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In Vitro Analysis of Multistage Carcinogenesis

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Several key events in the multistep process of neoplastic transformation of rat tracheal epithelium (RTE) are described. Whether tracheal epithelium is exposed *in vivo* to carcinogenic agents or whether primary tracheal epithelial cells are exposed *in vitro* to carcinogens, initiated stem cells† can be detected soon after the exposure by their ability to grow under selective conditions in culture. These initiated stem cells differ fundamentally from normal stem cells in their response to factors normally constraining proliferation and self-renewal. Thus, disruption of inhibitory control mechanisms of stem cell replication appears to be the first event in RTE cell transformation. While the probability of self-renewal (PSR) is clearly increased in initiated stem cells, most of the descendants derived from such stem cells differentiate and become terminal and do not express transformed characteristics.

Progression from the first to the second stage of RTE cell transformation, the stage of the immortal growth variant (IGV), is characterized by loss of responsiveness to the growth-restraining effects of retinoic acid. In the third stage of neoplastic transformation, the stage during which neoplastic growth variants (NGV) appear, a growth factor receptor gene is inappropriately expressed in some of the transformants. Thus, it appears that loss of growth-restraining mechanisms may be an early event, and activation of a growth stimulatory mechanism a late event, in neoplastic transformation of RTE cells.

Introduction

The combined epidemiological, clinical, and experimental evidence suggests that the development of cancer occurs in discrete stages. According to current models of carcinogenesis such as the one presented in Figure 1 (*1*), carcinogen exposure does not instantly transform the affected target cells to cancer cells, rather, it causes heritable changes in a small proportion of stem cells, converting them to a preneoplastic state (intermediate cells in Fig. 1). In the second stage of carcinogenesis, clonal expansion of the preneoplastic stem cell variants occurs. This can be brought about by exogenous promoting agents (such as phenobarbital, dioxins, or phorbol esters), by endogenous promoters (e.g., prolactin in mammary carcinogenesis), or it may occur without an identified stimulus. As a result of the proliferative expansion of the preneoplastic clone, a new cell variant with neoplastic characteristics arises. Additional steps may be required for various neoplastic

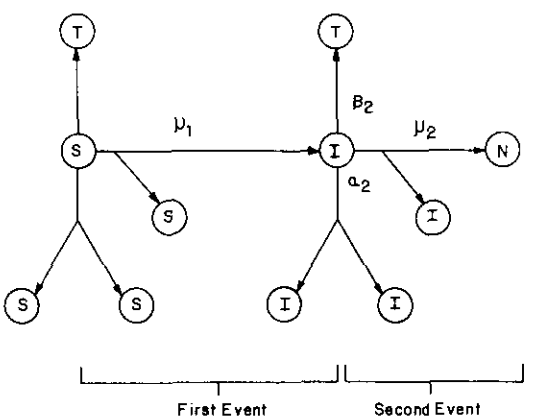

FIGURE 1. Two-stage model of carcinogenesis. S = normal stem cells; I = intermediate stem cells (these are usually called "initiated" cells; we consider them to be preneoplastic, as their probability to become neoplastic is increased); N = neoplastic stem cells; T = terminal cells. μ_1 = Rate at which first event occurs; μ_2 = rate at which second event occurs; α_2 = rate of division of I; β_2 = rate of commitment to terminal state of I. Modified from Moolgavkar and Knudson (*1*).

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†Terminology used: stem cell: a cell with self-replicating ability; we consider clonogenic cells to be stem cells. Initiated stem cell: the stem cell that has been heritably altered by exposure to a carcinogen and as a result of that exposure produces progeny with a greater than normal probability of becoming neoplastic. We consider the founder cell of the enhanced growth variant colony (EGV colony) to be an initiated stem cell. Preneoplastic cells: any cell whether appearing early or late during the multistage process of neoplastic transformation that has a greater than normal probability of giving rise to neoplastic offspring.

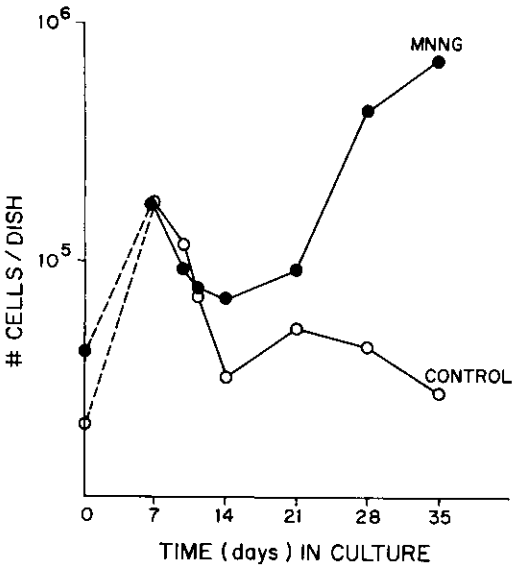

FIGURE 2. Growth of normal and transformed primary rat tracheal epithelial cells. (○) Normal cells; (●) cells exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, MNNG.

phenotypes to appear (e.g., invasiveness, metastatic ability, hormone independence). This model of carcinogenesis is, as one might imagine, not uncontested. However, in principle, this model is supported by a great wealth of experimental data and by studies on heritable neoplastic diseases in humans such as familial retinoblastoma (*2*). For the purposes of this presentation, it may serve to provide the overall perspective and the hypothetical framework for the discussion of our own investigations.

