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Age-dependent Alterations of c-fos and Growth Regulation in Human Fibroblasts Expressing the HPV16 E6 Protein

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Normal human cells in culture become senescent after a limited number of population doublings. Senescent cells display characteristic changes in gene expression, among which is a repression of the ability to induce the c-fos gene. We have proposed a two-stage model for cellular senescence in which the mortality stage 1 (M1) mechanism can be overcome by agents that bind both the product of the retinoblastoma susceptibility gene (pRB)-like pocket proteins and p53. In this study we determined whether the repression of c-fos at M1 was downstream of the p53 or pRB-like "arms" of the M1 mechanism. We examined c-fos expression during the entire lifespan of normal human fibroblasts carrying E6 (which binds p53), E7 (which binds pRB), or both E6 and E7 of human papilloma virus type 16. The results indicate a dramatic change in cellular physiology at M1. Before M1, c-fos inducibility is controlled by an E6-independent mechanism that is blocked by E7. After M1, c-fos inducibility becomes dependent on E6 whereas E7 has no effect. In addition, a novel oscillation of c-fos expression with an ~2-h periodicity appears in E6-expressing fibroblasts post-M1. Accompanying this shift at M1 is a dramatic change in the ability to divide in low serum. Before M1, E6-expressing fibroblasts growth arrest in 0.3% serum, although they continue dividing under those conditions post-M1. These results demonstrate the unique physiology of fibroblasts during the extended lifespan between M1 and M2 and suggest that p53 might participate in the process that represses the c-fos gene at the onset of cellular senescence.

INTRODUCTION

Human cells have a limited lifespan in culture and reach a quiescent state termed cellular senescence after a finite number of population doublings (Hayflick and Moorhead, 1961; Hayflick, 1965; Hayflick, 1977; Martin, 1977; Norwood and Smith, 1985). Senescent cells are viable but fail to divide in response to mitogenic stimulation. The number of doublings that can be achieved in culture depends on the cell type and is inversely proportional to donor age (Martin *et al.*, 1970). In the intact organism, cellular senescence may represent a balance between the benefits of tumor suppression caused by limited proliferation and the adverse effects of limiting the maintenance/repair capabilities of tissues (Martin, 1977; Wright and Shay, 1995). Telomere shortening has been proposed as a mitotic clock that regulates cellular senescence (Harley *et al.*, 1990; Harley, 1991; Allsopp *et al.*, 1992; Counter *et al.*, 1992; Levy *et al.*, 1992; Wright and Shay, 1992; Shay *et al.*, 1994). Telomerase, the enzyme that compensates for telomere shortening, is absent in most somatic cells but is found in immortal cultured cell lines and in most human tumors (Kim *et al.*, 1994). Cellular senescence occurs before the reactivation of telomerase when at least several kilobases of telomeric repeats are still remaining (Harley *et al.*, 1990; Allsopp *et al.*, 1992; Counter *et al.*, 1992; Shay *et al.*, 1993).

Many viral oncogenes allow human cells to bypass normal senescence and to gain an extended lifespan. These oncogenes do not directly immortalize human

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cells by de-repressing telomerase. Instead, they block the downstream antiproliferative effects of cellular senescence (Shay and Wright, 1989; Wright *et al.*, 1989; Counter *et al.*, 1992; Shay *et al.*, 1992). These oncogenes include the simian virus 40 (SV40) large T antigen, the E6 and E7 proteins of the human papilloma virus type 16, and the E1A and E1B proteins of the human adenovirus type 5 (Shay *et al.*, 1991; references therein). These three groups of oncogenes have the common property of inactivating two tumor suppressors, the p53 protein and the product of the retinoblastoma susceptibility gene (pRB). The latter is a member of a family of proteins that possess a common motif termed the "pocket," which includes pRB, p130, and p107. We have shown that human fibroblasts gain a full extension of their lifespan only when both p53 and a pRB-like pocket protein have been inactivated (Shay *et al.*, 1993). Such fibroblasts are not yet immortal. They lose additional telomeric repeats and eventually reach a state of crisis, in which cell division is apparently compensated by cell death (Shay and Wright, 1989; Counter *et al.*, 1992; Shay *et al.*, 1993). We have proposed a two-stage model for cellular senescence, in which the block to proliferation that is overcome by viral oncoproteins is called mortality stage 1 (M1) and the block that is probably caused by terminally shortened telomeres is called mortality stage 2 (M2) (Wright *et al.*, 1989; Wright and Shay, 1992; Shay *et al.*, 1994; Wright and Shay, 1995). The rare reactivation of telomerase (or other pathways that restore telomere length) in cells at crisis is thought to be the final event leading to the inactivation of the M2 mechanism and to immortalization.

