Rapid induction of senescence in human cervical carcinoma cells

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Expression of the bovine papillomavirus E2 regulatory protein in human cervical carcinoma cell lines repressed expression of the resident human papillomavirus E6 and E7 oncogenes and within a few days caused essentially all of the cells to synchronously display numerous phenotypic markers characteristic of cells undergoing replicative senescence. This process was accompanied by marked but in some cases transient alterations in the expression of cell cycle regulatory proteins and by decreased telomerase activity. We propose that the human papillomavirus E6 and E7 proteins actively prevent senescence from occurring in cervical carcinoma cells, and that once viral oncogene expression is extinguished, the senescence program is rapidly executed. Activation of endogenous senescence pathways in cancer cells may represent an alternative approach to treat human cancers.

Cancer is thought to be caused by the stepwise accumulation of genetic changes, resulting in progressive phenotypic abnormality. Infection of cervical keratinocytes with a high-risk human papillomavirus (HPV) such as HPV18 is the initiating event in the great majority of cervical carcinomas (1). The HPV E6 and E7 proteins are continuously expressed in cervical carcinoma cells and seem to play a role in tumor development and in maintenance of the malignant phenotype (1). The role of the E6 and E7 proteins in the progression from normal keratinocytes to transformed cells has been studied in cell culture. Normal human keratinocytes can undergo a limited number of cell divisions *in vitro* and then stop proliferating, a phenomenon referred to as replicative senescence (2–4). Telomere length progressively shortens during cell passage (5, 6), and it has been proposed that once the length of one or more telomeres falls below a critical threshold, an active genetic program is mobilized that culminates in senescence (7, 8). Cultured keratinocytes can escape senescence and become immortalized by inactivation of the retinoblastoma tumor suppressor pathway and activation of telomerase, the enzyme responsible for maintaining telomere length (9, 10). Expression of the HPV E6 and E7 proteins also can immortalize keratinocytes (11, 12), an activity that is consistent with the known biochemical activities of these proteins. The E7 protein neutralizes the retinoblastoma tumor suppressor pathway, and the E6 protein stimulates telomerase activity in keratinocytes (1, 6, 13). Although the mechanistic basis for this effect on telomerase activity is not known, this activity seems crucial for immortalization because E6 mutants defective for telomerase activation are immortalization-defective (10). In addition, host-cell mutations are required for HPV-immortalized keratinocytes to become tumorigenic and to acquire other malignant characteristics, such as metastatic potential (9, 14–20). The E6 and E7 proteins facilitate the acquisition of these mutations by causing accelerated degradation of p53 and $p105^{Rb}$, respectively, resulting in abrogation of DNA damage checkpoint control, elevated rates of mutagenesis, and genetic instability (1).

The results summarized above indicate that the HPV E6 and E7 proteins allow cervical epithelial cells to escape the senescent barrier and accumulate the mutations responsible for tumorigenesis. However, cervical cancer cells frequently retain wild-type p53 and $p105^{Rb}$ genes, suggesting that it may be possible to restore growth control in these cells by repression of HPV oncogene expression. To test this possibility, we developed a simian virus 40 (SV40)-based recombinant viral vector that efficiently expresses the bovine papillomavirus (BPV) E2 regulatory protein in cervical carcinoma cell lines (21). The E2 protein binds directly to the HPV early promoter and represses transcription of the E6 and E7 genes. Delivery of the E2 gene into several cervical carcinoma cell lines causes dramatic repression of HPV E6/E7 expression, followed shortly thereafter by reactivation of the endogenous p53 and retinoblastoma tumor suppressor pathways, repression of E2F-responsive genes required for entry into S phase, and profound growth inhibition (21–25). The E2 protein does not affect the proliferation of cells devoid of HPV DNA (22, 25), and E2-induced growth inhibition of cervical cancer cells is blocked by constitutive expression of the E6 and E7 genes (ref. 26; and E.C.G. and D.D., unpublished data). These and related studies indicate that the p53 and retinoblastoma tumor suppressor pathways are intact but dormant in these cancer cells and that these pathways can be resuscitated by repression of E6/E7 expression (refs. 22, 23, and 25–27; and E.C.G. and D.D., unpublished data). In this paper, we show that E2-mediated repression of the HPV E6/E7 genes induced cervical carcinoma cells to rapidly assume the phenotype of senescent cells.

