

Reversible Cellular Senescence: Implications for Immortalization of Normal Human Diploid Fibroblasts

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Received 22 November 1988/Accepted 30 March 1989

IMR-90 normal human diploid fibroblasts, transfected with a steroid inducible mouse mammary tumor virus-driven simian virus 40 T antigen, were carried through crisis to yield an immortal cell line. Growth was dependent on the presence of the inducer (dexamethasone) during both the extended precrisis life span of the cells and after immortalization. After dexamethasone removal, immortal cells divided once or twice and then accumulated in G1. These results are best explained by a two-stage model for cellular senescence. Mortality stage 1 (M1) causes a loss of mitogen responsiveness and arrest near the G1/S interface and can be bypassed or overcome by the cellular DNA synthesis-stimulating activity of T antigen. Mortality stage 2 (M2) is an independent mechanism that is responsible for the failure of cell division during crisis. The inactivation of M2 is a rare event, probably of mutational origin in human cells, independent of or only indirectly related to the expression of T antigen. Under this hypothesis, T-antigen-immortalized cells contain an active but bypassed M1 mechanism and an inactivated M2 mechanism. These cells are dependent on the continued expression of T antigen for the maintenance of immortality for the same reason that precrisis cells are dependent on T antigen for growth: both contain an active M1 mechanism.

Normal diploid cells exhibit a finite proliferative capacity in culture (13–16, 25, 32, 43), characterized by a decreasing mitogen responsiveness (33) and an eventual arrest in G1. The frequency with which cells from different species escape cellular senescence and give rise to immortal cell lines varies extensively (23). Whereas mouse cells immortalize with a high probability (6, 19, 24, 29), human diploid fibroblasts essentially never spontaneously evolve into immortal lines. Chemical carcinogens readily immortalize rodent cells but rarely immortalize human fibroblasts (28). A variety of oncogenes can immortalize mouse cells, but these oncogenes fail to immortalize human diploid fibroblasts (8, 44, 45), although there is one report that *ras* can immortalize human epithelial cells (51).

DNA tumor viruses such as simian virus 40 (SV40) and polyomavirus can immortalize human fibroblasts. Both appear to immortalize by expressing a protein called large T antigen. Plasmids expressing the SV40 early region containing the large T and small t antigens are able to immortalize human fibroblasts (5, 27). SV40 extends the life span of normal human diploid fibroblasts by about 20 population doublings (17, 22, 48), but then the cells enter crisis. Crisis is a period when cell number remains constant or declines because successful cell division is balanced by cell death. Stein has proposed that crisis represents senescence in SV40-infected cells (48). In some cases, a colony (focus) of immortalized cells appears, which can then be subcultured indefinitely. We have determined that approximately 50% of transfected clones expressing the SV40 early region can immortalize and that the frequency of immortal foci within each immortalization-competent clone is approximately 3×10^{-7} (J. W. Shay and W. E. Wright, *Exp. Cell Res.*, in press).

In rodent cells, temperature-sensitive SV40 T-antigen mutants have been used to demonstrate that T antigen is

necessary to obtain immortalization over the background of spontaneous events and that continued expression of T antigen is necessary for maintenance of the immortal phenotype (2, 3, 18, 26, 34, 36, 39, 41). The role of T antigen in the maintenance of immortality of human cells has not yet been determined. Because the mechanisms regulating senescence in rodent and human cells are different, this study was undertaken to examine this question. We report that the continued expression of T antigen is required for the proliferation of T-antigen-immortalized human fibroblasts and discuss a model suggesting why it is misleading to say that T antigen is necessary for the maintenance of immortality. A similar observation for a temperature-sensitive T antigen is presented in the accompanying paper (40).

