Induced Senescence in HeLa Cervical Carcinoma Cells Containing Elevated Telomerase Activity and Extended Telomeres[1]

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Abstract

Proliferation of normal somatic human cells in culture is limited by replicative senescence, a growth-arrested state that appears to be triggered by the erosion of telomeres. Tumor cells such as HeLa cervical carcinoma cells, which contain short telomeres, can be induced to undergo senescence by various manipulations including oncogene withdrawal. Repression of the human papillomavirus (HPV) type 18 *E6/E7* genes in HeLa cells by the bovine papillomavirus E2 transcriptional regulatory protein results in reactivation of the dormant p53 and p105^{Rb} tumor suppressor pathways in these cells, repression of telomerase, and profound growth arrest. Strikingly, the growth-arrested cells rapidly and synchronously acquired numerous characteristics of primary cells undergoing replicative senescence. To explore the role of telomerase and telomere length in induced senescence, we expressed an exogenous *hTERT* gene, which encodes the catalytic subunit of telomerase, to generate stable HeLa cell clones with elevated telomerase activity and extended telomeres. Expression of the E2 protein in these cells repressed HPV E6/E7 expression, activated tumor suppressor pathways, and induced senescence as assessed by growth arrest, morphological changes, senescence-associated β-galactosidase expression, and increased autofluorescence. Cells carrying the *hTERT* gene and control cells displayed identical responses to E2 expression. Therefore, HeLa cell senescence induced by HPV repression is not triggered by short telomeres or low levels of telomerase activity.

Introduction

Most normal somatic human cells cannot proliferate indefinitely *in vitro*. After months of continuous culture and expansion, cells gradually and asynchronously cease division and enter a permanent growth-arrested state called replicative senescence (1, 2). Senescent cells are viable and exhibit a number of characteristic phenotypic and biochemical changes, including an enlarged and flattened morphology, expression of SA-β-gal[3] activity, and increased autofluorescence (1, 3, 4). Certain exogenous treatments can induce cells to rapidly acquire characteristics of senescent cells. For example, early passage rodent or human fibroblasts can be induced to undergo "premature" senescence by expression of an activated *ras* oncogene (5). Other oncogenes, usually of viral origin, or somatic mutations allow human cells to escape replicative senescence and continue to proliferate until they enter crisis in which proliferation is opposed by increasing levels of cell death, and there is no net increase in cell number. These oncogene products are thought to bypass senescence, at least in part, by neutralizing the tumor suppressor proteins p53 and p105^{Rb} (6, 7). Clonal cell populations with an unlimited division potential may emerge from crisis in a process called immortalization.

Replicative senescence is, thus, the first barrier that prevents the indefinite proliferation of cultured cells, and it appears to be an important tumor suppressive mechanism in animals (8–10). Cellular senescence may also underlie certain aspects of organismal aging (11). Thus, there is considerable interest in determining the signal that causes cells to senesce and in unraveling the mechanisms responsible for executing the senescence program. Several lines of evidence indicate that telomerase and telomere length play an important role in replicative senescence. As normal somatic cells are passaged, telomeres generally shorten with cell division, and senescent cells typically have shorter telomeres than do primary cells (12–14). In addition, primary and senescent cells in general express low levels of telomerase, the enzyme responsible for synthesizing new telomeric DNA at the ends of chromosomes. In contrast, most immortal and tumor cells maintain telomere length and express telomerase (12, 15–18). These findings suggest that telomere erosion may be the mechanism by which cells record their replicative history, and that short telomeres may initiate replicative senescence (19–22). Indeed, constitutive expression of an exogenous telomerase gene that prevents telomere shortening allows the indefinite proliferation of some primary human cell types, such as fibroblasts and retinal pigment epithelial cells (23–28). Immortalization of other cell types, such as keratinocytes, requires both the expression of telomerase and additional events, such as inactivation of the retinoblastoma

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[3] The abbreviations used are: SA-β-gal, senescence-associated β-galactosidase; HPV, human papillomavirus; BPV, bovine papillomavirus; TRAP, telomeric repeat amplification protocol.

tumor suppressor pathway (29, 30). Conversely, inhibition of telomerase activity in immortal or cancer cells leads to telomere shortening and eventual senescence or cell death (31–34).

The *in vitro* life span of human keratinocytes can be extended by the high-risk *HPV E6* and *E7* oncogenes (35), although immortalization appears to require additional as-yet-undefined events (30, 36). The high-risk HPV E6 protein binds to p53 and targets it for accelerated ubiquitin-mediated degradation, and the high-risk HPV E7 protein binds to hypophosphorylated members of the retinoblastoma family, resulting in their destabilization and the disruption of Rb/E2F repressor complexes (37–41). In addition, the E6 protein induces telomerase activity in keratinocytes (42, 43). Genetic studies indicate that the ability of the E6 protein to activate telomerase is essential for keratinocyte immortalization (29), and the reduction in telomere length that occurs in keratinocytes during passage prior to immortalization is arrested or reversed in HPV-immortalized cells (36, 43). Furthermore, essentially all cervical carcinomas, which develop from keratinocytes, and all cervical carcinoma-derived cell lines constitutively express the *E6* and *E7* oncogenes from integrated HPV genomes (35).