The status of a large number of cell-cycle genes has been studied in senescent cells, and most changes are consistent with a block in late G1. The induction of early genes such as Ha-ras, c-myc, and ornithine decarboxylase is apparently normal, with the exception of c-fos. Transcription of the c-fos gene is transiently induced after the addition of mitogens such as phorbol esters, serum, or epidermal growth factor to young, quiescent human fibroblasts. However, the c-fos gene is not induced by the same mitogens in senescent cells (Seshadri and Campisis, 1990; Irving *et al.*, 1992). Although there is evidence that induction of FOS by growth factors is required for exit from G0 (Holt *et al.*, 1986; Nishikura and Murray, 1987; Riabowol *et al.*, 1988) and down-regulation of the c-fos gene has been suggested as a mechanism by which cellular senescence inhibits growth (Seshadri and Campisi, 1990; Irving *et al.*, 1992), situations exist in which c-fos is not required for proliferation (Kovary and Bravo, 1991; Hu *et al.*, 1994), and forced expression of c-fos does not significantly extend the lifespan of fibroblasts (Phillips *et al.*, 1992) or overcome the M1 mechanism (Shay *et al.*, 1991).

We chose c-fos as a marker for the part of the early mitogen–response pathway that is altered in senescent cells and asked whether the changes were downstream of the p53 or pRB-like "arms" of the M1 mechanism. Overexpressed p53 (Ginsberg *et al.*, 1991; Kley *et al.*, 1992) and pRB (Robbins *et al.*, 1990) have both been shown to repress the c-fos promoter in cycling cells. However, p53 levels do not change in senescent cells (Afshari *et al.*, 1993), although there may be some increase in the DNA binding activity of the p53 that is present (Atadja *et al.*, 1995). Similarly, pRB levels do not change in senescent cells and, as expected for noncycling cells, pRB is primarily in the hypophosphorylated form (Stein *et al.*, 1990; Futreal and Barrett, 1991). Our results suggest that p53 might participate in the process that represses the c-fos gene during cellular senescence. More importantly, they demonstrate a dramatic change in cellular physiology that occurs at the onset of M1, which produces age and papilloma virus E6 protein-dependent oscillations in FOS expression and reduced serum requirements for cell growth.

MATERIAL AND METHODS

Cell Culture

IMR90 normal diploid human-lung fibroblasts (ATCC no. 186; American Type Culture Collection, Rockville, MD) infected with defective retroviruses (see below) were cultured in a basal medium consisting of DMEM:199 (4:1) plus 10% defined supplemented calf serum (Hyclone Laboratories, Logan, UT). IDH4 cells (IMR90-D305.2H4) are a line of IMR90 fibroblasts immortalized with a dexamethasone (Dex)-inducible[1] SV40 large T antigen. In the absence of steroids, these cells stop dividing and reexpress a senescent phenotype (Wright *et al.*, 1989; Shay *et al.*, 1992). IDH4 cells were grown in basal media supplemented with charcoal-treated serum (to remove traces of glucocorticoid) to which 1 μM of Dex was added (to produce transformed IDH4 cells) or omitted (to yield senescent IDH4 cells).

Retroviral Vectors and Transfection

Retroviral vectors (Miller and Rosman, 1989) carrying a neomycin-resistance gene and expressing the human papilloma virus type 16 E6 gene (PLXSN16E6), E7 gene (PLXSN16E7), both genes (PLXSN16E6/E7), or no gene (parental vector PLXSN) were obtained from D. Galloway (Shay *et al.*, 1993). Viruses produced after electroporation of the ecotrophic-packaging cell line PE501 were then used to infect the amphotrophic-packaging line PA317. Culture supernatants of these cells were then used to infect young IMR-90 cells at population doubling level (PDL) 25. Infected cells were selected in media containing G418 (400 μg/ml) for 2 wk, after which point they were passaged without selection.

Serum Starvation and Stimulation

Subconfluent cells growing in media containing 10% serum were washed three times with serum-free medium and were fed medium containing 0.3% serum for 3 d. Cell density was adjusted so that the

[1] Abbreviations used: Dex, dexamethazone; SV40, simian virus 40; PBS, phosphate-buffered saline; pRB, product of the retinoblastoma susceptibility gene; PDL, population doubling level.

cells would be at 50–70% confluency at the end of the starvation period. Parallel series were established in 24-well dishes for the measurement of DNA synthesis and in 10-cm dishes for the analysis of FOS levels. In both cases, serum was added to a final concentration of 10% at different times so that all wells/dishes could be harvested at the same time.

Tritiated Thymidine Incorporation

The rate of DNA synthesis was measured by the level of incorporation of thymidine into acid-insoluble fractions. Determinations were done after a 3-h pulse with [^{3}H]thymidine (5 μCi/ml) and 5′-fluorodeoxyuridine (2.65 μg/ml), ending at the time of harvest. Wells were washed with 10% trichloroacetic acid, their contents were solubilized in an alkaline buffer (0.25 N NaOH and 1% SDS), and scintillation counted. Cells from duplicate wells were trypsinized, and their number was determined by a Coulter counter. The amount of thymidine incorporation per cell per 3 h was then calculated.

