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Senescence As a Mode of Tumor Suppression

by Ruth Sager*

Two independent lines of experimental evidence are presented in support of the hypothesis that senescence is a normal mechanism of tumor suppression, a homeostatic device designed through evolution to limit cell proliferation irreversibly and thereby to protect the organism against cancer. One set of experiments uses normal human foreskin fibroblasts, transfected at early passage with SV40 DNA and subsequently infected with the K-*ras* virus. If the cells are immortal prior to infection, they become tumorigenic and make large tumors in nude mice, whereas if they are not immortal, though expressing SV40 T-antigen, they make tiny tumors that senesce in the test mouse after as many doublings as similar cells make in culture. This result demonstrates that immortalization is essential for progressive tumor growth *in vivo*.

The second set of experiments demonstrate that normal human mammary epithelial cells can be immortalized by transfection with viral DNA from human papilloma virus 16 or 18, although these viruses have not been associated with breast cancer. The effective immortalization and other premalignant changes induced by human papilloma virus transfection are accompanied by chromosome changes that may contribute to the partially transformed phenotypes. None of the cloned or pooled transfectants have been tumorigenic in the nude mouse assay. Here, too, immortalization is experimentally separable from tumor-forming ability.

Introduction

Life is a balancing act. Good and evil, right and wrong, production and destruction are in opposition, at the social level. And at the biological level, networks of regulation interact to maintain the metabolic balance and offset proliferation with differentiation, inflammation with anti-inflammatory mechanisms, etc. In a word: homeostasis. The balance between opposing forces is part of the underlying biological design, a fundamental theme in cell and organism.

This theme is particularly evident in the cancer field, where genes encoding tumor-suppressor proteins are at least as important in tumor progression as those (i.e., oncogenes) with tumor-promoting activity. Losses of tumor-suppressor activity, as well as the activation of oncogenes, are driven by genomic instability leading to mutations, large deletions, rearrangements, and aneuploidy (*1*).

These progressive genomic changes include effects on cells that regulate cell proliferation and differentiation, enmeshing cancer onset and progression with systemic aging and with cellular senescence and immortalization. The long, multistage progression of cancer, as well as the acceleration of cancer incidence with age, both highlight the existence of multiple devices, cellular and systemic, that have evolved to protect us against cancer (*1*).

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At the cellular level, three general mechanisms of tumor suppression have been documented: the stability of the human genome, the mechanisms of proliferation control, and the process of terminal differentiation, which involves the irreversible loss of proliferative capacity. Senescence is a kind of terminal differentiation in which cells can no longer respond to any known proliferation stimulus, but do carry on metabolic activities. Current evidence implicates four dominant genes or complementation groups in senescence, implying that loss of these genes or their functions is essential for immortalization (*2*).

Blocking Tumor Growth by Senescence

In my laboratory a few years ago, we carried out experiments designed to examine directly the role of senescence in tumor suppression (*3*). Because of their relevance to current work, these experiments will be briefly reviewed here. The initial aim was to convert normal human diploid fibroblasts into tumor-forming cells by transfection with specific transforming genes. We used a plasmid containing SV40 early-region DNA to try to immortalize the cells, but we found after extensive studies that the integrated SV40 DNA, while coding for T-antigen, did not immortalize these cells, although some transfectants had an extended life span. The cells appeared morphologically transformed and

were used for the second step, namely, introduction of viral Kirsten *ras*.

The nonimmortalized SV40-transfected FS-2 cells, called FSSV, were compared with a human cell line, Va2, which had been selected as immortalized but not tumor-forming after infection with SV40 virus. Since SV40 virus immortalization of human cells is very rare, it was presumably the result of additional genetic changes in Va2 beyond SV40 DNA integration that determined their immortalization. FS-2, FSSV, and Va2 cells were each infected with viral K-*ras*, which had been pseudotyped with an endogenous baboon virus to facilitate entry into human cells (*4*). The cell populations before and after transfection or infection were carefully monitored to count population doublings.

