Human Squamous Cell Carcinoma in Culture: A Defect in Terminal Differentiation and Its Relation to Malignancy

James G. Rheinwald

ABSTRACT—Thirteen primary squamous cell carcinomas of the epidermis and of the oral and pharyngeal epithelium were cultured with a 3T3 fibroblast feeder layer, a system originally developed for clonal growth and long-term serial cultivation of normal human keratinocytes. Six of these tumors could be propagated indefinitely as established cell lines. They formed rapidly growing well-differentiated squamous cell carcinomas when injected sc into athymic (nude) mice. The squamous cell carcinoma lines possessed different aneuploid karyotypes. They displayed subtle differences in colony morphology such that they were visually distinguishable from one another as well as from normal keratinocytes. The lines also varied greatly in their dependence on the fibroblast feeder layer for clonal growth in surface culture. Only 1 line could form large colonies with high efficiency in semisolid medium; the others grew only abortively under this condition and eventually differentiated terminally to form cornified envelopes. Progressive growth in semisolid medium, therefore, was not a useful in vitro marker of malignant transformation for these cancer cells of keratinocyte origin. However, a property shared by all the squamous cell carcinoma lines was a subnormal rate of commitment to terminal differentiation during incubation in suspension culture. Deprivation of anchorage triggers commitment so rapidly in normal keratinocyte populations that no cells remain viable after 2 days in semisolid medium. In contrast, after this same period, all 6 lines retained more than 20% of their original colony-forming ability when replated in surface culture. This phenotype of increased survival capacity in semisolid medium promises to be a useful selective marker for the detection of rare malignant keratinocytes within large normal keratinocyte populations.—Natl Cancer Inst Monogr 60: 133–138, 1982.

Since the beginning of vertebrate cell culture over 50 years ago, much effort in this research area has been devoted to identification of altered cellular phenotypes associated with malignancy so that cancer cells can be recognized and the mechanism of malignant growth determined. Unfortunately, carcinomas and the normal epithelial cell types from which they are derived grow poorly in culture with media and methods that have been available until recently. Therefore, study of these clinically important cancers has been largely limited to examination of cell morphology and karyotype in short-term primary cultures (1, 2) or to analysis of rare, successfully cultured carcinoma lines, such as HeLa (3) from the cervix and MCF-7 (4) from the breast, that are unlikely to be representative specimens of these types of cancer.

Because of the inability to culture normal and malignant cells satisfactorily from most types of human tissues, the nature of the neoplastic state has been most intensively investigated during the past 20 years with cultured rodent fibroblasts as a model system and viruses or mutagenic chemicals as experimental transforming agents. In this system, anchorage-independent growth ability in culture is highly correlated with malignant growth ability in animal hosts (5, 6). Unfortunately, the cells from many types of naturally occurring human solid tumors do not grow in semisolid medium (7, 8), and this property may be a general characteristic of only certain classes of tumors, such as melanoma and ovarian carcinoma (9, 10).

Squamous cell carcinoma is a common cancer that arises in stratified squamous epithelial tissues, such as the epidermis and the oral, pharyngeal, esophageal, and exocervical epithelia. These tissues are formed by the keratinocyte cell type. Proper tissue size is normally maintained in stratified squamous epithelia by a balance between the rate at which cells in a proliferative compartment divide and the rate at which they leave this compartment and become committed to a program of terminal differentiation. A cell culture system has been developed that satisfies tissue-specific growth requirements and permits long-term serial cultivation of the normal human keratinocyte (11, 12). Normal keratinocytes retain important cell type-specific features of growth regulation in culture. They express a requirement for growth-promoting factors elaborated by connective tissue fibroblasts (11) and an inexorable tendency to lose division capacity and become committed to terminal differentiation by a mechanism subject to influence by EGF and anchorage (13, 14). These observations originally made on the epidermal keratinocyte in culture also apply to the keratinocytes from normal human cornea and conjunctiva (15), oral epithelium (16), and exocervix (17). Thus the culture of squamous cell carcinomas seemed feasible for the ascertainment of whether they are "mutants" in any aspect of growth regulation that might be causally related to their ability for malignant growth.