Protein Isolation and Immunoblotting

Cells were washed twice in ice-cold phosphate-buffered saline (PBS), scraped and lysed in a buffer containing 0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.5, 5% sucrose, and 1% SDS (0.4 ml/10-cm dish). The samples were passed 10 times through the tip of a Gilson P20-P200 to reduce viscosity and were then stored at −70°C. Protein concentration was determined by the use of fluorescamine. Two to ten microliters of each sample in 1 ml of 0.2 M sodium borate, pH 9.25, was mixed while vortexing with 0.5 ml of fluorescamine (30 mg/100 ml in acetone). Fluorescence was immediately measured with a fluorometer designed for fluorescent DNA analysis (TKO fluorometer; Hoeffer, San Francisco, CA) with an excitation wavelength of 390 nm and an emission wavelength set at 475–490 nm. Bovine serum albumin was used as the standard. The level of FOS was determined by Western blotting with the use of a monoclonal antibody (AB-1; Oncogene Science, Manhasset, NY) directed against the basic domain of FOS. In our hands, this antibody recognized FOS as a single band with an apparent molecular weight of 62 kDa and failed to detect other members of the FOS family (Fra-1, Fra-2, and FosB). One hundred micrograms of total protein, supplemented with 0.7 M β-mercaptoethanol and 0.01% bromophenol blue were boiled for 5 min, separated on an SDS–10% polyacrylamide gel (Laemmli, 1970), and electrotransferred to a charged nylon membrane (Immobilon P; Millipore, Bedford, MA). The membranes were incubated for 4 h in blocking buffer (PBS supplemented with 6% bovine casein and 1% polyvinylpyrrolidone-40, pH 7.6) and then overnight at 4°C with the anti-FOS antibody (1 μg/ml in blocking buffer). After three 5-min washes in washing buffer (PBS containing 0.3% Tween-20), the membranes were exposed for 1 h at room temperature to an alkaline phosphatase-conjugated goat antibody against mouse IgG (0.2 μg/ml in blocking buffer) (Southwestern Biotechnology Associates, Birmingham, AL). Membranes were washed once for 15 min and then four times for 5 min each in washing buffer. The membranes were then treated with Nitroblock (Tropix, Bedford, MA) to enhance cheminoluminescence signals, washed in assay buffer (1 mM $MgCl_2$, 50 mM Tris-HCL, pH 9.5), and then impregnated with disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (100 mM in assay buffer; Tropix). Membranes were then blotted dry and exposed to x-ray films.

RESULTS

At PDL 25, rapidly growing IMR90 fibroblasts were infected with defective retroviruses carrying a neomycin resistance gene and expressing various human papilloma virus type 16 proteins. These populations of cells reached a calculated PDL of 36 after 2 wk of selection in G418. Figure 1A demonstrates that, at PDL 38, E7-expressing cells (E7 alone or E6 plus E7) failed to growth arrest in low serum. Control fibroblasts and those expressing E6 alone became quiescent in 0.3% serum and were induced to synthesize DNA with equivalent kinetics after the addition of serum. Figure 1B shows that FOS was transiently induced by serum in the control cells and in those expressing E6 alone, whereas it was undetectable at any time after the addition of serum to E7-expressing cells (E7 alone or E7 plus E6). The failure of serum to induce FOS in E7-expressing cells cannot be a simple consequence of