Methods

HeLa and HT-3 cells were grown as described (22). To reduce heterogeneity in the cell population, HeLa cells were cloned by limiting dilution, and individual clones were assayed for their response to infection with the E2 virus (see below). All clones tested were efficiently inhibited by E2 expression, and for further experiments we used a clone, designated HeLa/sen2, that generated approximately one proliferating colony per 10^5 infected cells. HeLa/sen2 cells were aneuploid and contained numerous marker chromosomes specific for HeLa cells (unpublished results). Stocks of the recombinant SV40 virus expressing the BPV E2 protein (Pava-5′BΔS-RMC) or the null mutant E2Am containing an amber stop codon were prepared, titered, and used to infect cells at a multiplicity of 20 as described (22, 28). A portion of the stock expressing the E2 protein was inactivated by 4 J/cm^2 UV light irradiation. We also used mock-infected cells or cells infected with the virus expressing the defective E2 mutant as negative controls. For later time points, a second infection was done at 3 days to reduce the background caused by outgrowth of cells that escaped the first infection, and the growth medium was

Abbreviations: HPV, human papillomavirus; SV40, simian virus 40; BPV, bovine papillomavirus; SA β-gal, senescence-associated β-galactosidase.

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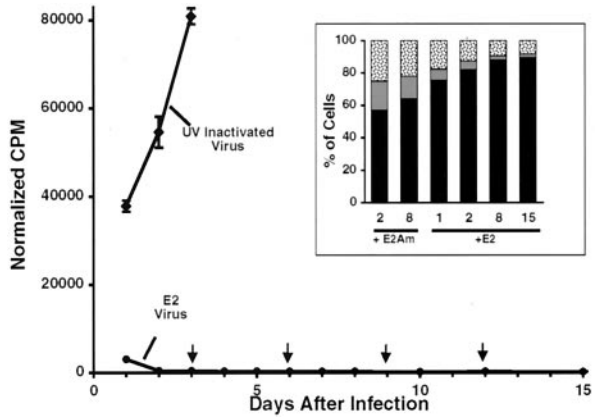

Fig. 1. Effect of E2 expression on cellular DNA synthesis and cell cycle progression. HeLa/sen2 cells were assayed for incorporation of [^{3}H]thymidine at the indicated days after infection with the E2 virus or treatment with UV-inactivated virus. cpm incorporated per hr of labeling time are shown, with the error bars indicating 2 standard deviations of the mean. The cells were fed with medium containing 10% FBS on the days indicated by arrows. (*Inset*) Cellular DNA content as determined by flow cytometry on the indicated days after infection with the E2 virus or the E2 amber mutant. The solid portion of each bar corresponds to cells with a G_0/G_1 phase DNA content, the gray portion to cells in S phase, and the stippled portion to cells in G_2/M phase.

changed every 3 days. DNA synthesis was measured in quadruplicate by incorporation of [^{3}H]thymidine as previously described (28).

For flow cytometry, 5×10^5 HeLa/sen2 cells were seeded per 100-mm dish and infected the following day. The cells were harvested by trypsinization, washed with medium, washed once at 4°C with PBS, and resuspended at 1–2 $\times$ 10^6 cells per ml. For each time point, control cells also were assayed in parallel to correct for minor variability in the assay. Analysis was performed with a FACS Vantage flow cytometer (Becton Dickinson) by using CELLQUEST software. After excitation at 488 nm, emission forward scatter and autofluorescence were collected through a 530/30-nm band pass filter. At least 15,000 cells were analyzed for each sample. For each sample, the number of cells displaying peak fluorescence was normalized to 100% to correct for the different numbers of cells in the various samples, and the raw data were smoothed with a five-point moving average. To determine DNA content, the cells were fixed in 70% ethanol, treated with 1 mg/ml RNase A for 30 min, and stained with 50 μg/ml propidium iodide before flow cytometry as above, except emission was measured with a 630/30-nm band pass filter.

To obtain fluorescent photomicrographs, HeLa/sen2 cells were plated on multichambered slides (Lab-Tek) 12 days after mock-infection or infection with the E2 virus and cultured for an additional 5 days. The cells then were washed in PBS and fixed in 4% neutral buffered formaldehyde for 5 min at room temperature. The cells were washed in PBS, and the slide was mounted in Gel/Mount (Biomeda, Foster City, CA). Fluorescent photomicrographs ($\times$200) were obtained by using a green H546 filter (Zeiss), with 60-sec exposure using Kodacolor ISO 100 Gold film.

