Suppression of tumor growth by senescence in virally transformed human fibroblasts

(immortalization/simian virus 40/Kirsten murine sarcoma virus/p21/chromosomes)

WENDY O'BRIEN, GÖRAN STENMAN*, AND RUTH SAGER†

Division of Cancer Genetics, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115

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ABSTRACT Normal human cells whether embryonic, neonatal, or adult are resistant to experimentally induced tumorigenesis in contrast to rodent or chicken cells. We showed previously that neither transformation with simian virus 40 DNA nor transfection with human mutant *HRAS* DNA immortalized FS-2 cells (diploid, neonatal human fibroblasts). Further, tumorigenicity was not induced, despite expression of the respective transforming gene products tumor (T) antigen or p21. Here we describe treatment of FS-2 and FSSV cells with baboon endogenous virus pseudotyped Kirsten murine sarcoma virus. FSSV cells were derived from individual foci of simian virus 40-transformed FS-2 cells. The retrovirus-treated FS-2 cells (called FSK) appeared heavily granulated and expressed viral p21 but senesced during passage in culture and were not tumorigenic. The retrovirus-treated FSSV-27 cells (called FSVK-27) expressed simian virus 40 tumor antigen, had elevated levels of viral p21 protein, and formed transient tumors in nude mice. Whether grown in culture or explanted from small tumors, the FSVK-27 cells senesced. The FSVK-46 cells senesced before tumor growth occurred. On the contrary, Kirsten murine sarcoma virus (baboon endogenous virus) treatment of immortalized nontumorigenic human fibroblasts expressing simian virus 40 tumor antigen (Va2 cells) led to consistent tumor formation. The results illustrate the importance of senescence in restricting the tumor-forming ability of human cells.

The resistance of normal human cells grown in culture to experimentally induced tumorigenesis is very well documented (e.g., refs. 1–5). Spontaneous transformation of explanted normal human cells has not been reported. Indeed, such cells maintain normal growth patterns until they senesce, after a variable number of population doublings that are influenced by nutrition and other conditions of cell culture. After senescence no transformed survivors remain from which cell lines can be established, in contrast to rodent cells. Extensive efforts to induce immortalization and tumor forming ability by treatment with chemical carcinogens or radiation have consistently failed with explanted normal human fibroblasts of embryonic, neonatal, or adult origin. Rare instances have been reported in one or a few exceptional clones following exposure of large fibroblast populations to chemicals (6, 7), x-rays (8), simian virus 40 (SV40) (9), or adenovirus 5 DNA (10), but none of these experiments have yielded consistent results to provide a basis for analysis of underlying mechanisms. Epithelial cells in culture may be slightly more susceptible to immortalization than fibroblasts (e.g., refs. 11 and 12).

The transforming genes of DNA tumor viruses such as SV40 can induce partial transformation of human fibroblasts, but not tumorigenesis (1, 13). This behavior contrasts sharply

with that of rodent cells, which routinely undergo crisis in cell culture and produce transformed survivors, as well as undergoing cell transformation after treatment with carcinogens, radiation, or any one of numerous viruses or viral transforming genes (14, 15).

With the availability of cloned retroviral oncogenes and their cellular homologs, it became possible to examine directly whether DNA sequences that are oncogenic in rodent cells might also be oncogenic in human cell cultures. Experiments addressing this question have been carried out in this laboratory with a population of neonatal (foreskin-derived) human diploid fibroblasts, designated FS-2 (2).

Foci of cells containing SV40 DNA and expressing tumor (T)-antigen were recovered after transfection with SV40 DNA defective in the late region. The cells appeared morphologically transformed, but did not form tumors in nude mice on repeated testing, nor were they immortalized (1). This result is consistent with the extensive clinical evidence that SV40 is not oncogenic for humans (16) and with previous reports that SV40-transformed human cells are not tumorigenic in the nude mouse assay (13). The rare examples of SV40-transformed human cell lines that are tumorigenic probably result from further unidentified genetic changes that occurred in culture.