Our interest is primarily focused on the compartment of intermediate preneoplastic transformants (Fig. 1). What experimental evidence do we have that preneoplastic stem cells exist? Can they be isolated? How do they differ in their behavior from normal stem cells? What is the molecular basis of their phenotype? What is involved in the conversion of the preneoplastic to the neoplastic cell variant, a process we call progression, and what factors can accelerate or inhibit that progression? Preneoplastic cells that have not yet acquired malignant characteristics presumably are present only in small numbers. Thus, it may be possible to prevent the progression of preneoplastic cells to malignancy or to eliminate them from the host altogether through chemopreventive measures. The studies to be discussed are concerned with the following topics: the detection, isolation, and quantification of early, preneoplastic stem cells in rat tracheal epithelium; manifestations of aberrant growth control in these early transformants and their abnormal self-renewal capacity; manifestations of progression, namely, the loss of responsiveness to negative growth regulators; and altered gene expression in late stages of neoplastic transformation. The examples used in this review to illustrate the manifestations of altered growth control of RTE cell transformants are taken from several *in vitro* transformation studies; however, transformants isolated from tracheal epithelium exposed *in vivo* to various types of carcinogens show the same growth alterations and the same sequence of phenotypic changes during progression from the preneoplastic to the neoplastic stages of transformation.

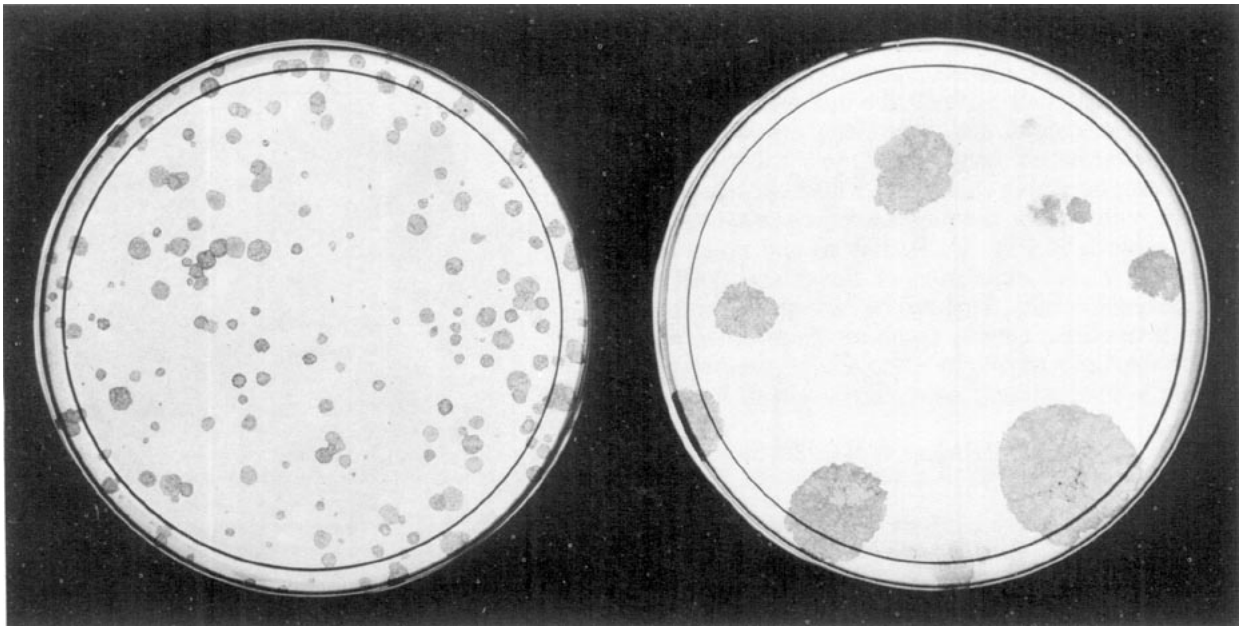

FIGURE 3. Normal and transformed rat tracheal epithelial cell colonies. Left, day-7 colonies. Right, transformed colonies after 5 weeks of selection.