CaSki cells were maintained in DMEM containing 10% FBS. AvBPVE2, a recombinant adenovirus that expresses BPV1 E2 under the control of the cytomegalovirus immediate-early promoter in place of the adenoviral E1 region, was purified by CsCl-density-gradient centrifugation and was free of E1A-containing replication-competent virus (unpublished results). CaSki cells (5×10^5) were plated in a 60-mm dish and infected the following day with AvBPVE2 at a multiplicity of infection of approximately 50 plaque-forming units per cell.

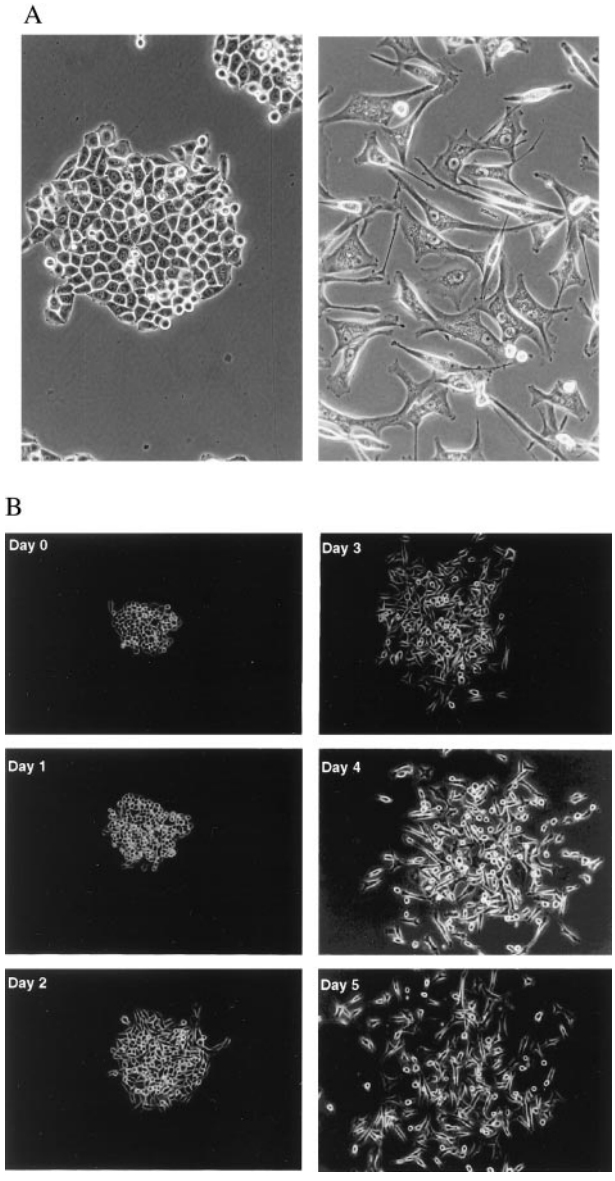

Fig. 2. Effect of E2 expression on cell morphology. (*A*) Phase contrast micrographs at $\times$200 magnification show a colony of cells 8 days after infection with the amber mutant (*Left*) and cells 15 days after infection with the E2 virus (*Right*). (*B*) HeLa/sen2 cells were sparsely seeded, and colonies grew for 1 week. The cells were then infected with the E2 virus at day 0, and a typical colony is shown from day 0 through day 5.

For biochemical analysis, 1.2 $\times$ 10^6 HeLa/sen2 cells were seeded in 150-mm dishes and infected the following day. Protein extraction and immunoblot analysis of total extracted protein were carried out as described (E.C.G. and D.D., unpublished data) with the following primary antibodies: 15801A (p53), 14001A (p105^{Rb}), 15126E (p16^{INK4a}), and 14841A (cyclin D) from PharMingen; sc-397 (p21), sc-965 (human mdm2), sc-318 (p107), sc-317 (p130), sc-40 (c-myc), sc-198 (cyclin E), and sc-8432 (pan-actin), from Santa Cruz Biotechnology; and anti-cyclin A, a gift from H. Zhang (Yale University). 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 Enhanced Chemiluminescence Plus (Amersham Pharmacia), and the signals were detected with Hyperfilm (Amersham Pharmacia). Protein extracts also were prepared 8 days after treatment