Prior to our studies (1, 2), all reported experiments to examine the oncogenic properties of mutant *ras* genes by transfection were carried out with rodent cells. We found no foci following transfection of FS-2 cells with the mutant human *HRAS* (*EJ*) gene, either in a simple pEJ plasmid or in the pSV2gptEJ plasmid (2). Drug-resistant clones recovered after transfection with pSV2gptEJ, though morphologically normal, contained the integrated *EJ* gene and expressed elevated levels of the *HRAS* gene product p21. These clones senesced in culture and were not tumorigenic in nude mice (1, 2). Our results were confirmed by microinjection experiments in which mutant p21 protein was shown to have no effect on human fibroblasts; whereas in companion injections of mouse or rat cells, morphological changes were induced, and DNA synthesis was initiated (17, 18).

In view of these negative results, we were interested in the report by Rhim *et al.* (12) describing immortalization of human foreskin epithelial cells following infection with an SV40-adenovirus chimeric construct, and subsequently tumorigenesis following infection with a retrovirus, Kirsten murine sarcoma virus (KiMSV), pseudotyped with baboon endogenous virus (BaEV) to facilitate entry into human cells. We report here the results of analogous experiments using human fibroblasts as recipients for KiMSV(BaEV).

Abbreviations: SV40, simian virus 40; T, tumor; KiMSV, Kirsten murine sarcoma virus; BaEV, baboon endogenous virus; PD, population doublings.

*Visiting Associate Professor from the Department of Oral Pathology, University of Göteborg, Göteborg, Sweden.

†To whom reprint requests should be addressed.

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MATERIALS AND METHODS

Cells. FS-2 cells were derived from a human foreskin as described (1, 2). FSSV-27 and FSSV-46 were derived from foci induced by transfection of FS-2 cells with λ SV-9, a cloned fragment in λ gtWES from the SV40-transformed mouse cell line SVT2/S containing an integrated copy of SV40 DNA with a deletion in the late region (19). Va2 is an SV40-transformed human W18 fibroblastic line selected for resistance to 8-azaguanine (13). FSK, FSVK-27, FSVK-46, and Va2K are cell populations recovered from infection of FS-2, FSSV-27, FSSV-46, and Va2, respectively, with KiMSV(BaEV) as described below.

Culture Conditions. Cells were grown at 37°C in humidified air containing 6.5% CO₂/93.5% air in the α modification of Eagle's minimal essential medium (MEM) with 2 g of glucose per liter/insulin at 10 μ g/ml/10% (vol/vol) fetal bovine serum (HyClone, Logan, UT)/1% human serum (GIBCO)/penicillin, streptomycin, and glutamine as described (20). To test for anchorage independence, plastic dishes were coated with 0.6% agar bases, and cells were added in suspension in 1.3% (wt/vol) methyl cellulose (Fisher Scientific, Medford, MA). Colonies containing more than 50 cells were counted.

Infection with KiMSV. Culture supernates containing KiMSV(BaEV) were obtained from J. Rhim and titered by him at approximately 1×10^4 focus-forming units/ml or higher. Cells were plated at 2×10^5 cells per 60-mm Petri dish for 24 hr, then the medium was removed and replaced with approximately 2.5×10^3 focus-forming units in a total of 500 μ l of medium containing a final polybrene concentration of 4 μ g/ml. After a 2-hr incubation in a moist chamber at 37°C with occasional shaking, 3.5 ml of fresh medium was added, and cells were incubated 24 hr, then washed, released from the culture dish with trypsin, replated for further growth, and then stored in liquid N₂.