We are exploring the consequences of repression of *HPV E6/E7* genes in cervical carcinoma cell lines, including HeLa cells, which contain HPV 18 DNA and harbor wild-type *p53* and $p105^{Rb}$ genes. This is accomplished by infecting cells with a SV40-based recombinant viral vector that expresses the BPV E2 regulatory protein, which binds the HPV early promoter and represses transcription of the *E6* and *E7* genes (44–46). This results in reactivation of the p53 and $p105^{Rb}$ tumor suppressor pathways and a rapid and profound growth arrest in the G_1 phase of the cell cycle (44, 47–50). The growth-arrested cells rapidly and synchronously acquired numerous characteristics of primary cells undergoing replicative senescence including changes in morphology, expression of SA-β-gal, increased autofluorescence, and inhibition of telomerase activity (51). Similar results were obtained in the HT-3 (HPV30) and CaSki (HPV16) cervical carcinoma cell lines, as well as in freshly established cervical carcinoma cell lines containing HPV16 DNA, which suggests that senescence may be a general response to oncogene withdrawal in HPV-containing cells (51, 52). The E2 protein induced growth arrest only in cell lines containing HPV DNA, and constitutive expression of HPV E6/E7 prevented growth arrest and activation of tumor suppressor pathways (47, 49, 53). Transfection of HeLa cells with an E2-expressing vector also generated cells that displayed senescent characteristics (54).

These data suggest that induced senescence in cervical carcinoma cell lines is the result of a specific program that is activated by repression of the HPV oncogenes. Repression of the *E6* and/or *E7* oncogenes may generate a senescent signal *de novo.* Alternatively, proliferating HeLa cells may contain a pro-senescent signal, but expression of HPV E6 and/or E7 prevents the cells from executing the senescence program in response to this signal. Because of the importance of telomerase and telomere length in replicative senescence, we decided to assess the role of these factors in induced senescence in cervical carcinoma cells. The parental HeLa cells used in these experiments express telomerase but have short telomeres of about 2 kb pairs, a length shorter than that at which normal keratinocytes senesce but within the range reported for different strains of HeLa cells (55). In addition, E2 expression caused a rapid reduction in telomerase activity (51). These observations are consistent with the hypothesis that the loss of the HPV oncogenes allows the cells to respond to a telomere-based signal that initiates senescence. To test this hypothesis, we expressed exogenous telomerase in HeLa cells to generate cell clones with telomeres as long as those in proliferating keratinocytes and determined the response of these cells to expression of the BPV E2 protein. The results of these experiments indicate that increased telomere length and maintenance of high-level telomerase activity do not protect HeLa cells against growth arrest or senescence induced by HPV oncogene repression, which implies that induced senescence in cervical carcinoma cells is not triggered by short telomeres or low-level telomerase activity.

Results

Generation of HeLa Cells Expressing a Transduced *hTERT* Gene. The experiments reported in this article were carried out to test whether the short telomeres in HeLa cells or the low level of telomerase activity after E6/E7 repression was required for induced senescence. For this purpose, we determined the consequences of expressing the BPV E2 protein in HeLa cells that stably overexpressed the catalytic subunit of human telomerase (hTERT) from a heterologous promoter. We used infection with a recombinant SV40 viral vector, designated here the “E2 virus,” to acutely express the BPV E2 protein in a subcloned line of HeLa cells, HeLa/sen2 cells, which efficiently and synchronously undergo senescence in response to E2 expression and E6/E7 repression (51). We chose to use these cells because they display a very low background of proliferating cells after E2 expression. Therefore, the growth-inhibited cells are not overgrown by cells that escaped E2-induced senescence, and it is possible to analyze the phenotype of the arrested cells for several weeks. However, it should be noted that E2-induced senescence occurs not only in HeLa/sen2 cells but also in uncloned HeLa cells and other cervical carcinoma cell lines containing HPV16 or HPV30 DNA.

The *hTERT* gene was transduced into HeLa/sen2 cells by using a retrovirus containing the wild-type hTERT cDNA and a gene coding for resistance to G418 (*LXSN-hTERT*). Control cells were generated by infection with the empty LXSN retrovirus that contained the drug resistance gene only. Individual G418-resistant colonies were expanded into cell lines, and the lengths of their telomeres were determined. Genomic DNA was isolated and digested with a mixture of restriction endonucleases that do not cut within the (TTAGGG)*n* telomeric repeats. The digested DNA was resolved by agarose gel electrophoresis, denatured, transferred to a solid support, and hybridized to a telomere-specific oligonucleotide. Fig. 1 displays the results obtained from representative LXSN and hTERT clones, as well as from the parental HeLa/sen2 cells. The broad smears are attrib-

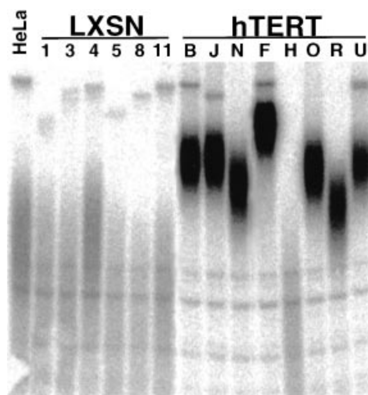

Fig. 1. Telomere lengths of cloned HeLa cell lines. Genomic DNA was prepared from parental HeLa/Sen2 cells (HeLa) and from clones generated with the LXSN vector or hTERT retroviruses. The DNA was digested with *Hin*fI and *Rsa*I, separated by electrophoresis, transferred to a nylon membrane, and probed with a telomere-specific oligonucleotide. *The seven lanes on the left* show the short telomeres present in the parental HeLa/Sen2 and the indicated LXSN cell lines. *The eight lanes on the right* show that the telomeres are considerably longer in the majority of the hTERT cell lines.

utable to the heterogeneous telomere lengths present even in these recently cloned cell lines. The telomeres from the HeLa/sen2 cells and LXSN clones are extremely short, ranging from 1.5 to 2.5 kb in length, far shorter than the length at which primary keratinocytes are reported to senesce. None of the seven control clones established with the empty vector exhibited an increase in telomere length relative to the starting cells. In contrast, the transduced *hTERT* gene caused a dramatic though variable increase in average telomere length in 15 of the 20 clones examined (Fig. 1 and data not shown). The intense hybridization signals obtained with the hTERT clones with extended telomeres are attributable in large part to the increased number of telomere repeats complementary to the probe in these telomeres and to the relative compression of the larger DNA fragments in the upper portion of the gel. The two hTERT clones with the longest telomeres, hTERT-F and hTERT-J, as well as the controls LXSN-1 and LXSN-4, were selected for further analysis. These four clones had similar growth rates with an average doubling time of 26 h (data not shown). Telomerase activity in these hTERT clones was markedly increased compared with the parental cells (see Fig. 5).

