Analysis of Growth Fractions and Stem Cell Compartments in Transformed Rat Tracheal Epithelial Cell Colonies


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ABSTRACT

The purpose of the studies described here was to define the biological behavior of the various clonally transformed colonies observed in cultures of carcinogen-exposed rat tracheal epithelial cells. As described in the preceding paper (H. Kitamura et al., Cancer Res., 46: 4631-4641, 1986), these colonies fall into four morphologically distinct categories. In the studies reported here we found that type I colonies had the smallest growth fraction (7%) and contained the lowest frequency of clonogenic cells (~10^3). Colonies of types II to IV had mean growth fractions of 21 to 28%, and the frequency of clonogenic cells was 2 to 5 x 10^7 when measured under growth-permissive conditions (3T3 feeders). When the clonogenic cell assays were performed under select conditions to identify cell variants which can grow without feeder support, the average frequency of such clonogenic cells in type I colonies was <4 x 10^3 and in colonies of types II to IV, between 5 x 10^4 and 10^5. In type IV colonies, the total number of cells per colony increased 8-fold between 5 and 12 wk postcarcinogen, but the clonogenic cell compartment increased 42-fold; the compartment of variant clonogenic cells, which are able to replicate on plastic, increased 139-fold during the same period of time. This indicated that major changes in the self-renewal capacity of the clonogenic cells were taking place during this early stage of transformation. Examination of the daughter colonies produced by replating colonies of types I to IV revealed that clonogenic cells with different growth potential existed within the same parent colony. Comparison of transformed colonies of the same type showed a marked degree of heterogeneity in the sizes of growth fractions and clonogenic cell fractions. These studies further indicated that, within all colonies, including the most advanced transformants, the majority of the cells were nonreplicating, terminal cells, suggesting that, at least during early stages of transformation, the transformed characteristics were not transferred from parent to daughter cells. With the exception of type I colonies, most of the colonies recognizable at 5 wk after carcinogen exposure progressed with time and acquired the morphological characteristics of type IV colonies, which were the most transformed phenotype. We conclude that transformation of rat tracheal epithelial cells is an asynchronous process and that the morphologically distinct types of rat tracheal epithelial cell colonies represent different stages of the clonal evolution of transformants.

INTRODUCTION

Neoplasia can be regarded as a stem cell disorder in which the normal growth restraints regulating self-renewal and production of terminal progeny are dysfunctional. A variety of biochemical and molecular mechanisms may underlie the disruption of growth control, depending on the target tissue and the nature and severity of the carcinogenic insult. Regardless of the molecular basis of the abnormality, an early phenotypic change at the cellular level appears to be an imbalance in cell population dynamics possibly resulting from a shift towards an increased self-renewal capacity. This could occur in two ways: either (a) the probability of self-renewal of stem cells is increased (see Ref. 1) (i.e., compared to normal cells, division of transformed stem cells results more often in production of stem cells than in production of cells committed to differentiation); or (b) the differentiating progeny retain replicative capacity instead of becoming terminal. This marked increase in growth capacity and stem cell renewal may be related to the process of escape from cellular senescence termed immortalization (2).

We have been interested in the clonal evolution of neoplastic cell variants for a number of years and have reported that the emergence of cells with increased clonogenicity and infinite replicative potential is an early and possibly essential event in neoplastic transformation of RTE cells (3, 4). Recent studies from our laboratory (5) suggested that the process of immortalization of RTE cell populations may occur in several steps, since it is possible during the early stage of transformation to discern cells with greatly different clonogenic potential. In the studies reported in the preceding paper (6), we describe the development of 4 different morphological types of colonies seen during early stages of clonal transformation of RTE cells. Two of the colony types, designated types I and II, were considered to be nontransformed or minimally transformed because of their small size and because they were composed mostly of large, seemingly inactive cells. Two classes of colonies, designated types III and IV, were considered to be transformed because they were large, obviously expanding, and composed of small, dark-staining cells. Particular emphasis was placed on identifying patterns of differentiation, and it was demonstrated that the transformed variants were morphologically characterized by the prevalence of small, poorly differentiated cells and keratinocytic cells.