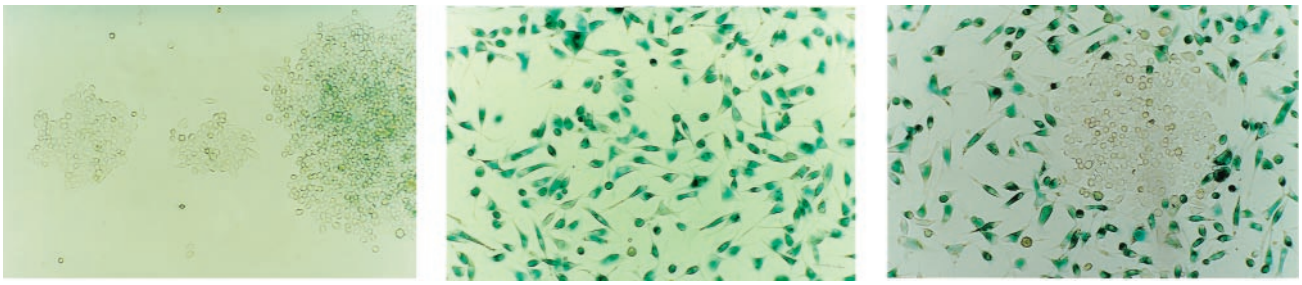

Fig. 3. Effect of E2 expression on SA β-gal activity. HeLa/sen2 cells were stained at pH 6.0 with X-Gal (5-bromo-4-chloro-3-indol β-D-galactopyranoside) as described (29) 10 days after mock-infection (*Left*) or 15 days after infection with the E2 virus (*Center* and *Right*) and photographed by using brightfield optics at ×100 magnification.

with UV-inactivated virus or at various times after infection with the E2 virus and assayed for telomerase activity by using a TRAPeze Telomerase Detection Kit (Intergen, Purchase, NY). The reaction products were resolved on 6% polyacrylamide minigel and detected and quantitated by using a Storm 840 PhosphorImager (Molecular Dynamics).

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 & Schuell), and crosslinked to the membrane with a Stratalinker (Stratagene). mRNAs were detected by hybridization with random-prime-labeled probes.

Results

Senescent cells exhibit a number of features that distinguish them from other nonproliferating cells (2). Unlike quiescent cells, senescent cells cannot be stimulated to proliferate by addition of growth factors or by plating at low density. In addition, senescent cells have increased volume and exhibit flattened morphology, and they contain elevated levels of an endogenous β-galactosidase active at pH 6.0 [senescence-associated β-galactosidase (SA β-gal)] and fluorescent pigments such as lipofuscin (29–31). Senescent cells also express altered levels of a number of cell cycle regulatory proteins including p53, p21, $p16^{INK4a}$, and cyclin D, and they generally express low telomerase activity.

For most of the experiments described here, we used HeLa/sen2 cells, a subclone of HeLa cervical carcinoma cells that express the HPV18 E6 and E7 proteins and contain wild-type p53 and $p105^{Rb}$ genes. The BPV E2 protein was expressed by infecting cells with a recombinant SV40 viral vector, designated here the E2 virus. DNA synthesis was measured in HeLa/sen2 cells infected with the E2 virus or treated with UV-inactivated virus (Fig. 1). The control cells exhibited high levels of thymidine incorporation until they became too confluent for meaningful analysis. In contrast, DNA synthesis in the E2-infected cells rapidly declined to background levels. The cells persisted in this nonreplicative state over the course of the 20-day experiment (and for at least 10 weeks), despite regular addition of fresh medium containing serum. DNA synthesis was not stimulated in the infected cells by replating at lower density or by incubation in complete keratinocyte growth medium. DNA content at selected time points is shown (Fig. 1 *Inset*), demonstrating that the growth-arrested cells persisted with G_1 DNA content. The cells did not display markers of apoptosis such as subG_1 DNA content or nuclear blebbing, they excluded trypan blue, and there was no decrease in viable cell number (data not shown).

E2 expression also caused a striking morphological change in HeLa/sen2 cells, as shown in Fig. 2*A*. After treatment with UV-inactivated virus, the cells formed tight colonies of small proliferating cells. In contrast, after infection with the E2 virus, HeLa/sen2 cells appeared much larger, flatter, and more granular than uninfected cells did, and they did not form stable cell–cell contacts. The maximum plating density of cells expressing the E2 protein was approximately 5% that of uninfected cells. Thus, E2-infected cells resembled primary keratinocytes that have attained senescence after extensive serial passage (3). We also plated uninfected HeLa/sen2 cells at low density, and after the formation of small colonies, the cells were infected with the E2 virus. A typical infected colony is shown in Fig. 2*B*, showing that previously established contacts between cells were dissolved by E2 expression, and the cells in the colony dispersed over the course of a few days.