Tumor Formation. Growing cells ($5\text{--}10 \times 10^6$ cells) were injected subcutaneously into the flank of BALB/c nude mice as described (20). Cell masses reaching 1 cm in diameter were scored as positive. Tumors exceeding this size that subsequently regressed are so indicated in Table 1. To demonstrate the human cell content of excised tumors, tissue sections were pretreated with normal goat serum to reduce background, then absorbed 1 hr with rabbit anti-human β_2 -microglobulin (Boehringer Mannheim), rinsed, and exposed

to fluorescein-conjugated goat anti-rabbit IgG. As controls, sections were treated as above except (i) with Dulbecco's phosphate-buffered saline instead of primary antibody or (ii) with mouse anti-BALB/c serum as primary antibody and fluorescein-conjugated goat anti-mouse IgG.

p21 Determination. Cells were labeled with [³⁵S]methionine for 1 day; 3×10^7 cpm of trichloroacetic acid-precipitable material was immunoprecipitated and redissolved (21) using the rat monoclonal antibody preparation Y13-259 from Mark Furth (22). Normal rat serum (Cappel Laboratories, Malvern, PA) served as control. Immunoprecipitates were electrophoresed in 12.5% polyacrylamide slab gels. Protein bands were identified by fluorography.

Chromosome Preparation. For chromosome analysis, $2\text{--}3 \times 10^6$ cells were plated into 75-cm² flasks and incubated 48–72 hr at 37°C. Cells were harvested after treatment with Colcemid at 0.05 μ g/ml (GIBCO) for 1–2 hr. The methods for chromosome preparations, as well as the G-banding technique employed, were essentially as described (23). From each of the strains investigated, 25–50 cells were analyzed. The nomenclature follows the recommendations of the International System for Human Cytogenetic Nomenclature (24).

RESULTS

Origin and Properties of SV40-Transformed FS-2 Cells. FSSV clones arose as foci following transfection of FS-2 cells with phage λ SV-9 containing SV40 DNA, defective in the late region but retaining the intact origin, early regulatory region, and T-antigen coding sequences. Cells from foci were typically transformed in morphology and expressed T-antigen. FSSV-27 cells are shorter and plumper than FS-2s, grow in the disoriented manner depicted in the photograph and contain T-antigen (Fig. 1).

Growth properties of the FSSV-27 cells compared with FS-2 and the Va2 line are shown in Table 1. The Va2 and FSSV-27 cells formed large colonies in medium with 1% serum at a frequency close to that in high serum. In methyl cellulose, the Va2s and FSSV-27s grew somewhat better than FS-2s and formed tight colonies at low plating efficiency. None of the untreated populations were tumorigenic in nude mice, a result consistent with our results (1, 2). FSSV-46 and FSVK-46 are omitted from the table since they are mixtures of T-antigen-positive and -negative cells as discussed below.

FIG. 1. The following confluent monolayers of normal and transformed strains are shown: A, FS-2; B, FSSV-27; C, FSSV-27 (T-Ag); D, FSK; E, FSVK-27; F, FSVK-27 (T-Ag). (A, $\times 100$; B, D, and E, $\times 160$; C and F, $\times 250$.)

Table 1. Properties of untreated and virus-transformed cells

Cells	Colony formation			Tumors*
	10% S	1% S	MC	
FS-2	21.7	2.7	0.8	0/12
FSSV-27	22.5	19.6	2.3	0/10
Va2	36.9	32.8	1.8	0/11
FSK	10.1	1.3	0.2	0/9
FSVK-27	16.4	12.8	1.5	20/32†
Va2K	31.6	29.4	2.0	6/6

S, fetal bovine serum measured as % (vol/vol); MC, methyl cellulose.

*Number of tumors found/total number of sites injected with $5-10 \times 10^6$ cells.

†Includes 12 tumors larger than 1 cm in diameter that regressed; 8 were excised for further study.

Retroviral Infection of Human Cells. Four cell populations (FS-2, FSSV-27, FSSV-46, and Va2) were each treated with KiMSV (BaEV). After treatment, the FS-2 cells, now called FSK, appeared very grainy and full of opaque and translucent granules (Fig. 1) but did not undergo a pronounced shape change. However, less than 10% appeared infected by passage 8 and less than 1% at passage 13. The infected FSSV-27 and FSSV-46 cells, now called FSVK-27 and FSVK-46 cells, were also very grainy and filled with dense particles initially and remained so during subsequent passage. The FSVK-27 supernates were negative for reverse transcriptase activity. During subculture, some cells were loosely attached to the plastic, and giant cells with large vacuoles were observed. The adherent populations were passaged until they senesced. Va2 cells appeared transformed before infection, and after infection the resulting cells, now called Va2K cells, were grainier than Va2s; some multinucleated cells were present. FS-2 cells infected with BaEV alone showed no morphological change and were nontumorigenic, but did produce virus as shown by reverse transcriptase assay.