In the studies reported here we analyzed these different morphological classes of colonies, which are presumed to be of clonal origin, in terms of cell replication and clonogenic potential. This was done in order to substantiate, if possible, our subjective morphological judgments with quantitative measurements of biological activity. The emphasis was placed on growth-related parameters because an enhanced and often unlimited growth capacity is the key phenotypic change in carcinogen-altered RTE cells, preceding development of the neoplastic phenotype (7). These studies extend our earlier observations in which we reported the emergence of clonogenic cells with different growth potential in carcinogen-altered RTE cell cultures containing morphologically transformed colonies (5). Our results with distinct morphological types of colonies show that clonal heterogeneity is a striking feature of early stages of neoplastic transformation and that a large proportion of the progeny derived from transformed stem cells is terminal, programmed for cell death. Furthermore, most of the morphologically distinct colonies seemed to represent stages in a developmental continuum since they acquired progressively more transformed characteristics with time in culture.

MATERIALS AND METHODS

Cell Culture. The culture medium used throughout these studies was Ham's F-12 containing 5% fetal bovine serum (GIBCO; Lot 31K),

4642

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: RTE, rat tracheal epithelial; CFU, colony-forming units growing on feeders; CFUpl, colony-forming units growing on plastic; EG, enhanced growth; LI, labeling index (indices); MNGN, -methyl-N'-nitro-N-nitrosoguanidine; N/C, nuclear/cytoplasmic ratio; PBS, Ca^2+- and Mg^2+-free phosphate-buffered saline.

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insulin (1 μg/ml), hydrocortisone (0.1 μg/ml), and 1% penicillin/streptomycin/Fungizone solution (GIBCO). All incubations were performed at 37°C in a humidified atmosphere of 5% CO₂. Persistent colonies, types I to IV (see preceding paper (6) for definition), were generated by exposure of primary RTE cells to MNNG as outlined in "Materials and Methods" of the preceding paper (6). At 5 wk after carcinogen exposure colonies were located on culture dishes and classified using a phase microscope. All cells not part of a clearly circumscribed epithelial colony, including residual feeder cells and rat fibroblasts, were then removed using sterile cotton swabs and rinsing the culture dish repeatedly with PBS. When required, dishes were prepared containing only one select colony, and all other colonies and extraneous cells were removed.

 Autoradiographic Studies with [³H]Thymidine. Dishes containing colonies belonging to one of the four morphological types were incubated for either 1 or 24 h with 4 ml of medium containing [methyl-³H]thymidine (0.5 μCi/ml; New England Nuclear, Boston, MA) and 1 μM thymidine. After removal of the radioactive medium, colonies were washed 3 times with PBS, 3 times with cold 5% trichloroacetic acid (1 min per wash), and twice with distilled water before being air dried. Dish edges were cut away, and the dishes were processed for autoradiography according to standard procedures using Kodak NTB2 emulsion (Eastman-Kodak, Rochester, NY). After a 7-day exposure at 4°C, the dishes were developed (4 min, 15°C, Kodak D-19 developer) and fixed (Kodak fixer, 5 min, 15°C) and then stained for 20 min with Wright's Giemsa (0.1% in PBS, pH 6.8). Labeling indices were determined by scoring labeled (>10 grains over the nucleus) and unlabeled cells. For type I colonies and the smallest type II colonies, all cells in the colonies were scored. For all other colonies more than 1000 cells were scored in numerous adjacent microscopic fields along the axes of maximum and minimum diameter.

 In some experiments cytospin preparations were made of dispersed cells obtained from specific colony types in the following manner. After 24-h [³H]thymidine labeling and subsequent rinsing with PBS of pre-selected colonies, cells were dissociated by incubation with trypsin-EDTA (GIBCO; 0.15% trypsin-0.06% EDTA) for 5 to 10 min at 37°C. The cells were collected into culture medium containing 1 μM thymidine and then centrifuged (~1000 rpm, 4°C, 10 min) after an aliquot was taken for cell counting (Coulter Counter). The cell pellet was triturated with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline (with 1 μM thymidine), and the cell density was adjusted to 3 × 10⁵ cells/ml. Aliquots of 0.2 ml were centrifuged onto glass slides in a cytotrip centrifuge (600 rpm, 5 min), and the cells were air dried fixed, in absolute methanol (30 min), then trichloroacetic acid treated, and processed for autoradiography as outlined above. The slides were exposed in the dark for 14 days at 4°C (preliminary studies indicated similar results for a 7-day exposure). Labeling indices were calculated after scoring 500 to 700 cells in random fields of view.