Effect of hTERT on HPV Gene Expression and Tumor Suppressor Pathways. We previously showed that infection of HeLa cells with the E2 virus led to greatly reduced levels of the HPV18 E6/E7 mRNAs, followed by activation of the p53 and p105^{Rb} tumor suppressor pathways. To determine whether the E2 protein had similar effects in the hTERT-transduced cells, we first tested if expression of the E2 protein repressed the endogenous HPV oncogenes. The LXSN and hTERT clones were either mock infected or infected with the E2 virus, and RNA was prepared 1 and 2 days after infection. As shown in the Northern blot in Fig. 2*A*, expression of the E2 protein caused rapid and complete repression of the *HPV18 E6/E7* genes in both the LXSN controls and hTERT clones. In both types of cells, HPV

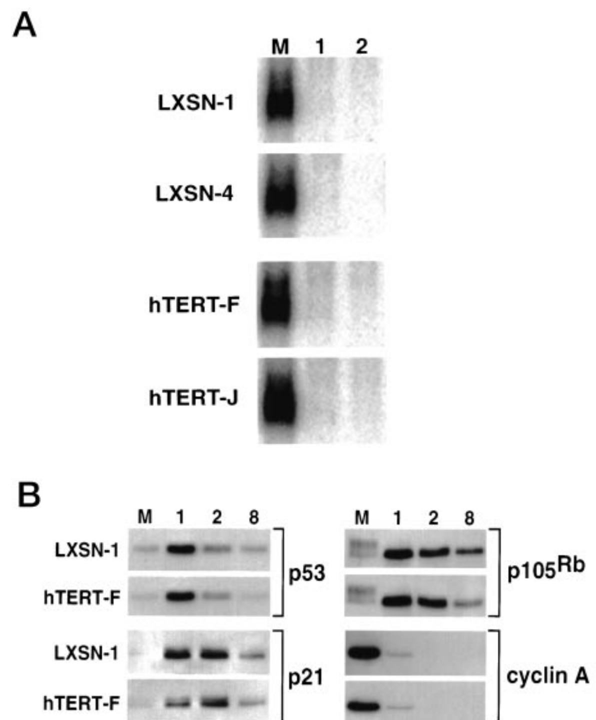

Fig. 2. HPV oncogene repression and activation of tumor suppressor pathways. In *A*, total RNA was isolated from the LXSN or hTERT cell lines 1 or 2 days after infection with the E2 virus, as indicated, or 2 days after mock infection (*M*). Five μg of RNA from each sample was electrophoresed in a formaldehyde denaturing gel, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled probe specific for the *HPV18 E6/E7* genes. Only the major band is shown here, but all of the other bands exhibited identical regulation after E2 expression. In *B*, total protein extracts were prepared from LXSN-1 or hTERT-F cells 1, 2, or 8 days after infection with the E2 virus, as indicated, or 2 days after mock infection (*M*). Five μg of protein from each sample was subjected to denaturing gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane and probed with antibodies specific for p53, p21, p105^{Rb}, or cyclin A. Hyperphosphorylated p105^{Rb} corresponds to the upper band in the mock lane, and hypophosphorylated p105^{Rb} is present in the lower band.

expression remained repressed 8 days after infection (data not shown).

We next determined whether expression of hTERT affected activation of the p53 and p105^{Rb} tumor suppressor pathways in response to the E2 protein. The p53 pathway was monitored by accumulation of p53 and by the induction of one of its transcriptional targets, the cdk inhibitor p21$^{sdi1/cip1/WAF1}$. Activation of the p105^{Rb} pathway was monitored by the increase of hypophosphorylated p105^{Rb} and by repression of cyclin A, the transcription of which is inhibited by the p105^{Rb}-E2F complex. Protein extracts prepared from mock-infected cells or from cells 1, 2, and 8 days after infection with the E2 virus were analyzed by immunoblotting. Fig. 2*B* presents the data from the LXSN-1 and hTERT-F cell lines, which display the greatest difference in telomere lengths. In both control and hTERT cells, p53 levels were greatly increased by 1 day after infection and then declined to mock-infected levels. p21 was also transiently induced in both cell lines although there was a slight delay in the hTERT-F cells. The E2 protein also induces mdm2 expres-

Table 1 Effect of the E2 protein on cellular DNA synthesis

LXSN and hTERT clones were assayed in quadruplicate for their ability to incorporate [^{3}H]thymidine 2 days after mock-infection or infection with a recombinant virus expressing the *BPV E2* gene. The average counts per minute (cpm) incorporated are shown in the first two columns.

