Regulation of Telomere Length and Telomerase in T and B Cells: A Mechanism for Maintaining Replicative Potential

Review

Nan-ping Weng,*† Karen S. Hathcock,†
and Richard J. Hodes*†‡
*National Institute on Aging
National Institutes of Health
Baltimore, Maryland 21228
†Experimental Immunology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

Prior to antigenic stimulation, the precursor frequency of T cells or B cells with receptor specificity for any given antigen is extremely low, and it is the proliferative response to antigen that generates clonally expanded populations of effector cells as well as populations of long-lived memory cells that are capable of additional clonal expansion on reencounter with the same antigen. Thus, the ability of lymphocytes to undergo repeated cell division is essential for effective immune function. This review will focus on evidence suggesting that T and B lymphocytes, antigen-specific cells of the immune system, have adapted a mechanism otherwise used by malignant cells and germline cells to extend the replicative capacity necessary for lymphocyte function.

In marked contrast to malignant cells and cells of the germline lineage, normal somatic cells have a finite capacity for cellular replication, as first demonstrated in the seminal observation of Hayflick that human fibroblasts cultured in vitro undergo a limited number of cell divisions before reaching a state termed replicative senescence, in which further cell division cannot occur (Hayflick, 1965). Recently, considerable attention has focused on the possible role of telomeres and telomere length regulation in determining the replicative capacity of normal somatic cells, transformed cells, and cells of germline lineage. The telomeric ends of chromosomes have been identified as a candidate for the "replicative clock" that monitors cell division and accounts for cessation of replication. Telomeres are complex DNA–protein structures at the ends of linear chromosomes; they are composed of hexameric DNA repeats, (TTA GGG)n in vertebrates, and a number of tolemere-associated proteins (reviewed in Blackburn, 1991; Greider, 1996). Telomeres appear to be important in maintaining the integrity of chromosomes, protecting against illegitimate fusion events such as the formation of dicentric chromosomes, mediating chromosomal localization in the nucleus, and possibly in mediating selective silencing of subtelomeric genes (reviewed in Greider, 1996). As proposed by Watson (Watson, 1972), the template priming requirement of DNA polymerases results in loss of terminal bases during lagging strand chromosomal replication, leading (in the absence of compensatory mechanisms) to the shortening of telomeres with each cell division. As a consequence, telomere length has the potential of acting as a mitotic clock, reflecting the summated outcome of prior chromosomal replication and providing a measure of the residual replicative capacity of cells prior to reaching a critically short telomere length, at which time processes are activated that result in clonal replicative senescence. Consistent with this model, telomere shortening has been identified in vivo in normal somatic tissues as a consequence of human aging as well as in in vitro cultured human fibroblasts (Harley et al., 1990; Hastie et al., 1990; Lindsey et al., 1991; Vaziri et al., 1994; Chang and Harley, 1995).

Transformed cells and germline cells appear to have an unlimited capacity to divide and proliferate, suggesting that a compensatory mechanism must exist capable of avoiding the consequences of telomere shortening. Indeed, one such mechanism is mediated by the ribonucleoprotein enzyme telomerase, a subject of intensive recent experimentation (reviewed in Blackburn, 1992; Greider, 1996). The enzymatic activity termed telomerase is capable of synthesizing terminal TTAGGG telomeric repeats, thus extending telomere length and compensating for loss that occurs during chromosmal replication. Recent discoveries have identified the genes encoding two mammalian telomerase components, one a catalytic subunit that belongs to the reverse transcriptase family (Harrington et al., 1997a; Meyerson et al., 1997; Nakamura et al., 1997; Nakayama et al., 1998) and the other an RNA template component (Blasco et al., 1995; Feng et al., 1995), which together are sufficient to reconstitute telomerase activity in vitro (Weinrich et al., 1997) (Figure 1). In addition, a number of telomere-binding or telomerase-binding proteins have been identified, some of which are functionally active in the regulation of telomere length (Chong et al., 1995: Harrington et al., 1997a and 1997b; Smith and de Lange, 1997; van Steensel and de Lange, 1997; van Steensel et al., 1998). This review will summarize recent experimental findings that establish a clear relationship between telomere length and differentiation stage in both T and B cell lineages and demonstrate that there is stringent regulation of telomerase activity during T and B lymphocyte activation. These observations are consistent with a model in which maintenance of telomere length, mediated at least in part through the activity of telomerase, may function to support the capacity of lymphocytes for extensive clonal expansion. In addition to considering the limitations of currently available data in addressing this hypothesis, this review will discuss the opportunities created by recent genetic discoveries for more direct analysis of this seminal issue.