Both the FSK and the FSVK cell populations recovered from the infection experiments senesced in cell culture after a number of doublings similar to those occurring in the uninfected cells, whereas the Va2K cells remained immortal. When tested in nude mice, the Va2K cells made large persistent tumors, whereas the FSVK line, containing SV40 and K-*ras* DNA, formed small tumors, which stopped growing after reaching about 1 cm diameter. It was estimated from the tumor volume that the number of doublings in the nude mice was comparable to the total doublings in culture prior to senescence (Table 1).

The lines infected with K-*ras* were shown to contain the phosphorylated form of the encoded p21 protein, demonstrating expression of the viral gene. The FSVK and Va2k cells also expressed T-antigen as shown by immunocytofluorescence.

Thus, when normal human fibroblasts, FS-2 cells, are infected with the Kirsten *ras* virus, they do not make tumors, despite expressing the viral *ras* gene. In FS-2 cells expressing both T-antigen and viral K-ras protein, i.e., FSVK cells, the tumorigenic effect of the viral ras was seen as the initial growth of small tumors in the nude mouse assay, subsequently blocked by putative senescence gene-encoded proteins. The Va2 cells, the positive controls, produced tumors on schedule following infection with the K-*ras* virus.

These studies provide a clear experimental dichotomy between immortalization and tumor growth. These experiments distinguish the differential effects of the two processes and show that both are required for effective tumor formation. Immortalization is shown to play a distinctive and essential role in tumorigenesis. Contrariwise, senescence is shown to block tumor formation in cells that have actually begun the process of tumor formation. Identification of senescence genes and their encoded protein products could provide an important set of gene products to test for their potential use in cancer therapy.

Table 1. Effect of KiMSV (BaEV) on immortalization and tumor formation.

Cells	Tumor formation[a]	Population doublings to senescence
FSK	0/9	≈ 66
FSVK-27	21[b]/29	≈ 60
Va2K	6/6	Previously immortalized

[a]Numbers are positive sites/total sites.
[b]Transient or excised for subculture.

Immortalization of Normal Human Mammary Epithelial Cells with Human Papilloma Virus

A few years ago my laboratory began a long-term investigation, applying molecular and cellular methods to the identification of genetic changes in human breast cancer. Initially there was no suitable medium for isolating and growing primary human mammary epithelial cells in culture. Thus, our first task was at the level of media development and cell culture. We have succeeded in establishing a new medium in which both normal cells and tumor cells grow well, at about the same growth rate (*5*).

The normal cells, derived from reduction mammoplasty surgery, senesce after 15 to 20 passages, representing 40 to 50 population doublings. It was decided to look for ways to immortalize these cells, both for convenience in long-range experiments and to study the molecular genetic basis of the senescence/immortalization switch. Among the potential methods of immortalization was transfection or infection with a DNA tumor virus, since several of them have been implicated in immortalization: SV40, polyoma, adenovirus, herpes virus, Epstein-Barr virus, and human papilloma virus (HPV) (*6*).

Previous studies with HPV (*7*) have demonstrated the existence of many types; so far about 65 have been recognized on the basis of differences in DNA sequence, measured by cross-hybridization. Only a few of these types have been associated with carcinomas, either genital or oral. The virus exhibits high host specificity for squamous epithelium. No reports are known of HPV in glandular epithelium.

Human papilloma virus has proven very difficult to grow in human tissues in the laboratory, but recently, with the availability of plasmids containing the full viral genome, linearized and unable to replicate autonomously, it has become possible to study HPV by transfection. Encouraged by positive results of other investigators with keratinocytes, the known cellular hosts of HPV (*8–11*), we used the same plasmids, containing HPV 16 and HPV 18, frequently associated with cervical carcinoma, to transfect normal human mammary epithelial cells growing in our novel DFCI-1 (Dana-Farber Cancer Institute) medium (*12*). This medium supports the growth of both normal and tumor-derived mammary epithelial cells and was therefore considered the medium of choice for selection of putative immortalized cells, which would have unknown growth factor requirements.