	Mock-infected	E2-infected	% inhibition[a]
LXSN-1	155106	7568	95.1
LXSN-4	167508	4127	97.5
hTERT-F	113652	2898	97.4
hTERT-J	146159	2320	98.4

[a] Calculated by subtracting the cpm from E2-infected cells from that of the mock-infected cells, dividing by the mock cpm, and multiplying by 100.

sion (47, 51), which is presumably responsible for the subsequent reduction in p53 and, in turn, p21 expression. In both cell lines, infection with the E2 virus also resulted in a rapid but transient increase in the levels of the hypophosphorylated, active form of $p105^{Rb}$ and a decrease in the hyperphosphorylated form, and cyclin A was repressed. The other retinoblastoma family members, p107 and p130, as well as several other E2F-responsive genes, responded identically to E2 expression in control and hTERT clones, despite the presence of long telomeres in the latter cells (data not shown). The LXSN-4 and hTERT-J lines also responded to E2 expression by transiently activating the p53 and retinoblastoma tumor suppressor pathways to a similar extent and with similar kinetics (data not shown). Thus, increased telomerase activity and extended telomeres did not affect the induction of tumor suppressor pathways after repression of HPV oncogene expression.

HeLa Cells Expressing Telomerase Remain Sensitive to E2-mediated Growth Arrest and Senescence. Because expression of the E2 protein repressed HPV18 E6/E7 expression and activated the p53 and $p105^{Rb}$ pathways in the hTERT clones, we determined its effect on the cellular phenotype. The transduced clones were infected with the E2 virus, and cellular DNA synthesis was determined 2 days later by measuring the incorporation of tritiated thymidine (Table 1). As expected, after infection with the E2 virus, the control LXSN clones incorporated only about 3% as much thymidine as did mock-infected cells. Expression of the E2 protein reduced DNA synthesis to a similar extent in hTERT clones, indicating that increased telomerase activity and longer telomeres offered no protection from the inhibition of DNA synthesis.

Although hTERT expression did not prevent E2-induced growth arrest, it remained possible that the arrested cells were not senescent. Therefore, we examined the phenotype of the arrested hTERT cells to determine whether they underwent senescence. Eight days after mock infection or infection with the E2 virus, cellular morphology was determined by phase-contrast microscopy (Fig. 3*A*). The mock-infected cells formed discrete, compact colonies of small cells. The E2 virus-infected cells did not form colonies. Instead, they failed to form tight cell-cell contacts, became motile, adopted an elongated morphology, and appeared larger and more granular, resembling the morphology of senescent cells. Importantly, the LXSN and hTERT cells responded identically to E2 expression.

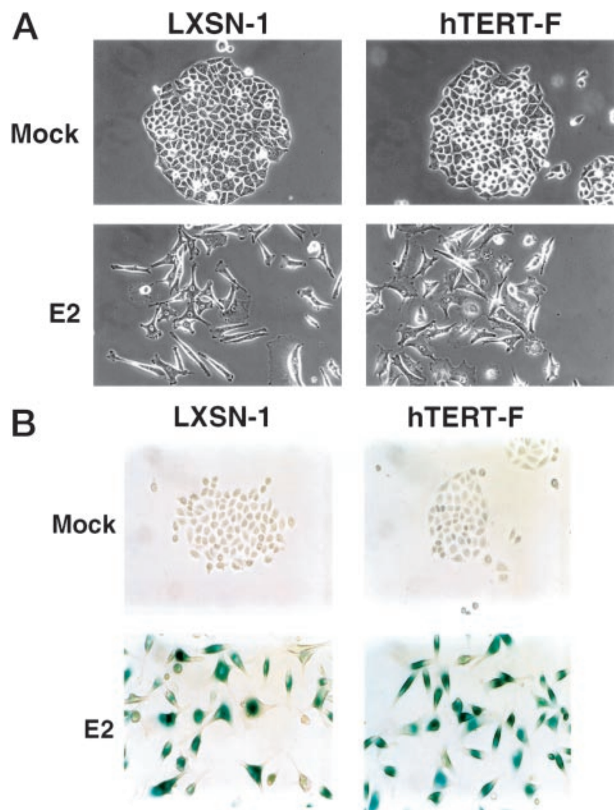

Fig. 3. Cellular morphology and SA-β-gal activity. *A*, phase micrographs at ×200 show colonies of LXSN-1 and TERT-F cells 8 days after mock infection (*top panels*) or the isolated cells 8 days after infection with E2 virus (*bottom panels*). Increased size, flattening, granular cytoplasm, and lack of intercellular contacts are observed in the treated cells. In *B*, LXSN-1 or hTERT-F cells were stained for SA-β-gal activity 15 days after mock-infection (*top panels*) or infection with E2 virus (*bottom panels*) and were photographed using brightfield optics at ×200.

SA-β-gal activity was determined by incubating cells with the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), followed by brightfield microscopy 15 days after mock infection or infection with the E2 virus. The mock-infected cells showed little staining, whereas the cells infected with the E2 virus showed intense, blue-green staining, indicative of high-level induction of SA-β-gal activity (Fig. 3*B*). Again, the LXSN and hTERT cell lines behaved identically.

Cells that have undergone replicative senescence show increased intrinsic fluorescence, called autofluorescence, which is thought to be caused by the accumulation of oxidatively damaged proteins and lipids. LXSN-1 and -4 cells and hTERT-F and -J cells were either mock infected or infected with the E2 virus. After 8 days, the cells were harvested, and autofluorescence was determined by flow cytometry. All four of the cell lines responded identically to infection with the E2 virus (Fig.4 and data not shown). Specifically, E2 expression induced the entire population of cells to uniformly shift to higher levels of autofluorescence, ruling out the possibility that a subpopulation of the transduced cells escaped senescence but was masked by greater num-