 To prepare autoradiograms of a cross-sectioned transformed colony, one type IV colony was incubated for 24 h with [³H]thymidine, washed 3 times with PBS, then fixed, washed, stained, and embedded as described under transmission electron microscopy procedures in the preceding paper (6). Thick sections (0.5 μm) were cut vertically to the dish base, placed on glass slides, and covered with Ilford L4 nuclear track emulsion (Ilford, Essex, England). After 2 to 4 wk of exposure in the dark at 4°C, the slides were developed (Kodak D-19), fixed (Kodak fixer), and stained with methylene blue.

 Determination of Clonogenic Cell Fractions. To determine the number of clonogenic cells present in various types of colonies, preselected colonies were trypsinized and dissociated (see above). Cells of colonies of types I and II, respectively, were pooled while cells obtained from individual colonies of types III and IV, respectively, were either pooled or kept separately. After collection of trypsinized cells into an excess volume of culture medium, cells were centrifuged and resuspended in fresh culture medium. Using an electronic cell counter, the total cell yield per individual colony or pooled sample was determined. Cells were replated in 5 ml of culture medium at a density of 2000 cells/60-mm dish onto irradiated 3T3 feeders (CFU) or at 5000 cells/60-mm dish onto plastic substratum (CFUpl), and cultures were fixed with absolute methanol and stained with 10% aqueous Giemsa after a 7-day incubation period. Five replicate dishes per group were used. The colony-forming unit frequencies were calculated by dividing the number of Day 7 colonies (>10 cells) per dish by the number of cells plated. The absolute number of clonogenic cells per cell pool or individual colony was calculated by multiplying the frequency of colony-forming units by the total number of cells present in the original cell suspension.

 RESULTS

 [³H]Thymidine Labeling of Various Colony Types. The aims of these experiments were (a) to determine the relative proliferative activity of the different colony types based on 1-h [³H]thymidine incorporation; (b) to obtain an estimate of the growth fraction in the different types of colonies, based on 24-h [³H]thymidine incorporation; and (c) to determine the location of labeled cells in various colonies. The LI after 1-h labeling were determined in colonies of types I to IV (Fig. 1A). The LI in all of the 49 colonies studied were below 15%. Most of the type I colonies had LI of <1.5% and were thus clearly distinguished from the other three colony types whose average LI ranged from 5.7 to 8.6%. The LI of colonies of types II to IV did not differ significantly from each other even though there appeared to be an upward trend with increasing colony grade (Fig. 1A). The estimated growth fraction of colonies was determined by incubating various types of colonies with [³H]thymidine for 24 h (Fig. 1B). For colony types II, III, and IV, the estimated mean growth fractions were 21, 28, and 27%, respectively, and for type I colonies the mean value was 7%. The range of growth fractions within a given class of colonies was considerable; e.g., among the 13 type IV colonies examined, values ranged from ~5% to ~40%. Surprisingly, even in the most transformed colonies (types III and IV), the majority of cells appeared to be noncycling or cycling very slowly, and in most colonies less than 35% of the cells were replicating.

 As described in the preceding paper (6), colonies of types III and IV contained stratified regions with some areas having 3 or more layers of cells. Potentially this could have affected the autoradiographic evaluation, leading to an underestimation of the labeling indices, since β-particles from labeled cells may have been unable to penetrate overlying cells and reach the photoemulsion. To determine whether such an error existed, we made autoradiograms of cytospin preparations of cells ob-