T Lymphocytes
Telomere Shortening In Vivo and In Vitro
Analysis of telomere length in human peripheral blood mononuclear cells reveals that telomere length decreases progressively with increasing age of the donor (Slagboom et al., 1994), including telomere reduction in both $CD4^+$ and $CD8^+$ T cells with age. Telomere length decreases in both naive ($CD45RA^+$) and memory ($CD45RO^+$) human $CD4^+$ T cells as a function of age at the rate of

‡ To whom correspondence should be addressed (e-mail: Richard_Hodes@nih.gov).

Telosome structure

Telomere   DNA-Protein complex   Telomerase

TTAGGGTTAGGGTTAGGGTTAGGG-3
AATCCCAATCCC-5   CAAUCCCAAUC

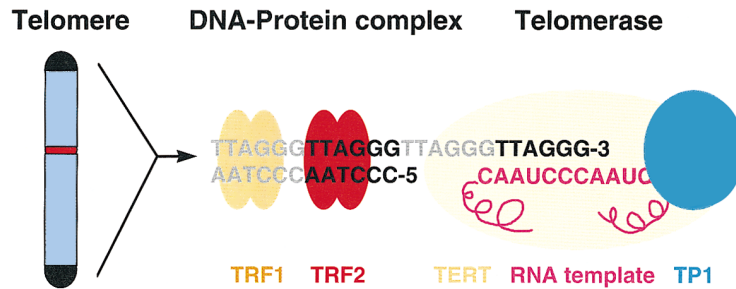TRF1   TRF2   TERT   RNA template   TP1

Chromosome

Figure 1. Telosome Structure
Telomeres are specialized structures at the ends of linear chromosomes. Telomeres consist of an array of highly conserved tandem hexamer repeats, (TTAGGG)n, and telomere binding proteins (TRF1 and TRF2). Telomerase is a multicomponent reverse transcriptase, with three telomerase-associated genes identified (hTERT, hTP-1, hTR).

approximately 33 base pairs (bp) per year (Weng et al., 1995). Strikingly, naive CD4$^+$ T cells have longer telomeres than those of memory T cells from the same individual, suggesting that differentiation from naive to memory cells reflects cell division in vivo; the difference in telomere length in naive and memory cells is remarkably consistent (1.4 ± 0.1 kb) at all ages, suggesting that the differentiation of naive to memory T cells involves a relatively constant number of cell divisions independent of the donor's age. A similar difference in telomere length is also observed in comparisons of human CD8$^+$ T cell subsets with CD28$^-$CD8$^+$ T cells, which may be derived from CD28$^+$CD8$^+$ T cells, exhibiting shorter telomeres than CD28$^+$CD8$^+$ T cells (Monteiro et al., 1996). Collectively, these results suggest that telomere length may correlate with the replicative history of T cells during in vivo activation and/or differentiation.

Previous studies of human fibroblasts indicated that telomere shortening during in vitro culture correlates with the induction of replicative senescence and have been interpreted to suggest that telomere shortening is in fact causally related to senescence (Harley et al., 1990; Hastie et al., 1990). A similar phenomenon occurs during culturing of human lymphocytes (Vaziri et al., 1993). When CD4$^+$ naive and memory T cells are grown in vitro by stimulating with immobilized anti-CD3 + anti-CD28 MAbs, naive T cells are capable of substantially more extensive division than memory T cells before reaching replicative senescence (Weng et al., 1995), a difference that corresponds to a 128-fold greater replicative capacity in naive CD4$^+$ T cells. Telomeres shorten at a rate of 50–100 bp per mean population doublings (MPD) for both naive and memory T cell subsets.

