Tumorigenic Keratinocyte Lines Requiring Anchorage and Fibroblast Support Cultured from Human Squamous Cell Carcinomas

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ABSTRACT

We have established cell lines from six human squamous cell carcinomas (SCC) of the epidermis and tongue, using culture methods previously developed for clonal growth and serial cultivation of normal keratinocytes. The SCC lines all form rapidly growing, well-differentiated SCC's or progressively carcinomas (SCO of the epidermis and tongue, using culture methods previously developed for clonal growth and serial cultivation of normal keratinocytes. The SCC lines all form rapidly growing, well-differentiated SCC's or progressively growing squamous cysts in nude mice. In contrast to normal keratinocytes, SCC cells form unstratified or very poorly stratified colonies and do not require epidermal growth factor for sustained growth. The SCC lines vary in their requirement for a fibroblast feeder layer to support clonal growth, from complete independence to a dependence as absolute as normal keratinocytes possess. Only one line forms large, progressively growing colonies at high efficiency in semisolid medium; the other five lines exhibit only a small amount of abortive growth in semisolid medium, after which the cells appear to rapidly degenerate. These results demonstrate that SCC's often grow as established lines in culture, but they frequently possess in vitro growth requirements similar to those of normal keratinocytes. Consequently, neither semisolid medium nor standard surface culture media are appropriate for initiating primary SCC cultures or for selecting transformants out of carcinogen-treated keratinocyte populations, because they do not provide conditions permissive for the growth of many malignant keratinocytes.

INTRODUCTION

Cell culture has long been perceived as an excellent system in which to identify intrinsic differences between normal cells and cancer cells and to determine how an alteration manifested as abnormal behavior in culture gives rise to malignant behavior in vivo. Much effort has been devoted to the analysis of experimentally induced variants of nontumorigenic cell lines in an attempt to correlate the acquisition of newly acquired phenotypes in culture with the ability to form tumors in experimental animals. Beginning with studies of the growth properties of virus-transformed fibroblasts (12), much attention has been given to anchorage-independent growth ability as an important marker of cancer cells. Studies of virally and chemically transformed rodent fibroblast lines (2, 22) have demonstrated a strong correlation between anchorage-independent growth in culture and tumorigenicity in experimental animals. However, although cells from some types of naturally occurring human solid tumors, such as melanoma (4), generally form colonies in semisolid medium, cells of many types of carcinoma (epithelial tissue-derived cancers) often do not (13, 24). Alternative markers are therefore needed for identifying and selecting neoplastic variants of epithelial cell types in culture. Some established cell lines have been derived from virtually every type of carcinoma, but the success rate has been extremely low (5, 9), suggesting that only unusual forms of these tumors have generally been studied in culture. Progress in understanding the biology of any type of carcinoma has been impeded by the inability until recently to culture adequately either normal or malignant cells from most epithelial tissues.

The development of a cell culture system that satisfies tissuespecific growth requirements has permitted long-term serial cultivation of the human keratinocyte (the cell of stratified squamous epithelia) (19), which is able to divide only a few times in standard culture media. Tissue size and architecture is normally controlled in stratified squamous epithelia by the maintenance of a balance between the rate at which cells in a proliferative compartment divide and the rate at which daughter cells leave this compartment and become committed to a program of terminal differentiation. Normal human keratinocytes retain this pattern of growth regulation in culture. During their finite culture lifetime, they express a specific requirement for growth-promoting factors elaborated by connective tissue fibroblasts (15, 19) and an inexorable tendency to lose division capacity and become committed to terminal differentiation via a mechanism subject to influence by EGF (14, 20, 27).

The most important malignant variant of the keratinocyte is SCC. The expression of cell type-specific growth-regulatory mechanisms by normal keratinocytes in culture suggested to us the feasibility of culturing SCC's to determine the necessary conditions for their growth and to ascertain whether they consistently are "mutants" in any aspect of growth regulation that might be causally related to their malignant growth ability. We report here the isolation of cell lines representing the tumorigenic populations of 6 human SCC's and a comparison of their growth requirements in culture with those of normal keratinocytes. Because the culture environment was fully permissive for the growth of normal keratinocytes, some of the tumor cells that we were able to culture were more similar to normal cells in certain respects than SCC lines that had previously been isolated; some of the lines retained a strong dependence upon fibroblast feeder support, and most were unable to grow progressively in semisolid medium.