METHODS

Cells and media. IMR-90 normal human fibroblasts (ATCC 186) were maintained in a 4:1 mixture of Dulbecco modified Eagle medium-medium 199 supplemented with 10% defined supplemented bovine calf serum (Hyclone Laboratories, Logan, Utah). When necessary, serum was depleted of steroids by being stirred overnight at 4°C with 5 mg of sterile activated charcoal (Norit A) per 100 ml of serum (10). After removal of the charcoal by spinning at $25,000 \times g$ for 60 min, the serum was decanted and stored at -10°C .

Plasmids and transfection. A plasmid containing the SV40 early region under control of the mouse mammary tumor virus promoter was generously provided by Michael Crow. This plasmid, pMTV-D305, was constructed by deleting a *Bam*HI-partial *Hind*III fragment containing the DHFR sequence from plasmid pMTVdhfr (21) and replacing it with a *Bam*HI-partial *Hind*III fragment from plasmid pD3-05 (4). This fragment contains the SV40 early region and has a deletion (0.585 to 0.54 map units) that eliminates most of the small-t-antigen-coding region. pMTV-D305 expresses increased amounts of large T antigen in response to steroids such as dexamethasone.

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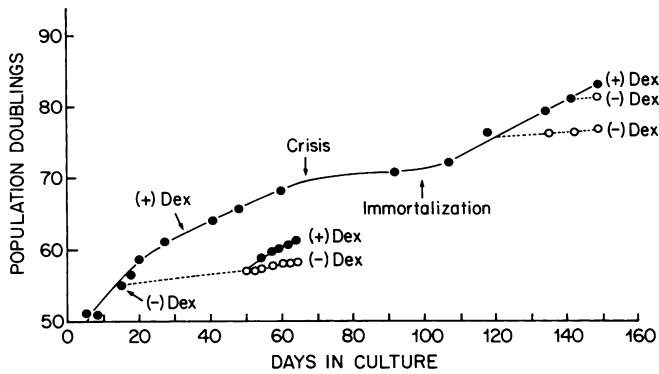


FIG. 1. Immortalization of IMR90-D305. IMR-90 human diploid fibroblasts at PDL 25 were cotransfected with a selectable marker and a plasmid containing an SV40 early region driven by the steroid-inducible murine mammary tumor virus promoter. One clone positive for T antigen by immunostaining was subcultivated in the presence of 10^{-6} M dexamethasone (Dex) through crisis until an immortal line was obtained. The growth rate, expressed as the PDL obtained, is presented for the entire period. ---, Behavior of sister cultures initiated at different times and maintained in the absence of dexamethasone. Growth was dependent on steroids during both the pre- and postcrisis phases.

Rapidly dividing IMR-90 fibroblasts at population doubling level (PDL) 25, plated at 200,000 cells per 10-cm^2 dish, were cotransfected with $1\ \mu\text{g}$ of a selectable marker (PSV2neo) (47) and $10\ \mu\text{g}$ of pMTV-D305, using Polybrene as described by Morgan et al. (30). The next day, the cells were fed cloning medium (MCDB 202 [11]) supplemented with $400\ \mu\text{g}$ of G418 per ml and 10^{-6} M dexamethasone and placed in modular incubators (gassed with 1% oxygen–5% carbon dioxide–94% nitrogen to increase the cloning efficiency [49]). Two of six clones that survived selection in G418 exhibited nuclear staining. One of these reached crisis before it could be expanded and was therefore lost. The remaining T-antigen-positive clone was expanded and designated IMR90-D305. Unless otherwise indicated, IMR90-D305 was maintained in $1\ \mu\text{M}$ dexamethasone. Cells were counted at each passage, and the increase in PDL was calculated as the \log_2 of the fold increase in cell number. Cells were expanded so that approximately 20 150-cm^2 dishes, each containing 1×10^6 to 4×10^6 cells, were available when crisis was reached.