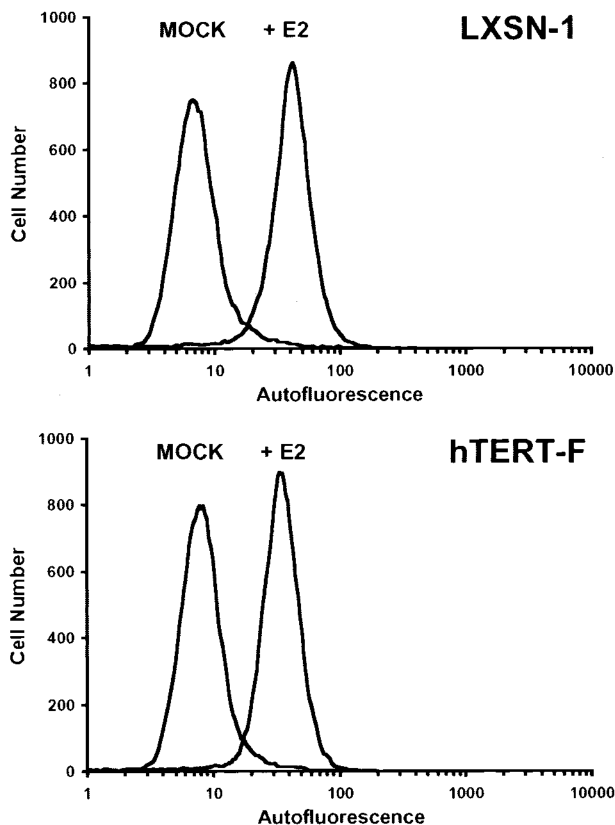

Fig. 4. Effect of the E2 protein on autofluorescence. LXSN or hTERT cell lines were either mock infected or infected with the E2 virus, and cells were collected by trypsinization 8 days later. The cell suspension was analyzed by flow cytometry as described in the "Materials and Methods" section. Histograms of the log of the autofluorescent signal are shown for mock-infected or E2-infected samples, as indicated.

bers of sensitive cells. Taken together, these results indicate that senescence is still induced by HPV repression in the hTERT-transduced HeLa cells that harbored greatly increased telomere lengths.

Effects of the E2 Protein on Telomere Length and Telomerase Activity. To determine the effects of the transduced *hTERT* genes on telomerase activity, we assayed telomerase activity in extracts prepared at various times after infection with the E2 virus or mock infection. As shown in Fig. 5*A*, 100 ng or 10 ng of protein from the LXSN or hTERT cell lines, respectively, were subjected to an *in vitro* TRAP assay, and the amplified products were separated on a nondenaturing polyacrylamide gel. Because of the high amount of telomerase activity in the hTERT cell lines, 10 ng of protein from these cells were assayed to maintain the linearity of the assay. "C" marks the position of the internal control amplification product that allows quantitative comparison between the samples, whereas the intensity of the more slowly migrating ladder of bands is a measure of telomerase activity. HeLa cells and LXSN controls contained readily detectable telomerase activity, and extracts of uninfected hTERT-F and -J lines exhibited about 50-fold more telomerase activity than did the LXSN-1 samples (Fig. 5*A*). The sample containing heat-inactivated protein extract and the sample without extract did not display activity, demonstrating the specificity of the assay. As was the case for the parental HeLa/sen2 cells, telomerase activity rapidly declined in both the LXSN clones and in the hTERT-transduced cells after infection with the E2 virus. Fig. 5*B* shows the results normalized to the amount of protein used in the TRAP assay to allow ready comparison between the LXSN-1 and hTERT-F samples. This analysis revealed that despite the reduction in activity after infection, the absolute levels of telomerase activity of the senescent hTERT-F cells 8 days after infection were still greater than the activity of proliferating uninfected LXSN-1 cells. A Northern blot demonstrated that the levels of mRNA from the transduced *hTERT* gene did not decline over the 8 days after infection with the E2 virus, which indicated that the decline of telomerase activity was posttranscriptional (data not shown).

We also determined telomere lengths of mock-infected and E2-infected cells 8 days after infection and compared them with those of proliferating primary keratinocytes (Fig. 5*C*). Infection with the E2 virus and the induction of senescence had no effect on the length of the telomeres. For comparison, the telomere lengths of primary keratinocytes from two different sources are also shown. DNA was purified from early passage foreskin and ectocervical keratinocytes that were not yet senescent, and the telomere lengths were determined. Both types of keratinocytes had telomeres that were much longer than those of the parental HeLa/sen2 cells or LXSN clones. The hTERT clones have telomeres that are longer than those in proliferating ectocervical keratinocytes, and the hTERT-F telomeres are about the same length as those present in newborn foreskin keratinocytes. Thus, although the hTERT clones possess telomeres as long as, or longer than, those in actively proliferating keratinocytes, they readily underwent E2-mediated senescence, as did control HeLa/sen2 cells, which contain very short telomeres.

Discussion

There is considerable interest in studying the molecular basis of cellular senescence because of its potential role in organismal aging and tumor suppression. The correlation between replicative senescence and telomere erosion led to the formulation of the telomere hypothesis, which proposes that the gradual reduction in telomere length cause by successive rounds of DNA replication and cell division causes the length of one or more telomeres length to fall below a certain threshold, resulting in the initiation of senescence (19–22). The strongest evidence in support of this hypothesis is the finding that ectopic expression of telomerase and extension of telomeres in primary human cells can delay or prevent senescence (24, 25, 28). Furthermore, in the case of human keratinocytes, the ability of the high-risk HPV E6 protein to induce telomerase activity appears important for extending the replicative life span of the cells and inducing immortalization in combination with HPV E7 or other events that inactivate the retinoblastoma pathway (29). However, there are situations in which short telomeres are not sufficient to trigger cell senescence. For example, in human fibroblasts expressing hTERT or human keratinocytes expressing HPV

