Growth and Differentiation of Human Esophageal Carcinoma Cell Lines

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ABSTRACT

Human esophageal carcinoma cell lines (8 cell lines) differed from their normal counterpart in terms of their morphological appearance, growth properties, and the expression of certain differentiated functions, namely keratin proteins and cross-linked envelopes. In contrast to normal human esophageal keratinocytes, the carcinoma cells were pleomorphic and tended to pile up in an unorganized fashion. When grown under optimal growth conditions the carcinoma cells generally grew to a higher saturation density than their nontransformed counterpart; their generation times were variable. Transformed cells grew better under stringent growth conditions (e.g., decreased serum and no additional growth factors except hydrocortisone) than did nontransformed human esophageal keratinocytes but their growth was still restricted under these conditions. The carcinoma cells retained a requirement for a 3T3 feeder layer when grown at clonal densities (5 × 10^3 cells/60-mm dish) but could be passaged and maintained without a feeder layer if plated at higher than clonal densities (10^4 cells/60-mm dish). All cell lines grew in an anchorage-independent fashion in soft agarose although the colony forming efficiency and size of the colonies varied among the different cell lines. Not all anchorage-independent cell lines were tumorigenic. Tumorigenic potential was greatly augmented by the use of cell lines derived from soft agarose selected clones. Altered expression of keratin proteins and cross-linked envelopes was observed in the carcinoma cell lines and generally reflected those changes seen in primary esophageal carcinomas. In two cell lines (HCE-4 and HCE-6), the synthesis of the Mr 44,000 (analogous to Rheinwald's Mr 40,000 keratin) and 52,000 keratins was suppressed coincident with the appearance of the 67 Kd keratin in tumors derived from these cell lines. These keratin patterns were once again reversed in cell lines recultured from these tumors, suggesting that the expression of these specific keratins is subject to extrinsic growth regulation. Another feature of terminal differentiation in keratinocytes, cross-linked envelope formation, was found to be significantly altered (reduced) in most but not all human esophageal carcinoma cell lines.

INTRODUCTION

Human esophageal cancer occurs worldwide and is characterized by large variations in incidence even within the same country (1–6). Environmental and nutritional factors as well as cultural habits are thought to be important contributing factors to human esophageal carcinogenesis although the true etiology of this disease remains unknown (2–4, 7–13). Despite efforts at early diagnosis and treatment, the prognosis of esophageal cancer is poor.

We have developed cell lines from human esophageal tumor specimens to facilitate examination of the biological behavior of this tumor type in vitro, thereby contributing to our understanding of esophageal carcinogenesis as well as cellular regulation and differentiation. Because of the difficulty in establishing cell lines from human tumors (success rates varying usually from 6 to 11%) for a wide variety of tumor types (14, 15), only a limited number of continuous human esophageal carcinoma cell lines have been reported in the literature (16–22). In general, they have been established in countries marked by a high incidence of esophageal cancer, namely South Africa (15 cell lines), China (3 cell lines), and Japan (15 cell lines). For the most part, studies with these cell lines have involved morphological examination of the cells at the light microscopic and ultrastructural level, chromosome analysis, and tumorigenicity in nude mice.

In the present study, we have established a number of different cell lines from human esophageal carcinomas (tumor type, squamous cell carcinomas) and characterized their morphological appearance, growth properties, and the expression of certain characteristics associated with differentiation, namely keratin proteins and cross-linked envelopes. These human esophageal carcinoma cells were found to differ both morphologically and biochemically from their normal counterpart and in general many of the changes were reflective of those observed with primary esophageal carcinomas. Of particular interest were two cell lines that demonstrated a reversible modulation of keratin (Mr, 44,000, 52,000, and 67,000) expression during transition from the cell culture to the tumor state.

MATERIALS AND METHODS

Human Tissue and Tumor Procurement and Cell Culture of Human Esophageal Cells. Human esophagus was obtained either at "immediate autopsy" (i.e., within 2 h of death of the donor) or from surgery specimens (normal appearing epithelium adjacent to the tumor site) and human foreskin was obtained from circumcision of newborn infants. Human esophageal carcinomas were primary tumors obtained from tumor resections at the time of surgery.

