Expression of Catalytically Active Telomerase Does Not Prevent Premature Senescence Caused by Overexpression of Oncogenic Ha-Ras in Normal Human Fibroblasts

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ABSTRACT

All normal cells in culture display a limited capacity to divide and eventually undergo an irreversible growth arrest known as replicative senescence. The development of cellular immortality has been implicated as an important factor in the progression of human cancers. Expression of telomerase has been shown to elicit a bypass of senescence and the acquisition of an extended life span. Although oncogenic Ras induces malignant transformation in most immortal cells, it has been shown to cause a senescence-like cell cycle arrest in presenescent human fibroblasts. To test the relationship between the senescence-inducing effect of Ras and the senescence-bypassing activity of telomerase, we used retroviral vector infection to introduce the catalytic subunit of human telomerase into normal human lung fibroblasts. Cell clones displaying in vitro telomerase catalytic activity and life span extension were observed. However, these cells still became senescent after infection with a retrovirus vector expressing oncogenic Ha-Ras. No differences in premature senescence phenotypes between normal and telomerase-expressing cells were observed. A small number of colonies were recovered after the introduction of Ha-Ras into either normal or telomerase-expressing cells, but in all cases, these clones failed to express the exogenously introduced Ras. We propose that even in the presence of active telomerase, the cellular senescence machinery remains intact and can be activated by appropriate signals. Consequently, interventions aimed at the activation of the latent senescence program may be a fruitful approach in cancer therapy.

INTRODUCTION

Normal cells in culture display a limited capacity to divide and eventually undergo a process termed replicative cellular senescence (1). A number of senescence-associated phenotypes have been described to date (reviewed in Ref. (2); (a) irreversibility of the growth arrest; (b) maintenance of viability for extended periods of time (3); (c) increased cell volume and a distinct flat morphology (4); (d) the presence of SA-β-gal3 activity (5); (e) the absence of serum-dependent induction of the c-fos gene (6); (f) elevated expression of the Cdk inhibitor proteins p16INK4a and p21CIP1/WAF1 (7, 8); and (g) overexpression of plasminogen activator-inhibitor type 1 (9).

Telomere shortening has been proposed to constitute the mitotic clock by which cells count the number of elapsed cell divisions (10). The most direct evidence in support of this model is the observation that the introduction of active telomerase into normal human cells prevents telomere shortening and results in apparent immortality (11). It has been proposed that critical telomere shortening may generate a DNA damage-like signal transmitted in part by the tumor suppressor protein p53 (12) that triggers a cell cycle arrest mechanism in which the Cdk inhibitor protein p21CIP1/WAF1 plays a key role (13). Primary mouse fibroblasts lacking either p53 (14), p21CIP1/WAF1 (15), the p16INK4a/p19ARF locus (16), or p19ARF (17) immortalize with very high frequency. Likewise, interference with the p53 and Rb pathways in human cells has been shown to confer an extended life span and thus aid immortalization (18).

The Ras proto-oncoprotein is activated in approximately 30% of human cancers (19). Oncogenic Ras can transform established rodent cell lines but fails to transform primary cells (20). Transformation of primary cells also requires the cooperation of genes such as c-myec, dominant-defective forms of p53, adenosine E1A, or SV40 large T antigen, which facilitate the immortalization of cells (21). More recently, it has been shown that expression of Ha-Ras in normal human fibroblasts results in a G1 arrest that is correlated with an increased expression of p16INK4a and p21CIP1/WAF1 and that the arrest is phenotypically indistinguishable from replicative cellular senescence (22). In this study, we examine the relationship between the senescence-inducing effect of Ha-Ras and the senescence-bypassing activity of telomerase.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The human embryonic lung fibroblast cell strain LF1 was established and cultured as described previously (13). Cells were passaged 1:4 at 80–90% confluence, and each passage was considered as 2 PDs. The amphotrophic retrovirus packaging cell line PA317 was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum. In all of the experiments presented in this study, strains 9B3 and 9B5 were used between passages 4 and 6 (PD = 8–12; see Fig. 1A). Strain LF1 was used between passages 24 and 30, which corresponds to approximately the middle of its replicative life span (13). The human foreskin fibroblast cell strain BJ expressing the catalytic subunit of human telomerase (hTERT) clone B52 (11) was a kind gift of W. Wright (University of Texas Southwestern Medical Center, Dallas, TX). Cultures were propagated in DMEM supplemented with 10% fetal bovine serum. Strain BJ-B52 was used at passage 125 or higher (11).