 ![Fig. 1. Labeling indices of individual colonies of types I to IV. At 5 wk after MNNG exposure, colonies were incubated with [³H]thymidine, and the fraction of labeled cells was determined by autoradiography. A, 1-h labeling index (relative proliferative activity); B, 24-h labeling index (estimated growth fraction). Horizontal bars represent mean values.](image-url)
obtained from dissociated colonies of types III and IV after the colonies had been labeled for 24 h with \(^{3}H\)thymidine. Evaluation of these cytopsin preparations, in which the cells were homogeneously distributed regardless of the original location in the colonies, revealed growth fractions of 33.5\% (±10.8\%) and 23.0\% (±1.2\%) for colony types III and IV, respectively. Since these values were rather similar to those obtained for intact colonies (Fig. 1), we concluded that the stratification of cells in colony types III and IV did not introduce a significant error in the estimation of labeling indices and growth fractions.

Autoradiograms of intact colonies showed that distribution of labeled cells in the horizontal plane was highly irregular. Some colonies showed predominantly peripheral labeling, others predominantly central labeling, and others showed no recognizable pattern at all. As described in greater detail in the preceding paper (6), small cells with high N/C were the predominantly labeled cell type. In stratified areas of the type IV colony examined in cross-section, labeled cells were observed in basal, intermediate, and superficial cell layers.

Estimation of Size of Clonogenic Cell Compartments in Colonies of Various Types. The purpose of these experiments was to determine the frequency of clonogenic units in the four different morphological types of colonies 5 wk after carcinogen exposure. We quantitated the clonogenic units by dissociating the various types of colonies enzymatically and replating the cells onto two different substrata: 3T3 feeder layers, which maximizes the colony-forming ability of RTE cells; and plastic, which selects for variant cells with altered substrate requirements (5). These variant cells are not present among normal RTE cells but develop between 2 and 5 wk in carcinogen-exposed cultures and appear to be the key cell variant responsible for immortalization (5). Since colony types I and II contained only a few thousand cells, it was necessary to pool cells collected from large numbers of isolated colonies in order to quantitate the clonogenic units. Colonies of types III and IV contained a sufficiently large number of cells so that they could be assayed individually, although we also assayed pooled populations. Fig. 2 summarizes the relevant findings of one experiment in which we studied 50 pooled type I colonies, 32 pooled type II colonies, 10 individual type III colonies, and 9 individual type IV colonies. Estimates of the average number of cells per colony obtained from dissociated colonies (Fig. 2A) were as follows: type I, \(10^3\) cells; type II, \(10^4\) cells; type III, \(10^5\) cells; and type IV, \(10^6\) cells. These results are similar to those obtained by morphometric analysis [see accompanying paper (6)].

The colony assay performed under permissive growth conditions on feeders (CFUf) showed that the average clonogenic cell frequency in type I colonies was \(3 \times 10^{-3}\); for colony types II and III, the average frequency was \(2.1 \times 10^{-2}\), 7-fold greater than for type I colonies; and for type IV colonies the average frequency was \(5.3 \times 10^{-3}\), \textit{i.e.}, 17-fold greater than for type I colonies (Fig. 2B). A great colony-to-colony variation was observed in the size of the clonogenic cell fractions (this information is available only for colony types III and IV). When the colony assay was performed without feeder cells, \textit{i.e.}, under growth conditions selective for cells with altered substrate requirement (CFUpl), the estimated frequencies of these clonogenic cells in pooled type I and pooled type II colonies were \(<4 \times 10^{-5}\) and \(\sim 2 \times 10^{-3}\), respectively (Fig. 2C). Of the 17 colony types III and IV tested individually, only 4 (\textit{i.e.}, 24\%) had a frequency of \(\geq 1 \times 10^{-3}\); three of these were type IV colonies. In individual colonies there was no consistent relationship between the frequencies of cells able to form colonies on feeders and plastic.