ABBREVIATION: EGF = epidermal growth factor.


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3 Division of Cell Growth and Regulation, Sidney Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115; and the Department of Physiology, Harvard Medical School, Boston.

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MATERIALS AND METHODS

Preparation and propagation of squamous cell carcinoma cultures.—Detailed methods, reported elsewhere (18), were essentially as published for preparing keratinocyte cultures from normal skin (11, 12). A portion of each tumor biopsy was fixed, sectioned, and stained so that the diagnosis of squamous cell carcinoma was confirmed. The remaining portion was minced and either disaggregated into single cells with trypsin and collagenase or was distributed directly to culture dishes for initiation of explant outgrowth cultures. The tumor material was always cultured with a mitomycin C-treated feeder layer of the Swiss mouse embryonic fibroblast line 3T3. Growth medium was Dulbecco's modification of Eagle's medium plus 20% fetal calf serum and 0.4 μg hydrocortisone/ml.

The tumor cell populations were serially passaged with feeder cells at 7- to 10-day intervals by the subculturing of preconfluent cultures that had been initiated with 3 × 10⁵ to 10⁶ cells/60-mm dish. To assess feeder layer-independent growth ability, we plated 10⁵ cells/60-mm dish in M199 plus 20% fetal calf serum and 0.4 μg hydrocortisone/ml or in the same medium previously conditioned by incubating it for 24 hours with a confluent culture of 3T3 cells.

Assays for anchorage-independent growth and suspension-induced terminal differentiation.—Medium was made semisolid for cell suspension experiments by the addition of methylcellulose (4,000 centipoise, Fisher Scientific Co., Pittsburgh, Pa.) to a final concentration of 1.3% as described elsewhere (12, 19). For assessment of anchorage-independent growth, cells were inoculated into 60-mm plastic bacteriological petri dishes (not chemically modified for cell attachment) at a density of 5 × 10⁴ cells/ml in 10 ml methylcellulose medium. We estimated the number of colonies per culture and thus the colony-forming efficiency by counting colonies in 10 random fields as observed through the inverted microscope and by relating the area of the magnified visual field to the surface area of the dish.

For assessment of suspension-induced terminal differentiation, cells growing exponentially in a monolayer culture were trypsinized, resuspended at 10⁵ cells/ml in 3T3 conditioned medium made semisolid with methylcellulose, and returned to the incubator. Aliquots were withdrawn at various times thereafter and the cells were recovered by tenfold dilution with regular medium, pelleting with low-speed centrifugation, and resuspension in regular medium. The proportion of cells retaining colony-forming ability was measured by the replating of cells in surface culture with a 3T3 feeder layer. For determination of the proportion of cells that had formed the cornified envelope, cells were heated to 90° C in a phosphate-buffered, neutral isotonic saline solution containing 1% sodium dodecyl sulfate and 20 mM dithiothreitol. The resistant envelopes, appearing as hollow refractile spheres, were then counted in a hemocytometer with the aid of phase contrast optics (20).

RESULTS

Normal human keratinocytes cannot be grown from low density platings or be serially subcultured in standard media in the absence of a fibroblast feeder layer. However, when cultured with a feeder layer of 3T3 fibroblasts, keratinocytes form large, tightly packed, stratifying epithelial colonies from single cells (11) (fig. 1A). These colonies can be disaggregated to single cells, subcultured with a fresh feeder layer, and grown again the same way. However, after several passages, colony-forming efficiency becomes low, the colonies that do initiate depart from exponential growth when they are still small, and their constituent cells are unable to reinitiate growth when subcultured.

The addition to the medium of 20 to 30 ng EGF/ml causes a striking increase in colony morphology and greatly improved growth (13). In the presence of EGF, colonies stratify little and, in secondary and subsequent cultures, they do not stop growing at a small size but continue to grow progressively until adjacent colonies coalesce and the culture vessel is confluent. Colony-forming ability remains relatively high. Growth with EGF apparently helps the cells to avoid terminal differentiation so that they continue to divide through more subcultures until they are finally halted by replicative senescence (13, 14).