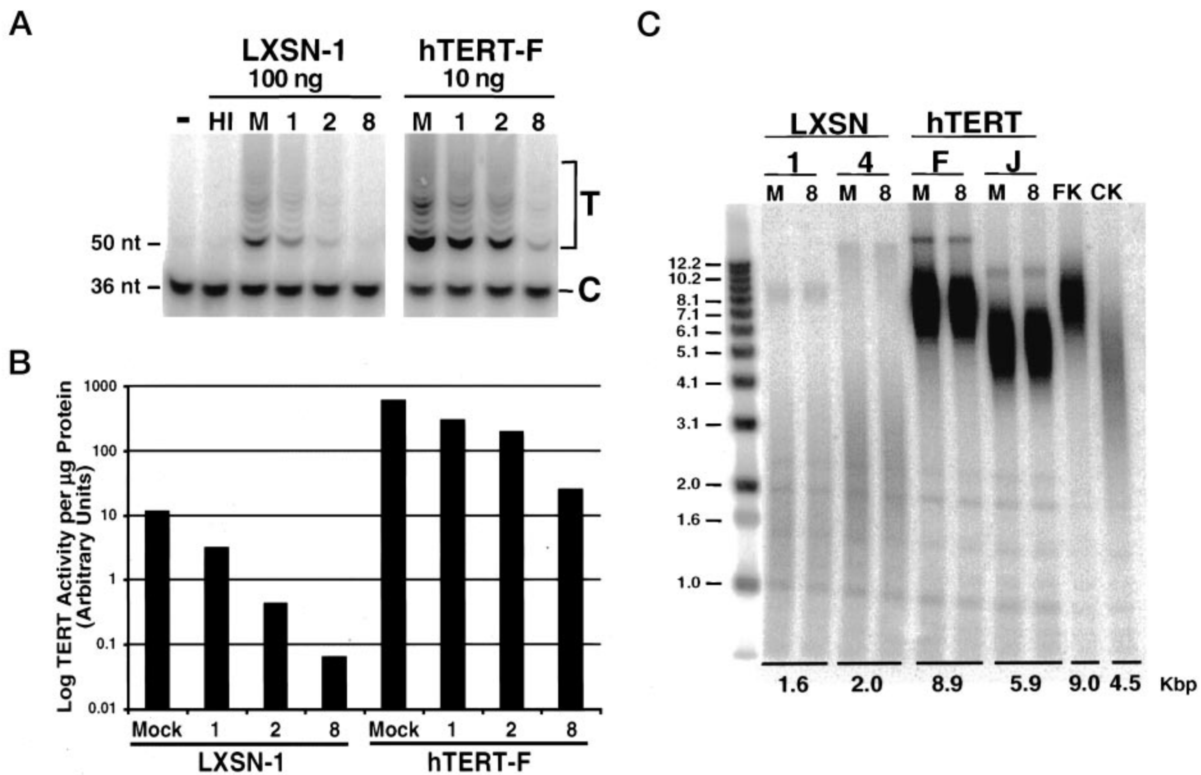

Fig. 5. Effect of the E2 protein on telomerase activity and telomere length. In *A*, protein extracts were prepared from mock (*M*) or E2-infected cells after 1, 2, or 8 days, as indicated, and telomerase activity was determined by a modified TRAP assay using 100 ng of LXSN-1 and 10 ng of hTERT-F cell protein. The lower band (*C*) represents the 36 nucleotide internal control that allows quantitative comparisons between different samples, and the upper bands (*T*) are the result of *in vitro* telomerase activity adding variable numbers of the six nucleotide telomere repeats. The shortest of these bands is a 50-nucleotide product containing three telomere repeats. *The two lanes on the left* are negative control reactions containing no extract (−) or 100 ng of heat-inactivated extract from LXSN-1 cells (*HI*). In *B*, a phosphorimager was used to quantitate the telomerase activity relative to the internal control, and the values were normalized to the amount of protein used in each assay. The results are shown on a log scale to facilitate the comparison between samples with markedly different telomerase activity. *C*, telomeres were visualized as in Fig. 1 for LXSN-1 and -4, and hTERT-F and -J either 2 days after mock infection or 8 days after infection with the E2 virus. For comparison, the telomeres in proliferating human foreskin keratinocytes (*FK*) and human cervical keratinocytes (*CK*) are also shown. *In the first lane*, the DNA size markers along with their size in kbp; *numbers at the bottom of the other lanes*, the modal telomere length in kbp for each sample.

16 E6/E7, telomeres stabilize at lengths even shorter than those in cells undergoing crisis (36, 43, 56). Furthermore, many cancer cells, including the uninfected HeLa cells studied here, harbor short telomeres but, nevertheless, actively proliferate (55, 57, 58).

Although replicative senescence was originally defined as occurring after extended passage of primary cells, several exogenous treatments can cause early-passage cells to rapidly acquire various phenotypes characteristic of senescent cells. Such premature senescence can be triggered in primary fibroblasts by the introduction of activated *ras* alleles and activation of the *raf*/mitogen-activated protein (MAP) kinase signaling cascade; sublethal levels of hydrogen peroxide or DNA-damaging agents; high-level expression of cdk inhibitors, E2F1, p14^{ARF}, p105^{Rb}, or p53; or treatment with other chemical or physical agents (*e.g.,* as seen in Refs. 5 and 59–61). In these situations, the cells undergo senescence long before they would have reached the senescence barrier during continuous passage. Because these early-passage cells contain relatively long telomeres, and senescence ensued too rapidly for telomeres to undergo significant shortening, it appears that premature senescence is independent of telomere length. Indeed, introduction of the *hTERT* gene did not block *ras*-induced senescence (62). These results imply that diverse stimuli can initiate the senescent program in early-passage cells.