Human epidermal and esophageal epithelial cells were isolated and grown from human foreskin epidermis and esophageal epithelium, respectively, by cocultivation with an irradiated layer of mouse 3T3 cells using procedures described in detail elsewhere (23, 24). Human esophageal carcinoma cells in cell culture were initiated by either tissue explantation (25) or trypsinization (23).

Radio labeling, Immunoprecipitation, and Resolution of Keratin Proteins on Polyacrylamide Gels. Normal human epithelial tissues (small fragments, approximately, 6 mm), tumors (minced), and cultured cells were radiolabeled with [35S]methionine (specific activity, >600 Ci/mmol) in medium containing 10% of the usual methionine concentration and 5% FCS (23) Radiolabeled keratin proteins were extracted from human

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2 The abbreviations used are: FCS, fetal calf serum; HCE, human carcinoma of the esophagus; NM, nude mouse.
epithelial tissues and cultured cells (normal and neoplastic) by a modification (26) of the high salt extraction method described by Franke et al. (27). Keratin protein patterns on polyacrylamide gels were unaffected by the inclusion of protease inhibitors [phenylmethyl sulfonyl fluoride or Trasylol (Sigma Chemical Co., St. Louis, MO)] in the extraction buffers. Keratin proteins were selectively immunoprecipitated from these labeled cell extracts by a modification (28) of the method of Kessler (29) using antiserum prepared against whole keratins purified from human stratum corneum (30). An equivalent number of cpm was electrophoretically separated on an 8.5% polyacrylamide gel and prepared for autoradiography as previously described (26, 31).

Immunofluorescent Detection of Keratin Proteins in Human Esophageal Epithelial Cells and Carcinoma Cell Lines. Cells on coverslips were fixed and permeabilized for 5 min with 100% methanol (−20°C) and stained with antiserum to keratin purified from human stratum corneum using the technique of indirect immunofluorescence as described in detail elsewhere (26, 32).

Ionophore-induced Terminal Differentiation of Normal and Transformed Human Esophageal Epithelial Cells. Either normal human esophageal epithelial or carcinoma cells (approximately, 10^6) were induced to terminally differentiate using the calcium ionophore, X-537A, and the percentage of total cells containing cross-linked envelopes was determined as described in detail elsewhere (33, 34).

Anchorage-independent Growth of Normal Human Esophageal Epithelial Cells and Esophageal Carcinoma Cells in Soft Agarose. Duplicate 60-mm dishes were inoculated with 5 × 10^4 or 5 × 10^5 cells suspended in 2 ml of 0.3% Seakem ME agarose (FMC Corporation, Rockland, ME) in growth medium and overlaid on top of 6 ml of 0.6% agarose base (35). Cultures were incubated at 37°C for 8 wk and were fed every 3 to 4 days with 1 or 2 ml of growth medium. The cultures were monitored weekly for colony formation.

Tumorigenicity Studies. Cells (approximately 5 × 10^6 to 5 × 10^7) obtained from cultures of either normal human esophageal epithelial cells or esophageal carcinoma cells were injected s.c. into athymic nude mice. The animals were monitored for tumor formation. If tumors did form, they were subjected to various biochemical analyses, reestablished in cell culture, and transferred to other nude mice. In the case of normal human esophageal epithelial cells, nodules formed within 1 to 2 wk which subsequently regressed. When the nodule had reached a maximum size, the animals were sacrificed by cervical dislocation and the nodules were excised surgically and processed for histological examination.

Keratin Nomenclature. Based on selective immunoprecipitation with anti-M, 40,000 antiserum, generously provided by Dr. James Rheinwald, the M, 40,000 keratin described by Wu et al. (36) and Moll et al. (37) appears to correspond to the keratin with a relative molecular weight of 44,000 in our gel system. Keratins designated as 4, 5, 6, and 13 appear to correspond to the keratin with a relative molecular weight of M, 40,000 keratin described by Wu et al. (36) and Moll et al. (37) of the high salt extraction method described by Franke et al. (26, 32).