MATERIALS AND METHODS

Tumors. Biopsies of SCC's of the tongue and pharynx were obtained from the Head and Neck Tumor Clinic of the Sidney Farber Cancer Institute. Portions of surgically excised SCC's...
of the epidermis were obtained from Peter Bent Brigham Hospital, Boston, Mass. SCC-4, SCC-9, SCC-15, and SCC-25 were derived from SCC’s of the tongue. SCC-4 was from a 55-year-old male who had had radiation and methotrexate treatment for the tumor for 16 months before the biopsy was taken for culture. SCC-9, SCC-15, and SCC-25 were from a 25-year-old, a 55-year-old, and a 70-year-old male, respectively. These tumors were first detected less than 3 months before the biopsy was taken for culture, and no treatment had been given to the patients prior to biopsy.

SCC-12 and SCC-13 were cultured from SCC’s of the facial epidermis. SCC-12 was from a 60-year-old male kidney transplant recipient who had been treated with immunosuppressive drugs for the previous 7 years. SCC-13 was from a 56-year-old female who had received a series of radiation treatments for the tumor for several years before its surgical removal.

**Preparation and Propagation of SCC Cultures.** Culture conditions and procedures were essentially those published previously for preparing keratinocyte cultures from normal skin (15, 18, 19). Biopsies and portions of surgically excised tumors were placed aseptically into culture medium for transport to the laboratory, where processing was begun within 2 hr of removal. Samples were rinsed thoroughly with serum-free medium containing Mycostatin or Fungizone (both from Grand Island Biological Co., Grand Island, N. Y.) and then were cut into pieces approximately 2 mm in diameter. Several pieces were fixed, sectioned, and stained with hematoxylin and eosin in order to confirm that the sample indeed contained SCC. The remaining fragments were minced with scissors into pieces less than 1 mm in diameter and then either were transferred to a stir flask at 37°C with 0.2% trypsin plus 0.2% collagenase to disaggregate into single cells or were distributed directly to culture dishes to initiate explant outgrowth cultures. In either case, the tumor material was cultured with a mitomycin C-treated feeder layer of the Swiss mouse embryonic fibroblast line 3T3 (31). Growth medium was Dulbecco’s modified Eagle’s medium plus 20% fetal calf serum plus hydrocortisone, 0.4 µg/ml. Serum lots were selected for optimal growth promotion of normal keratinocytes.

Primary cultures prepared by either method were subcultured after about 2 weeks, at a time when individual colonies had attained a size of 10³ to 10⁴ cells and before neighboring colonies had merged to make a confluent monolayer. Cultures were first relieved of the 3T3 feeder cells and any living human keratinocytes. SCC-12 and SCC-13 were cryogenically preserved as were cell populations at apochromat immersion objectives.

**RESULTS**

**Establishment of Cell Lines from Tumor Biopsies.** Of 22 biopsies of SCC placed in culture, 7 were heavily contaminated with yeast and were discarded. Two others proved not to have contained tumor cells by histological examination. Of the remaining 13 samples, 4 yielded only keratinocyte colonies of normal morphology, and these populations senesced after 3 or 4 passages. Three of the 13 yielded a mixture of colonies with normal and abnormal morphology in the primary culture, but abnormal colonies were not detected after the second passage, and these populations senesced after 3 or 4 passages as well. The other 6 tumor samples also yielded normal and abnormal colonies in the primary culture, but in these cases the abnormal colony-forming cells persisted in subsequent passages and behaved like established cell lines. The proportion of tumors that grew in culture as established lines varied with the tissue of origin: 4 of 4 SCC’s of the tongue; 2 of 5 SCC’s of the epidermis; and none of 4 SCC’s of the pharynx.

The abnormal colonies from all tumors were different from normal keratinocyte colonies in that stratification was absent or greatly reduced. The cells appeared to adhere less tightly or more reversibly to one another, since they showed more heterogeneity in shape and packing density and tended to round in culture as a control in these experiments.
up more above the level of the surrounding monolayer when in mitosis (compare Fig. 1a and Fig. 1b). Indirect immunofluorescence with an antisera specific for human stratum corneum keratin proteins (28) showed that the cells of all 6 lines contained a dense cytoplasmic matrix of keratin filament bundles (Fig. 1c) consistent with their epithelial origin (7, 29, 30). The cells growing in early-passage cultures of the different tumors had distinguishing morphological characteristics (Fig. 2), such as a tendency to stratify slightly and abnormally in large colonies or at confluence (SCC-9) and the rare (SCC-4) or frequent (SCC-25) presence of loosely adherent, rounded cells. The lines also displayed differences in their 2-dimensional packing densities in growing colonies (i.e., how flattened the cells appeared). These characters were preserved by the respective cell populations indefinitely through many subcultures, suggesting that the long-term cultured populations were descended without change from the major colony-forming cells in the primary cultures.