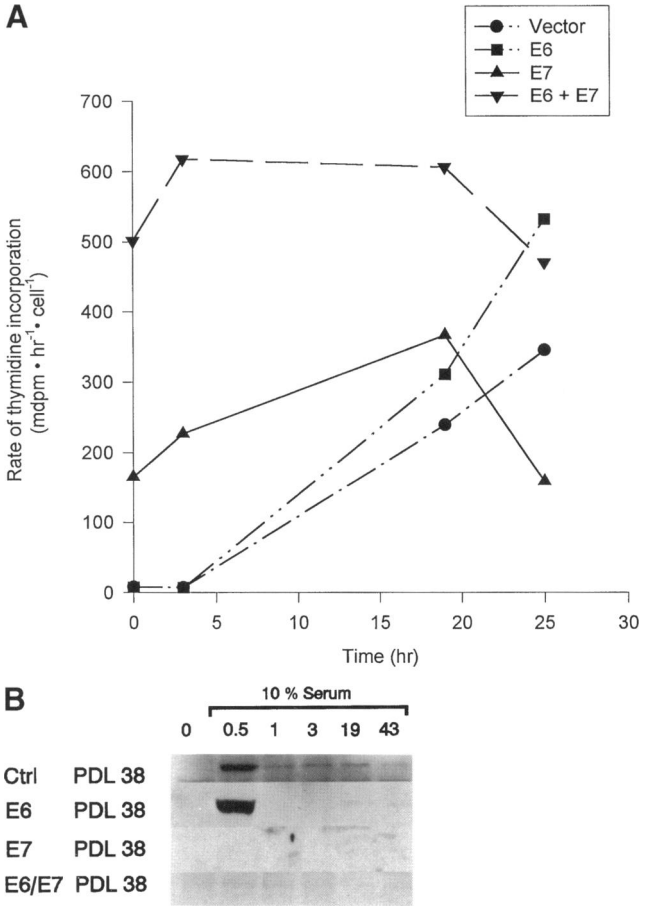

Figure 1. Characterization of fibroblast populations expressing E6, E7, or both oncogenes before the onset of M1. At PDL 38, subconfluent IMR90 fibroblasts infected with retroviruses expressing E6, E7, E6/E7, or no oncogene were serum starved for 3 days in media containing 0.3% serum. Serum was then added to 10%, and cell samples were harvested both before (time 0) and at various times (0.5, 1, 3, 19, and 43 h) after the addition of serum. (A) Rate of thymidine incorporation. (B) FOS levels. One hundred micrograms of total proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a charged nylon membrane in which FOS was revealed by chemoluminescence with the use of a mouse monoclonal antibody directed against human FOS.

their failure to growth arrest in low serum. First, c-fos is not a cell-cycle–regulated gene, and both its basal level and its inducibility by serum remained unchanged along the cell cycle (Bravo *et al.*, 1986). Second, our E6-expressing fibroblasts continued to show serum-inducible FOS expression after M1 although they had become resistant to growth arrest (see below).

Very different results were obtained when the cells were reexamined at higher PDLs. All four cell populations were passaged until they had reached the end of their lifespans. Control cells infected with the backbone vector reached normal senescence at PDL 50, at which point cells were viable but failed to incorporate thymidine after serum stimulation. All three populations of cells carrying viral oncogenes exhibited an extended lifespan. As reported previously (Shay *et al.*, 1993), cells expressing only E6 or E7 showed a partial extension of lifespan and, in the present experiment, stopped proliferating at PDL 64 and PDL 74, respectively. Cells expressing both E6 and E7 exhibited the full extension of lifespan and reached crisis at PDL 89. Near the end of their lifespans, all three populations of cells carrying an oncogene were capable of incorporating thymidine in media containing 0.3% serum (Figure 2). For those cells carrying E6 alone, this observation represents a drastic change in behavior, because these cells at PDL 38 became quiescent and failed to incorporate thymidine in low-serum conditions.

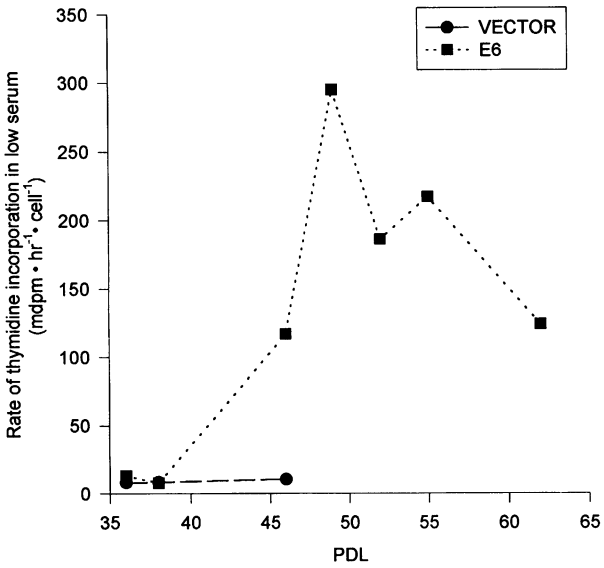

Figure 2. Rate of thymidine incorporation in low serum as a function of PDL in E6-expressing cells. Subconfluent fibroblasts expressing E6 alone (E6) or carrying no oncogene (Vector) were serum starved for 3 days in media containing 0.3% serum. Then their rate of thymidine incorporation in low serum was measured. Both cell populations were examined at different PDLs.

The expression of FOS was reexamined in all four cell populations as they approached the end of their lifespans (Figure 3). As expected, little-to-no FOS was induced in the control fibroblasts as they approached M1 (at PDL 46). Cells expressing E7 alone continued to show the absence of FOS expression at PDL 64. However, FOS was induced by serum in the old population of fibroblasts expressing E6 alone (at PDL 56). Surprisingly, an induction of FOS was also seen, at PDL 89, in the old cells expressing both E6 and E7, a combination that showed no FOS induction at PDL 38. Moreover, the kinetics of the FOS induction in the old populations of cells expressing E6, alone or with E7, was altered. The initial peak of FOS expression was not always seen within the first hour after serum addition and was sometimes delayed until the third hour post-serum. Because of sample to sample variations, this early peak of FOS expression was sometimes missed in the limited set of time points used, as is the case for the E6-expressing fibroblasts displayed in Figure 3. In addition, a novel late peak of FOS expression was sometimes observed at 19 h after serum addition. The kinetics of appearance of these late peaks of FOS expression was further explored by analyzing samples every 30 min for 8.5 h, starting 13.5 h after the addition of serum (Figure 4). In both populations of old cells (E6 alone or with both E6 and E7), FOS levels were found to oscillate, with dramatic changes in FOS levels occurring within one-half hour. The apparent period of the oscillation was close to 2 h.