RESULTS

Morphology of Human Esophageal Carcinoma Cell Lines. Cultures of normal human esophageal epithelial cells and esophageal carcinoma cell lines, representative examples of which are shown in Fig. 1, were morphologically distinguishable when examined by phase contrast microscopy. Normal human esophageal epithelial cells were uniform in appearance and polygonal whereas esophageal carcinoma cells were pleomorphic, varying greatly in size and shape. In two cell lines, HCE-5 and HCE-8 (data not shown), some of the cells were less epithelioid and more spindle shaped in appearance. Dispase-detached sheets of carcinoma cells revealed a more limited and less orderly stratification process than normal esophageal keratinocytes (data not shown), similar to previous findings with virally transformed human epidermal keratinocytes (38). One of the cell lines, HCE-1, senesced on the third passage. The remaining cell lines (HCE-3 through -9 have been passaged more than 20 times (greater than 100 cell generations).

Growth Characteristics of Normal and Malignant Human Esophageal Epithelial Cells. Growth characteristics of human esophageal carcinoma cells and their nontransformed counterparts were examined under both optimal growth conditions (Medium 199 containing 10% FCS, 5 × 10^{-7} M hydrocortisone, epidermal growth factor (20 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), and 10^{-10} M cholera toxin (39)) and more stringent conditions.

S. P. Banks-Schlegel and H. Green, unpublished data.
growth conditions (with Medium 199 containing only 2% FCS and 5 x 10\(^{-7}\) M hydrocortisone). The results are summarized in Table 1. When grown under optimal growth conditions, human esophageal carcinoma cell lines often grew to higher saturation densities (approximately 5 to 8 x \(10^6\) cells/60-mm dish) than normal human esophageal epithelial cells (2.8 x \(10^6\) cells/60-mm dish). Their doubling times ranged anywhere from 27 h (a shorter doubling time than the 33 h of normal human esophageal epithelial cells) to 66 h, depending on the cell line. Because transformed cells have been reported to exhibit reduced serum and growth factor requirements (38, 40–42), we also examined the ability of the cells to grow under more stringent growth conditions. Although carcinoma cells grew better than the normal cells under stringent growth conditions, they grew much more slowly (in most cases) than they had under optimal growth conditions, and reached lower saturation densities (somewhere between 0.5 and 2 x \(10^6\) cells/60-mm dish). These results suggest that most human esophageal carcinoma cells are still dependent on serum and growth factors for optimal cell growth.

Unless grown under special conditions (43, 44), most human epithelial cells require a layer of irradiated 3T3 fibroblasts to support multiplication at clonal densities (24). When we examined the requirement of human esophageal carcinoma cells for a 3T3 feeder cell layer at clonal densities, we found that all of the carcinoma cell lines examined still required a 3T3 feeder layer for growth. When 5 x \(10^5\) cells were plated onto 60-mm dishes, even in the presence or absence of a 3T3 feeder cell layer, no colonies formed on the dishes without feeders (data not shown). However, the human esophageal carcinoma cells did not exhibit as stringent a requirement for 3T3 feeder support as did their normal counterpart in that they could be routinely passaged and maintained in the absence of any 3T3 cells if inoculated at \(10^5\) cells/60-mm dish.

To test for anchorage-independent growth, normal esophageal cells or carcinoma cells were suspended in 0.3% agarose as described in “Materials and Methods.” CHES, cultured human esophageal epithelial cells. Cells underwent only 2 to 3 doublings, stopped growing, and then began to detach from the dish.

