Role of Spontaneous Transformation in Carcinogenesis: Development of Preneoplastic Rat Tracheal Epithelial Cells at a Constant Rate

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ABSTRACT

The rate of spontaneous transformation of normal rat tracheal epithelial (RTE) cells to preneoplastic enhanced growth (EG) variants was estimated in serum-free culture. Spontaneous transformation of RTE cells has previously been observed, but an accurate estimation of the rate of change has not been possible due to the use of serum and feeder cells in the cultures which prevents both unlimited RTE cell proliferation and an accurate determination of the number of cells at risk. RTE cells were plated in serum-free medium and were switched to serum-containing medium at various times during the first 23 days of culture. In serum-containing medium, normal RTE cells cease proliferation, while EG variants continue to proliferate. The fraction of RTE cell colonies which developed into EG variants increased with time to a maximum of 15% when selection was imposed 5 to 23 days after plating. The number of cells per culture also increased during the same time, suggesting a role for cell proliferation in the spontaneous generation of EG variants. In contrast to the time-dependent increases in cell number and the frequency of EG variants, the rate of development of spontaneous EG variants remained constant with time and was estimated to be $7.5 \pm 4.1 \times 10^{-6}$ variants/cell generation. The rate of spontaneous preneoplastic transformation of normal epithelial cells reported here, the rates of spontaneous progression of preneoplastic and neoplastic cells reported elsewhere, and the association between cell proliferation in vitro and increased cancer risk are consistent with the hypothesis that spontaneous changes play a role in the multistep progression of cells to cancer.

INTRODUCTION

Neoplastic transformation in vivo and in vitro is a progressive, multistep process (1-3), but definitive information on the nature, origin, and number of the required specific cellular changes is lacking. The role of carcinogenic agents in neoplastic transformation has been studied extensively, and many changes which occur during preneoplastic progression can be attributed to interactions of these agents with cells in vivo or in vitro. However, changes can occur during the progression of cells to neoplasia which cannot be attributed to carcinogen exposure. These changes have been described as spontaneous (4). Spontaneous changes have most frequently been described in cell culture, where the prolonged passage of cultured normal rodent cells (4) usually results in the emergence of neoplastic cells. In contrast, normal human cells cultured under similar conditions rarely (5), if ever (6), undergo spontaneous neoplastic transformation. In some cases, changes initially described in cell culture as spontaneous have been attributed to specifically identifiable environmental factors (4). However, the development of spontaneous changes in vivo and in vitro can be affected by the fidelity of DNA replication and the maintenance of normal DNA structure and activity. The roles of DNA repair, replication, and recombination in spontaneous mutagenesis have been well documented (7). Similarly, their roles in neoplastic transformation should be considered. An understanding of the origin of spontaneous changes and their role in progression to neoplasia is important in the interpretation of models and mechanisms of carcinogenesis.

Quantitative studies on the rates of spontaneous transition between normal and preneoplastic cells or between preneoplastic and neoplastic or malignant cells can provide basic information needed to determine the role(s) of spontaneous changes in the multistep process of carcinogenesis. Such rates have been estimated in vivo (8) from the age-specific tumor incidence data of individuals who have a dominantly inherited cancer syndrome such as retinoblastoma or polyposis coli. These estimates are expressed as rates of tumor development per year or per (human) generation but not per cell at risk, since the number of cells at risk in vivo cannot be accurately estimated. However, many in vitro cell transformation systems permit determination of the number of cells at risk. Thus, rates of change per cell at risk can be determined. Determinations have previously been made using preneoplastic or neoplastic cell lines in culture for the rates of spontaneous change from anchorage dependence to independence (reviewed in Ref. 9) from nontumorigenic to tumorigenic (10), and from nonmetastatic to metastatic (11).