RESULTS

Figure 1 shows the proliferative history of clone IMR90-D305. This clone was at PDL 50 by the time it had been transfected and expanded. IMR-90 senesces at about PDL 45 to 50, and it is probable that this clone would have been senescent at this time in the absence of T antigen. SV40 T antigen extends the life span of IMR-90 by about 20 PDL, and clone IMR90-D305 entered crisis at approximately PDL 70. After 2 months, eight foci were observed in several dishes. One focus differing slightly in morphology from the others was picked and designated IMR90-D305.2. The remaining foci were pooled and designated IMR90-D305.1. Although the two clone groups behaved similarly, IMR90-D305.2 was used because of its more vigorous growth. Both precrisis and postcrisis cells were dependent on the presence of dexamethasone for continued proliferation.

The concentration of dexamethasone required for growth

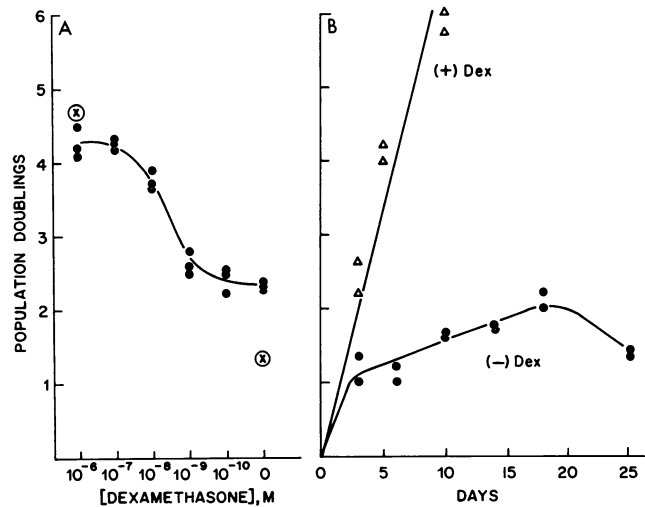


FIG. 2. Dexamethasone dependence for cell growth. (A) Growth curves for postcrisis IMR90-D305.2 cells (PDL 90) plated in triplicate in various concentrations of dexamethasone (Dex). One set of dishes was counted the next morning to determine the number of attached cells. The remaining cells were counted 6 days later. The fold increase in cell number was obtained by dividing the final cell counts by the number initially attached. Some growth occurred even in dexamethasone-free medium. In a second experiment, the serum was treated with activated charcoal to remove endogenous steroids. ⊗, Growth was still good in dexamethasone-supplemented medium containing charcoal-treated serum but the number of doublings was reduced twofold in the absence of steroids contributed by the serum. (B) Growth curves for postcrisis IMR90-D305.2 cells (PDL 106) in charcoal-treated serum with or without 10^{-6} M dexamethasone. An initial doubling of cell number in the absence of dexamethasone occurred within the first few days, after which cell number stayed relatively constant for at least 3 weeks.

is shown in Fig. 2A. Treating serum with activated charcoal to remove residual steroids resulted in a twofold reduction in growth compared with growth obtained by using untreated serum, indicating that the serum contained sufficient glucocorticoid activity to support some proliferation of the IMR90-D305.2 cells. Figure 2B presents growth curves in medium containing 10% charcoal-treated serum in the presence versus the absence of dexamethasone. In dexamethasone-free medium, the cells divided once during the first 4 days, whereafter cell number remained almost constant before beginning to decline after 3 weeks. Immunoprecipitation of metabolically labeled T antigen indicated a more than 20-fold decrease in the rate of T-antigen synthesis in the cells grown steroid free (Fig. 3).