Table 1

<table>
<thead>
<tr>
<th>Cell or cell line</th>
<th>Saturation density (cells x (10^6)/60-mm dish)</th>
<th>Generation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHES</td>
<td>No growth</td>
<td>33</td>
</tr>
<tr>
<td>HCE-3</td>
<td>33</td>
<td>73</td>
</tr>
<tr>
<td>HCE-4</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>HCE-5</td>
<td>80</td>
<td>156</td>
</tr>
<tr>
<td>HCE-6</td>
<td>27</td>
<td>90</td>
</tr>
<tr>
<td>HCE-7 (NM)</td>
<td>53</td>
<td>67</td>
</tr>
<tr>
<td>HCE-8</td>
<td>66</td>
<td>298</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Cell or cell line</th>
<th>Colony-forming efficiency (%)</th>
<th>Size of colonies ((\mu m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHES</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>HCE-3</td>
<td>7</td>
<td>36–127</td>
</tr>
<tr>
<td>HCE-4</td>
<td>30</td>
<td>115–769*</td>
</tr>
<tr>
<td>HCE-5</td>
<td>10</td>
<td>45–72</td>
</tr>
<tr>
<td>HCE-6</td>
<td>41</td>
<td>45–72</td>
</tr>
<tr>
<td>HCE-7 (NM)</td>
<td>25</td>
<td>54–118</td>
</tr>
<tr>
<td>HCE-8</td>
<td>6</td>
<td>72–118</td>
</tr>
</tbody>
</table>

* CHES, cultured human esophageal epithelial cells.

Table 3

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of athymic nude mice given injections</th>
<th>% of athymic nude mice forming tumors</th>
<th>Latency period (mo)</th>
<th>Histological appearance (degree of differentiation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCE-3</td>
<td>10</td>
<td>30</td>
<td>8</td>
<td>pd-md* SQC</td>
</tr>
<tr>
<td>HCE-4</td>
<td>10</td>
<td>20</td>
<td>7</td>
<td>pd-md SQC</td>
</tr>
<tr>
<td>HCE-5</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>md-wd SQC</td>
</tr>
<tr>
<td>HCE-6</td>
<td>9</td>
<td>100</td>
<td>2</td>
<td>md-wd SQC</td>
</tr>
<tr>
<td>HCE-7 (NM)</td>
<td>10</td>
<td>50</td>
<td>2</td>
<td>md-wd SQC</td>
</tr>
<tr>
<td>HCE-8</td>
<td>Not done</td>
<td></td>
<td></td>
<td>psd-SGC</td>
</tr>
<tr>
<td>HCE4-SAC22</td>
<td>10</td>
<td>100</td>
<td>0.1</td>
<td>psd-SGC</td>
</tr>
</tbody>
</table>

* pd, poorly differentiated; md, moderately differentiated; wd, well differentiated; SQC, squamous cell carcinoma; SAC, soft agarose selected colony.

Colonies of esophageal, the remaining cell lines formed microscopically visible colonies. Tumorigenicity of human esophageal carcinoma cell lines was evaluated by injection of approximately 5 x \(10^6\) to 107 cells s.c. into athymic nude mice. Control cultures of normal esophageal epithelial cells were similarly injected into nude mice. Four of the seven cell lines examined, HCE-3, HCE-4, HCE-6, and HCE-7 (NM) formed tumors in nude mice with an efficiency of 10, 20, 100, and 50%, respectively (Table 3). The tumors grew to a large size (approximately 2 cm) and could be serially passaged into other nude mice. The morphology of the tumor is summarized in Table 3; the histological appearance of one of the tumors, HCE-3, is shown in Fig. 2 and exhibits features characteristic of a poorly to moderately differentiated squamous cell carcinoma. The histological appearance of the tumors formed by HCE-4 and HCE-7 (NM) cells were similar to the former, whereas HCE-6 tumors also exhibited areas of "squamous pearl" formation (data not shown). We also examined the tumorigenic potential of cell lines derived from anchorage-independent soft agarose colonies. While HCE-4 cells produced tumors in 20% of the animals at 7 mo, agarose colonies of HCE-4 produced tumors in 100% of the animals in less than 1 mo (Table 3). These results suggest that attainment of tumors in nude mice, at least in some cases, is augmented through the use of cell lines established from cells exhibiting the potential to grow in an anchorage-independent fashion (namely, from agarose-selected colonies). We are currently establishing other cell lines in culture from these "anchorage-independent" agarose colonies and reevaluating their abilities to form tumors in nude mice.