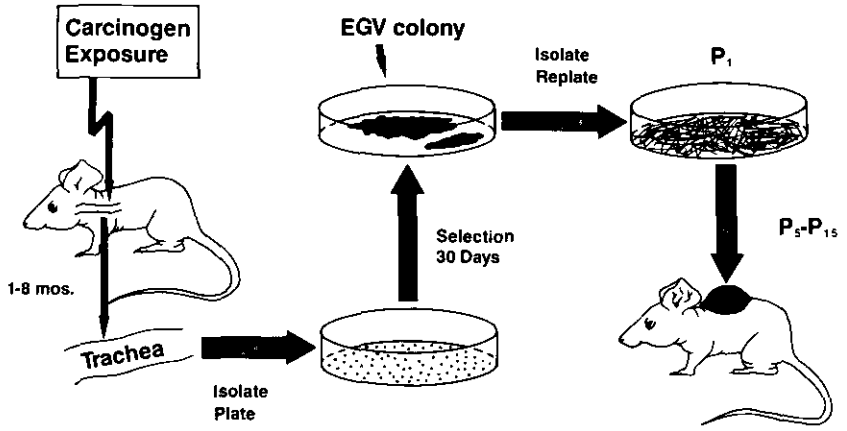

FIGURE 4. *In vitro* assay for detection of preneoplastic stem cell variants formed *in vivo*, following exposure of tracheas to carcinogens. Rats are exposed to a tracheal carcinogen. At different times after carcinogen exposure tracheas are removed, the epithelium is dissociated and plated on irradiated 3T3 feeder cells. Four days after plating, feeder cells are removed to select for transformed stem cells, which form large colonies within 3 to 5 weeks after start of selection (enhanced growth variant colonies = EGV-colonies). The EGV-colonies are isolated, replated, and subcultured repeatedly. Different passages (P5–P15) are inoculated into compatible hosts to examine the neoplastic potential.

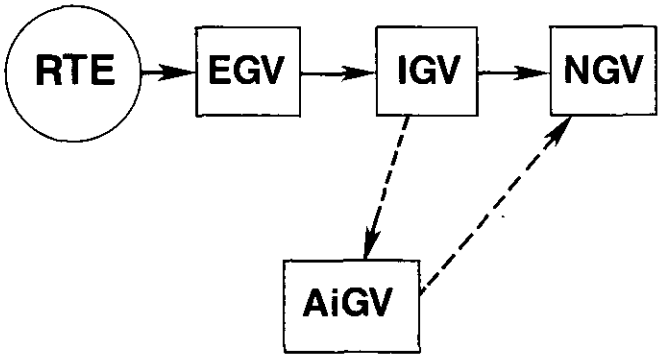

FIGURE 5. Schematic representation of multistage carcinogenesis of rat tracheal epithelial cells. The same phenotypic changes are observed in cells initiated *in vivo* or *in vitro*. RTE = normal rat tracheal epithelial cells; EGV = enhanced growth variant; IGV = immortal growth variant; NGV = neoplastic growth variant; AiGV = anchorage independent growth variant; the AiGV is not an obligatory preneoplastic phenotype.

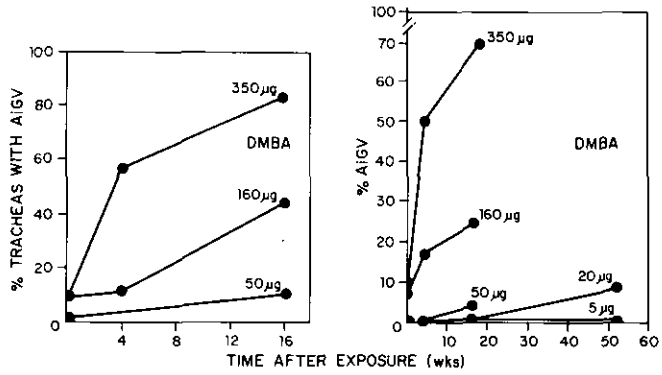

FIGURE 6. Effect of carcinogen dose and time after the end of exposure on the rate of appearance of advanced preneoplastic cell variants. Tracheas were exposed *in vivo* to various amounts of 7,12-dimethylbenz[a]anthracene for a period of 2 weeks. At differrent times after the end of exposure 10 to 20 tracheas were removed and the number of tracheas containing transformed stem cells giving rise to anchorage independent growth variants as well as the relative frequency of such transformed stem cells (i.e., AiGV/EGV %) was determined (*3,4*).

Detection, Isolation and Quantification of Early Preneoplastic Transformants

Carcinogen exposure causes cellular and molecular changes that, months and years later, result in the development of tumors. To investigate the clonal expansion and progression of the putative initiated stem cells during this long latency period, one must be able to isolate the stem cells and study their descendants over many cell generations. More than 10 years ago, studies in our laboratory (*3,4*) led to the discovery that tracheas of rats exposed to carcinogens contain a small proportion of cells exhibiting a fundamental derangement in growth control. This becomes evident when the cells from such tracheas are isolated and cultured. Epithelial cells obtained from tracheas of normal rats undergo approximately 10 population doublings in primary culture (Fig. 2); thereafter, they senesce and become terminal. In contrast, cells that have been exposed to carcinogens either *in vivo* or *in vitro* proliferate indefinitely. If the cells are seeded at clonal densities (Fig. 3), one can observe many small colonies around 8 to 10 days after plating; however, subsequently, most of these senesce and only a few (0.5–2.0%) continue to expand and can be scored as "enhanced growth variant (EGV) clones" several weeks later (*5*). This is regarded as the first stage of RTE cell transformation (Fig. 4). These enhanced growth variant colonies can be isolated and subcultured. Approximately one-half of these clones