Preliminary experiments demonstrated that E2 expression induced the activity of SA β-gal in uncloned HeLa cells (32). Here, control or infected HeLa/sen2 cells were assayed for SA β-gal activity. Fig. 3 *Left* shows the faint staining of control cells that proliferated to form colonies. E2-infected HeLa/sen2 cells exhibited increased staining as compared with controls as early as 3 days postinfection, with the staining intensity gradually increasing at later times. As shown in Fig. 3 *Center*, virtually every cell stained intensely by 15 days after infection. The extremely rare proliferating colonies that arose after E2 expression looked like colonies of uninfected cells and did not stain, whereas the cells surrounding such colonies stained intensely and displayed the morphological changes noted above (Fig. 3 *Right*). We presume these colonies arose from cells that escaped infection or that were resistant to the growth-inhibitory effects of the E2 protein.

The lysosomes of senescent cells accumulate lipofuscin, an autofluorescent pigment that is thought to be a heterogeneous mixture of lipid and protein products of peroxidation (30). Fluorescence microscopy was performed on HeLa/sen2 cells 17 days after mock-infection or infection with the E2 virus. There was faint autofluorescence in control cells, but the infected cells displayed intense cytoplasmic, granular fluorescence (Fig. 4*A*). The autofluorescence of infected cells in comparison to mock-infected samples analyzed in parallel was quantitated by flow cytometry (Fig. 4*B*). As early as 1 day after infection, there was a discernable increase in fluorescence that became progressively more dramatic at later times. These data show that the entire population of infected cells synchronously shifted to higher fluorescence. In addition, the cells began displaying this senescent marker soon after or concomitant with the inhibition of DNA synthesis. Within 1 day, the E2 protein also caused increased forward scatter, an optical parameter that correlates with cell diameter, and measurements made with a Coulter Counter revealed a 3.1-fold increase in cell volume at 8 days after infection (data not shown).

We also measured telomerase activity at various times after infection (Fig. 5). Fig. 5 *Inset* shows the characteristic ladders formed in a telomeric repeat amplification protocol (TRAP) assay in response to telomerase activity, and demonstrated that telomerase activity was decreased in E2-infected HeLa/sen2

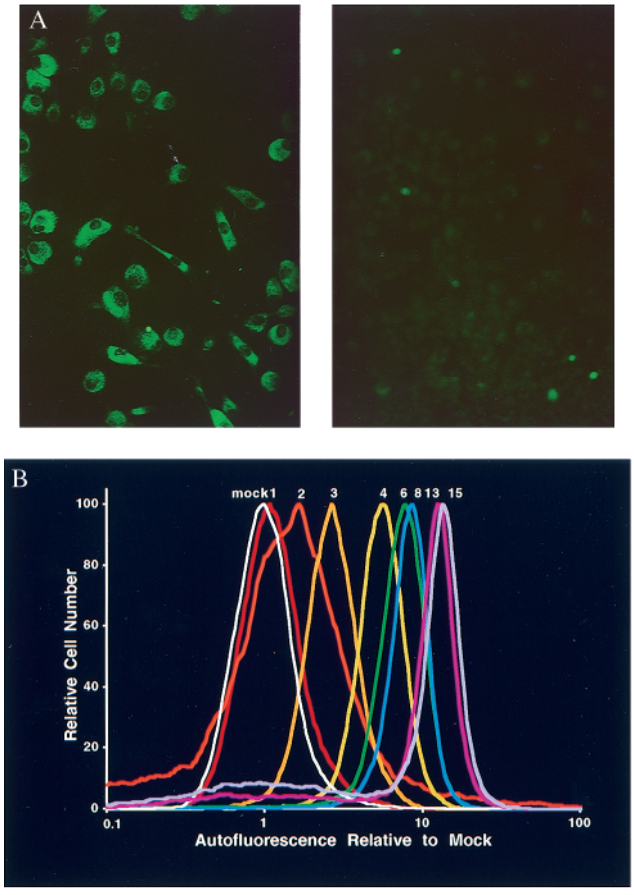

Fig. 4. Effect of E2 expression on autofluorescence. (*A*) Fluorescent photomicrographs (×200) of HeLa/sen2 cells 17 days after mock-infection (*Right*) or infection with the E2 virus (*Left*). (*B*) HeLa/sen2 cells were infected with the E2 virus and cultivated for the number of days indicated at the top before being analyzed by flow cytometry in parallel with uninfected HeLa/sen2 cells. The fluorescence values of each sample were divided by the peak fluorescence value of the matched control to yield -fold increase in autofluorescence relative to mock-infected cells.

cells as compared with cells treated with UV-inactivated virus. The graph in Fig. 5 quantitates the signals obtained by assaying extracts from infected cells and various amounts of extract from control cells and demonstrates the rapid and profound decline in telomerase activity in response to E2 expression.