The possibility that the growth dependence of IMR90-D305.2 might have been due to effects of dexamethasone other than the regulation of T antigen was eliminated by providing an alternate source of SV40 T antigen. IMR90-D305.2 cells were transfected with either pSV3gpt, a plasmid containing the bacterial xanthine-guanosine phosphoribosyltransferase gene and the SV40 early region, both under control of the SV40 promoter-enhancer (31), or pSV2gpt, the closely related plasmid which lacks the SV40 early region. In the presence of dexamethasone, 9 pSV2gpt- and 12 pSV3gpt-transfected clones per 6×10^5 cells were obtained. No pSV2gpt-transfected clones were observed in the absence of dexamethasone, whereas five clones of pSV3gpt-transfected clones were obtained. The steroid dependence was therefore due to a direct effect on T-antigen expression, since provid-

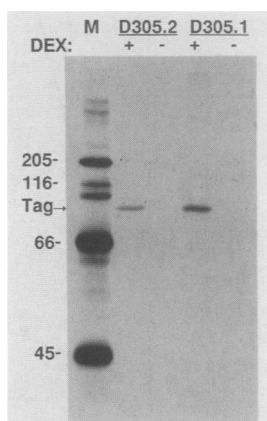


FIG. 3. Synthesis of T antigen by IMR90-D305.2 cells. Cells grown for 1 week in the presence (+) or absence (-) of 10^{-6} M dexamethasone (DEX) were labeled for 3 h with [35 S]methionine in methionine-free medium to which 3 μ g of cold methionine per ml had been added; the cells were lysed and then immunoprecipitated by using the monoclonal antibody 101 as described by Ericson et al. (7) and Opperman et al. (35), with minor modifications. Immunoprecipitates were loaded into 10% polyacrylamide gels on the basis of equivalent cell numbers (determined by counting duplicate flasks). The position of T antigen (Tag) is indicated. Densitometry of the autoradiogram indicated that at least 20-fold more T antigen was made in the presence than in the absence of dexamethasone. M, Molecular weight markers (indicated in thousands on the left).

ing an independent source of T antigen abrogated the steroid dependence.

The DNA contents of pre- and postcrisis cells were analyzed by fluorescence-activated cell sorting (Table 1). In the presence of dexamethasone, most of the cells exhibited a G1 content of DNA (consistent with their relatively slow [36 h] doubling time), and this fraction increased after 7 days in the absence of steroids. In addition, the percentage of cells in mitosis decreased by an order of magnitude in cultures grown steroid free. Most of the cells that had more than the G1 DNA content in the absence of dexamethasone are therefore extremely unlikely to represent cycling cells; rather, they are probably aneuploid cells with a greater than normal DNA content (the chromosome content of IMR90-D305.2 was bimodal, with most cells having an average of 75

TABLE 1. Cell cycle parameters plus or minus dexamethasone

Cell type	% DNA content ^a		No. of mitotic cells/total counted ^b	Mitotic index (%)
	G1	>G1		
Pre-crisis PDL 60				
IMR90-305, +dex	58	42	54/3,058	1.8
IMR90-305, -dex	71	29	3/3,008	0.1
Post-crisis PDL 86				
IMR90-D305.1, +dex	50	50	21/3,026	0.7
IMR90-D305.1, -dex	60	40	2/3,007	0.07
Post-crisis PDL 180				
IMR90-D305.2, +dex	51	49	93/3,130	3.0
IMR90-D305.2, -dex	70	30	9/3,012	0.3

^a Determined by propidium iodide staining (using a modification of the procedure described by Krishan [20]) of cells grown for 1 week in charcoal-treated serum in the presence (+dex) or absence (-dex) of 10^{-6} M dexamethasone. DNA content analysis was performed in an Ortho 50H fluorescence-activated cell sorter (Ortho Diagnostics Institute, Westwood, Mass.).

^b Determined by Giemsa staining of sister dishes grown as described in footnote *a*. Approximately 3,000 cells were counted for each condition.

chromosomes [range, 60 to 81], but about 20% had a much greater number [range, 125 to 140]). Despite a greatly reduced rate of T-antigen synthesis (Fig. 3), cells grown in the absence of dexamethasone for 1 week continued to be T-antigen positive by immunofluorescence. The residual T antigen present after 1 week in the absence of dexamethasone, due either to its long half-life (38 h [1]) or a low basal level of synthesis by the mouse mammary tumor virus promoter, was apparently sufficient to cause only minimal cell cycling. Collectively, these results imply that steroid removal causes the deinduction of T antigen and the accumulation of most of the cells in the G1 phase of the cell cycle.