Keratinocytes rapidly lose their division potential and differentiate terminally when they are detached from their culture substratum and held in suspension medium (13, 14). Under these conditions, normal keratinocyte populations irreversibly lose, with a half-life of 3 hours, their ability to reinitiate growth when replated in surface culture with a 3T3 feeder layer. Within 24 hours thereafter, the influx of Ca²⁺ into the dying cells activates a cytoplasmic transglutaminase to cross-link special proteins positioned beneath the plasma membrane into an insoluble, detergent-resistant cornified envelope (20–23). This envelope is the ultimate product of keratinocyte terminal differentiation. Thus the normal keratinocyte in culture expresses a stringent requirement for products elaborated by the fibroblast feeder layer [presumably the in vitro manifestation of its requirement for mesenchymal support in vivo (24)], and its growth potential is subject to strict regulation by its capacity to differentiate terminally.

We sought to determine whether the malignant variant of the keratinocyte, squamous cell carcinoma, differed from the normal cell with respect to expression in culture of either of these tissue-specific growth control systems. Details of these studies are published elsewhere (18, 25). Biopsies of primary squamous cell carcinoma of the epidermis, tongue, and hypopharynx from 20 patients were placed in culture with 3T3 feeder cells. Colony morphology was observed, and the cultures were serially passaged with feeder layers until the populations either senesced or revealed that they were immortal (i.e., behaved as established lines). Table 1 shows that tumors from the three tissues behaved differently in culture. Colonies of abnormal morphology, i.e., unstratified and less tightly adherent (fig. 1B) grew in the primary cultures from 9 of the 13 tumors that were not lost to contamination. Six continued to grow indefinitely in serial passages. The tumor-derived cell populations that did not grow as established lines senesced between the second and fourth passages, about the same time as keratinocyte populations cultured from normal adult tissues.

The 6 squamous cell carcinomas that grew as established lines were studied from the 3d to 20th passage (about 20 to 200 cell generations) in culture. Each line had a unique
FIGURE 1.—A) Normal human epidermal keratinocyte colony growing with 3T3 feeder cells in the absence of EGF. Note close packing of cells and stratification, which is most pronounced toward the colony center. Phase contrast. × 63. B) Colonies of the line SCC-4, which originated from a squamous cell carcinoma of the base of the tongue. Note the absence of stratification and the occasional cells that become less adherent to their neighbors and round up, appearing more refractile. Phase contrast. × 63. C) Section of tumor which formed 34 days after the sc injection of \(3 \times 10^6\) cells of line SCC-15 (originated from a squamous cell carcinoma of the tongue) into a nude mouse. Tumor is a progressively growing, well-differentiated squamous cell carcinoma. Hematoxylin and eosin. × 100
TABLE 1.—Growth of squamous cell carcinomas in primary culture and as established lines

<table>
<thead>
<tr>
<th>Behavior in culture</th>
<th>Site of tumor</th>
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<tbody>
<tr>
<td></td>
<td>Epidermis</td>
</tr>
<tr>
<td>Lost to contamination</td>
<td>2</td>
</tr>
<tr>
<td>“Normal” colonies only</td>
<td>2</td>
</tr>
<tr>
<td>Abnormal, but senescing cells</td>
<td>1</td>
</tr>
<tr>
<td>Abnormal cells, established line</td>
<td>2</td>
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aneuploid karyotype (Eberling E, Kitchin R, Rheinwald J: Unpublished observations), contained abundant keratin filaments as detected by indirect immunofluorescence with antiserum against human callus keratin, and formed large, progressively growing, well-differentiated squamous cell carcinomas when injected sc into athymic mice (fig. 1C).

Occasionally during serial subcultivation with the 3T3 feeder layer, the squamous cell carcinoma lines were plated at clonal density in the absence of 3T3 feeder cells, either in regular medium or in medium conditioned by 3T3 cells. The 6 lines varied greatly in their ability to form colonies under these conditions. One line grew almost as poorly as normal keratinocytes in the absence of 3T3 feeder cells; 1 could not grow in regular medium, but grew moderately well in 3T3 conditioned medium; 3 could form progressively growing colonies in regular medium, but their growth was substantially stimulated by 3T3 conditioned medium; and 1 was indifferent to 3T3 conditioning factors and grew optimally in regular medium. The squamous cell carcinoma lines retained their characteristic degrees of fibroblast dependence through at least 200 cell generations of serial culture with 3T3 feeder cells.