We also examined two other cervical cancer cell lines, HT-3 cells and CaSki cells, that express the E6 and E7 genes from endogenous HPV30 and HPV16 genomes, respectively. An adenovirus vector was used to express the E2 protein in CaSki cells, because the recombinant SV40 virus did not efficiently express the E2 protein in these cells. In both HT-3 and CaSki cells, E2 expression repressed HPV E6/E7 expression and inhibited DNA synthesis (although inhibition was less complete than in HeLa or HeLa/sen2 cells) (ref. 22 and data not shown). After mock-infection or infection with a virus expressing the E2 protein, the cells were stained for SA β-gal activity. As shown in Fig. 6, there was low-level staining in the control cells, whereas expression of the E2 protein led to significantly increased staining. Staining was somewhat patchy, consistent with the less efficient E2-induced growth inhibition and the outgrowth of uninhibited cells in these uncloned cell populations.

We previously showed that expression of p53 and the p53-responsive genes p21 and mdm2 dramatically rise within the first day after HPV E6/E7 expression is repressed in uncloned HeLa

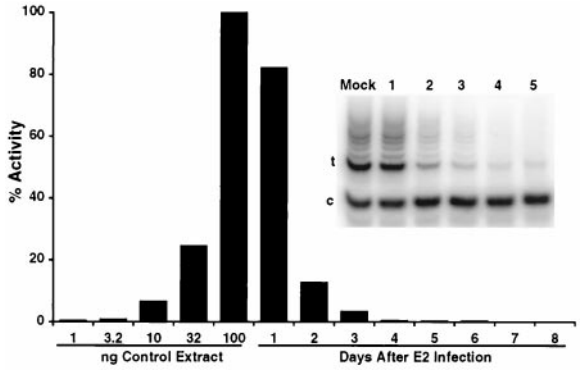

Fig. 5. Effect of E2 expression on telomerase activity. Telomerase activity was measured as described in *Methods*. (*Inset*) The primary data for control extract and extracts from cells 1 to 5 days after infection with the E2 virus, as indicated. The signal from the major amplication product generated by 100 ng of extracted protein (t) was normalized to the signal from the internal control product (c) for each sample and expressed as a percentage of the signal obtained from 100 ng of protein from the control cells. The graph also shows the results obtained by titrating 1 ng to 100 ng of protein from the control cells.

cells (ref. 23; and E.C.G. and D.D., unpublished data). Levels of retinoblastoma family members also are elevated at this time, and $p105^{Rb}$ exists in the hypophosphorylated form. Consistent with these changes, several genes regulated by the E2F family of transcription factors are repressed. Immunoblotting was used to assay the levels of proteins at different times after infection (Fig. 7). HeLa/sen2 cells displayed high-level induction of p53, p21, mdm2, p107, and hypophosphorylated $p105^{Rb}$ within the first day after infection, and p130 was induced with slower kinetics. Strikingly, after the initial induction, the level of these proteins declined substantially relative to total cell protein. The actin blot demonstrated that equal amounts of protein were loaded for each time point. Hypophosphorylated $p105^{Rb}$ also accumulated in CaSki and HT-2 cells (ref. 22 and data not shown). p21 expression was induced in CaSki cells, but not in HT-3 cells, which contain transactivation-defective p53 (22).

Cyclin A, p107, c-myc, cdc25A, and E2F1 (Fig. 7 and data not shown), products of E2F-responsive genes, were constitutively expressed in mock-infected HeLa/sen2 cells and were rapidly repressed as infection proceeded (after a transient induction in the case of p107). Cyclin D and cyclin E were repressed with