The senescent phenotype can also be elicited in immortal or cancer cells by expression of the tumor suppressors p53, p105^{Rb}, p27^{KIP}, p21, or p16^{INK4} (63–71), by thermal inactivation of a temperature-sensitive SV40 large T antigen (72–74), by treatment with transforming growth factor β (75) or chemotherapeutic agents (76), or, as reported here and elsewhere, by repression of the HPV oncogenes in cervical carcinoma cell lines (51, 52, 54). In contrast to early-passage cells undergoing premature senescence, these cells have proliferated far beyond the senescence barrier, and, in many cases, telomere erosion has already occurred, and the cells harbor short telomeres. Indeed, telomeres in the parental, proliferating HeLa cells are far shorter than they are in early-passage human keratinocytes, and HPV E6/E7 repression causes a rapid decline in telomerase activity. These results suggested that expression of the HPV E6 and/or E7 proteins

prevented transduction of the senescent signal from the short telomeres in the cells, and that once E6/E7 expression was extinguished, the cells sensed the presence of short telomeres and initiated the senescence program. Alternatively, the low level of telomerase after E6/E7 repression may signal senescence. To test these possibilities, we generated HeLa cell clones with increased telomere length and elevated telomerase activity. Elimination of HPV E6 and E7 expression in these HeLa cell derivatives resulted in the acute activation of the p53 and retinoblastoma tumor suppressor pathways and the rapid acquisition of the senescent state. Thus, the presence of long telomeres and active telomerase did not protect HeLa cells from induced senescence after E6/E7 repression.

It is unclear whether the level of telomerase or the length of telomeres controls replicative senescence. In normal human oral keratinocytes, onset of replicative senescence correlates better with telomerase levels than with telomere length, and in other systems, telomerase expression, rather than telomere maintenance, appears important for the bypass of senescence (23, 77). However, in other cell types, telomere maintenance is a better predictor of proliferation than is telomerase activity (27, 78). The results reported here indicate that the proximal signal that induces senescence in HeLa cells is neither short average telomere length nor a low absolute level of telomerase activity. However, these results do not rule out a role for telomerase or telomere length in induced senescence. It is possible that a particular telomere remains short in the hTERT-expressing cells, although the average length of telomeres is greatly extended, or that the cells sense the decline in telomerase activity, rather than absolute activity. Alternatively, short telomeres may affect the transcription of telomeric genes involved in senescence or induce structural chromosomal abnormalities. If these changes persist despite the reexpression of telomerase, they may initiate senescence when the HPV oncogenes are repressed.

It is also possible that E6/E7 repression can initiate a senescence program independently of telomerase and telomere length. E6/E7 repression induces expression of p53, p21, and hypophosphorylated p105^{Rb}, all of which have been implicated in inducing senescence in various cell systems. In the E2 transfection experiments of Wells *et al.*, p21 was proposed to mediate induced senescence (54). However, E2-expression also induces senescence in p53-minus HT-3 cervical carcinoma cells in the absence of p21 induction (49, 51), which indicates that the induction of p53 and p21 is not required for senescence in this system. *p16^{INK4}* and activated oncogenes can also induce senescence in some settings. Expression of p16^{INK4} is constitutively high in HeLa cells and persists during induced senescence (51); therefore, repression of E6/E7 may allow the cells to undergo senescence in response to the elevated levels of p16^{INK4}. HeLa cells may also contain activated oncogenes, which may induce senescence once E6/E7 are repressed. Experiments are currently underway to test these possibilities.

The rapid and synchronous onset of induced senescence is in apparent contrast to the long time required for replicative senescence to occur during continuous passage of primary cells, raising the possibility that induced senescence is mechanistically distinct from replicative senescence. Moreover, expression of exogenous telomerase and telomere extension do not prevent induced senescence, in contrast to the protection that telomerase expression affords against replicative senescence. However, cells undergoing replicative and induced senescence display indistinguishable phenotypes, which suggests that these processes share a mechanistic basis. In fact, it is possible that an individual cell undergoing replicative senescence during passage progresses rapidly through the senescent program. According to this view, the slow and asynchronous nature of replicative senescence results from the heterogeneous length of time before different cells enter this pathway. This heterogeneity may arise from the different lengths of telomeres in the starting cell population, or from cell-to-cell variability in generation time, or the rate of telomere shortening during passage. In the case of induced senescence, this program is initiated synchronously in all of the cells.

HPV E6 expression induces telomerase activity in early passage keratinocytes, an effect that is thought to be attributable to E6-mediated activation of the hTERT promoter (42, 43, 79, 80). Telomerase activity declined in hTERT cells infected with the E2 virus, although the *hTERT* gene was expressed from a retroviral promoter, and there was no apparent loss of hTERT mRNA expressed by the transgene. This result implies that E2 expression and HPV E6/7 repression exert a posttranscriptional effect on the expression or activity of telomerase. The basis of this effect is currently unknown, but it may reflect the down-regulation of telomerase activity induced by growth arrest or by p105^{Rb} expression in other systems (16, 71).

We previously suggested that induction of senescence in cervical carcinoma cells by inhibition of the expression or activity of the HPV oncogenes may represent a novel therapeutic approach for this cancer (51). The results reported here suggest that this approach may not be restricted to those cancers with short telomeres. The pathway leading to induced senescence in cancer cells warrants further study, because manipulations that activate this pathway may limit the *in vivo* growth of many types of cancers, including those of nonviral etiology.