Retroviral Vectors and Virus Infection. The pBabe puromycin-resistant retrovirus vector expressing human Ha-RasV12 cDNA was obtained from S. Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (22). The hTERT cDNA was obtained from R. Weinberg (Whitehead Institute, Cambridge, MA) and subcloned into the pWZL blasticidin-resistant retrovirus vector (obtained from J. Morgenstern, Ariad Pharmaceutical, Cambridge, MA). PA317 cells were electroporated with 100 μg of retrovirus vector DNA; after 48 h, they were selected with 3 μg/ml blasticidin. After 7 days, the cultures were pooled and immediately used for the production of viral supernatants. LF1 cells were infected with 8 μg/ml polybrene; after 48 h, they were selected with 3 μg/ml blasticidin. After 12–14 days, individual colonies were ring-cloned and expanded into clonal cell strains. Because it is not possible to precisely follow PD during the growth of colonies and the subsequent expansion, the passage number for each clone was arbitrarily set to 0 at the point when the clone was first expanded into a 10-cm dish.

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3 The abbreviations used are: SA-β-gal, senescence-associated β-galactosidase; Cdk, cyclin-dependent kinase; PD, population doubling; hTERT, human telomerase reverse transcriptase; TRAP, telomeric repeat amplification protocol; BrdUrd, bromodeoxyuridine.
Single Colony Growth Curves. Cultures of LF1 cells, their derivatives, or BJ-B52 were infected with pBabe Ha-Ras viral supernatants as indicated above and selected with 2 μg/ml puromycin. After 48 h of puromycin treatment, floating cells were washed away, and single drug-resistant cells were chosen randomly for microscopic observation. The culture vessels were marked such that individual cells could be identified, and the growth of cells into colonies was monitored by time-lapse photography at 12-h intervals.

Histochemical Procedures. Subconfluent cultures were labeled for 3 h with 15 μM BrdUrd and processed for histochemical staining as described previously (23). SA-β-gal activity was detected as described previously (5).

Assay of In Vitro Telomerase Activity. The TRAP assay was performed as described by Kim et al. (24), using the modifications described by Wright et al. (25).

Immunoblotting. Cells were washed twice with PBS and harvested by rapid lysis in Laemmli sample buffer (26). Aliquots (50 μg) of total cell protein were separated on 10% SDS-PAGE gels and transferred to Immobilon-P (Millipore) membranes. Immunoblotting was performed by standard procedures (27). Signals were detected using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham). The following primary antibodies were used: (a) p21 SC-397 (Santa Cruz Biotechnology); (b) p16 SC-759 (Santa Cruz Biotechnology); and (c) Ras 06–403 (Upstate Biotechnology).

RESULTS

Expression of Telomerase in Normal Human Fibroblasts. The hTERT cDNA was cloned into the blasticidin-resistant retrovirus vector pWZL, and high-titer supernatants generated in an amphotropic packaging cell line were used to infect early-passage normal human fibroblasts. Empty vector was used as the negative control. Clonal cell strains were established and screened using the TRAP assay to identify clones with the highest telomerase catalytic activity (Fig. 1B, Lanes 9 and 11). Two clones (9B3 and 9B5) were chosen on this basis, and subsequent passaging revealed that both displayed a robust degree of life span extension (Fig. 1A). Relative to a HeLa cell control (Fig. 1B, Lane 7), both clones displayed a similar level of TRAP activity.

Ha-Ras Causes Premature Senescence in Both Normal and Telomerase-expressing Human Fibroblasts. Ha-Ras was introduced into cells using a retrovirus vector carrying the puromycin drug resistance gene. After infection, cells were incubated in the absence of drug for 24 h to allow the expression of genes encoded by the vector. Puromycin was added to the medium, and incubation was continued for 48 h. Because the kinetics of cell killing elicited by puromycin are very rapid, at this point, essentially all noninfected, puromycin-sensitive cells were dead and could be removed by simply changing the medium. Infection with empty vector was used as the control.

To monitor the induction of premature senescence, random single cells were selected for observation 24 h after the addition of drug. Selected cells were monitored by microscope, and their growth was recorded by time-lapse photography (Fig. 2). The majority of empty vector-infected cells divided rapidly and grew into colonies (Fig. 2, A and C). In contrast, the majority of cells infected with the Ha-Ras-expressing vector ceased division by day 5 of drug selection (Fig. 2, B and D). Virtually all surviving cells acquired the enlarged morphology characteristic of senescent cells. Pulse-labeling with BrdUrd revealed an almost complete absence of incorporation (Fig. 3A). Histochemical staining for SA-β-gal activity was positive (Fig. 3B). Immunoblotting confirmed the expression of exogenously introduced Ha-Ras and showed a strong
The telomere hypothesis states that telomere attrition caused by DNA replication comprises the molecular clock that keeps track of cell divisions in somatic cells (10). In agreement with this hypothesis, it has recently been demonstrated that ectopic expression of active telomerase in presenescent human cells causes a very significant extension of the in vitro replicative life span (11). A large volume of evidence indicates that the molecular machinery that triggers and maintains the growth arrest characteristic of the senescent state is critically dependent on p53 and Rb function (18). Specifically, both the p16INK4a and p21CIP1/WAF1 Cdk inhibitors have been implicated in senescence (13, 28). However, the signal generated by the telomere clock and the manner in which it regulates the senescence machinery remain poorly understood (12).