Only 1 line could form large colonies (>50 cells) at relatively high efficiency (1%) in semisolid medium. The other 5 lines showed at best abortive growth at a much lower efficiency to a maximum clone size of 6 to 40 cells. These 5 lines differentiated terminally and formed cornified envelopes in the semisolid medium in the same manner as normal keratinocytes deprived of anchorage but at reduced rates. Under this condition, the squamous cell carcinoma lines lost their ability to reinitiate growth when replated in surface culture with a half-life of 24 to 144 hours, as opposed to 3 hours for normal keratinocytes. Thus the rate at which squamous carcinoma cells became committed to terminal differentiation was slower than that of normal cells by a factor of 8 to 50. The properties of normal keratinocytes and these cell lines are summarized in table 2.

DISCUSSION

The data described above lead to several conclusions about the biology of squamous cell carcinoma and indicate some directions for future research. Only 6 of the first 13 tumors we placed in culture (that did not fail because of contamination) behaved as established cell lines. These were not randomly distributed among the three tissues of origin. Thus we were able to study 4 of 4 of the tongue, 0 of 4 of the hypopharyngeal, and 2 of 5 of the epidermal squamous cell carcinomas. However, the number of tumors we studied is too small to permit a firm conclusion about tissue differences for this phenotype. We did not study the properties of the other tumors because it was not easy to expand their cell populations by serial cultivation to a large enough size before they senesced. Replicative immortality of a tumor's cells in culture did not appear to be correlated with the age of the lesion, age of the patient, or previous clinical treatment. There have been many reports that human carcinomas do not generally behave as established lines in culture (26, 27). However, in most studies, culture conditions permissive for growth of the normal epithelial cell type of origin were not used. Thus the limited growth observed might not have resulted from a true replicative senescence (28) but rather from the cells' failure to thrive in a nonpermissive environment. In the experiments described here, cells from every biopsy grew for at least 2 passages in the feeder layer system. In three instances, colonies with abnormal morphology similar to that of the 6 immortal squamous cell carcinoma lines appeared in the primary and sometimes in the secondary cultures, but the populations still failed to grow progressively. Therefore, our results tend to favor the idea that escape from replicative senescence in culture is not a necessary attribute of cells that had grown malignantly in vivo.

The 6 lines grew about equally well with a 3T3 feeder layer but varied greatly in their ability to grow without it. Some of our lines grew so poorly without 3T3 fibroblast support that they probably could not have been isolated in standard medium. Loss of fibroblast dependence does not,

<table>
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<tr>
<th>Character</th>
<th>Normal keratinocytes</th>
<th>Squamous cell carcinoma</th>
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<tbody>
<tr>
<td>Replicative life-span</td>
<td>Flexible, modulated by EGF</td>
<td>Immortal</td>
</tr>
<tr>
<td>Karyotype</td>
<td>Diploid</td>
<td>Aneuploid</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Epithelioid, close-packed, stratified</td>
<td>Epithelioid, less tightly adherent, unstratified</td>
</tr>
<tr>
<td>Cornified envelope formation</td>
<td>+</td>
<td>+ (some with abnormal expression)</td>
</tr>
<tr>
<td>Requirements for fibroblast support</td>
<td>Absolute, for clonal growth</td>
<td>Varies (− to +)</td>
</tr>
<tr>
<td>Colony-forming efficiency</td>
<td>≤10%</td>
<td>15−50%</td>
</tr>
<tr>
<td>Doubling time</td>
<td>About 24 hr</td>
<td>About 24 hr</td>
</tr>
<tr>
<td>Growth in suspension</td>
<td>No</td>
<td>Varies (− to +), most grow abortively</td>
</tr>
<tr>
<td>Survival in suspension</td>
<td>Poor (T₃ = 3 hr)</td>
<td>Good (24 hr ≤ T₃ ≤ 144 hr)</td>
</tr>
<tr>
<td>Growth in nude mice</td>
<td>No; small transient cysts formed</td>
<td>Yes; well-differentiated squamous cell carcinomas formed</td>
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therefore, appear to be necessary for a keratinocyte to grow malignantly. Only 1 of our 6 lines could grow progressively in semisolid medium, even when supplied with 3T3 conditioned medium. The other 5 showed some evidence of anchorage-independent growth ability because they underwent several divisions at low efficiency. Normal keratinocytes cannot undergo even a single division under similar conditions. However, most squamous cell carcinomas are likely to be too susceptible to suspension-induced terminal differentiation to grow in semisolid medium as well as HeLa or a virus-transformed fibroblast line does. Therefore, as has been recently suggested (29), any in vitro assay for tumorigenic cells that depends on colony formation in semisolid medium, such as that developed for testing anticancer drugs on cells from tumor biopsies (30), is not likely to be useful for squamous cell carcinomas.