Materials and Methods

Cell Lines and Viruses. HeLa/sen2 cells are a subclone of HeLa cells that respond rapidly and uniformly to infection with the E2 virus (51). The LXSN control retrovirus was obtained by transfecting amphotropic Bing packaging cells with the pLXSN vector and collecting a cell-free supernatant 2 days later. A cell line producing LXSN-hTERT retrovirus was kindly provided by Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA). The hTERT cDNA was cloned from a HeLa cell library, inserted into pLXSN, and packaged using the PG13 cell line.[4] Retrovirus-containing supernatants were used to infect HeLa/sen2 cells at a low

[4] Denise Galloway, unpublished observations.

multiplicity, and transduced cell lines were cloned from individual colonies after 2 weeks of selection in 1 mg/ml G418. Cells were maintained in complete media with 0.5 mg/ml G418, but G418 was omitted during all of the experiments.

High titer stocks of the recombinant SV40 virus expressing the BPV E2 protein (pPava-5′BΔS-RMC, designated the E2 virus) were prepared, titered, and used to infect cells at a multiplicity of 20 as described previously (47, 49). For later time points, a second infection at the same multiplicity was done at 3 days to reduce the background attributable to the outgrowth of cells that escaped the first infection. The growth medium was changed every 3 days. For biochemical analysis, the HeLa-derived cell lines were seeded in 150-mm dishes and infected the following day. Washed cells were scraped and collected into aliquots by centrifugation and stored as frozen cell pellets until processing.

Telomere Length. Genomic DNA was prepared from cell pellets using a Qiagen DNeasy kit including the RNase digestion according to the manufacturer's instructions. One μg of genomic DNA was then digested with *Rsa*I and *Hin*fI and resolved by electrophoresis in a 0.7% agarose gel with DNA size markers. The DNA was partially depurinated in 0.25 M HCl for 30 min and was then fragmented and denatured in 3 M NaCl- 0.4 M NaOH for 30 min. The gel was equilibrated in 3 M NaCl-8 mM NaOH; the DNA was transferred to a Nytran Supercharge membrane (Schleicher and Schuell) and neutralized in 0.2 M sodium phosphate buffer (pH 6.8). The DNA was UV-cross-linked to the membrane with a Stratalinker 1800 (Stratagene), prehybridized in 5× SSPE [1× SSPE is 150 mM NaCl, 10 mM sodium phosphate (pH 7.4), and 1 mM EDTA], 5× Denhardt's solution (Sigma Chemical Co.; 50× Denhardt's is a 1% solution of BSA, ficoll, and polyvinyl pyrrolidone), and 0.5% SDS for 1 h at 37°C. Then 15 pmol of the ^{32}P-5′ end-labeled, $(TTAGGG)_3$ telomere-specific oligonucleotide was added, and the mixture was hybridized overnight. The membrane was washed three times in 2× SSPE-0.5% SDS at room temperature and the bands visualized with a phosphorimager. The membrane was subsequently hybridized with random-primed-labeled probes for the DNA size markers to estimate the lengths of the telomeres.

Cell Analysis. DNA synthesis was measured in quadruplicate by incorporation of [^{3}H]thymidine. Cells (2.5×10^4) were seeded per well in a 24-well dish and were infected with the E2 virus the next day. Two days later, the labeling and measurement of incorporation were performed as described previously (51). For determination of cell morphology and SA-β-gal activity, 5×10^4 or 4×10^2 cells were seeded in 35-mm wells for experimental and mock-infected samples, respectively. The cells were infected or mock infected the next day and again 3 days later, and subsequently were refed every 3 days. Cells were photographed through a phase microscope 8 days after the first infection, to document changes in morphology. SA-β-gal activity was determined at pH 6.0 (3) 15 days after the initial infection with the E2 virus- or mock-infection and documented by photography with brightfield optics.

For flow cytometry, 2×10^5 cells were seeded per 100-mm dish, infected the following day with the E2 virus. Matched cells for mock infection were seeded at 2×10^4 per dish and maintained in parallel. The cells were harvested by trypsinization after 8 days, washed with complete medium, washed once at 4°C with PBS and resuspended in PBS at $\sim 1 \times 10^6$ cells/ml. Data were collected using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with CellQuest software. After excitation at 488 nm, autofluorescence was measured after passage through a 530/30-nm band pass filter and listmode data files were analyzed with WinMDI 2.8 software. At least 20,000 cells were analyzed for each sample.

Biochemical Analysis. LXSN or hTERT cell lines were seeded in 150-mm dishes and infected the following day. Washed cell pellets were frozen at various times after infection, and immunoblot analyses of total extracted protein were carried out as described previously (51) with the following primary antibodies: 15801A (p53) and 14001A (p105^{Rb}) from PharMingen; sc-397 (p21), sc-318 (p107), sc-317 (p130), and sc-8432 (pan-actin), from Santa Cruz Biotechnology; and anti-cyclin A, a gift from H. Zhang (Yale University, New Haven, CT). After washing, filters were incubated with a 1:20,000 dilution of species-specific donkey antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch). Immunoblots were incubated with ECL+Plus (Amersham), and the signals were detected using Hyperfilm (Amersham).

Total RNA was prepared by using the RNeasy Mini kit (Qiagen). RNA was denatured, resolved on a 1% agarose-formaldehyde gel, transferred to Nytran Supercharge (Schleicher and Schuell), and cross-linked to the membrane using a Stratalinker 1800 (Stratagene). mRNAs were detected by hybridization with random-prime labeled probes.

Telomerase activity was assayed in protein extracts prepared at various times after infection with the E2 virus by using a TRAPeze Telomerase Detection kit (Intergen, Purchase, NY). The reaction products were resolved on 6% polyacrylamide minigel and were detected and quantitated using a Storm 840 (Molecular Dynamics, Inc.). The signal from the entire ladder of bands attributable to telomerase activity was normalized to the signal from the internal control band and to the amount of cellular protein analyzed. We previously showed that the signal was proportional to the amount of extract assayed (51).

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