Cells expressing E6 were reexamined at intermediate passage levels to determine when the changes in tritiated thymidine incorporation and FOS expression occurred. Cells expressing E6 alone gained a capacity to synthesize DNA and to grow in low serum as they approached M1 (Figure 2). At PDL 36 and 38, the population failed to incorporate thymidine in low serum, but significant incorporation was detected at PDL 46 and beyond. Similar results were also obtained

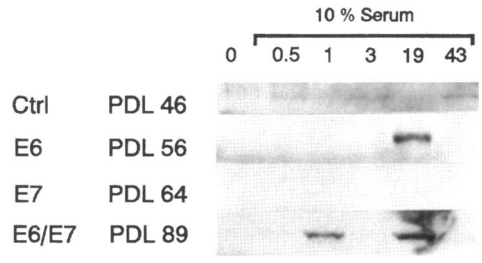

Figure 3. Expression of FOS in the human fibroblast populations expressing E6, E7, or both oncogenes after the induction of M1. Subconfluent fibroblasts carrying E6 (at PDL 56), E7 (at PDL 64), both oncogenes (at PDL 89), or no oncogene (at PDL 46) were serum starved for 3 days in media containing 0.3% serum. Serum was then added to 10%, and cell samples were harvested both before (time 0) and at various times (0.5, 1, 3, 19, and 43 h) after the addition of serum.

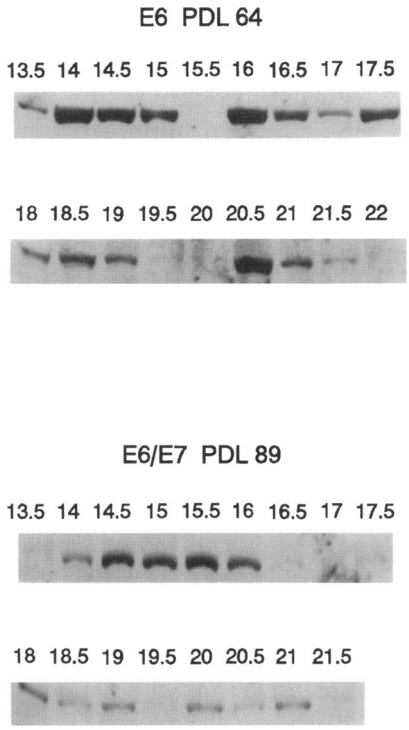

Figure 4. Characterization of the late peaks of FOS expression observed near crisis in fibroblasts carrying E6 alone or with E7. Subconfluent fibroblasts expressing E6 (at PDL 64) or both E6 and E7 (at PDL 89) were serum starved for 3 days in media containing 0.3% serum. Serum was added to 10%, and cell samples were collected every 30 min starting 13.5 h after serum addition until 22 h postserum.

Table 1. A change in cellular physiology accompanies the onset of M1 in E6-expressing fibroblasts

Cells	Properties	pre-M1	post-M1
Vector	Growth in low serum	N	N
	Serum inducibility of FOS	Y	N
	FOS oscillations	N	N
E6	Growth in low serum	N	Y
	Serum inducibility of FOS	Y	Y
	FOS oscillations	N	Y
E7	Growth in low serum	Y	Y
	Serum inducibility of FOS	N	N
	FOS oscillations	N	N
E6/E7	Growth in low serum	Y	Y
	Serum inducibility of FOS	N	Y
	FOS oscillations	N	Y

Our results show that a dramatic change in cellular physiology occurs as cells approached M1. E6-expressing cells behaved identically to the controls (Vector) until M1 was reached, after which point they failed to repress the serum-dependent inductions of FOS, produced oscillations in FOS levels, and grew well in low serum, properties that they kept until the end of their lifespans. Fibroblasts expressing E7 kept a unique phenotype until the end of their lifespans characterized by a complete absence of FOS and a capacity to grow in low serum. Cells expressing both E6 and E7 had a composite phenotype: they first displayed properties of the E7 fibroblasts but later behaved like the post-M1 fibroblasts expressing E6. Y/N, Yes/No.

from a second population of fibroblasts independently infected with the E6-retrovirus and examined at both PDL 38 and PDL 62. Oscillations in FOS levels were also first detected at the time when the control cells approached M1. Thus, the oscillations were absent at PDL 36 and 38 but were seen at PDL 46, 49, 52, 55, and 64. Cells expressing both E6 and E7 showed high levels of thymidine incorporation in low-serum conditions at all PDL examined with no age-related changes. The induction of FOS in cells expressing both E6 and E7 was undetectable at PDL 38 and at the time of M1 (at PDL 51) but developed into serum-induced oscillations during the extended lifespan (at PDL 74 and 89) (Figure 4).