76N cells, previously described (*5*), were transfected by the calcium phosphate procedure with either HPV

16 or 18, as well as pSV*neo* used for selection. Our recovery was about 10^{-5} *neo*-resistant colonies, all of which when tested contained the HPV plasmid. Several individual clones as well as pools of colonies from single plates of both HPV types were subsequently shown to contain one or multiple copies of the HPV genome, integrated into cellular DNA, with no evidence of free episomes.

Chromosome counts and karyotypic analysis revealed that some clones were hypodiploid and showed minimal chromosome rearrangements, whereas several others were aneuploid and rearranged, much as has been reported for HPV-transfected keratinocytes (*13*). The hypodiploid clones also contained on average a single copy of HPV per cell.

The expression of HPV-encoded protein E7 was demonstrated with the use of antibody preparations against HPV 16/E7, a gift from D. Galloway and J. McDougall, and against HPV 18/E7, from L. Gissman and H. zur Hausen. The proteins were expressed in clonally isolated as well as pooled samples (*12*).

The changes in growth factor requirements were of particular interest. Although the normal 76N cells used in these experiments grow well in DFCI-1 medium, they do not form colonies on plastic. The cells will grow, but at a slower rate, on D2 medium, which lacks fetal calf serum and bovine pituitary extract. The HPV transfectants grow at the same rate on both of these media and plate with about 15% plating efficiency on each of them. In addition, the transfectants grow and plate on a medium, D3 + epidermal growth factor (EGF), in which the supplemental growth factors insulin, hydrocortisone, triiodothyronine, and cholera toxin have been removed.

Finally, with respect to immortalization and tumorforming ability, the parental 76N cells senesce after 15 to 20 passages in culture, whereas all of the tested clonal and pooled transfectants have grown 200 or more passages with no decrease in growth rate. In extensive testing in the nude mouse assay, injecting 10^7 cells per site subcutaneously into the mammary fat pad, no tumors have appeared in 4 to 6 months in 50 mice, using cells from several of the transfectants.

These results provide a clear and dramatic demonstration of the successful immortalization of human mammary epithelial cells with either HPV 16 or 18. Numerous questions arise. Does the mechanism of immortalization involve the retinoblastoma protein to which E7 protein has been shown to bind (*14*), or the p53 protein, to which E6 binds (*15*)? Since these genes seem to be involved in regulation of the cell cycle, i.e., quiescence versus cycling, one wonders whether the same mechanisms regulate quiescence and senescence, with senescence representing the constitutive expression of some gene(s) normally under regulation.

It should also be emphasized that chromosome changes have been seen in all experimentally transfected cell lines, as well as in HPV-associated tumors. Are there specific, nonrandom gene changes—loss or mutation—required for effective HPV-induced phenotypic changes? The hypodiploid cells recovered in these experiments provide a unique opportunity to examine this question. Whatever the answer, the genomic changes associated with HPV transfection or infection suggest that loss of suppressor genes may be a requisite for immortalization.

At another level, our results impinge on genes involved in mammary carcinoma and whether HPV or a related DNA tumor virus may be implicated. As with cervical carcinoma, where HPV seems to be involved but not a sole determinant of tumorigenesis, might HPV play a similar role in breast cancer? In view of the multiple types of HPV and the difficulty of identifying new types, this question may remain moot for some time. It should be emphasized, however, that the changes seen in the transfected 76N cells require not only the expression of HPV-encoded genes, but also their interaction with cellular gene products. Thus, host specificity is expressed at the level of host cell recognition of viral gene products, and the ability of mammary epithelial cells to show this recognition is what really suggests that HPV might play a role in breast cancer.

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