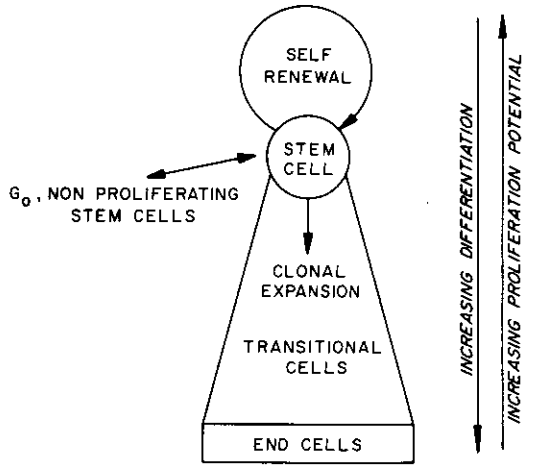

FIGURE 7. Model of stem cell differentiation. From Buick and Pollack (*8*).

become permanent cell lines, i.e., they undergo immortalization (IGV = immortal growth variants) and between 30 and 50% of the immortalized clones become neoplastic (NGV = neoplastic growth variants) after 5 to 20 passages. The anchorage-independent growth variant (AiGV), which is frequently observed when the tracheal epithelium is initiated *in vivo*, is not an obligatory preneoplastic phenotype. Thus we have identified three different stages of neoplastic transformation in cells isolated from rat tracheas exposed *in vivo* to carcinogens (EGV, IGV, NGV), and we can say with confidence that the early EG-variant clones are preneoplastic because they have an increased probability of becoming neoplastic (Fig. 5).

Preneoplastic stem cells with EGV characteristics can be detected in the tracheal epithelium within a few days after *in vivo* carcinogen exposure. Obviously it is of great interest to learn about the fate of this cell compartment during the tumor latency period. Following a single carcinogen exposure, which causes an approximate 20% tumor incidence at about 24 months, the preneoplastic stem cell population expands (Fig. 6). As a function of time, the number of tracheas containing transformants increases and the number of transformants per trachea also increases. The growth of the transformed stem cell pool is carcinogen-dose dependent. It is important to note that the expansion of preneoplastic cells in the tracheal epithelium occurs in the absence of any exogenous stimulus and many months before tumors arise.

In summary, we showed that early preneoplastic cell variants can be isolated from carcinogen-exposed tracheal epithelium of rats and can be quantitated in cell culture; the number of these early transformants increases as a function of carcinogen dose and as a function of time after exposure; and their clonal descendants undergo secondary changes, most importantly, immortalization and neoplastic transformation. Other laboratories have observed similar findings: cells with characteristics of EG-variants have been isolated from a number of organs in rats (*6*) and from initiated mouse skin (*7*).

Analysis of the Aberrant Self-Renewal of EG-Variant Clones

According to the two-stage model of carcinogenesis presented by Moolgavkar and Knudson (*1*), neoplastic

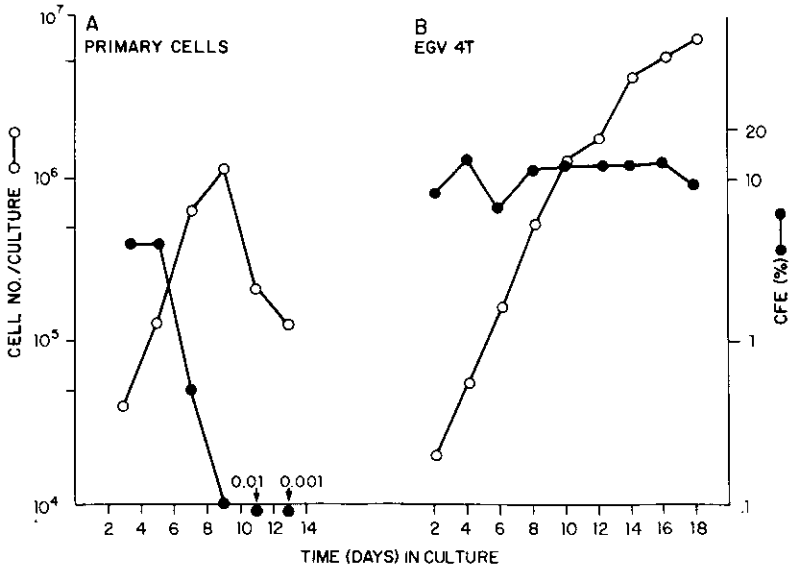

FIGURE 8. Effect of cell density on growth and stem cell compartment size. (*A*) Cultures of normal and (*B*) of transformed rat tracheal epithelial cells. (○) Cell number per culture; (●) percent clonogenic cells. (CFE = colony forming efficiency was assayed on 3T3 feeder cells to maximize expression of colony forming ability.)