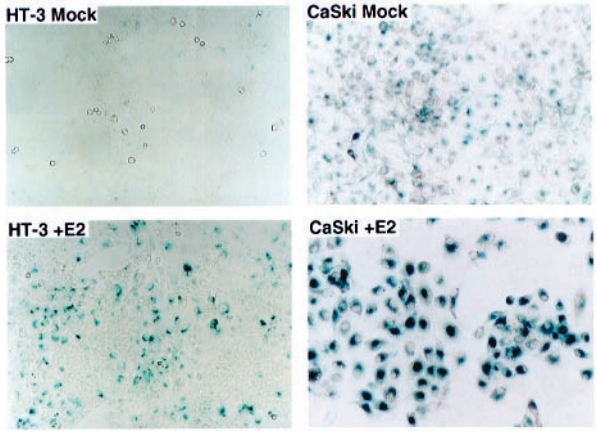

Fig. 6. Other cervical cancer cell lines exhibit E2-induced senescence. HT-3 and CaSki cells were mock-infected or infected with viruses expressing the E2 protein and assayed for SA β-gal activity after 12 (HT-3) or 5 (CaSki) days.

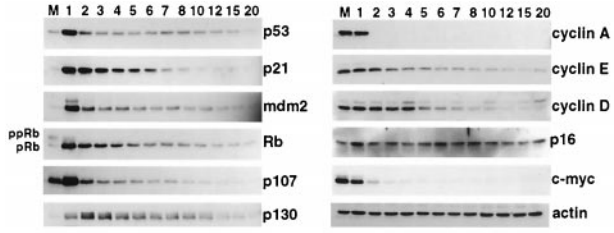

Fig. 7. Immunoblot analysis of cell cycle proteins. Immunoblot analysis was carried out on 5 μg of extracted protein for each sample. The numbers above the lanes indicate the number of days after infection with the E2 virus, and lanes with samples from mock-infected cells are labeled M. ppRb and pRb indicate hyperphosphorylated and hypophosphorylated $p105^{Rb}$, respectively.

slower kinetics. The cyclin-dependent kinase (cdk) inhibitor $p16^{INK4a}$ was constitutively expressed, and its expression varied little over the course of the experiment. Cdc25A and cyclins A and D stimulate the activity of cyclin/cdk complexes involved in phosphorylation of retinoblastoma family members, and $p16^{INK4a}$ inhibits the activity of these complexes. Accordingly, after infection, $p105^{Rb}$ was exclusively hypophosphorylated.

As shown in the Northern blot in Fig. 8, E6/E7 mRNA remained repressed over the course of the experiment, so the decline in p53 and $p105^{Rb}$ levels was not caused by reexpression of the viral oncogenes. Two representative E2F-responsive mRNAs, cyclin A and E2F1, also remained repressed, in contrast to $p105^{Rb}$ mRNA, which did not vary. Probing for ubiquitin mRNA demonstrated that similar amounts of RNA were present in each lane. Overall, these results suggest that a transient growth-inhibitory signal was sufficient to cause durable repression of E2F-responsive genes.

Discussion

E2-mediated repression of the HPV E6 and E7 genes induced human cervical carcinoma cells to express numerous phenotypic markers of senescence, including changes in cell size and morphology, sustained growth factor-resistant G_1 arrest, greatly increased SA β-gal activity and autofluorescence, and dramatically reduced telomerase activity. We did not carry out a systematic analysis of molecular markers of senescence, because in contrast to the situation with fibroblasts, such markers have not been clearly defined for keratinocytes. Although the E2 protein can induce apoptosis in other systems (27, 33), we did not detect apoptosis in our experiments (unpublished results). Furthermore, E2-induced senescence was inhibited by constitutive expression of the HPV16 E6 and E7 genes and did not occur in HPV-negative cells (refs. 21 and 22; and unpublished results), indicating that senescence is not a nonspecific toxic response to the E2 protein. Thus, repression of HPV E6/E7 expression caused cervical carcinoma cell lines to behave like cells that had never encountered the viral oncogenes, but rather had reached the end of their normal lifespan (3). Our results show that the cellular mutations responsible for immortalization, tumorigenicity, and metastasis in a naturally occurring virus-induced human cancer did not prevent the cells from mounting a vigorous senescent block once viral oncogene expression was extinguished. This behavior is similar to that of human fibroblasts immortalized *in vitro* by a temperature-sensitive SV40 large T antigen, which also acquire a senescence-like phenotype when T antigen is inactivated (34, 35).

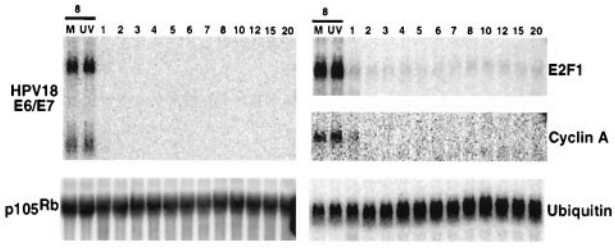

Fig. 8. Effect of E2 expression on mRNA levels. Five micrograms of RNA from mock-infected cells or cells treated with UV-inactivated virus (lanes labeled M and UV, respectively) or from cells infected with the E2 virus for the indicated number of days were hybridized to the indicated probes.