DISCUSSION

Ide et al. (17) infected a long-lived strain of human fibroblasts with a temperature-sensitive SV40 T-antigen mutant and isolated infected clones before normal senescence. They demonstrated that the cells were not temperature sensitive for growth during presenescence but were temperature sensitive during the extended life span before crisis. They did not obtain any immortal cells and therefore could not determine the temperature sensitivity of the immortal state. The findings presented here and in the accompanying paper (40) extend these results and show that postcrisis T-antigen-immortalized human cells remain dependent on the expression of T antigen for continued proliferation.

A simple description of the data in Fig. 1 is that continued expression of T antigen is required for the maintenance of immortality in human fibroblasts. Although it is correct as a descriptive statement, we believe that this conclusion is misleading and disguises the underlying mechanisms involved. We propose the following two-stage model for cellular immortalization to explain these and other results (Fig. 4).

Mortality stage 1 (M1) involves a loss of mitogen responsiveness, the production of a protein inhibitor of DNA synthesis, and arrest in G1 and is the process commonly viewed as *in vitro* cellular senescence (at approximately PDL 50 in IMR-90 cells; Fig. 4A). The cellular DNA synthesis-stimulating activity of SV40 T antigen overcomes or bypasses the M1 mechanism so that the entire population of cells expressing T antigen escapes from the M1 limit and divides until mortality stage 2 (M2) is initiated (at approximately PDL 70; Fig. 4B). T antigen does not inactivate the M1 mechanism; it simply bypasses it. Consequently, the deinduction of T antigen in postsenescence but precrisis cells results in reexpression of the M1 phenotype (senescence; Fig. 4E).

M2 involves an entirely independent mechanism that limits proliferation. The onset of M2 produces a phenomenon known as crisis, in which the cells attempt to divide but most die in the process. It is the inactivation of M2 that results in the final immortalization event and the escape from crisis (Fig. 4C). The M2 mechanism is inactivated as a very rare event, probably of mutational origin, and its inactivation represents a loss of function, since hybrids between immortal and normal cells are mortal (37, 38). T antigen plays no direct role in M2, although the induction of chromosomal aberrations by T antigen might help eliminate or inactivate the gene for the protein(s) responsible for M2.

Under this hypothesis, SV40 does not inactivate the mechanism of M1 but rather bypasses or overcomes it. Since the inactivation of M2 is postulated to be a mutational event

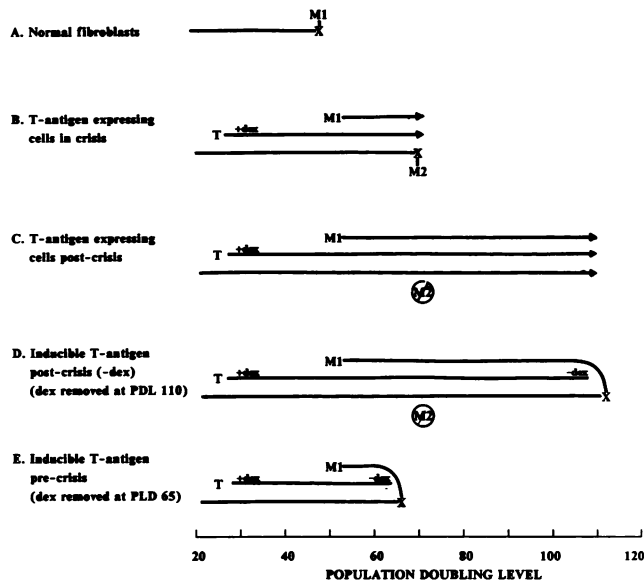


FIG. 4. Schema of the two-stage immortalization hypothesis. Normal human IMR-90 fibroblasts senesce because of the M1 mechanism at about PDL 50 (A). Fibroblasts transfected with a mouse mammary tumor virus promoter-driven SV40 T antigen at PDL 25 and maintained in the presence of dexamethasone (+dex) express T antigen, which bypasses M1 so that cells divide until the M2 mechanism causes crisis at PDL 70 (B). Because of the inactivation of M2, an immortal line appears (C). Because M1 exists after PDL 50 in both pre- and postcrisis cells, deinduction of T antigen (-dex) causes these cells to stop dividing, for example, at PDL 110 (D) or PDL 65 (E).