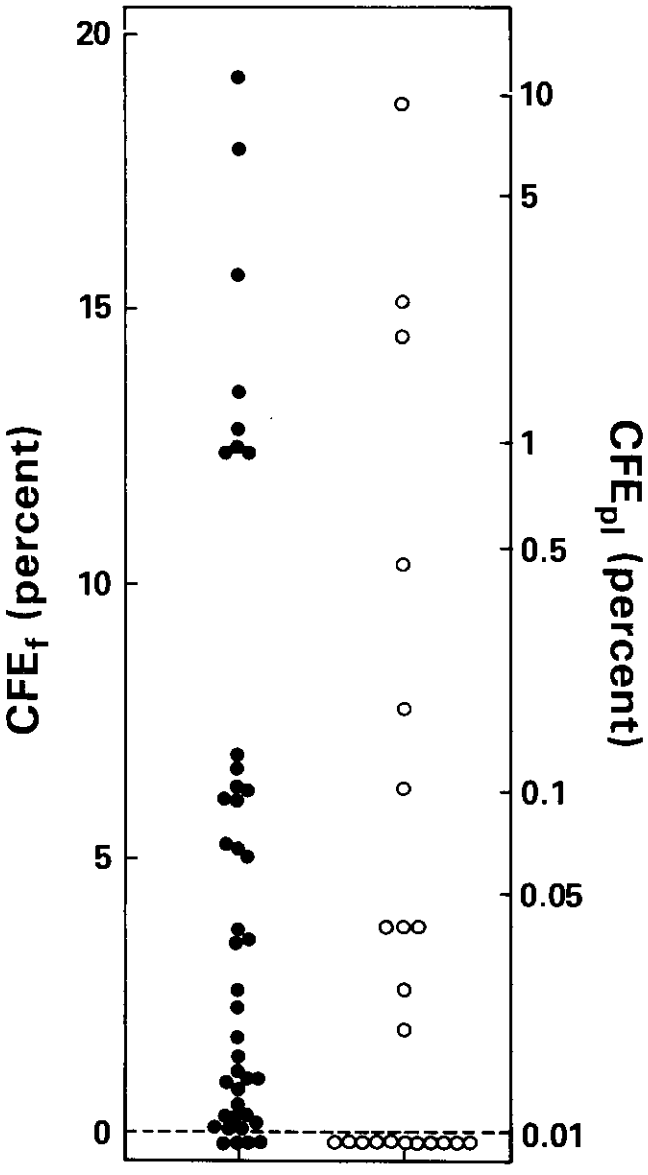

FIGURE 9. Frequency of clonogenic units (stem cells) in primary EGV clones 5 weeks after MNNG exposure. More than 40 individual EGV colonies were tested under optimized growth conditions on feeder cells (○) CFEf, and under selective growth conditions on plastic (●) CFEpl. Each circle represents one EGV colony containing ~10^5 cells. From Fitzgerald et al. (*10*).

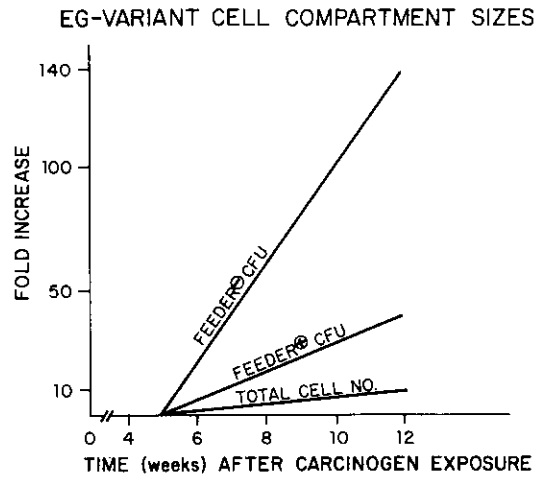

FIGURE 10. Relative growth rate of stem cell compartments in EGV colonies. The growth of EGV colonies (average number of cells/colony) was measured in primary culture between 5 and 12 weeks after MNNG exposure. Also measured was the size of the clonogenic cell compartments under permissive (feeder$^+$ = in presence of 3T3 feeder cells) and under selective (feeder$^-$ = in absence of 3T3 feeder cells) conditions. CFU = colony-forming units. The CFU which replicate on plastic in the absence of feeder cells are thought to be responsible for immortalization of the EGV clones (EGV → IGV). From Fitzgerald et al. (*10*).

transformation is a stem cell disorder. If this hypothesis is correct, we should be able to detect abnormal stem cell growth kinetics during early preneoplastic stages of neoplastic transformation. Before we describe the proliferation kinetics of EG-variant clones, it may be useful to briefly review a simple model of cell replication in differentiating tissues (Fig. 7). This model (*8*) postulates three main cell compartments: a compartment of cells endowed with the capacity for self-renewal (so-called stem cells); a compartment of committed, replicating cells responsible for expansion of the clone; and a compartment of terminally differentiated cells, carrying out the main differentiated functions of the respective tissue.

In normal steady-state epithelia, most of the stem cells are believed to remain in G_o and thus are noncycling. When they enter cell cycle, their probability for self-renewal (PSR) is 50%. During nonsteady-state conditions such as regenerative growth or neoplastic disease, the PSR increases and the stem cell compartment increases in size. In cell culture, one determines the size of the stem cell compartment by means of clonogenic assays that measure the proportion of clone-forming cells in a cell population. Typically, during the growth of normal primary rat tracheal cells (Fig. 8*A*), the number of clonogenic cells, or stem cells, in the population amounts to only a few percent during the early logarithmic growth phase, and as the clonal cell density increases and the cultures approach plateau of growth, the stem cell compartment decreases rapidly in size. Simultaneously the cultures produce terminally differentiated cells. Ultimately cell replication ceases and the cultures senesce.