Presence of Viral p21 Protein (Fig. 2). The expression of the RAS gene product, p21, was detected with the rat monoclonal antibody Y13-259, which binds to the *HRAS* and *KRAS* p21s, both viral and cellular (22). Elevated levels of viral p21 were

found in FSK, in FSVK-46 (data not shown), and in FSVK-27, as well as in K-HOS, a human KiMSV-infected tumor-derived line used as a positive control. Only the cellular p21 was expressed in FSSV-27 cells [and in FS-2 cells (1)]. The phosphorylated form of viral p21 was not detected in ^{35}S -labeled immunoprecipitated proteins from Va2K cells although the viral gene was seen in the DNA by Southern blot hybridization (data not shown). Similar gels contained immunoprecipitates of proteins prelabeled with ^{32}P . The phosphorylated form of p21 was present in proteins from FSK and FSVK-27 cells but not from FS-2, FSSV-27, Va2, or Va2K cells (data not shown).

Chromosomal Characteristics of Transfected and Infected Cells. The chromosomal findings are summarized in Table 2. A detailed description of the banding patterns will be reported elsewhere. The FS-2 cells were diploid, whereas after retrovirus treatment, both diploid and aneuploid FSK cells were present, consistent with the morphological evidence of a partially infected population. No characteristic markers were detected in the FSK aneuploid cells.

FSSV-27 cells were essentially hypotetraploid with a modal range of 80–90 chromosomes. The FSVK-27 cells, which formed transient tumors, had a narrow mode of 86–88 chromosomes but a wider overall range than the FSSV-27s. The FSSV-46 population contained both diploid and aneuploid cells. By subcloning, the T-antigen-positive cells were found to be aneuploid, and the T-antigen-negative cells were diploid. After retroviral treatment, as expected from the results with FS-2s, the frequency of diploids greatly decreased.

The FSVK-46s were very heterogeneous, and it was impossible to discern a modal number or any consistent markers. Despite the presence of extensive structural rearrangements including dicentric, ring chromosomes, and fragments, the FSVK-46s did not form tumors. Both FSSV-27 and -46 contained dicentric chromosomes in about 50% of the aneuploid cells (Fig. 3), as well as other types of markers. The characteristic appearance of dicentric after SV40-viral infection has been reported (25).

The Va2 cells were aneuploid with a modal number of 60–65 chromosomes. The only major difference detected between the Va2 and Va2K cells was that a large submetacentric marker found in Va2 had increased in frequency and was present in over 50% of the Va2K cells. Both the Va2 and Va2K cells contained dicentric and ring chromosomes.

Table 2. Chromosomal characteristics of infected, transfected, and untreated cells

Cells	Passage number*	Modal chromosome number	Comments
FS-2	13	46	Diploid
FSK	4	46	80% diploid/20% aneuploid with 70–80 chromosomes
FSSV-27	12	80–90	95% aneuploid with 68–92 chromosomes/5% diploid
FSVK-27	6	86–88	95% aneuploid with 62–110 chromosomes/5% diploid
FSSV-46	9	46	70% diploid/30% aneuploid with 49–150 chromosomes
FSVK-46	6	None	>90% aneuploid with 64–250 chromosomes/<10% diploid
Va2	—	60–65	100% aneuploid with 50–80 chromosomes
Va2K	8	62–67	100% aneuploid with 46–80 chromosomes

*Passage number after infection for KiMSV-treated cells and after SV40 transfection and cloning for FSSV cells.