Recently, it was proposed that depletion of CD4$^+$ T cells occuring during HIV infection reflects the destruction and ultimate replicative exhaustion of the CD4$^+$ T cell lineage (Ho et al., 1995). If such clonal exhaustion and senescence occurs, it might be reflected in a critical level of telomere shortening in CD4$^+$ cells from HIV-infected donors. In contrast to this prediction, it was found that CD4$^+$ T cells from HIV$^+$ donors have telomeres that are equal to or slightly longer than those from HIV$^-$ donors (Wolthers et al., 1996; Palmer et al., 1997). Consistent with these findings, the replicative capacity of CD4$^+$ cells from HIV$^+$ patients is undiminished (Palmer et al., 1997), which fails to support the

contention that CD4$^+$ T cells undergo clonal exhaustion in HIV patients. (However, these studies do not exclude the possibility that CD4$^+$ T cells in HIV patients are predominantly a new cohort of cells recently derived from stem cells with long telomeres.) In contrast to the behavior of CD4$^+$ T cells, telomeres in CD8$^+$ T cells from HIV$^+$ donors are shortened relative to HIV$^-$ donors (Effros et al., 1996; Palmer et al., 1997), suggesting that HIV infection is associated with some alterations in the dynamics of T cell subpopulations. In other disorders, accelerated telomere loss has been reported in lymphocytes from individuals with Down's syndrome (Vaziri et al., 1993) and ataxia telangiectasia (Metcalfe et al., 1996), suggesting that telomere loss may somehow relate to the immune dysfunction in these disorders.

Although telomeres from human and from the mouse species *Mus musculus* and *Mus spretus* all consist of (TTAGGG)n repeats, these species differ in the average length of their telomeres. Telomeric restriction fragments (TRF) from *M. musculus* have mean lengths of 25–150 kb, whereas *M. spretus* have mean TRF lengths similar to those in human (5–15 kb) (Kipling and Cooke, 1990). Changes in telomere length with age or in vitro culture have generally not been detected in *M. musculus* somatic cells, perhaps reflecting the difficulty in detecting small changes in the large telomeres of this species. For this reason several studies have examined telomere length with in vivo aging and in vitro culture for shorter *M. spretus* telomeres (Prowse and Greider, 1995; Coviello-McLaughlin and Prowse, 1997). These studies have demonstrated that telomeres of early passage *M. spretus* fibroblasts shorten with in vitro culture in the absence of detectable telomerase activity. At approximately 60 population doublings (PD) the fibroblast lines appear to undergo crisis as characterized by phenotypic changes, after which the cell lines express telomerase and their telomere lengths stabilize. Significant telomere shortening is detected in vivo as a function of age in *M. spretus* spleen and brain but not in several other tissues. No more specific analysis of lymphoid tissues has been reported in mice or other nonhuman species. The potential for analyzing telomere length regulation during defined developmental events or immune responses has therefore not yet been fully realized in animal model systems.
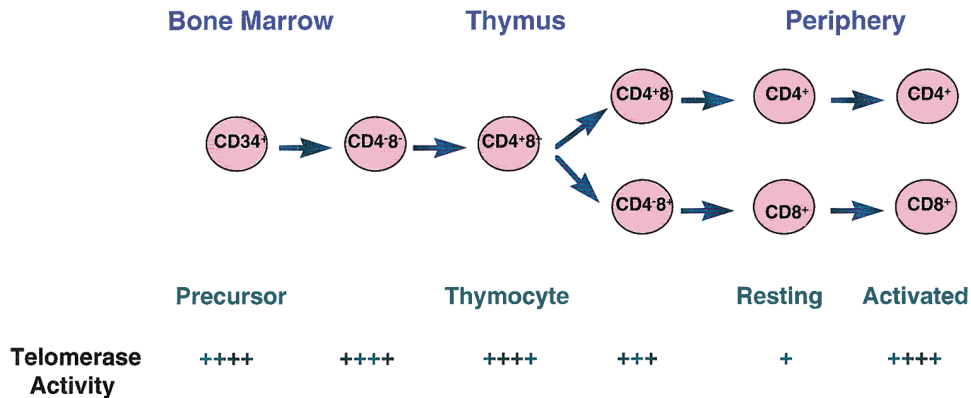
T lymphocyte development