Keratin Proteins. To establish their epithelial nature, human
esophageal carcinoma cell lines were stained for keratin by indirect immunofluorescence using antiserum prepared against whole keratins purified from human stratum corneum as described in "Materials and Methods." All of the esophageal carcinoma cell lines examined stained positively for keratin (Fig. 3). Compared to normal human esophageal keratinocytes the cells were more weakly stained in general and rather than exhibiting the predominantly perinuclear staining pattern of normal esophageal keratinocytes, human esophageal carcinoma cells frequently displayed a more diffuse cytoplasmic staining pattern and/or one in which staining was often concentrated at the cell borders. The pattern of staining varied among the different cell lines, ranging from a fine lace network of filaments in some cell lines to a thicker, more cobwebby appearance in others. These results were confirmed by ultrastructural studies demonstrating bundles of tonofilaments arranged perinuclearly in cultured normal esophageal keratinocytes in contrast to carcinoma cells (such as HCE-3) which contained scant amounts of poorly developed tonofilament bundles scattered throughout the cytoplasm and associated with desmosomes.4

To assess alterations in the profile of keratin proteins in the human esophageal carcinoma cell lines, radiolabeled keratin proteins were extracted using high salt and detergent and selectively immunoprecipitated with keratin antiserum as described in "Materials and Methods." In contrast to results with primary tumors where there was a characteristic reduction or complete loss of the major M, 52,000 and 61,000 keratin proteins (45), the esophageal carcinoma cell lines exhibited a greater variability in the spectrum of keratin proteins associated with malignant transformation. Human esophageal carcinoma cell lines, HCE-3 (Fig. 4A) and HCE-1 (Fig. 4B), both exhibited either a dramatic reduction or complete loss of the major M, 52,000 keratin, analogous to results with tumor masses. Esophageal carcinoma cell line HCE-1 also synthesized a M, 67,000 keratin [characteristic of keratinizing stratified squamous epithelium such as human epidermis and cultured epidermal keratinocytes but not found in esophageal epithelium or cultured esophageal keratinocytes (23)]. In the case of 4 other esophageal carcinoma cell lines examined, namely HCE-4, -5, -6, and -7 (NM) (a human esophageal carcinoma cell line established from a nude mouse tumor derived originally from the same esophageal tumor as that from which cell line HCE-4 was established), a different spectrum of keratins was found to be synthesized than was previously noted with the other two cell lines. All of these cell lines contained significant levels of the M, 52,000 keratin usually present in low

4 S. P. Banks-Schlegel and T. S. Traika, unpublished data.

Fig. 2. Morphology of the tumor formed by injection of esophageal carcinoma cell line HCE-3 into athymic nude mice. Cells (5 x 10⁶ to 10⁷) from just confluent cultures of the HCE-3 cell line were injected s.c. into athymic nude mice. The cells gave rise to progressively growing tumors which were serially transplantable. Histological examination of the tumor revealed features characteristic of a poorly (A) to moderately (B) differentiated squamous cell carcinoma. A and B, different areas within the same tumor; arrow, a mitotic figure; bar, 25 μm.