In this paper, the rate of spontaneous preneoplastic transformation is determined for primary RTE cells in serum-free culture, using the Luria-Delbrück fluctuation analysis (12). As previously described (13), treatment of RTE cells with carcinogens in vivo (14) or in vitro (13, 15) induces heritably altered, preneoplastic cells, termed EG variants, which have the capacity to proliferate in vitro under conditions where normal RTE cells fail to do so. These variants are nontumorigenic but can become tumorigenic with additional time in culture (13, 15). Spontaneous transformation of RTE cells to EG variants was observed, and it was proposed that the frequency of spontaneous EG variants was a function of cell proliferation (13). However, an accurate estimation of the rate of this spontaneous transformation was not possible because of the use of serum and the presence of feeder cells. In the present study, a serum-free and feeder cell-free culture system for the clonal proliferation of normal RTE cells was developed to eliminate the inhibitory effects of serum in long-term cultures and to eliminate the use of feeder cells (13). Selection for preneoplastic EG variants was imposed by switching cultures from serum-free to serum-containing medium to mimic previously used selective conditions which consisted of selective removal of feeder cells from the cultures and the same serum-containing medium used here (13). Using these conditions, the rate of spontaneous transformation to EG variants can be estimated. The hypothesis that spontaneous changes can play a role in the multistep progression of cells to cancer is examined with respect to this rate of spontaneous preneoplastic transformation, rates of spontaneous progression of preneoplastic and neoplastic cells, and the association between cell proliferation in vivo and increased cancer risk.

MATERIALS AND METHODS

Cells and Cell Culture. RTE cells were obtained from 7- to 8-wk-old male Fischer 344/NCR rats (specific pathogen free; Animal Products

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The abbreviations used are: RTE, rat tracheal epithelial; EG, enhanced growth.
tion, Frederick Cancer Research Facility, Frederick, MD) as previously described (13). Primary RTE cells were cultured in serum-free medium composed of Ham’s F-12 medium (GIBCO) modified by increasing the Ca\(^{2+}\) to 0.8 mM and adding 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer (Utrol; Calbiochem-Behring) and supplemented with 1% (v/v) bovine pituitary extract (16), bovine serum albumin (500 μg/ml) (essentially globulin free; Sigma), 10\(^{-4}\) M cholera toxin (Sigma), epidermal growth factor (5 ng/ml) (Collaborative Research, Waltham, MA), hydrochloric acid (0.1 M), insulin (5 μg/ml) (Calbiochem, La Jolla, CA), 5 × 10\(^{-5}\) M ethionine and hydrocortisone (0.1 M), hydrocortisone (0.1 M), and antibiotics (13). All cultures were fed twice weekly with the appropriate medium.

Transformation Assays. The development of EG variants with time in culture was examined by plating RTE cells in serum-free medium and then selecting for EG variants by switching, at various times after plating, to serum-containing medium which will not support the continued proliferation of normal RTE cells. RTE cells were plated in 60-mm-diameter tissue culture dishes (Lux, Naperville, IL) at 6000 (Experiment 1) or 2500 (Experiments 2 and 3) cells per dish. At the specified times after plating in serum-free medium, 5 to 20 dishes of RTE cells were switched to serum-containing medium for an additional 4 wk to select for EG variants. The number of cells per culture continued to increase for several days after switching from serum-free to serum-containing medium, increasing the number of cells at risk for spontaneous change. Estimates of the rate of spontaneous transformation to EG variants were, therefore, based on the maximum number of cells at risk in each set of cultures. A determination of the number of cells per culture was made on representative cultures on the day of switching to serum-containing medium (Table 1; Fig. 1A) and was determined as described (13) 10 days after the RTE cells were plated. The frequency of spontaneous EG variants was determined for each set of cultures switched to serum-containing selective medium at different times after plating. The frequency was calculated as the fraction of RTE cell colonies which formed in serum-free medium by Day 10 of culture which became EG variant colonies 4 wk after being switched to selective medium.