Further evidence for the heterogeneity of type IV colonies in terms of clonogenic cell pool sizes was obtained in a series of experiments carried out over several months in which individual type IV colonies were isolated and tested at 5 wk after MNNG exposure. The data from these experiments are compiled in Fig. 3 and combined with the type IV colony data summarized in Fig. 2. Ninety \% of the colonies contained \(>10^{-3}\) clonogenic units when tested under growth-permissive conditions; about 50\% contained between \(10^{-2}\) and \(7 \times 10^{-2}\) colony-forming units, while 20\% contained between \(10^{-3}\) and \(2 \times 10^{-1}\) colony-forming units. This latter group appeared to be distinct from the majority of the colonies, thus suggesting that (at least) 2 separate classes of type IV colonies may exist at 5 wk after carcinogen exposure. Of the 22 colonies tested under selective conditions, 50\% contained \(<4 \times 10^{-3}\) colony-forming units able to grow on plastic (CFUpl), and 50\% of the colonies contained between \(3 \times 10^{-4}\) and \(10^{-1}\) CFUpl, again illustrating the great heterogeneity of this class of transformed colonies.
As we described earlier (Fig. 2; Ref. 6) the size of the various colony types in terms of number of cells per colony increased with increasing colony grade. It was therefore important to examine the relationships between cell population size, grade of colony, and the absolute size of clonogenic cell populations. The absolute size of the clonogenic cell compartments was estimated by multiplying the cell number per colony with the frequency of clonogenic units (CFUf). A log-log plot of colony cell number versus total clonogenic cell number is presented in Fig. 4. These data indicated that the clonogenic cell compartment sizes increased exponentially with increasing cell population size and with increasing grade of the colonies.

**Study of Daughter Colony Subtypes Present in CFUf Assay.** In addition to quantitating clonogenic cell fractions in the various colony types, we determined whether qualitative differences existed among the clonogenic cells derived from 5-wk-old colonies of types I to IV. We examined the morphology of the daughter colonies produced in the 7-day colony-forming assays on feeder layers. A number of morphologically distinct daughter colony subtypes were indeed noted and were subsequently categorized according to colony size and cellular composition (see Fig. 5). Designated subtype A (Fig. 5, a to c) includes those daughter colonies which were of considerable size (0.6- to 1.8-mm diameter at 7 days) and were composed of numerous small, often stellate cells showing focal stratification. These colonies appeared to be the most proliferative daughter colony type. Designated subtype B colonies (Fig. 5d) were often smaller than subtype A colonies (0.3- to 1.3-mm diameter) and were composed of uniformly arranged polygonal cells with no evidence of cell stratification. These colonies were somewhat reminiscent of 7-day-old colonies formed by normal primary RTE cells (8). Designated subtype C colonies (Fig. 5, e and f) were usually smaller (0.2- to 1.0-mm diameter) and contained comparatively fewer cells than colony subtypes A or B; furthermore, they were characterized by the prevalence of large pale-staining cells within the colonies. These daughter colonies were similar in many respects to remnant colonies in late primary RTE cell cultures.\(^1\)

The prevalence of these 3 morphologically distinct daughter colonies was determined after replating cells from transformed colony types I to IV. In most cases, 50 to 100 daughter colonies were analyzed (Fig. 6). However, only a few daughter colonies derived from type I parent colonies were available for study; these were mostly very small, subtype C colonies. Clonogenic cells from type II parent colonies produced ~30% subtype A colonies, ~20% subtype B colonies, and ~50% subtype C colonies. Among the individual parent colonies of types III and IV, the subtype profile was very heterogeneous, ranging from 100% of a single subtype to varied proportions of 2 or 3 of the subtypes. Interestingly, clonogenic cells from 1 of 9 type III colonies and from 2 of 9 type IV colonies produced exclusively subtype C colonies. Clonogenic cells of individual type IV colonies tended to generate daughter colonies of mostly subtypes A and B while cells from type III colonies produced a mixture of all 3 morphological subtypes, thus indicating a shift towards a more homogeneous subtype profile in the more transformed parent colonies.