Fig. 4. Keratin proteins extracted from human esophageal carcinoma cell lines HCE-3 and HCE-1 and selectively immunoprecipitated with keratin antiserum. [³⁵S]Methionine-labeled keratin proteins were extracted from human foreskin epidermis (HFE), human esophageal epithelium (HEE), a moderately differentiated human esophageal carcinoma (HET), cultured human epidermal and esophageal epithelial cells (CHE and CHES, respectively), and cell lines HCE-3 and HCE-1, immunoprecipitated selectively with keratin antiserum and analyzed on 8.5% polyacrylamide gels as described in "Materials and Methods." Molecular weight markers, shown to the left of the gels, are phosphorylase b (92,000), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (30,000). Molecular weights ascribed to the keratins of HEE and HCE-1 (A and B, respectively), shown to the right of the gels, were derived from the best fitting curve for the standards. Similar to the primary human esophageal carcinoma (HET), the esophageal carcinoma cell lines HCE-3 and HCE-1 showed either a dramatic reduction or complete loss of the major M, 52,000 keratin, normally present in HEE and CHES. HCE-1 was also characterized by the presence of a M, 67,000 keratin characteristic of cornified squamous epithelia, such as human epidermis (HFE) and cultured epidermal keratinocytes (CHE) but not normally found in noncornifying epithelia such as esophageal epithelium (HEE) and their cultured keratinocytes (CHES). Note the distortion of the keratin bands in the M, 48,000 to 52,000 region of the gels, especially prominent in B, due to the large amount of unlabelled IgG heavy chain in the immunoprecipitate. K, thousands.
or undetectable amounts in all esophageal tumors thus far examined (45) (Fig. 5). As anticipated, cell lines HCE-4 and HCE-7 (NM), derived ultimately from the same starting material, exhibited identical keratin patterns. Interestingly HCE-5 and HCE-6 showed a significant reduction in the $M_r$ 57,000 keratin, previously observed to be the most conserved keratin of the intermediate sized keratin class ($M_r$ 57,000 to 61,000) in several primary esophageal carcinomas examined (45). Occasionally higher levels of this keratin were found in these cell lines. In contrast to the previous cell lines, one additional cell line, HCE-8, was unique in that it possessed mainly smaller sized keratins ($M_r$ 46,000 to 50,000), similar to the pattern observed with primary esophageal carcinomas which were poorly differentiated based on histological criteria (Fig. 6) (45). Examination of keratin patterns in four nude mouse tumors established from different primary esophageal carcinomas, [designated HET-NM1 through NM4 (Fig. 5)] revealed keratin patterns reflective of the original tumor masses (Fig. 4A, MET) (45). Specifically the major $M_r$ 52,000 and 61,000 keratins, characteristic of normal human esophageal epithelium, were missing in these tumors. Interestingly when cell lines such as HCE-4 and HCE-6 were injected into athymic nude mice, the tumors which formed showed a reduction or loss of the $M_r$ 44,000 [analogous to Rheinwald’s $M_r$ 40,000 keratin (36); see “Materials and Methods”] and $M_r$ 52,000 keratins normally expressed in these cells concomitant with the appearance of a $M_r$ 67,000 keratin, characteristic of keratinizing squamous epithelium and consistent with the presence of keratinization in the tumor observed on histological examination (Fig. 7). The presence of a $M_r$ 67,000 keratin in some primary human esophageal tumors and nude mouse tumors derived therefrom has also been confirmed by $[^{14}C]$formaldehyde labeling of keratin-enriched extracts followed by selective immunoprecipitation with keratin antiserum and analysis on gels (Fig. 8). Upon reestablishment of cell lines from these tumors, the $M_r$ 44,000 and 52,000 keratins were found to be reexpressed and the amount of $M_r$ 67,000 keratin was usually greatly diminished (Fig. 4). Specifically the major $M_r$ 52,000 and 61,000 keratins, characteristic of normal human esophageal epithelium, were missing in these tumors. Interestingly when cell lines such as HCE-4 and HCE-6 were injected into athymic nude mice, the tumors which formed showed a reduction or loss of the $M_r$ 44,000 [analogous to Rheinwald’s $M_r$ 40,000 keratin (36); see “Materials and Methods”] and $M_r$ 52,000 keratins normally expressed in these cells concomitant with the appearance of a $M_r$ 67,000 keratin, characteristic of keratinizing squamous epithelium and consistent with the presence of keratinization in the tumor observed on histological examination (Fig. 7). The presence of a $M_r$ 67,000 keratin in some primary human esophageal tumors and nude mouse tumors derived therefrom has also been confirmed by $[^{14}C]$formaldehyde labeling of keratin-enriched extracts followed by selective immunoprecipitation with keratin antiserum and analysis on gels (Fig. 8). Upon reestablishment of cell lines from these tumors, the $M_r$ 44,000 and 52,000 keratins were found to be reexpressed and the amount of $M_r$ 67,000 keratin was usually greatly diminished (Fig. 7).