Figure 2. Telomerase Expression in Human T Cell Lineage Development and Activation

T lymphocytes derive from bone marrow stem cells, differentiate in the thymus, and migrate and circulate in the periphery. Telomerase activity was measured in the course of T cell development and activation. The number of pluses reflects the relative levels of telomerase activity.

Telomerase expression

Until recently, it was proposed that telomerase is expressed in germline and malignant cells but not in normal somatic cells (Shay and Wright, 1996). However, a number of studies have tested the possibility that lymphocytes might use the expression of telomerase as an adaptive strategy for extension of their replicative capacity and have found that telomerase can be expressed at high levels in both T and B cells. Indeed, telomerase expression in T and B cells is highly regulated, as it is, for example, during T cell development in the thymus. High levels of telomerase activity are detected in the $CD4^-8^-$, $CD4^+8^+$, and $CD4^+8^-$ subsets of human thymocytes, with intermediate levels in $CD4^-8^+$ thymocytes (Weng et al., 1996) (Figure 2). Low or undetectable levels of telomerase are expressed in mature peripheral blood T cells, and higher levels in tonsil T cells, suggesting that telomerase may be induced in mature T cells in response to antigenic stimulation (Broccoli et al., 1995; Hiyama at al., 1995; Buchkovich and Greider, 1996; Igarashi and Sakaguchi, 1996; Weng et al., 1996). Telomerase is induced in peripheral blood $CD4^+$ T cells stimulated in vitro with anti-CD3, anti-CD3 + anti-CD28, or PMA/ionomycin. Interestingly, the ability of stimuli to induce telomerase in T cells in vitro is closely correlated with the ability of stimuli to induce entry into cell cycle (Buchkovich and Greider, 1996; Weng et al., 1996). Although induction of telomerase in peripheral blood T cells requires that these predominantly quiescent G0 cells enter cell cycle, telomerase activity once induced does not appear to be restricted to a particular cell cycle phase (Weng et al., 1996). Anti-CD3/CD28-induced telomerase expression in $CD4^+$ T cells requires new RNA and protein synthesis and requires signals mediated via protein tyrosine kinases (Weng et al., 1996). Cyclosporin A (CsA) completely blocks telomerase induction in $CD4^+$ T cells stimulated with anti-CD3 alone or with PMA/ionomycin, but it only partially blocks induction in T cells that had been stimulated with anti-CD3 + anti-CD28. Thus, CD28-mediated signals are critical for a CsA-resistant pathway of telomerase induction in combination with CD3-mediated signals. These results identify proximal signaling events that are required for telomerase induction in response to stimulation through CD3/CD28. However, it is not clear whether these requirements are specific for telomerase regulation or are linked to cell cycling events in general.

Although a number of human telomerase components have been identified, only one component, telomerase RNA template (hTR), has been analyzed in T cells (Weng et al., 1997a). hTR is ubiquitously expressed in all tissues tested regardless of the status of telomerase activity (Feng et al., 1995). However, levels of hTR are higher in thymocytes than in peripheral blood T cells, and in vitro activation of peripheral blood $CD4^+$ T cells leads to a significant, albeit modest, 2- to 5-fold up-regulation of hTR expression (Buchkovich and Greider, 1996; Weng et al., 1997a). Thus, hTR expression is regulated to some degree in normal T cell development and activation, correlating with telomerase activity in these T cells. It will be important to analyze during T cell development and activation expression of other telomerase genes, such as hTERT, the expression of which is highly regulated in germline and malignant cells. A more complete understanding of telomerase gene expression should shed light on how telomerase activity is regulated in normal cells and should provide a basis for future experimental interventions designed to modify telomerase activity.