Colony-forming efficiency in low-density platings with the feeder layer was 15 to 40% in the secondary and subsequent cultures, much higher than that displayed by normal keratinocytes (19, 20). Cultures grew with population-doubling times of about 24 hr. In contrast to normal diploid keratinocytes (20), EGF was not required by any of the SCC lines to permit continued exponential growth to confluence or to effect a high colony-forming efficiency at subculture. All of the lines grew progressively and at only slightly slower rates when the serum supplement was reduced to 5%. The lines also grew satisfactorily with the majority of fetal calf serum lots tested, in contrast to the fastidious requirement of normal keratinocytes for a 20% supplement of only certain lots.

Each line was found to have a unique aneuploid karyotype with model chromosome numbers ranging from 50 to 80. Five of the 6 lines have undergone at least 200 and the most recently isolated line, SCC-25, has undergone about 60 cell generations in culture to date without significant alterations in the properties described below.

**Tumor Formation in Nude (Athymic) Mice.** When $3 \times 10^6$ cells were injected s.c. into nude mice, all 6 SCC lines produced rapidly growing tumors in every animal inoculated. Growth was visually apparent within 10 days, and the tumor diameters increased to 5 to 10 mm by 3 to 6 weeks. SCC-4, SCC-9, and SCC-15 formed well-differentiated SCC’s with characteristic small nests of differentiated cells surrounded by several layers of dividing cells, intermixed with connective tissue (compare Fig. 1d and Fig. 1e). SCC-13 tended to form large differentiating cysts with a more organized structure as well as regions of histologically typical SCC. SCC-12 regularly formed one or 2 large well-organized differentiating cysts (Fig. 1f), growing to a maximum diameter of 5 to 10 mm, accumulating fluid in the center, and remaining for the life of the mouse. SCC-25 formed a single large differentiating cyst similar to that of SCC-12 but without fluid accumulation. None of the SCC lines grew invasively beyond the dermis but remained encapsulated by a thin sphere of mouse dermal connective tissue. In contrast, diploid keratinocytes cultured from normal epidermis formed only a small 1- to 2-mm-diameter lump that regressed and disappeared after 2 to 3 weeks. Tumors formed from 3 of the lines (SCC-4, SCC-9, and SCC-13) were placed back in culture. Cells with the distinctive morphology and karyotype of

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3 E. Eberling, R. Kitchin, and J. Rheinwald, unpublished observations.

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>3T3 feeder layer</th>
<th>Medium 199</th>
<th>3T3 CM</th>
<th>Maximum colony size</th>
<th>Colony-forming efficiency (%)</th>
</tr>
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<tr>
<td>Strain N</td>
<td>++++</td>
<td>±</td>
<td>±</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SCC-4</td>
<td>++++</td>
<td>+</td>
<td>++</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>SCC-9</td>
<td>++++</td>
<td>±</td>
<td>+++d</td>
<td>4-15&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td>SCC-12</td>
<td>++++</td>
<td>++a</td>
<td>++</td>
<td>40</td>
<td>0.02</td>
</tr>
<tr>
<td>SCC-13</td>
<td>+++++</td>
<td>±</td>
<td>+</td>
<td>15-40&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td>SCC-15</td>
<td>+++++</td>
<td>+</td>
<td>+</td>
<td>4-15&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>SCC-25</td>
<td>+++++</td>
<td>+</td>
<td>+</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> CM, conditioned medium.

<sup>b</sup> Medium supplemented with EGF, 20 ng/ml.

<sup>c</sup> Varied in different experiments.

<sup>d</sup> Less than one-fifth as many colonies as in cultures grown with a 3T3 feeder layer.
began degenerating at this time. In some experiments, no growth was observed from plating $5 \times 10^4$ cells of SCC-9 or SCC-15. SCC-12 and SCC-13 produced colonies with an efficiency of about 0.02% that grew to a maximum size of about 40 cells, although most colonies stopped growing at a smaller size. Normal diploid epidermal keratinocytes did not divide at all in the same methylcellulose medium, while HeLa cells formed colonies larger than 100 cells with an efficiency of about 2%.