Table 1 summarizes the results we obtained after examining all four cell populations both before and after the induction of M1 (at PDL 50). On the basis of three criteria (the presence of serum-dependent induction of FOS, the presence of oscillation in FOS level, and the capacity of cells to grow in low serum), clearly there is dramatic change in cellular physiology as E6-expressing cells (E6 alone or E6 plus E7) enter their extended lifespans and approach crisis. E6 binds and inactivates p53 by stimulating its ubiquitin-directed degradation (Huibregtse *et al.*, 1993; Scheffner *et al.*, 1993). Western blots were performed with an anti-p53 antibody (pAB421, Oncogene Science) to determine if any of the changes with PDL could be explained by alterations in the level of p53. Although the steady-state level of the p53 protein was highly reduced in cells expressing E6 (with or without E7), no significant change was seen as a function of passage level. This result shows that the change in cellular physiology that occurs at M1 in the presence of E6 is not a simple consequence of age-dependent changes in p53 level.

The oscillations in FOS level seen as cells approached crisis were unanticipated. To determine whether this pattern was unique to cells expressing E6 and whether it persisted after immortalization, we examined FOS expression in IDH4 cells (Wright *et al.*, 1989). IDH4 cells were derived from IMR90 fibroblasts that had been immortalized with a steroid-inducible SV40 large T antigen. The transcription of T antigen in these cells is controlled by the Long Terminal Repeats (LTR) of the mouse mammary-tumor virus whose activity depends on the presence of glucocorticoids such as Dex. In the absence of Dex, the expression of T antigen is greatly reduced, and the cells stop dividing and display a senescent phenotype (Shay *et al.*, 1992). Under these conditions, the reversibly senescent IDH4 cells failed to incorporate significant amounts of tritiated thymidine and also failed to express FOS after

serum stimulation (Figure 5A). IDH4 cells expressing T antigen (grown in medium containing Dex) behaved like E6/E7-expressing cells and incorporated high amounts of tritiated thymidine in both 0.3% and 10% serum. As with the old cells carrying E6/E7, serum stimulation produced an early peak in FOS level followed by later peaks of expression (Figure 5A). The detailed kinetics of this response showed an oscillation with the same ~2 h periodicity as had been observed in the E6 or the E6/E7 cells at the end of their lifespans (Figure 5B).

The dependence of FOS expression on T antigen in IDH4 cells was further examined during the reversal of the senescent phenotype that follows the reinduction of T antigen by Dex (Figure 6). When Dex was added to cells maintained in steroid-free media containing 10% serum, a unique peak of FOS expression was detected 11 h later. The experiment was repeated twice with the same results. Eleven hours corresponds to the approximate time of half-maximal accumulation of T antigen after Dex addition to IDH4 cells (Shay *et al.*, 1992). When Dex was added to IDH4 cells maintained in steroid-free media containing 0.3% serum, FOS failed to be detected at all time points (Figure 6). This result shows that the presence of T antigen in senescent IDH4 cells can permit the induction of FOS and that this expression of FOS is still dependent on the presence of 10% serum. Interestingly, a single peak of FOS was observed, and no oscillations or later peaks were seen. These results contrast with those obtained with IDH4 cells continuously exposed to Dex and then serum stimulated. They indicate that the initial FOS peak depends on the presence of both T antigen and 10% serum but is not dependent on the specific timing of serum addition, whereas the later oscillations are dampened if T antigen is not already present at the time of serum stimulation.

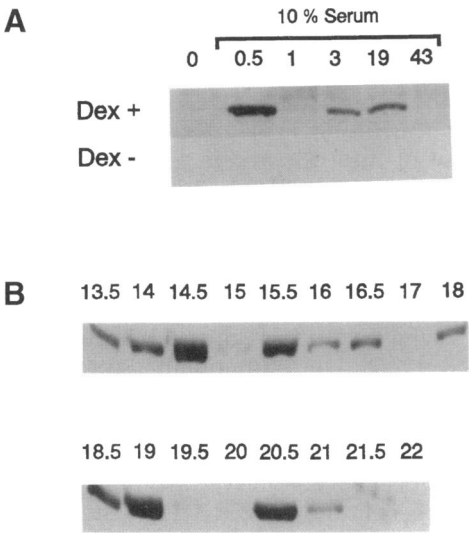

Figure 5. FOS levels in IDH4 grown with or without Dex. IDH4 cells were first grown for 4 days in media containing 10% charcoal-treated serum to which 1 μM Dex was added (Dex +) or omitted (Dex −). The cells were then serum starved for 3 days in media containing 0.3% charcoal-treated serum with (Dex +) or without (Dex −) Dex. Charcoal-treated serum with (Dex +) or without (Dex −) Dex was then added to 10%, and cell samples were collected at various times and analyzed for fos expression. (A) Samples were collected both before (time 0) and after (0.5, 1, 3, 19, and 43 h) the addition of serum. (B) Samples were collected every 30 min starting at 13.5 h after serum addition to Dex + cells.