FIG. 2. KiMSV-infected FS-2, FSSV, and Va2 cells have p21 protein. Lysates from ^{35}S -labeled cells were immunoprecipitated with a rat monoclonal antibody Y13-259 (lanes A) or normal rat serum (lanes B). Positions of p21s (shown by arrows) were determined from molecular weight standard markers.

FIG. 3. (A) G-banded karyotype of a hypotriploid FSVK-27 cell with 5 marker chromosomes is shown. (B) Selected G-banded dicentrics and rings of different size and morphology observed in FSVK-27 and -46 cells are shown. ($\times 1600$ – 1900 .)

However, these marker types were much less frequent here than in the FSSV and FSVK cells.

Tumor Formation by FSVK-27 Cells. Of the cell strains tested for tumor formation, only FSVK-27 and Va2K were tumorigenic (Table 1). With FSVK-27, 32 sites were injected, and at 8 of them vascularized tumors arose that were excised for further study. At 12 other sites, tumors over 1 cm in diameter formed and then regressed; they were scored as positive. Those that regressed before reaching this size were scored as negative. Some growth occurred at every injection site, in contrast to results with the other strains, where little or no detectable growth occurred after injection.

The infection of nontumorigenic Va2 cells with KiMSV induced consistent tumor formation. This result reflects the effect of viral infection as well as the preimmortalized status of the Va2 cells. It is not evident why the phosphorylated form of p21 was not detected in ^{35}S - or ^{32}P -labeled immunoprecipitates.

FSVK-27- and Va2K-derived tumors were excised, sectioned, and tested for the presence of human cells by immunofluorescence using β_2 -microglobulin to detect human antigens as well as mouse antiserum to detect mouse cells. All tumors examined were composed largely of human cells.

Neither FSSV-46 nor FSVK-46 formed tumors. However, FSSV-46 was subcloned and shown to contain T-antigen-negative as well as -positive cells. When the subclones were separately treated with KiMSV(BaEV), they senesced before they could be tested in nude mice. The uncloned population of FSVK-46 cells also senesced without forming tumors.

Senescence (Table 3). To study senescence, cell populations were split 1:4 at each passage or 1:2 as cells reached confluence. Senescent cells remained attached to the plastic but stopped dividing.

FSSV cells selected as foci (2) were 20 population doublings (PDs) older than FS-2s when infected with KiMSV but still retained enough residual PDs to form transient tumors. Had they retained the same life span as uninfected FSSV cells, they could have formed large tumors in the mice, and explants would have grown further before senescing. On the contrary, as shown in the table, KiMSV infection decreased the life-span of FSVK cells.

FSVK-46 cells did not form the transient tumors seen with FSVK-27 cells, probably because the FSSV-46 populations were a mixture of T^+ and T^- cells, as shown by immunofluorescence. Both subcloned types senesced rapidly, whether or not infected with KiMSV. The uncloned FSVK-46 population did not form transient tumors presumably because the T^- cells were not tumorigenic (resembling FSK), and T^+ cells, which grew poorly, also required extra doublings to make up for the T^- cells in the mixture.

DISCUSSION

This paper shows that normal human fibroblasts, FS-2 cells, infected with KiMSV(BaEV), do not become tumorigenic despite expressing the v-Ki-ras-encoded p21 protein. On the other hand, if these cells are first transfected with SV40 DNA (defective in the late region) leading to expression of T-antigen, they remain nontumorigenic and nonimmortalized, but become capable of responding to KiMSV(BaEV) infection by making transient tumors. Their life-span is reduced by

Table 3. Growth and senescence of virus-transformed human fibroblasts

Cells	PD at time of KiMSV infection, no.	PD at time of mouse injection, no.	PD at senescence, no.*
FS-2	—	—	65
FSK	20	31–37	70
FSSV-27	—	—	95
FSVK-27	42	52†	60
FSSV-46	—	—	77
FSVK-46 uncloned	42	52; 60; 65	71
FSVK-46-I diploid, T^-	61	Senesced	69
FSVK-46 aneuploid, T^+	60	Senesced	64

*Cultured at high cell density with a split ratio of 1:4 at confluence.