**Changing Frequency of Clonogenic Cells with Time in Culture.** The above studies have examined clonogenic cell fractions of the colony types I to IV at 5 wk after carcinogen exposure, i.e., at the same time at which the colonies were characterized morphologically (preceding paper (6)). We wished to determine whether the sizes of the clonogenic cell fractions in the transformed colonies changed as a function of time. Previous studies using carcinogen-exposed RTE cell cultures (conglomerates of colony types I to IV) had revealed a time-dependent increase in frequency of clonogenic cells (5). In the present study, type IV colonies only were examined, and particular attention was given to clonogenic cells able to grow on plastic (CFUpl), since we believe that they may represent a more advanced transformed phenotype (5). It was necessary to pool cells from the smaller, early colonies (i.e., 3 wk) to have enough cells for the colony-forming assays; therefore the same procedure was adopted throughout the experiment. Table 1 summarizes the changes in clonogenic cell fractions occurring in type IV colonies. At 3 to 5 wk after carcinogen exposure, the frequency of CFUpl was \(\sim 1 \times 10^{-3}\), and at 9 and 12 wk, \(\sim 4 \times 10^{-2}\) and \(14 \times 10^{-2}\), respectively. The frequency of cells forming colonies on feeders (CFUf) also increased over this period of time. Table 1 also shows that with time the CFUpl, as a proportion of CFUf,
increased from 14% at 5 wk to 45% at 12 wk. To estimate the absolute size of the two clonogenic cell compartments, the clonogenic cell frequencies were multiplied by the average cell number per colony. These data, presented in Table 2, show that while the overall colony size increased 8-fold over this period of time, the CFUf and CFUpl compartments increased 42-fold and 139-fold, respectively, indicating selective expansion of these cellular subpopulations.

Fate of Different Colony Types as a Function of Time in Culture. To characterize the biological behavior of the 4 colony types and to understand their role in the transformation process of RTE cells, it was essential to determine their fate as a
GROWTH RATIOS AND STEM CELLS IN RTE CELL COLONIES

Fig. 6. Prevalence of daughter colony subtypes observed after replating 32 pooled type II parent colonies and 9 individual type III and 9 individual type IV parent colonies onto 3T3 feeders. See text and Fig. 5 for description of morphological subtypes. Daughter colonies: subtype A (solid bar); subtype B (hatched bar); subtype C (open bar).

Table 1 Changes in relative compartment size of clonogenic cells in type IV colonies with time in culture

<table>
<thead>
<tr>
<th>Age of colonies at replating (wk)</th>
<th>No. of colonies pooled</th>
<th>Frequency of CFUpl (x 10^2)</th>
<th>Frequency of CFUpl/CFUf (x 10^-2)</th>
<th>CFUpl/CFUf ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>6</td>
<td>1.0 ± 0.1*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>0.8 ± 0.2</td>
<td>5.5 ± 1.2</td>
<td>0.14</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>4.2 ± 0.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>18</td>
<td>13.8 ± 1.0</td>
<td>29.9 ± 2.0</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* Mean ± SD of 5 replicate dishes.

ND, not determined.

Table 2 Changes in total cell population and clonogenic cell compartment sizes in type IV colonies between 5 and 12 wk after carcinogen exposure

<table>
<thead>
<tr>
<th>Time after carcinogen exposure (wk)</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell population</td>
<td>2.4 x 10^3</td>
</tr>
<tr>
<td>CFUfl</td>
<td>1.3 x 10^6</td>
</tr>
<tr>
<td>CFUpl</td>
<td>1.8 x 10^4</td>
</tr>
</tbody>
</table>

* Values are average cell number per colony.

<table>
<thead>
<tr>
<th>Cell compartment</th>
<th>After 5 wk</th>
<th>After 12 wk</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell population</td>
<td>2.4 x 10^3</td>
<td>1.8 x 10^6</td>
<td>8</td>
</tr>
<tr>
<td>CFUfl</td>
<td>1.3 x 10^6</td>
<td>5.4 x 10^5</td>
<td>42</td>
</tr>
<tr>
<td>CFUpl</td>
<td>1.8 x 10^4</td>
<td>2.5 x 10^5</td>
<td>139</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Compartments</th>
<th>After 5 wk</th>
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<td>139</td>
</tr>
</tbody>
</table>

* Values are average cell number per colony.

DISCUSSION

The primary purpose of the studies described in this paper was to analyze biologically the cell populations comprising the 4 colony types (I to IV) which can be distinguished in RTE cell cultures undergoing neoplastic transformation [see accompanying paper (6)]. In the studies presented here, we compared these EG variant colonies in terms of DNA synthesis, clonogenicity, and growth, and we also monitored the progression of individual colonies as a function of time. We found that our morphological classification was in general appropriate, reflecting differences in "developmental" status since most colonies assumed the characteristics of type IV colonies as a function of time, the major exception being the type I colonies.