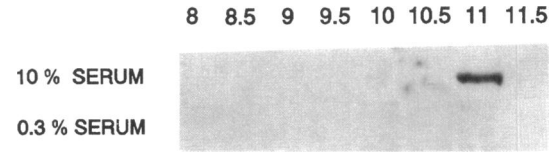

Figure 6. Induction of FOS in senescent IDH4 cells after the reexpression of T antigen by Dex. Subconfluent IDH4 cells were plated for 1 wk in the absence of Dex in media containing 10% or 0.3% of charcoal-treated serum. Dex was then added to 1 μM, and cell samples were collected every 30 min from 8 until 22 h after the addition of Dex. Only those samples spanning the 8 to 11.5 h time points are shown.

DISCUSSION

The general repression of the c-fos gene in senescent fibroblasts is a well-established phenomenon (Seshadri and Campisi, 1990; Irving *et al.*, 1992). In this study we determined whether this repression was altered by any of the viral oncogenes that block parts of the M1 mechanism and extend the lifespan of human cells. The expression of FOS was examined in normal IMR-90 fibroblasts expressing the E6 and/or E7 proteins of human papilloma virus type 16 and in the SV40 T antigen–immortalized IDH4 cells. The results indicate a dramatic change that occurs with cellular senescence in both cellular physiology and the regulation of FOS expression (Table 1). Before the onset of M1, E7 blocks the serum inducibility of FOS, whereas E6 has no effect. The action of E7 is dominant during this period, because cells expressing both oncogenes fail to express FOS. These relationships change at the PDL corresponding to the appearance of the M1 mechanism in control cells. E6 blocks the mechanism by which FOS inducibility is repressed in senescent cells and, in addition, allows a novel oscillation of FOS levels to be observed. Because these phenomena are also observed near crisis in cells expressing both E6 and E7, it is clear that, in the extended lifespan, E7 no longer behaves as a dominant repressor of FOS and that E6 is now the dominant oncogene (Table 1).

The two-stage model of cellular senescence predicts that the M1 mechanism is still activated in cells immortalized by agents such as HPV E6/E7 or SV40 T

antigen. These agents do not prevent the induction of the M1 mechanism but, rather, block its downstream antiproliferative effects. This is accomplished through their interactions with both p53 and the pRB-like pocket proteins (Hara *et al.*, 1991; Shay *et al.*, 1991). The persistence of the M1 mechanism can be demonstrated in cells immortalized by temperature-sensitive (Radna *et al.*, 1989) or inducible SV40 T antigen, such as IDH4 cells (Wright *et al.*, 1989; Shay *et al.*, 1992). De-induction of SV40 T antigen in IDH4 cells leads to growth arrest and to the reexpression of a senescent phenotype. In the present study, the persistence of M1 is also demonstrated by the repression of FOS induction that occurs when the expression of T antigen is reduced in IDH4 cells. The persistence of an "M1 physiology" is further reinforced by the observation that the same changes observed after the appearance of M1 in precrisis E6 or E6/E7-expressing cells are also seen in IDH4 cells immortalized by SV40 T antigen. Even after immortalization and the reexpression of telomerase, oscillations of FOS levels in IDH4 cells are observable, a phenomenon first detected in mortal cells only after the induction of M1.

The E6 protein from the type 16 virus displays multiple binding properties and has been shown to interact with E6-AP to form a complex that recruits p53 molecules for ubiquitin-directed degradation (Huibregtse *et al.*, 1993; Scheffner *et al.*, 1993). Thus, E6-expressing cells are expected to display a phenotype that includes properties of p53 null mutants. p53 null mutants would not be expected to have a phenotype unless the p53 system is engaged first. Such an activation is thought to occur at M1, and that may explain why E6-expressing cells first diverged from the controls only when the control cells had become senescent. The failure of E6 fibroblasts to repress FOS expression at M1 and during their extended lifespan is compatible with the interpretation that p53 may be directly or indirectly responsible for the repression of FOS expression in senescent cells.