†With 57–59 PDs at time of maximum tumor size.

KiMSV infection, and the tumors terminate by senescence. In contrast, when Va2 cells, which are immortal but nontumorigenic human fibroblasts, are infected with KiMSV-(BaEV), they make persistent tumors in nude mice.

These results raise a number of important questions. First, and of most general significance, does senescence play an important role in protection of humans against cancer, as suggested by these experimental results? Second, how different are human and rodent cells in their response to tumor-inducing agents? And third, what are the respective roles of SV40 DNA and of KiMSV in contrast to *HRAS* in human cells?

First, are these results relevant to the understanding of human cancer? It has been argued that the size of lethal tumors can be reached within the limits of senescence. If a 1-cm tumor contains 10^9 cells representing about 30 PDs, then a total of 50–60 PDs could clearly provide more than enough cells (10^{17}). However, this calculation is very misleading because it does not take into account the ongoing cell lethality that is a consistent feature of tumor growth (26). The net proliferation rate of tumors is dependent on the cellular doubling time, on the cycling fraction, and on the lethal fraction, which can be high enough to abort incipient tumors. Thus, while the population age of cells in growing tumors cannot be ascertained with present methods, the extensive lethality associated with tumorigenesis may be sufficient to propel the surviving cells into senescence.

Rhim *et al.* (12) used keratinocytes that had become immortalized following infection with an adeno-SV40 chimeric virus. Since the frequency of immortalization was not reported, it remains unclear whether the presence of SV40 T-antigen, the adenovirus DNA, aneuploidy, or immortalization *per se* were responsible for the susceptibility of this epithelial cell line to KiMSV-(BaEV)-induced tumorigenicity.

The effect of the SV40-encoded T-antigen is clearly different in human cells from rodent cells. In experiments using the SV40 large T gene in a defective retroviral expression vector, it was shown that in primary rat cells expressing elevated levels of the large T-antigen, neither morphological changes nor tumorigenicity had been induced (27) although the transfected cell populations behaved as established cell lines (28). In contrast, the SV40-transformed human fibroblasts produced in our laboratory were morphologically transformed but not immortalized.

Similarly, the *ras* genes have been shown to transform rodent cells much more effectively than human cells both in transfection studies (1, 2, 29) and by microinjection (17, 18). These studies suggest that human cells may have evolved means to modulate the transforming effect of mutant *RAS* genes, perhaps at the level of the p21 substrate or beyond it, in contrast to the pronounced effect of mutant p21 in inducing DNA synthesis in rodent cells.

As shown in this report, the resistance of human cells to SV40 and to *RAS* gene products has been genetically separated from the resistance of human cells to senescence. Although immortalization has been separated from other transforming properties in various rodent cell studies (e.g., ref. 30) the significant feature of human cells revealed in our studies is the powerful nature of the senescence control. With rodent cells, immortalization occurs rapidly in culture and does not provide a brake on tumor-forming ability. In sharp contrast, with human cells, senescence is a key limiting factor in tumorigenesis. These differences between human and rodent cells also serve to emphasize the caution required in extrapolating from rodent experiments to human cancer.

Our results do not establish whether SV40 transformation *per se* is a prerequisite for KiMSV-induced tumor growth or whether a different agent inducing immortalization might also be effective. Human cell lines that are nontumorigenic but

immortalized are rare. The Va2 line used here became immortal subsequent to SV40 transformation. The fact that Va2 cells produced persistent tumors after infection with KiMSV whereas nonimmortal FSSV cells made only transient tumors is a further confirmation of the important control over tumor growth provided by senescence.

In conclusion, we have shown that normal human fibroblasts are resistant to tumorigenesis induced by effective infection with KiMSV and that prior transformation of these cells with SV40 DNA (defective in the late region) makes the cells susceptible to transient tumor forming ability induced by KiMSV infection. We propose that senescence or resistance to immortalization plays a key role in the protection against human cancer at the cellular level. Identification of human genes involved in senescence could provide an important set of gene products (31, 32) for use in cancer protection and therapy.

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