Telomerase expression has also been examined in HIV-infected individuals. As discussed above, there is no significant decrease in telomere length in $CD4^+$ T cells from HIV^+ donors (Wolthers et al., 1996; Palmer et al., 1997). Thus, if there is an excessive proliferation of lymphocytes in HIV-infected individuals (Ho et al., 1995), the absence of telomere shortening could result from activation of telomerase in vivo in HIV-infected individuals. However, when telomerase activity was analyzed in $CD4^+$ and $CD8^+$ T cells from HIV-discordant monozygous twins ex vivo and after in vitro stimulation, no
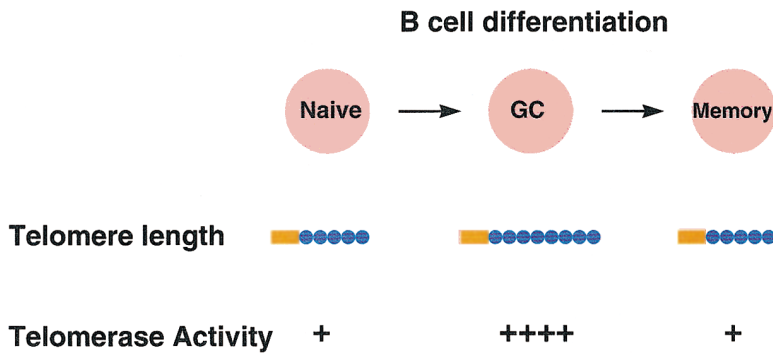
B cell differentiation

Telomere length

Telomerase Activity   +   ++++   +

significant difference was found either in the basal levels or in induced telomerase activity in CD4$^+$ and CD8$^+$ T cells from HIV$^+$ or HIV$^-$ monozygotic twins (Palmer et al., 1997).

The relationship between telomere length regulation and telomerase expression in human lymphocytes has been further studied in experiments that measured telomere length and telomerase activity in parallel during long-term stimulation of T cells. Stimulation of T cells induces both a rapid initial increase and a high peak level of telomerase activity. However, the magnitude and duration of telomerase activity decreases in long-term cultures with time and decreases with every subsequent stimulation. Strikingly, telomere shortening is not detected early after initial stimulation, when population doubling is extensive, but when telomerase activity is highest. At the later phases of long-term culture, when telomerase activity is significantly diminished, accelerated telomere reduction appears to occur. Similar patterns are observed in long-term cultures of human CD4$^+$ and CD8$^+$ T cells (Bodnar et al., 1996; Weng et al., 1997b). Thus, the relationship of telomere shortening to population doubling appears to be more complex in telomerase-expressing T lymphocytes than in human fibroblasts, which have no detectable telomerase activity and in which telomere shortening appears to be a more constant function of population doubling. These findings suggest, but do not directly demonstrate, that telomere length in activated CD4$^+$ and CD8$^+$ T cells may be stabilized by high levels of telomerase that are induced in these cells and may serve to prolong the capacity for extensive clonal expansion that is critical to physiologic T cell function.

In the mouse, high levels of telomerase activity are expressed in hematopoietic stem cells isolated from bone marrow and fetal liver (Blasco et al., 1995; Chadeneau et al., 1995; Prowse and Greider, 1995; Morrison et al., 1996). Telomerase expression is also detected in lymphoid organs including spleen and thymus (Prowse and Greider, 1995; Chadeneau et al., 1995; Blasco et al., 1997; Ogoshi et al., 1997). As was observed for human T cell populations, stimulation of mouse T cells with mitogens or with anti-TCR MAbs results in induction of telomerase activity (Ogoshi et al., 1997). In potentially more physiologic responses to antigen-specific stimulation, in vitro induction of telomerase activity is dependent upon both TcR and CD28/B7 costimulatory signals, requirements that parallel those for induction of T cell proliferation, suggesting that telomerase activity is induced in activated T cells coincident with proliferation

and might in principal modulate immune responses by extending the replicative capacity of antigen-activated T cells (Hathcock et al., 1998). Telomerase is also activated in vivo in response to antigen-specific stimulation, coincident with the clonal expansion that follows antigen-specific challenge (Hathcock et al., 1998). These findings indicate that induction of telomerase occurs in T cells during immune responses in vivo and may mediate a physiologic function in this setting.