Determination of the Rate of Spontaneous Transformation. The rate of spontaneous transformation was estimated for each time point using the equation from Luria and Delbrück (12).

\[ r = a N_{0} \ln \left( \frac{C N_{0}}{N_{t0}} \right) \]

where \( r \) is EG variants per culture, \( a \) is rate of spontaneous transformation of RTE cells to EG variants (variants/cell generation), \( N_{0} \) is maximum number of cells per culture, and \( C \) is number of cultures. Since \( r \), \( N_{0} \), and \( C \) are known values, the equation is solved for \( a \) using the method of Capizzi and Jameson (17) which has a computer-generated table for calculation of \( a \). The rate of spontaneous transformation is expressed as EG variants/cell generation, where one cell generation is defined as the production of two cells from a single cell by mitosis. Although all cell lineages do not contribute equally to the total number of cells in a population due to unequal rates of cell division, the total number of cell generations (mitoses) which have given rise to a cell population is theoretically equal to the total number of cells at the time of testing minus the initial number of colony-forming cells.

RESULTS

When selection for EG variants is imposed (13) by switching cultures from serum-free to serum-containing medium, the number of cells per culture increases 1- to 14-fold during the subsequent 0 to 7 days (average, 3.6 ± 2.6 days), after which the RTE cells flatten and begin to slough from the dishes. The numbers of RTE cells in cultures on each day of switch to serum-containing medium are shown in Table 1, and the maximum numbers of cells in these same cultures are shown in Fig. 1A and Table 1. The maximum number of cells per culture increased exponentially during the first 10 to 11 days after plating with a population doubling time of approximately 20 h, after which a plateauing was seen on Days 11 to 23.

After being switched to serum-containing medium at specified times, cultures were maintained for an additional 4 wk and the fraction of RTE cell colonies becoming EG variant colonies was determined (Table 1, Fig. 1B). The fraction of colonies becoming EG variants steadily increased from Days 5 to 11, doubling approximately every 1.5 days. From Days 13 to 23, the fraction of colonies becoming EG variants increased much more slowly, doubling only about once a week.

The rate of spontaneous transformation of RTE cells to EG variants was estimated for each day of switch to serum-containing medium (Table 1; Fig. 1C) and was based on the maximum number of cells present in the cultures after switching to serum-containing medium (Table 1; Fig. 1A). To determine if variations in the rate were time dependent, the hypothesis that the rate remained constant with time was tested by regression analysis. The null hypothesis that the slope of the regression line versus time was significantly different than zero was tested for rates calculated from Days 5 to 23. A probability of \( P < 0.05 \) would result in rejection of the null hypothesis. The slope of the regression line did not differ significantly from zero for Days 5 to 23 (\( P = 0.39 \)), suggesting that the rate of spontaneous transformation of RTE cells to EG variants remained constant with time. The average rate of change for Days 5 to 23 was 7.5 ± 4.1 × 10^{-6} EG variants/cell generation. Rates could not be accurately estimated at earlier times due to large errors in determining cell numbers at early times and due to the small number of variants obtained.

DISCUSSION

The development of spontaneous preneoplastic EG variants from normal RTE cells in culture has been analyzed to gain insight into the role of spontaneous transformation events in carcinogenesis. The occurrence of spontaneous changes in cells is theoretically dependent on cell proliferation for the generation and amplification of stable genetic variants. In the development of spontaneous EG variants described here, the fraction of colonies becoming EG variants increased with time when selection was imposed 5 to 23 days after plating (Table 1; Fig. 1B). The number of cells per culture also increased during the same period of time (Table 1; Fig. 1C), suggesting a role for cell proliferation in the spontaneous generation of EG variants. In contrast to the time-dependent increases in cell number and the frequency of EG variant colonies, the rate of development of spontaneous EG variants remained constant with time (Fig. 1C; Table 1) and was estimated to be 7.5 ± 4.1 × 10^{-6} EG variants/cell generation. New EG variant cells arose at a constant rate within expanding colonies of normal RTE cells, resulting in the increased frequency of colonies scored as EG variants with time. It is important to note the high frequency of EG variant colonies (>10%) which resulted from a relatively low rate (<10^{-6}/cell generation) of spontaneous change. The combined effects of cell proliferation, cell accumulation, and a constant rate of spontaneous change at the cellular level yielded high frequencies of variant colonies at the population level, which
can make a significant contribution to transformation in cell culture and theoretically in vivo.