Cross-Linked Envelopes. The extent of terminal differentiation in cultured normal human esophageal epithelial and esophageal carcinoma cell lines was assessed by inducing terminal differentiation with the calcium ionophore X-537A (33) and then determining the percentage of cells containing cross-linked en-
Fig. 7. Reversible modulation in the expression of $M_r$ 44,000, 52,000 and 67,000 keratins formed by human esophageal carcinoma cell line HCE-6 during transition from cell culture to the tumor state. ($^3$H)methionine-labeled keratin enriched protein fractions were extracted from human esophageal carcinoma cell line HCE-6, from a nude mouse tumor derived from the cell line (designated HCE-6 NM1), and from a cell line reestablished in culture from the nude mouse tumor [designated HCE-6 (NM1)] and were selectively immunoprecipitated with keratin antiserum and analyzed by one-dimensional polyacrylamide gel electrophoresis as described in "Materials and Methods." Synthesis of the $M_r$ 44,000 and 52,000 keratins was suppressed coincident with the appearance of a $M_r$ 67,000 keratin in tumors derived from human carcinoma cell line HCE-6. These keratin changes were once again reversed in cell lines reestablished from these tumors. Note the distortion (blurring) of the $M_r$ 52,000 keratin in lanes HCE-6 and HCE-6 (NM1) due to the presence of large keratins (Mₗ > 60,000) in the cell extract. The results, shown in Table 4, indicate that human esophageal carcinoma cells often exhibit a reduced capacity to form cross-linked envelopes (generally in the range of 1 to 30%) compared to normal cells (ranging from 70 to 90%), thus suggesting that these carcinoma cells frequently exhibit a defect in the pathway of terminal differentiation as assayed by this marker. However, at least one of the cell lines, HCE-9, did not exhibit this defect in the terminal differentiation pathway but rather formed cross-linked envelopes at levels approximating those of normal esophageal keratinocytes.

**DISCUSSION**

We have characterized a number of different human esophageal carcinoma cell lines in terms of their morphological appearance, growth properties, and the expression of certain differentiated functions, specifically keratin proteins and cross-linked envelopes. In comparison to keratinocytes grown from normal human esophagus, the carcinoma cells were characterized by marked alterations in morphological and growth properties as well as in the expression of specific differentiated functions.

Studies involving both epithelial and nonepithelial cell types (42, 46–54) indicate that there is not always a good correlation between the ability of a cell line to grow in an anchorage-independent fashion in soft agar or agarose, tumorigenicity in nude mice, and malignant potential. Nevertheless normal cells do not possess these properties and their acquisition by tumor cell lines still serves as a useful indicator of neoplastic transformation. All of the human esophageal carcinoma cell lines established in this study exhibited a capacity for anchorage-independent growth, although the efficiency of colony formation and size of the colonies varied among the different cell lines. However, despite this ability to grow in an anchorage-independent fashion, their abilities to form tumors in athymic nude mice varied from highly tumorigenic to apparently nontumorigenic. Once estab-
lished, the tumors passaged with high frequency (usually 100% of animals) and short latency period (1 mo). Interestingly the establishment of cell lines from soft-agarose selected clones seemed to augment the oncogenic potential of the carcinoma cell line. Similar to the results of Robinson and Maistry (22) using human esophageal carcinoma lines, we found no correlation between tumorigenicity, latency periods, and the extent of histological differentiation. One cell line derived from a nude mouse tumor [HCE-7 (NM)] was found to be tumorigenic at early passage but nontumorigenic upon subsequent challenge with late-passaged cells. A decrease in colony forming efficiency and the size of colonies in soft agarose paralleled this loss in tumorigenic potential.5 While the esophageal carcinoma cell lines of Kasai et al. (18) and Nishihera et al. (20) did form masses in nude mice (morphology, squamous cell carcinoma), they were resected within 10 to 14 days when the masses were a very small (5 mm) size. Since even normal human esophageal cells form nodules of an equivalent size which subsequently regress at this time period, their true tumorigenic potential cannot be assessed.