This dynamic relationship between clonogenic cells, committed cells, and terminally differentiated cells is fundamentally altered in transformed cell populations. To emphasize this change we have chosen to illustrate the growth dynamics of a preneoplastic, immortalized growth variant (IGV) (Fig. 8*B*); the growth rate is similar to that of normal cells during the logarithmic growth phase. However, subsequently, two crucial differences are noted: growth continues in spite of the high cell density being reached, and the clonogenic cell compartment does not decrease in response to high cell density (the same phenomenon is seen in early trans-

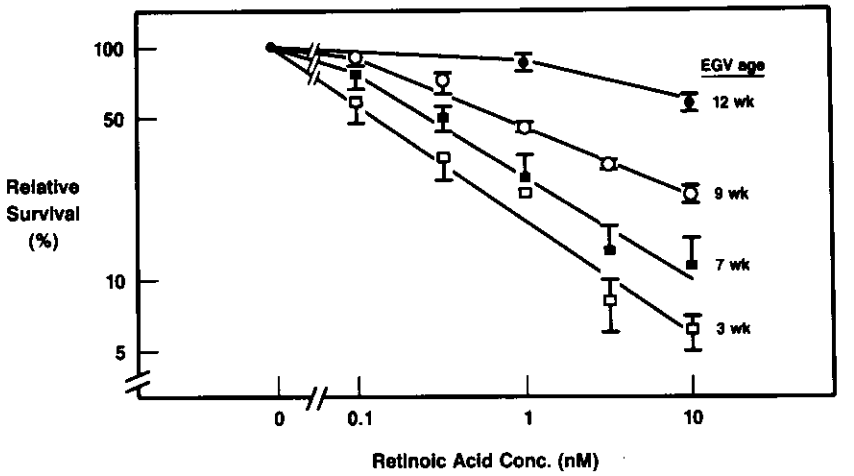

FIGURE 11. Loss of retinoic acid (RA) sensitivity of early RTE cell transformants as a function of time. EGV colonies were isolated at different times after MNNG exposure, dissociated and replated at clonal density, and treated with different concentrations of RA. The effect of RA on the colony-forming ability of the replated cells was determined after 7 days. From Fitzgerald et al. (*13*).

Table 1. Quantitation of c-*onc* expression in EGV-T cell lines.[a]

	fms	H-*ras*	K-*ras*	*raf*	*fos*	L-*myc*	c-*myc*	N-*myc*, *abl*, *erb*-B, *fes*, *myb*, *ros*, *sis*, *kit*
Normal rat tracheal epithelial (RTE) cells	1	1	1	1	1	1	1	No detectable expression
EGV_4T	5*	3*	2	2	≤1	≤1	≤1	
EGV_5T	5*	3*	2	2	≤1	≤1	≤1	
EGV_6T	19*	3*	≤1	2	≤1	≤1	≤1	
$EGV_{10}T$	≤1	≤1	≤1	≤1	ND	≤1	ND	

ND = Not determined.

[a] Dot blot analysis of cytoplasmic RNA of independently transformed, tumor-derived RTE cell lines and normal RTE cells grown in primary culture.

* Significant ($p \leq 0.05$).

formants, EGV clones). It is clear that the regulation of the size of the stem cell compartment in these transformants is altered. It is important to note, however, that even in the immortal growth variant, only 10% of the cells are clonogenic, i.e., a substantial proportion of cells in these IG-variant cultures differentiate and become terminal (*9,10*). Thus, the transformed phenotype is transmitted to only a fraction of the descendants of the transformed stem cell. In the early transformants, namely the EG-variants, the stem cell compartment is even smaller (Fig. 9). Most of the transformed clones have ≤ 1% cells with clonogenic potential, and at most 10 to 20%. Autoradiographic studies have shown that the growth fraction, i.e., all cells able to proliferate, is not more than ~30%, and a large proportion of cells shows ultrastructural evidence of terminal differentiation (*9*). However, as the EG-variant clones progress and acquire unlimited growth capacity (i.e., become immortal) (Fig. 10), the fraction of EGV-stem cells increases disproportionately within each clone (*10*). The significance of this steady increase in the size of the preneoplastic stem cell compartment seems obvious: the larger the pool of proliferating stem cells, the greater the chance for a new cell variant to arise. This brings us to the third topic of our discussion, namely, the manifestations of progression.

Manifestations of Progression in Early Preneoplastic Clones: Loss of Responsiveness to Retinoic Acid

One can view neoplastic growth behavior as a consequence of abnormal growth stimulation (e.g., autocrine growth stimulation) or as a result of diminished growth restraints. There is in fact evidence for both mechanisms operating in neoplastic transformation. We are especially interested in exploring the possible breakdown of negative growth control mechanisms as a causal event in early stages of transformation. One key growth and differentiation regulator of normal RTE cells *in vivo* is vitamin A and related retinoids. Withdrawal of vitamin A from the diet results in increased cell proliferation and squamous metaplasia of the tracheal epithelium (*11*). Thus, retinoic acid (RA) can be regarded as a physiological, negative growth regulator. It has also been shown to inhibit the development of skin, mammary, and bladder tumors in mice and rats (*12*).