Measurements of the replicating cell fraction by incorporation of [3H]thymidine showed that type I colonies possessed a comparatively small replicating cell pool. However, only modest differences in proliferative activity existed between cell populations of colony types II, III, and IV as measured by [3H]thymidine labeling indices. This was surprising since, as shown in the accompanying paper (6), type II colonies were composed primarily (90%) of large, nonkeratinocytic cells which we considered proliferatively inactive, based on the fact that, in type IV colonies, only ~10% of the large, nonkeratinocytic cells were labeled after incubation with [3H]thymidine for 24 h. This suggested that cells which are ultrastructurally similar in different types of colonies may not necessarily have similar biological activities.

Recent studies in other laboratories (9, 10) have shown that it is possible to isolate the clonogenic cells from human epider-
mis and urothelium; these cells are small in size relative to other cells in the same tissues. Whether a similar relationship between cell size and clonogenicity exists in the cell populations comprising transformed RTE cell colonies is not clear. Determinations of the relative compartment sizes of clonogenic cells on feeders showed that type I colonies contained a very small fraction of clonogenic cells (3 x 10^{-3}), while colonies of types II and III contained on the average 2 x 10^{-2}, and type IV colonies 5 x 10^{-2}, clonogenic cells. As described in the preceding paper (6), type IV colonies contained on average 20% small, poorly differentiated cells, while type III colonies had only 6%, and type II colonies, 1%. In spite of these 3- to 20-fold differences in the percentage of small cells in colony types II to IV, cells isolated from the 3 types of colonies had roughly similar colony-forming efficiencies; this suggests that the small cell is not the primary clonogenic unit. Studies using cell separation techniques are required to further address this problem.

Daughter colonies, which were distinct in size, cellular arrangement, and cell morphology, were produced in the 7-day colony assay (CFUf) using cells from parent colonies of types I to IV. Our studies suggested that the clonogenic cells even within the same parent colony produced different progeny, illustrating cellular heterogeneity during this very early stage of neoplastic transformation. Such cellular heterogeneity is known to be common in established neoplasias (11–13). The clonogenic cells from type IV parent colonies seemed to produce the most proliferative daughter colonies, while the clonogenic cells from type I parent colonies produced the least proliferative offspring.

Based on the data available from these and the previous studies (4–7), the following events seem to play a major role in the process leading to immortalization of carcinogen-altered RTE cells. The first change to occur after carcinogen exposure is a decreased responsiveness of surviving cell clones to growth-arresting signals. Normal cell clones cease to proliferate and senesce shortly after removal of feeder cell support. This suggests that normal stem cells rapidly lose their self-renewal capacity when growth-restrictive conditions prevail. Contrary to the normal stem cells, carcinogen-altered stem cells retain their self-renewal potential for an extended period of time resulting in continuous expansion of clones. The wide variation in clone sizes noticed at 5 wk after MNNG exposure, which led us to distinguish 4 different types of colonies, probably reflects differences in self-renewal capacity of stem cells which are at different stages of transformation. Type I and some type II colonies seemed to derive from stem cells which cannot retain sufficient self-renewal potential to assure long-term survival of the clones. On the other hand, the self-renewal potential of stem cell populations seemed to be maintained in the type III and the type IV colonies and half of the type II colonies, since these colonies continued to expand. However, the clonal growth was not just an equal expansion of all cellular elements; e.g., the clonogenic cell compartment, which we presume to represent the self-renewing stem cell compartment, increased 42-fold between 5 and 12 wk when compared to only an 8-fold increase in the total population size (type IV colonies; Table 2). This must be a very important aspect of the altered growth behavior of the transformed RTE stem cells, since in normal RTE cell cultures the trends are in the opposite direction; i.e., as the size of the normal RTE clone increases, the fraction of clonogenic cells rapidly decreases (8). Thus a key feature typical of normal stem cell renewal was changed in the transformed clones: the probability of self-renewal of stem cells (1) was increasing with increasing clone size, instead of decreasing.