The relationship between Ras-induced premature senescence and senescence observed during the physiological aging of cultures is not completely understood. It is known that under different conditions, Ras can elicit a variety of responses: (a) proliferation; (b) cell cycle arrest; or (c) terminal differentiation (29). Likewise, senescence-like phenotypes have been observed in response to a variety of treatments including DNA-demethylating drugs (30), ceramides (31), or inhibitors of histone deacetylation (32). Because it has been known for some time that senescence may be regulated by multiple pathways (33), the growing list of senescence-inducing agents should not come as a surprise.

Of all of the premature senescence phenomena, Ras-induced senescence is known to include the broadest range of senescence-associated markers; for example, in addition to the phenotypes discussed here, Ras-induced senescence is also characterized by the lack of serum-dependent induction of c-fos and the overexpression of plasminogen activator-inhibitor type 1 (22). Of particular interest are the key roles played by the p16INK4a and p21CIP1/WAF1 Cdk inhibitors. It can be argued that Ras-induced senescence represents an amenable experimental system with which to investigate the mechanisms that regulate the expression of p16INK4a and p21CIP1/WAF1, an issue of crucial functional importance for the understanding of physiological senescence.

The mechanism of Ras-induced premature senescence appears to be mediated by the mitogen-activated protein kinase pathway (34);...
of the three Ras effector loop mutants that interact preferentially with specific Ras effector proteins (35), only H-RasV12/S35, which interacts with Raf-1 but not phosphatidylinositol 3'-kinase or Ral guanidine nucleotide dissociation factor, was able to induce premature senescence. However, it should be noted that in our experimental setting, as well as that of Lin et al. (34) and Serrano et al. (22), there is significant (5–10-fold) overexpression of introduced Ha-Ras relative to endogenous Ras. Because Ras is very rarely amplified in tumors, the significance of the premature senescence phenotype to the physiological mechanisms of oncogenesis and tumor progression remains to be established.

Two basic hypotheses can be invoked to explain telomerase-induced life span extension: (a) telomerase expression may generate a signal that interferes with the senescence machinery; or (b) telomerase expression may delay or eliminate the generation of a senescence-triggering signal. We show here that the expression of active telomerase does not interfere with Ras-induced premature senescence. Furthermore, the Ras-induced senescence behavior arises so quickly that it is unlikely to be associated with telomere-length reduction. We therefore propose that the cell division clock and the machinery that executes senescence are mechanistically distinct and that activation of telomerase does not actively interfere with senescence mechanisms. This is in contrast to the life span extension and immortalization events elicited by oncoproteins such as SV40 large T antigen or adenovirus E1A, which are known to bypass the senescence program by directly subverting the activity of key components (18). However, it is of interest to note that even after oncogene-assisted immortalization events, the senescence machinery remains intact and, given the right conditions, potentially active (36).

In summary, we present evidence that during life span extension elicited by the expression of active telomerase, the intrinsic senescence machinery remains intact and can be activated by appropriate signals. The search for such senescence-activating signals may thus be a fruitful line of investigation in our search for therapies directed against telomerase-expressing cancer cells.

Fig. 3. A, BrdUrd incorporation after the introduction of Ha-Ras. BrdUrd incorporation was performed on pooled cultures on day 7 of drug selection. Empty vector was used as the control. A minimum of 300 cells were counted in each sample. B, in situ histochemical staining for SA-β-gal activity after the introduction of Ha-Ras into strain 9B5. Staining was performed on day 7 of drug selection. Strains LF1 and 9B5 behaved indistinguishably from strain 9B5. Cells infected with empty retrovirus vector were uniformly SA-β-gal negative. C, in situ histochemical staining for SA-β-gal activity after the introduction of Ha-Ras into strain BJ-B52.

Fig. 4. A, expression of Ha-Ras, p16INK4a, and p21CIP1/WAF1 in pooled cultures on day 7 of drug selection. Cells from twelve 10-cm dishes were harvested and analyzed by immunoblotting as indicated. Strain 9B3 behaved indistinguishably from strain 9B5. B, expression of Ha-Ras in breakthrough colonies. The few colonies arising after infection with Ha-Ras (Fig. 2, B and D) were cloned, expanded, and analyzed by immunoblotting. Strain 9B3 behaved indistinguishably from strain 9B5. A total of 15 colonies for each of the LF1, 9B3, and 9B5 strains were analyzed; Ha-Ras expression was not detected in any of the colonies.
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REFERENCES

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