T-cell populations seen in the elderly are the progeny of cells that have undergone extensive cell division. The results of the present study suggest that attempts at immune reconstitution to enhance repertoire diversity might require novel immunomodulatory approaches to physically remove senescent T cells whose presence may inhibit clonal expansion and repopulation by more functional cells.

In conclusion, our studies provide the first documentation that replicative senescence in CD8+ T cells is correlated with resistance to apoptosis. The demonstration that T cells showing many of the characteristics of replicative senescence can be isolated from the peripheral blood in a variety of clinical situations underscores the need to further investigate the underlying mechanism of this stage-specific resistance to apoptosis.

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