B Lymphocytes
Telomere Length Dynamics
During B cell differentiation in secondary lymphoid organs, naive B cells undergo substantial cell division and clonal expansion in the course of differentiating into germinal center (GC) B cells and then memory B cells (Kelsoe, 1996). If cell division and an accompanying loss of telomeres occurs during this differentiation, it would be expected that telomeres would be longest in naive B cells and shorter in the GC and memory B cell populations. Unexpectedly, however, telomeres are longer in human tonsil GC B cells than in naive and memory B cells (Weng et al., 1997c) (Figure 3). Thus, it appears that, in contrast to telomere shortening that occurs during conversion of naive to memory CD4$^+$ T cells, telomere length is significantly increased during differentiation of naive B cells into descendent GC B cells. This represents a potentially novel indication that telomere elongation can occur in normal somatic cells and should lead to examination of the compensatory mechanisms that operate to elongate telomeres in GC B cells. Unfortunately, the lack of effective culture systems for long-term maintenance of proliferating normal B cells has precluded analysis of telomere length during replication of non-transformed B cells. However, telomere length reduction occurs with cell division in telomerase positive Epstein-Barr virus (EBV)-infected B cells at a rate similar to that observed in T cells cultured in vitro (100 $\pm$ 20 bp/MPD) (Guerrini et al., 1993; Counter et al., 1994).

Telomerase Expression
Like T cells, quiescent B cells isolated from peripheral blood express low to undetectable levels of telomerase (Norrback et al., 1996). Expression of telomerase is highly regulated during activation and differentiation of mature human B cells that occurs in GC in vivo and provides a mechanism for the observed increase in telomere length in GC B cells. In human tonsil B cell subsets, telomerase is low to undetectable in phenotypically naive B cells, becomes detectable at low levels during the

transition from naive to GC cells, and reaches high levels in GC centroblasts and centrocytes, then falling back to low levels in memory B cells (Norrback et al., 1996; Hu et al., 1997; Weng et al., 1997c) (Figure 3). The expression pattern of telomerase during B cell differentiation is intriguing in view of the observation that the longest telomeres are found in GC B cells, the same subset in which telomerase activity is highest, suggesting that telomerase may actually elongate telomeres in GC B cells to preserve the replicative lifespan of progeny memory B cells.

Telomerase activity is regulated in B cells, as it is in T cells, by signals mediated through a number of cell surface receptors. Telomerase is induced in human tonsil naive and memory B cells stimulated in vitro by the mitogen *Staphylococcus aureus* Cowan strain (SAC) or by engagement of the B cell receptor (BCR) in combination with additional signals, such as anti-IgM + anti-CD40, or anti-IgM + rIL-4 (Igarashi and Sakaguchi, 1997; Weng et al. 1997c). These stimulation conditions also result in B cell proliferation. Stimulation with anti-CD40, rIL-4 alone, or anti-CD40 + rIL-4, conditions that do not induce significant proliferation, fail to induce telomerase. Treatment of naive and memory tonsil B cells with either dexamethasone or γ-irradiation, conditions which induce apoptotic death of B cells, also fail to up-regulate telomerase activity (Weng et al., 1997c). Thus, these data suggest that telomerase can be induced by signals that induce proliferation and survival (Mandal and Kumar, 1997) but not by apoptotic signals. This linkage of telomerase expression to the induction of proliferation is again consistent with an adaptive and compensatory role in preserving the proliferative capacity of clonally expanding B cells.

When hTR expression was examined in B cell subsets, it was found that levels of hTR expression are approximately 2-fold higher in GC B cells than in naive and memory B cells and are increased 2- to 3-fold in naive and memory B cells after SAC treatment (Weng et al., 1997c). The high expression in GC B cells is further confirmed by in situ hybridization (Yashima et al., 1997). These results suggest that hTR is regulated and may contribute to overall activity of telomerase in B cells, although the magnitudes of observed differences in hTR expression are generally much less than the corresponding differences in telomerase activity.