Another dramatic indication of the altered physiology that occurs at M1 is the change in the serum requirements for the growth of E6-expressing cells. Before the onset of M1, E6 had no growth-stimulating properties, and cells expressing E6 became quiescent when subjected to serum starvation. However, after the induction of the M1 mechanism, the cellular response to E6 changed; now the cells grew well in both 0.3% and 10% serum. A possible explanation for this result derives from experiments showing that low levels of DNA damage can stimulate scheduled DNA synthesis in normal fibroblasts in low serum (Cohn *et al.*, 1984). One hypothesis for the induction of M1 predicts that one or a few of the 92 telomeres in a normal diploid cell may lack sufficient numbers of telomeric repeats to protect the end of the chromosome at a time when most telomeres still have several kilobase pairs of repeats remaining (Harley *et al.*, 1990; Allsopp *et al.*, 1992; Counter *et al.*, 1992; Shay *et al.*, 1993). This lack of protection would then produce a DNA damage signal that would activate p53, which, in turn, would increase p21 expression and induce the M1 mechanism. It is possible that by causing the degradation of p53, E6 blocks the antiproliferative effects of the DNA damage signals that may occur at M1 and thus uncovers their DNA synthesis-stimulating effects.

The E6 protein has been shown to have transforming properties (growth in soft agar and anchorage-independent growth) that could not be mimicked by a dominant negative mutant of p53 (Sedman *et al.*, 1992). Despite its small size, it is a multifunctional protein that interacts not only with E6-AP, p53, and a number of other cellular proteins (Huibregtse *et al.*, 1993; Scheffner *et al.*, 1993; Keen *et al.*, 1994) but also binds DNA and acts as an activator or suppressor of the transcription of some genes (Etscheid *et al.*, 1994). The activity or identity of the cellular partners of E6 may change with aging, producing pleiotropic variations at the onset of M1. Some of these changes, unrelated to the state of the p53 protein, could also be responsible for the altered regulation of FOS and growth by E6 in the post-M1 E6-expressing cells. Western blots of p53 levels in our E6-expressing cells showed an approximately fivefold reduction in steady-state levels that did not change throughout the normal and extended lifespan.

Although FOS expression was always tightly regulated by serum in E6 fibroblasts, its pattern of expression was modified after the onset of M1 and now included late peaks of expression. Surprisingly, these later peaks represented oscillations in FOS levels, a behavior never described before. Continuous expression of the c-fos mRNA has been seen by others under specific conditions (Naranjo *et al.*, 1990; Schönthal and Feramisco, 1993), but no evidence of oscillations or of late peaks of FOS expression have been documented by others. In the present study, FOS oscillations were observed in the E6-expressing cells but were also seen in the fibroblasts expressing both E6 and E7 and in the T antigen expressing immortalized IDH4 cells. Our results also indicate that the presence of oscillations is dependent on the induction of the M1 mechanism and that it requires E6-like functions. The FOS oscillations could be the consequence of an "out-of-context" presence of FOS in cells experiencing the M1 mechanism in which other alterations have occurred as a result of aging. One interpretation is that E6 may block only some of the effects of the M1 mechanism and allow FOS to be expressed without restoring all the pathways targeted by M1. Regulatory mechanisms have been identified that control FOS expression at the level of transcription and degradation of both the protein and the messenger RNA (Angel and Karin, 1991). We have not yet determined at which level of FOS expres-

sion the oscillations are generated, but one possibility is that they result from the alteration of a negative-feedback loop known to exist, which normally restricts the induction of FOS to a single peak (Angel and Karin, 1991).

The repression of FOS by E7 before M1 was unexpected, because E7 has been shown to activate FOS expression (Morosov *et al.*, 1994). However, we suspect that the repression of FOS by E7 in our experiments reflects the same long-term result as that observed with the serum stimulation of FOS. Under conditions of serum starvation, exposure to serum stimulates a transient peak of FOS protein, which then declines to very low basal levels that do not increase if the cells are fed fresh serum. Thus, either through changes in receptors or intermediates in the induction pathway, the cells become adapted to high serum. The above experiments showing an induction of FOS by E7 involved the induction of E7 and did not examine FOS levels or inducibility under steady-state conditions. We suspect that the repression of FOS that we observed reflects the down-regulation of pathways for FOS expression in young cells that constitutively express E7. This interpretation is consistent with the concept that a different pathway for the induction of FOS is present during the extended lifespan revealed by the effects of E6. Thus, although E7 is able to down-regulate the serum-response pathway for FOS induction in young cells, it is a different, novel pathway not blocked by E7 that appears after M1 and produces FOS oscillations late during the extended lifespan period in cells expressing both E6 and E7.

In summary, we have shown that the senescent block to FOS expression is likely to be a result of the p53 arm of the M1 mechanism. Furthermore, our results indicate that changes in cellular physiology at M1 produce dramatic alterations in the response to HPV16 proteins. Not only do cells expressing E6 then acquire the ability to grow in low serum and exhibit oscillations in FOS levels, but cells expressing E7 lose the ability to dominantly repress the inducibility of FOS. Knowledge of the detailed mechanisms underlying these effects should contribute to our understanding of the molecular mechanisms regulating cellular senescence.

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