that is independent of M1. SV40-immortalized cells would still have an active M1 mechanism. The deinduction of T antigen in these immortalized cells would result in reexpression of the M1 phenotype (inability of the cells to proliferate) even though their M2 mechanism had been inactivated (Fig. 4D). Continued expression of T antigen would be necessary for the maintenance of immortality only to the extent that it is required to overcome M1. Since this is exactly the same effect and the same mechanism that is observed in precrisis cells (which are not immortal; Fig. 4E), we believe that it is misleading to describe T antigen as being necessary for the maintenance of immortality.

Since M1 is postulated to be the mechanism responsible for normal cellular senescence, and senescent cells are arrested in G1 (9, 42, 46, 50), this model predicts a similar arrest point for IMR90-D305.2 cells grown in the absence of dexamethasone. Our results indicate that most of the cells accumulated in G1 in the absence of steroids, consistent with this model. Radna et al. (R. L. Radna, Y. Caton, K. K. Jha, P. Kaplan, G. Li, F. Traganos, and H. L. Ozer, unpublished observations) observed that human fibroblasts immortalized with an origin-defective SV40 transformant containing a temperature-sensitive large T antigen became growth arrested as hyperploid cells containing progressively increasing amounts of DNA. Since small t antigen is expressed in these cells at the nonpermissive temperature, whereas small t antigen has been deleted in our construct, one possible explanation for the difference in behavior is the expression of small t antigen.

The two-stage model of cellular senescence can also explain the rarity with which chemical carcinogens immortalize human fibroblasts. Since two independent mechanisms

are involved, the probability of having two mutational events in both required targets within one cell is too small to have been obtained except in exceptional circumstances (28). This model predicts that chemical carcinogens should increase the frequency of immortalization of precrisis T-antigen-expressing cells, in which T antigen bypasses M1 so that only one target site remains. Experiments testing this hypothesis are in progress. This model also predicts that rodent cells either lack M2 or have an M2 mechanism that can be inactivated by epigenetic rather than mutational mechanisms. Since they lack a functionally important M2, they are readily immortalized by chemical carcinogens, spontaneously immortalize with a high frequency, and exhibit little if any crisis when infected with SV40.

This model of cellular senescence predicts that single oncogenes would be unlikely to immortalize human diploid fibroblasts, since both M1 and M2 would need to be bypassed by the same oncogene. Some oncogenes might overcome M1 and extend the life span of fibroblasts such as IMR-90, and these oncogenes should thus complement T antigen in IMR90-D305.2 cells and render them dexamethasone independent. Although M2 represents a dominant mechanism (since immortality is recessive), it is possible that some oncogenes might repress or overcome the M2 mechanism. These oncogenes, however, could be detected only in studies of cells in which the M1 mechanism had been inactivated by T antigen or other oncogenes. The two-stage model for cellular senescence thus provides predictions and explanations for the different behaviors of human and rodent cells and should prove very useful in the design of experiments probing the mechanisms regulating cellular senescence, immortalization, and oncogenesis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AG07992 (W.E.W. and J.W.S.), AG01228 (W.E.W.), PO1-AG07123 (O.M.P.S.), and CA40065 (J.W.S.) from the National Institutes of Health.

We thank J. Butel for the pAB101 anti-T-antigen monoclonal antibody and M. Crow for plasmid pMTV-D305.

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