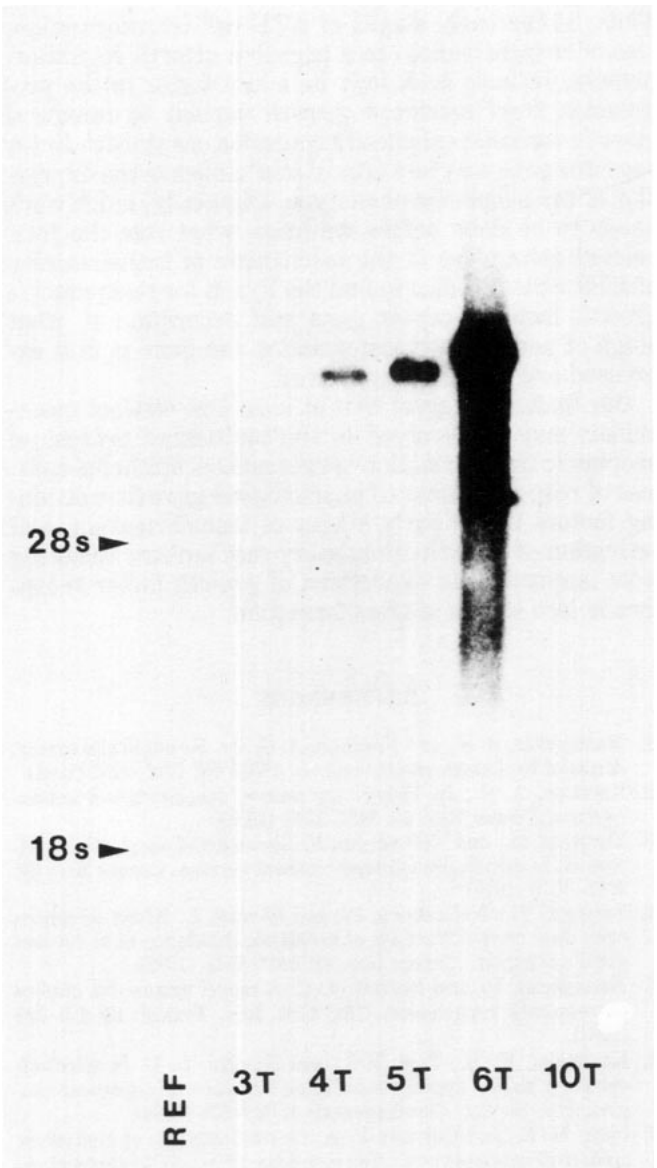

FIGURE 12. Northern analysis of RNA from several tumor-derived RTE cell lines using a v-*fms* probe. Samples of poly A^+ cytoplasmic RNA from rat embryo fibroblasts (REF) or EGV-T cell lines (4T, 5T, 6T, and 10T are independently transformed, tumor-derived RTE cell lines) were separated by electrophoresis in formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to 3′ v-*fms* probe. From Walker et al (*14*).