**DISCUSSION**

Behavior of Many Cultured SCC's as Established Lines. A large proportion of human SCC's of the epidermis and of the oral cavity and pharynx (the "head and neck" clinical category) could be cultured as established cell lines that expressed their tumorigenicity in nude mice. Grown in primary culture with a 3T3 feeder layer, the carcinoma cells possessed a characteristic abnormal morphology in colonies initiated from single cells or from explanted tumor fragments. They were therefore easily distinguished from the well-organized stratified colonies formed by any normal keratinocytes that were present in the tumor biopsy. Similar morphological differences between normal and neoplastic keratinocytes have been documented in previous studies of SCC's of a number of tissue types in primary culture (1, 25). Each of the 6 SCC lines described here retained its unique aneuploid karyotype and particular distinctive morphological characteristics indefinitely from the first several passages through subsequent serial cultivation. Although this fact does not constitute conclusive evidence, it is consistent with the notion that these lines represent the major stem populations of their respective tumors instead of being founded by rare variants that arise in culture and are selected for during serial cultivation. Thus, the 3T3 feeder layer, a permissive culture system for normal keratinocytes, also appears to support the growth of the malignant stem cells of SCC without imposing selection pressure to favor fitter variants having properties different from those of the original tumor cell population.

Nevertheless, our success rate for culturing SCC's as established lines varied substantially among the 3 anatomical locations studied (epidermis, tongue, and pharynx), in spite of the fact that keratinocyte colonies of normal morphology grew in the primary cultures of all tumors. We are continuing to place new tumors in culture to determine whether this pattern is sustained. We do not know why some of the biopsies that contained tumor tissue yielded no cells of abnormal appearance in culture. The tumor stem population may have been too small in those cases, and the physical association with a large number of normal keratinocytes in culture may have inhibited the growth or suppressed the abnormal morphology of the SCC cells. Abnormal SCC-like colonies were detected in the primary cultures of 2 of the 4 pharyngeal tumors and one of the 3 epidermal tumors that still senesced after several passages. The abnormal colonies disappeared from these populations during serial cultivation at least as rapidly as did the colonies with normal morphology. Limited culture life span appears to be the rule rather than the exception for most human solid tumor types (5, 9, 11). The 7 of 13 tumors in our study that did not yield established lines senesced before a sufficient number of cells could be obtained to permit comparison of their properties with those of the 6 immortal tumor cell populations described here.

Tumorigenicity in Nude Mice. When injected s.c. into nude mice, 3 of the lines formed progressively growing tumors with histology characteristic of well-differentiated SCC (i.e., small nests of cells with central cell enlargement and differentiation intermixed with a network of host connective tissue). The other 3 lines tended to form only one or 2 large, progressively growing, differentiating squamous cysts. There were no apparent correlations between the histological organization of the nude mouse tumor formed by an SCC line and either the degree of squamous differentiation in the original biopsy from which the line was derived or the growth requirements of the line in culture. It is possible that the SCC lines vary in their ability to induce a stromal proliferative response in the host to the extent that some fail to become well infiltrated with dermal connective tissue.

Growth Requirements of SCC Cells in Culture. Cell lines have been isolated without fibroblast feeder layer support from human (9) and mouse (32) SCC's and after in vivo transformation of primary mouse epidermal cultures (3, 6, 8, 23). Thus, malignant transformation of the keratinocyte can be accompanied by loss of a stringent requirement for fibroblast support. However, the culture systems used in the previous studies could not have supported the growth of a class of carcinoma cells that retained normal keratinocyte growth requirements. Not all established mouse epidermal lines selected for independence from fibroblasts are tumorigenic (3), and many that are give rise to anaplastic tumors rather than differentiating SCC's (3, 23). This indicates that loss of the fibroblast feeder requirement is not sufficient to permit malignant growth by a keratinocyte and that selection for this phenotype may yield atypical neoplastic variants.

By initially culturing carcinoma cell populations under non-selective conditions, we isolated SCC lines that proved to vary greatly in their requirements for fibroblast feeder support from complete dependence (e.g., SCC-13) to complete independence (e.g., SCC-25). SCC-13 and SCC-9 would probably not have been isolated under standard culture conditions. Even some lines that undoubtedly could have been isolated in the absence of fibroblast feeder support (e.g., SCC-4 and SCC-15) nevertheless are stimulated substantially by 3T3 conditioned medium or a 3T3 feeder layer (Table 1). It is likely that serial cultivation of such lines in the absence of fibroblast support would have caused these populations to become enriched in more rapidly growing variants, and the lines would have eventually evolved indifference to fibroblast feeding. Change in this direction does not seem to have occurred in these lines over the course of at least 200 cell generations of cultivation with a 3T3 feeder layer. SV40-infected human diploid keratinocyte populations are transformed into longer-lived lines that gradually evolve to independence from fibroblast support (26), but the culture conditions used may have selected for rare calls with that property.