Estimates of the rate of spontaneous transformation will be affected by a number of factors. Estimates will be affected by errors in determining the number of cells at risk per culture. The maximum number of cells potentially at risk for spontaneous transformation will also remain constant when cell numbers change are much less than the overall standard error (55%) would decrease estimates of the rate of spontaneous transformation by an equivalent amount (i.e., < 10%). However, these changes are much less than the overall standard error (55%) for the rates estimated using only attached cells as the total number of cells at risk and would, therefore, not significantly affect the results.

The rates and frequencies reported here may also be slightly underestimated. Each EG variant colony is assumed to arise from one spontaneous transformation event within an expanding RTE cell colony. However, more than one RTE cell in a colony could transform into a variant cell, although only one EG variant colony would develop, resulting in an underestimate of the rate of spontaneous transformation. Such multiple transformation events in single colonies are not likely to occur too frequently, since > 85% of RTE cell colonies do not become EG variant colonies and thus have not even had single transformation events.

If the development of EG variants depends on RTE cell proliferation as proposed here, effects of cessation of proliferation should be considered. When normal RTE cell proliferation stops, the number of cells per culture will remain constant or slowly increase as rare EG variant colonies continue to expand. Similarly, the fraction of RTE cell colonies which become EG variant colonies will remain constant after all variant cells which arose before proliferation stopped have become EG variant colonies. Finally, calculated rates of spontaneous transformation will also remain constant when cell proliferation stops, since the rate is based on the total number of RTE cells plated per culture and the colony-forming efficiencies for Experiments 1 to 3 were, respectively, 6000 and 2.2%, 2500 and 6.2%, and 2500 and 1.5%.

Maximum number of cells per culture after switch to serum-containing medium and the day on which that number of cells was observed.

Table 1. EG variants and estimated rates of their spontaneous development in cultures of RTE cells as a function of time of switch to serum-containing selective medium

Primary RTE cells were plated in serum-free medium. At various times after plating, sets of cultures were switched to 5% serum-containing medium and were scored for EG variants 4 wk later. The number of colony-forming cells per culture was determined 10 days after plating. The frequency of spontaneous EG variants was determined by dividing the number of EG variants found per culture at the end of the experiment by the number of colonies found per culture on Day 10. The maximum number of cells per culture and the day on which that number of cells was found were also determined for calculation of rates of spontaneous transformation. The rates of spontaneous transformation of RTE cells to EG variants were estimated for each day of switch to serum-containing medium, using the equation \( r = \frac{n}{N \cdot N_c} \), where \( r \) is EG variants/culture, \( a \) is rate of spontaneous transformation of RTE cells to EG variants (variants/cell generation), \( N_c \) is maximum number of cells per culture, and \( C \) is number of cultures.
of cells and EG variant colonies, both of which will have become constant. Thus, calculations of rate at late times when there are no increases in the numbers of RTE cells or EG variants will be artificial and will only reflect the steady state which has developed and not a dynamic state of new variant development. The experiments reported here represent a dynamic state of EG variant development, since there are increases in the numbers of cells at risk [see (number of cells/culture on day of switch) versus (maximum number of cells/culture), Table 1] and the fraction of colonies which have become EG variants (Table 1; Fig. 1B) over most of the time period examined.

Although it is difficult to demonstrate unequivocally a role for spontaneous transformation events in carcinogenesis in vitro or in vivo, an examination of factors important for the development of spontaneous changes will be useful in evaluating such a role. The estimated rates of spontaneous change presumably related to neoplastic transformation reported here and elsewhere (see below) can be used to make first order estimates of frequencies of changes with these rates in vitro or in vivo. In addition, the relationship between cell proliferation and carcinogenesis in vivo will be examined, because of the relationship between cell proliferation and spontaneous change in vitro.