A further step, leading to the immortalization of the transformed clone, was the generation of the aforementioned secondary stem cell variant which was characterized by its ability to grow at low cell density on plastic substratum without feeder support. This new cell variant seemed to have a considerable growth advantage over other cells; in type IV colonies, studied between 5 and 12 wk post-MNNG, there was a disproportionate increase in the frequency of this variant clonogenic cell type in comparison to the clonogenic cells which grew on feeders (Table 1). In terms of absolute compartment size, these variant cells increased 139-fold between 5 and 12 wk as compared to the 8-fold increase in total clone population size (Table 2).

Our findings were similar in some respects to observations made by other investigators studying immortalization of cell populations. For example, SV-40 immortalization of human fibroblasts consists of two phases. Initially all the infected cells have an extended life span, but only a few variant immortal cell lines arise from these cells even though all of the cells express viral antigens (14). Carcinogen treatment of human mammary epithelial cells induces a significant number of variant cells with an extended life span (possibly similar to the EG variant of RTE cells) but only rarely do immortal cell lines result from these carcinogen-altered cells (15). Matsumura et al. (16) recently described in great detail the multiphasic immortalization process in cultured rat liver cells, and our results with RTE cells are analogous to their findings. These authors described a “transitional phase” in which a clonal cell population as a whole maintains its proliferative capacity, but the proliferative activity of individual cells within the clone varies greatly, similar to EG variants of RTE cells. Subsequently, more uniformly proliferative variant cells arise within a clone which then becomes immortal. The nature of the second step in the immortalization process is unknown, but, at least in human fibroblasts, immortalization is a recessive trait and thus does not behave as a single dominant gene mutation (14). This is in contrast to reports that certain oncogenes, for example myc and the oncogene encoding Mr, 53,000 protein, function as dominant immortalization genes (17, 18). Our results suggest that the process of carcinogen-induced immortalization of RTE cells is complex and involves more than a single gene mutation.

A major objective of the studies presented in this and the preceding paper (6) was to elucidate the role of the different morphological types of colonies which develop in RTE cell cultures following carcinogen exposure. Should all of them be regarded as transformed clones which will ultimately produce neoplastic cells? What is the significance of the rather striking differences in size and morphology? The cell proliferation and clonogenicity studies indicated that there were no marked differences in the percentage of small cells in colony types II to IV, and type II and III contained on the average 2 x 10^{-2}, and type IV colonies 5 x 10^{-2}. The cell proliferation activity of individual cells within the clone varies greatly, similar to EG variants of RTE cells. Subsequently, more uniformly proliferative variant cells arise within a clone which then becomes immortal. The nature of the second step in the immortalization process is unknown, but, at least in human fibroblasts, immortalization is a recessive trait and thus does not behave as a single dominant gene mutation (14). This is in contrast to reports that certain oncogenes, for example myc and the oncogene encoding Mr, 53,000 protein, function as dominant immortalization genes (17, 18). Our results suggest that the process of carcinogen-induced immortalization of RTE cells is complex and involves more than a single gene mutation.

One important fact, which we wish to stress, seems to emerge clearly from these studies on early stages of RTE cell transformation: the alteration in growth behavior induced by the carcinogen in some of the RTE stem cells surviving the exposure was not “inherited” by the vast majority of their progeny. Even the most transformed EG variant clones (type IV) had growth fractions of no more than 30%, and their estimated clonogenic
cell fractions were usually less than 10%. Our data suggest that a significant proportion of the cells in the EG variant clones was terminal, and as the ultrastructural studies in the accompanying paper (6) indicated, keratinocyte differentiation is probably the ultimate fate of many of the cells. This is supported by the finding that, among EG variant colonies, typically 5 to 30% of the cells have formed a cross-linked envelope (data not shown), which is a marker of keratinocyte differentiation (19). Thus, whatever damage the original target cell sustained from the exposure to carcinogen, it does not seem to be transmitted in a heritable fashion to the progeny. The lack of transference of transformation characteristics from parent to the majority of daughter cells is common also to many frank neoplasms (11, 13).

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REFERENCES

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