On the other hand, squamous carcinoma cells become committed to terminal differentiation while deprived of anchorage at a much slower rate than normal keratinocytes. Thus these cells can be readily selected over their normal counterparts on the basis of their longer retention of division potential, rather than division itself, in semisolid medium. After 48 hours in methylcellulose medium, normal, cultured, keratinocyte populations lose more than 4 logs of their initial colony-forming ability. In contrast, after the same period each of the 6 lines retains more than 20% of its initial colony-forming ability when recovered from methylcellulose medium and replated in surface culture with a 3T3 feeder layer. This property of increased survival in suspension should be used as a selective marker for detection of potentially malignant transforms among cultured keratinocyte populations treated with carcinogenic agents. It may also be useful as a highly sensitive diagnostic assay for the presence of rare carcinoma cells in biopsies of tissues that are at too early a stage of neoplastic development to be recognizable histologically.

It is worth considering what role this defect in the terminal differentiation system plays in the malignant transformation of stratified, squamous epithelial tissues. The acquisition of such a lesion by a keratinocyte may be the first step of neoplastic development. It could provide the means for a cell to expand its clone within the dividing compartment of the epithelium at the expense of its normal neighbors whose progeny lose division potential and differentiate terminally at a higher rate than progeny of the variant cell. Cairns (31) has hypothesized that a mutation permitting clone expansion would be a powerful facilitating event for the development of cancer in stem cell renewal tissues. Premalignant lesions of the epidermis, oral epithelium, and cervix may contain cells with this differentiation defect. On the other hand, inasmuch as a mutation that reduces the rate at which cells permanently depart from the cell cycle would be strongly selected at any stage of tumor development and growth, it is likely to be a later step. Because every squamous cell carcinoma line we examined displays this defect, we can conclude that, whenever it occurs, it is probably a necessary alteration for a keratinocyte to grow malignantly.

Squamous carcinoma cells may sometimes acquire other heritable alterations in the expression of differentiated function as well. Three of the 6 squamous cell carcinoma lines possess, in addition to the 46,000- to 58,000-molecular weight keratin proteins present in normal cultured, human epidermal and oral keratinocytes, a novel small (40,000 mol wt) keratin (32). This small keratin shares some antigenic determinants with the larger normal keratins but also has some unique antigenic determinants. Its expression may be a frequent, though not inevitable, result of a widespread dysfunction of transcriptional control or of mRNA processing in squamous carcinoma cells. As such, the protein would play no role in the malignant activity of the cell. Its appearance may be analogous to the remarkable changes in expression and regulation of liver-specific differentiated functions that have been described in hepatomas (33).

To determine the mechanism of malignant growth, one must distinguish between altered cellular phenotypes that directly contribute to this ability and those that are by-products of events that led to the transformed state. Systematic analysis of a sufficiently large number of squamous cell carcinomas should indicate which growth control mechanisms and differentiated functions are consistently altered in this type of cancer. The order of appearance and the time course of these alterations during progression from the initiating event to frank malignancy might be determined from study of the cells cultured from premalignant lesions. Various phenotypic variants selected from carcinogen-treated keratinocyte populations can be assessed for tumorigenicity in nude mice. These lines of research should lead to the identification of those particular changes in keratinocyte properties that directly confer the ability for malignant growth.

REFERENCES