After E6/E7 repression, the cells began to express the senescent phenotype with remarkable synchrony and rapidity. Evidently, cervical carcinoma cell lines are poised to undergo senescence without the requirement for rare stochastic events or the accumulation of additional damage. These results strongly support a variety of studies indicating that senescence is the consequence of a defined genetic program (2, 4, 11, 36). However, these results may require reassessment of the conclusion, based on cell fusion and other studies (14, 36), that HeLa cells have suffered mutations in a gene that is required for senescence. Rather, the HPV E6 and E7 proteins appear to actively block the execution of the senescent program.

Short telomeres and induction of $p16^{INK4a}$ seem to be important events in mediating the replicative senescence that occurs in keratinocytes during *in vitro* passage (6, 9, 10, 13, 37, 38). Although telomerase activity markedly declined after E6/E7 repression, possibly as a consequence of the inhibition of E6 or c-myc expression (39, 40), we did not detect a decrease in telomere length (unpublished results), and growth arrest and senescence occurred rapidly. In contrast, inhibition of telomerase in other cell types results in cessation of proliferation and, in some cases, cell death after a significant number of cell divisions (41–44). Thus, further erosion of telomere length does not seem to trigger senescence after E2 expression in cervical carcinoma cells. However, the karyotypic abnormalities of HeLa cells suggest that they have already suffered an episode of chromosomal instability characteristic of cells with short telomeres before the reactivation of telomerase that occurred during carcinogenic progression. After repression of E6/E7 expression, the cells may be free to respond to the short telomeres or the other DNA damage they harbor and undergo senescence. $p16^{INK4a}$, which is constitutively highly expressed in HeLa cells and other cells expressing HPV E7 (45, 46), may help trigger the senescence program once the E6 and E7 proteins are absent. For example, constitutive expression of $p16^{INK4a}$, combined with E2-induced repression of cyclins and cdc25A and induction of p21, seems to be sufficient to maintain $p105^{Rb}$ hypophosphorylation and repression of E2F-responsive genes in the absence of further $p16^{INK4a}$ induction.

SA β-gal activity was induced in p53-negative HT-3 cells, even though p21 was not (22, 32). Thus, the senescence signaling pathway activated in cervical carcinoma cells may be p53-independent. Indeed, other studies suggest that induction of p53 is not important for replicative senescence of human keratinocytes, and some E6 mutants defective for p53 inactivation are immortalization-competent (47–50). However, the ability of a dominant-negative p53 mutant to substitute for the HPV16 E6 protein in a keratinocyte immortalization assay (24) implies that alternative pathways can lead to keratinocyte immortalization.

In summary, the cumulative genetic changes that occurred in cervical cancer cells are not sufficient to overcome the senescence barrier in the absence of continued expression of the viral proteins. These results suggest that activation of the endogenous senescence program in cancer cells should be explored as a therapeutic approach. Because several cell cycle components were only transiently induced, treatments that transiently restore tumor suppressor function may be sufficient to trigger senescence in cervical carcinoma cells. In addition, the reagents and cellular behavior described here may allow high-throughput,

cell-based screens for useful compounds. Screens could be developed to identify compounds that induce senescence in HeLa/sen2 or other carcinoma cells, for example by inhibiting HPV E6/E7 expression or activity. Conversely, compounds that inhibit E2-induced senescence may block the pathways leading to replicative senescence and cellular aging.

This paper is dedicated to the memory of Daniel Nathans. We thank R. Carbone for carrying out flow cytometry, B. Sawicki and P. Bray-Ward for examining the karyotype of the HeLa/sen2 cells, S. Mella for assistance with the Coulter Counter analysis, and J. Sedivy and J.-Y. Lee for helpful discussions. E.C.G. was supported by a postdoctoral fellowship from the Anna Fuller Fund for Cancer Research. E.Y. was supported by a predoctoral training grant from the National Institutes of Health. This work was supported by grants from the National Institutes of Health (CA16038), the Korea Science and Engineering Foundation (1999-1-208-002-3), and the Korean Ministry of Health and Welfare (97-M-2-0020).

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