It is important to note also that serial cultivation of SCC cells with the 3T3 feeder layer did not seem to impose selection pressure in the direction of increased fibroblast dependence; early-passage SCC-4 and SCC-25 cells did not grow better when plated in the absence of feeder cells than they did after more than 200 cell generations of continuous culture with the feeder layer. It is therefore unlikely that feeder-independent cells were initially important constituents of the tumors that
gave rise to SCC-13 and SCC-9 but that they were selected against in culture in favor of cells that were more dependent on the feeder layer.

Of the 6 SCC lines studied here, only one (SCC-4) formed large progressively growing colonies in semisolid medium. The other lines were able to undergo at most 2 to 5 divisions at a very low frequency. Because normal diploid keratinocytes are unable to divide even once in suspension, the ability of all the SCC lines to undergo at least some abortive growth is a significant deviation from normal keratinocyte behavior, implying a somewhat reduced requirement for anchorage. However, the low frequency and small size of the colonies produced by most of the SCC lines and their tendency to degenerate quickly probably would have precluded the use of semisolid medium to select for the malignant stem cells in primary cultures of the original tumors. A similar conclusion was reached by Rupniak and Hill (21), who recently reported that none of 17 primary lesions of SCC of the head and neck yielded colonies from single cells in primary soft-agar suspension cultures. However, the presence in the disaggregated biopsies of viable tumor cells with colony-forming ability in surface cultures was not determined in that study.

As reported elsewhere (16), the SCC lines other than SCC-4 retain the keratinocyte property of being triggered to terminal differentiation by anchorage deprivation (10, 20), although they become committed to differentiate (with the concomitant loss of division potential) at a slower rate than do normal keratinocytes. This suggests that SCC cells may have a somewhat reduced requirement for anchorage but that suspension of the cells in semisolid medium is an inadequate method for detecting this attribute, because the cells differentiate terminal before much, if any, growth can occur.

Our data indicate that many human SCC’s behave like established lines in culture and express their tumorigenic potential in nude mice. However, they can also retain the normal keratinocyte requirements for factors produced by fibroblasts and for anchorage in order to grow in culture. Therefore, the in vitro growth and selection conditions that have been demonstrated to be useful for some human tumor types and for experimental fibroblast transformation are not appropriate for human SCC.

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REFERENCES

J. G. Rheinwald and M. A. Beckett

Fig. 1. Human squamous cell carcinoma in culture and in vivo. a, colony of normal human epidermal keratinocyte strain N growing with a 3T3 feeder layer and EGF. All the cells are tightly adherent to one another and to the culture dish. The colony is stratified with an upper layer of very flattened cells the boundaries of which are in a different focal plane and are therefore indistinct. Normal oral epithelial keratinocytes form colonies of identical morphology. Phase-contrast, × 160. b, colony of human SCC line SCC-15 growing with a 3T3 feeder layer. Cells are less tightly adherent, often adopt a rounded morphology, and do not stratify. Occasionally large end-stage cells are formed. Phase-contrast, × 160. c, fluorescence of a 3-cell colony of the SCC-4 line stained with rabbit anti-keratin antiserum and rhodamine-conjugated goat anti-rabbit IgG. The cells contain a network of keratin filament bundles in a pattern similar to that found in normal keratinocytes. Filament bundles insert perpendicularly into the desmosome attachment plaques that form at regions of cell contact. Apparent differences in keratin content among cells may be an illusion caused by differences in the degree of cell spreading. × 630. d, section of a portion of the human tumor biopsy from which SCC-9 was derived. This is a typical well-differentiated SCC, with nests of carcinoma cells becoming enlarged and showing signs of nuclear degeneration toward the centers of the nests. The clusters of carcinoma cells are interspersed with lighter-staining connective tissue. H&E, × 80. e, section of the tumor formed in a nude mouse by cultured SCC-9 cells. SCC-9 cells were serially cultured for about 70 cell generations and then injected s.c. into a nude mouse. A large well-differentiated SCC had formed 26 days later. H&E, × 80. f, section of the tumor formed in a nude mouse by cultured SCC-12 cells. SCC-12 cells were serially cultured for about 50 cell generations and then injected s.c. into a nude mouse. The tumor consisted of 2 well-organized cysts with central differentiation in the form of multiple layers of flattened cells 37 days later. The centers of the cysts contained fluid. H&E, × 80.
Fig. 2. Colony morphology of human SCC lines. 

- a, SCC-4, 25th passage, × 200;
- b, SCC-25, 16th passage, × 200;
- c, SCC-13, 6th passage, × 200;
- d, portion of large colony of SCC-9, 35th passage, showing several abnormally stratifying regions × 200.
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