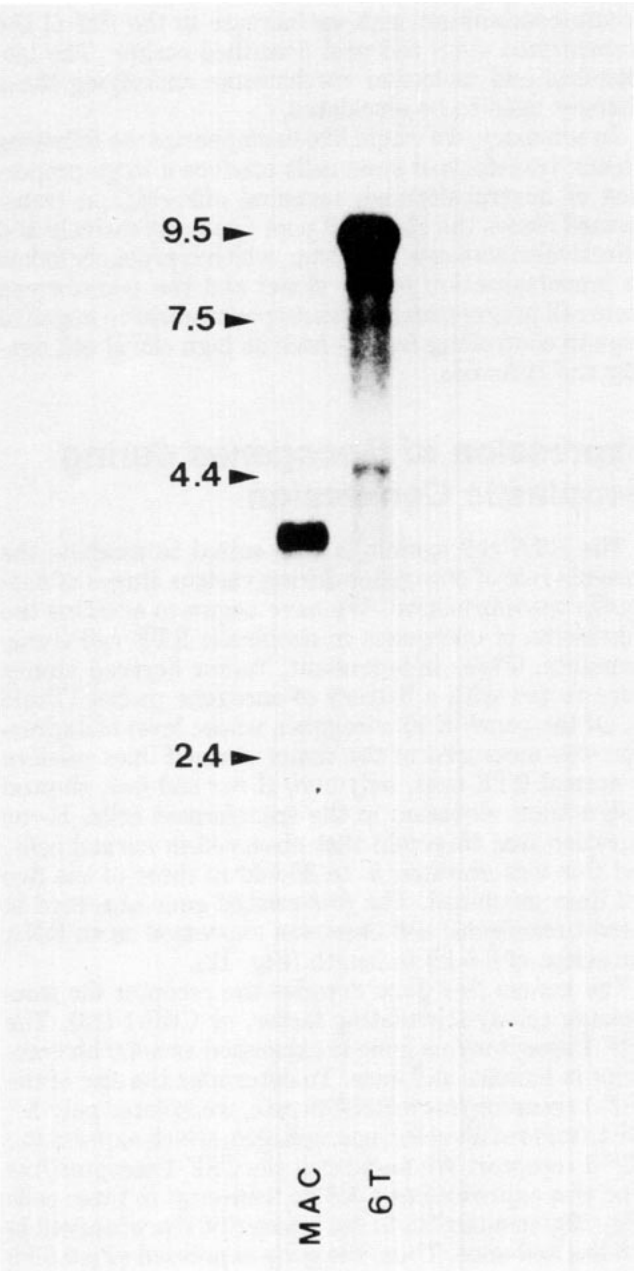

FIGURE 13. Northern analysis of RNA from EGV6-T and rat macrophages using a v-*fms* probe. Samples of poly A^+ cytoplasmic RNA from EGV6-T and normal rat alveolar macrophages (MAC) were separated by electrophoresis in formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to 3′-v-*fms* probe. From Walker et al. (*14*).

In our studies we found that RA inhibits growth of normal RTE cells and inhibits the development of RTE cell transformants when the cultures are treated as early as 1 day or as late as 20 days after carcinogen exposure (*13*). What is perhaps more interesting is that the sensitivity to RA of clonogenic cells isolated from transformed colonies (EG-variant colonies) decreases markedly with time (Fig. 11). The proliferation of cells isolated from 3-week-old transformants is readily inhibited at low doses (the IC_{50}, the dose needed to inhibit colony formation by 50%, is 0.1 nM); however, clonogenic cells from 9- and 12-week-old transformants become increasingly resistant ($IC_{50} > 10$ nM). We believe that this loss of RA sensitivity represents an important step in the progression of the early EG-variant to become an IG-variant, which escapes the growth regulatory function of RA. It is noteworthy that this change

occurs concomitant with an increase in the size of the transformed stem cell pool described earlier. The biochemical and molecular mechanisms underlying these changes need to be elucidated.

In summary, we would like to emphasize the following points: transformed stem cells produce a large proportion of nontransformed, terminal offspring; in transformed clones the stem cell pool size progressively and selectively increases with time, which is probably linked to immortalization of the clone; and the transformed stem cell progressively loses responsiveness to negative growth controlling factors such as high clonal cell density and retinoids.

Expression of Oncogenes during Neoplastic Conversion

The RTE cell system is well suited to examine the possible role of oncogenes during various stages of neoplastic transformation. We have begun to examine the expression of oncogenes in neoplastic RTE cell transformants. Five, independent, tumor-derived clones were tested with a battery of oncogene probes (Table 1). Of the panel of 15 oncogenes whose level of expression was measured in the tumor-derived lines relative to normal RTE cells, only two, H-*ras* and *fms*, showed a significant elevation in the transformed cells. H-*ras* elevation was threefold that observed in normal cells, and *fms* was elevated 5- to 20-fold in three of the five cell lines examined. The *fms*-related gene observed in these tumorigenic cell lines was expressed as an RNA transcript of 9.5 kb in length (Fig. 12).

The human *fms* gene encodes the receptor for macrophage colony-stimulating factor, or CSF-1 (*15*). The CSF-1 receptor/*fms* gene is expressed as a 4.0 kb transcript in humans and mice. To determine the size of the CSF-1 receptor/*fms* mRNA in rats, we isolated poly A^+ RNA from rat alveolar macrophages, which express the CSF-1 receptor. We found that the CSF-1 receptor/*fms* gene was expressed as a 3.8 kb transcript in these cells (Fig. 13), comparable to the transcript size observed in humans and mice. Thus, the gene expressed as a 9.5 kb transcript in the epithelial cell lines is related to, but distinct from, the CSF-1 receptor/*fms* gene. We speculate that this gene encodes a related growth factor receptor, possibly for another hematopoietic growth factor.

Because epithelial cells retain in their normal repertoire the capacity to synthesize and secrete various hematopoietic growth factors such as granulocyte colony-stimulating factor, if the *fms* related gene encodes the receptor for one of these growth factors, the possibility of autocrine transformation of cells expressing the *fms* related gene exists. This could be an important clue to the mechanisms underlying the neoplastic behavior of these cells. Conceivably, the growth factor responsible for the unregulated growth of these cells may be secreted by the neoplastic cells themselves. Thus, at the early stages of RTE cell transformation, loss of responsiveness to a negative growth regulator, namely, retinoic acid, may be a key factor in the progression from enhanced growth variant to immortal growth variants; enhanced expression of a growth factor receptor gene may be a crucial mechanism in the expression of the neoplastic phenotype. Obviously, much work needs to be done before we know what role the *fms*-related gene plays in the mechanism of transfomation of RTE cells. Studies to find the ligand for this putative growth factor receptor gene and determine at what stage of neoplastic transformation the gene is first expressed are currently underway.

Our findings suggest that at least two distinct mechanisms may be involved in the multistage process of neoplastic transformation of rat tracheal epithelial cells: loss of responsiveness to physiological growth restraining factors in the early stages of transformation, and activation of growth stimulatory mechanisms resulting from inappropriate expression of growth factor receptors in late stages of transformation.

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