Rates of spontaneous change for other neoplasia-related changes occurring in vitro have been estimated. Rates of spontaneous change from anchorage dependence to independence were $3 \times 10^{-7}$ to $1 \times 10^{-6}$/cell generation, depending on the type of cells (mouse, rat, hamster, diploid or tetraploid) and the kind of semisolid medium which were used (9). The rate of spontaneous development of tumorigenic variants from a line of nontumorigenic, anchorage-independent mouse (CAK) fibroblasts was $10^{-7}$ variants/cell generation (10). Finally, the rate of spontaneous development of metastatic variants from the tumorigenic, nonmetastatic mouse KHT sarcoma cell line was $10^{-5}$/cell generation (11). These rates were calculated by the method described here, although the assays used to identify variants were clearly different and are a likely source of variability.

These rates represent probabilities of errors that may occur as a function of cell division regardless of environment and could, therefore, be used as estimates of risk for spontaneous change in vivo. They can be used to predict the number of spontaneously arising tumors expected in an individual with an inherited gene for retinoblastoma under the assumption that one additional change is necessary and sufficient for tumor development (18). Using the equation $m = u \times N/\ln 2$, where $m$ is number of tumors per individual, $u$ is rate of spontaneous change [$10^{-7}$ tumor variants/cell generation (10) or $7.5 \times 10^{-5}$ EG variants/cell generation found here], and $N/\ln 2$ is number of cell generations at risk in two eyes [$N = 4 \times 10^6$ cells at risk in two eyes (19)], the predicted number of tumors in an individual with heritable retinoblastoma is 0.3 or 17 compared to an observed number of 3 to 4 tumors per individual (20). Thus, in the case of heritable retinoblastoma, spontaneous changes occurring during normal retinal development and having rates similar to those found in vitro for neoplasia-related changes could account for the observed tumor incidence.

If spontaneous changes related to neoplastic transformation occur as a function of cell proliferation, then cell proliferation in vivo may increase neoplastic or malignant cell development. Table 2 lists examples of cell proliferation in vivo and in vitro which are associated with an increased likelihood of subsequent neoplastic development. It should be emphasized, however, that each of these examples of cell proliferation may not always lead to cancer. Cancer development is a multistage process, and the spontaneous occurrence of neoplasia-related changes in a proliferating cell population will only lead to cancer if other essential change(s) occur or have occurred in the same cells. Cell proliferation increases the likelihood that spontaneous changes will occur and, as shown in Table 2, is often associated with neoplastic development. The association of cell proliferation with increased risk for both spontaneous change in vitro and neoplastic development in vivo suggests that spontaneous change(s) may play a significant role in carcinogenesis.

The immense complexity of cells and organisms and the changes or adaptations they can undergo make an absolute determination of a mechanism or a unique pathway of carcinogenesis theoretically impossible (21). A multivariate mathematical model which deals with some of the complexities of human carcinogenesis has been proposed (22) and encompasses roles for both spontaneous and induced events and for growth and differentiation of cells in vivo. The demonstration here that normal RTE cells in culture undergo spontaneous preneoplastic transformation at a constant rate which results in high frequencies of altered cells supports a proposed role for spontaneous transformation events in carcinogenesis. The similarly high frequency of presumed spontaneous change leading to tumor development in individuals with hereditary retinoblastoma (23) further emphasizes the potential importance of spontaneous changes in carcinogenesis. Finally, the relationship between increased cell proliferation and increased risk of neoplastic transformation in vivo and in vitro suggests a role for spontaneous change(s) in this process. The multistage process of neoplastic transformation is likely, therefore, to be governed by a balance between changes induced by exogenous or endogenous carcinogens, changes which occur spontaneously at a constant rate in dividing cells, and forces in vivo which act to expand, eliminate, or modulate neoplastic and preneoplastic cell populations.
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