Experimental Modification of Telomerase Activity and Telomere Length

Observed correlations between telomere length or telomerase activity and developmental stage or activation state are consistent with the hypothesis that telomere length and telomerase enzymatic activity play a functional role in the immune system. Conditions which induce proliferation of B or T cells induce expression of telomerase, which may serve to slow the rate of telomere shortening. Maintenance of telomere length may in turn conserve the residual capacity for further cell division. More definitive testing of this hypothesis will require experiments that directly manipulate telomere length and telomerase activity. To this end, a telomerase deficient mouse (mTR$^{-/-}$) has recently been derived by

germline deletion of the mouse telomerase RNA gene, allowing for the first time a direct examination of the role of telomerase function in vivo (Blasco et al., 1997; Lee et al., 1998). Mice bred as homozygous telomerase-deficient animals exhibited gradual telomere shortening in embryonic fibroblasts with each generation, with little, if any, expressed phenotype detected in these mice for the first several generations of breeding, perhaps because of the initially long telomeres present in the *M. musculus* mice studied. In later mouse generations, somatic cells from these mice showed progressive telomere shortening accompanied by increased aneuploidy and chromosomal abnormalities. By generation 6 (G6), these mice show evidence of impaired reproductive function associated with decreased germ cell production, decreased hematopoietic stem cell colony-forming capacity, and a decreased in vitro proliferative response of spleen cells to stimulation with Con A, PMA + ionophore, or anti-CD3 + anti-CD28 (Lee et al., 1998). When compared to activated spleen cells from control mice, activated cells from G6 telomerase-deficient mice had equivalent proportions of cells in [G2, S, M] phases of the cell cycle, but an increased proportion of these activated cells were apoptotic. Despite impaired proliferative responses to mitogenic and TCR-specific stimulation, histological and flow cytometric analysis of bone marrow, spleen, and thymus from the G6 telomerase-deficient mice did not differ from wild-type controls. G5 telomerase-deficient mice that were immunized in vivo and challenged with *Listeria monocytogenes* had survival and recovery rates similar to control mice, evidence of a normal and effective memory response.

Recently, it has been demonstrated that transfection of telomerase-negative human fibroblast or epithelial cell lines with the gene encoding the telomerase catalytic component results in expression of telomerase activity by these cells (Bodnar et al., 1998). In addition, these cells exhibit substantial increases in replicative capacity as well as telomere length. Manipulations such as this may also be applicable to cells of the immune system, allowing an analysis of telomerase function in maintenance of telomere length or through other as yet unappreciated functions of this unique enzyme.

Conclusion

Recent studies have established that telomere length is altered during differentiation of both T and B lymphocytes. There exists a clear and strong correlation of replicative capacity with telomere length in normal somatic cells. In human T cells, this correlation extends to differences in telomere length and replicative capacity in subsets such as naive and memory CD4 cells, or CD28$^-$positive and negative CD8 T cells. In addition, telomerase, a unique reverse transcriptase that is capable of extending telomeric length, is highly regulated during development and activation of both T and B lymphocytes. Together, these findings have provided a basis for hypotheses linking telomere length regulation to a functional role in sustaining the capacity for extensive clonal expansion in antigen-specific lymphocytes. In addition to providing insights into basic immune function, manipulation of telomere length has potential therapeutic

applications as well. For example, the ability to extend the replicative capacity of cells such as hematopoietic stem cells or mature lymphocytes through telomerase induction by transfection could be critical to therapeutic approaches to adoptive cell transfer or reconstitution. In assessing the feasibility of such approaches, it will be critical not only to measure extension of the capacity for cell division but also to consider other possible consequences such as enhanced susceptibility to malignant transformation through dysregulated telomerase activity. Conversely, the proposed use of telomerase inhibition as a modality for anticancer therapy should consider the possible impact of such intervention on any telomerase-dependent aspects of immune function. The rapid pace of gene discovery and genetic engineering, in combination with a richness of available systems for studying immune cell biology, should allow vigorous pursuit of these remaining questions.

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