We were interested in whether the transformation-associated changes in keratin proteins which we observed in a previous study of primary esophageal carcinomas (45) would be maintained in established cell lines. Similar to our previous findings with primary esophageal carcinoma, a reduction or loss of the M, 52,000 keratin was observed in some cell lines, although other cell lines [HCE-3, -5, -6, and -7 (NM)] exhibited significant levels of the M, 52,000 keratin. When the HCE-4 and HCE-6 cell lines were injected into nude mice, synthesis of the M, 44,000 and 52,000 keratins was found to be suppressed in the carcinomas which formed, coincident with the appearance of large keratins (M, 65,000 to 67,000). Upon reestablishment of cell lines from these tumors, the M, 44,000 and 52,000 keratins were again reexpressed in the cells and the M, 67,000 keratin was suppressed. Similar concerted modulation in the expression of these keratins in cell culture have been observed in normal human epidermal, conjunctival, and vaginal keratinocytes, presumably effected by vitamin A in the serum [e.g., depletion of vitamin A from the serum leads to a loss in expression of the M, 40,000 (relative mobility of M, 44,000 in our gel system) and M, 52,000 keratins coincident with the appearance of the M, 67,000 keratin (55)]. Moreover in a previous study of nine human squamous cell carcinomas cell lines derived from the epidermis and oral epithelium, Wu and Rheinwald (36) found that while the pattern of keratin proteins in the cell lines was virtually identical to that of normal keratinocytes cultured from the same epithelia, three cell lines were characterized by the presence of an additional keratin of M, 40,000. Modulations were observed in the expression of this M, 40,000 keratin (reduced) and a M, 67,000 keratin (increased) in nude mouse tumors derived from these cell lines and their reversible expression when regrown in culture, comparable to that which we noted for the M, 44,000, 52,000, and 67,000 keratins in some of our esophageal carcinoma cell lines. While the lack of expression of the M, 52,000 keratin was a consistent finding with most primary esophageal carcinomas, primary carcinomas established in athymic nude mice, and athymic nude mouse tumors established from various esophageal carcinoma cell lines, the coordinate expression of the M, 67,000 keratin (as measured by radiolabeling and immunoprecipitation with specific keratin antiserum) was noted in some but not all cases.

Despite the fact that human esophageal epithelium is a noncornifying tissue and therefore devoid of an anucleate stratum corneum the cells acquire involucrin (32), a precursor protein to the cross-linked envelope structure found just beneath the plasma membrane of stratum corneum cells during the terminal stages of differentiation (33, 56–59). Human esophageal epithelial cells continue to express this protein in cell culture. A survey of a wide variety of normal and neoplastic tissues (60–63) has demonstrated involucrin to be a specific marker for squamous differentiation and its presence in both normal and neoplastic tissues showed a positive correlation with differentiation. Involucrin-positive cells can be induced to form cross-linked envelopes by means of a calcium ionophore and this assay serves as a reliable measure of the fraction of differentiated cells in the population (32, 33, 64, 65). In most cases, the human esophageal carcinoma cells were found to have a lower capacity to form cross-linked envelopes, reflective of a shift from a more differentiated to a more proliferative population of cells. Similar defects in the terminal differentiation pathway (66) have been noted with human squamous cell carcinoma cell lines of the skin and oral cavity. However, another essential property manifested by the esophageal carcinoma cells noted above is their ability to maintain continually a proliferative fraction of cells refractory to terminal differentiation. Hence although some carcinoma cell lines may even exhibit high levels of envelope-forming capabilities (e.g., in the case of HCE-9, 63% of the cells formed cross-linked envelopes), they continue to maintain a refractory proliferative component of cells not sustained by normal keratinocytes.

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S. P. Banks-Schlegel, unpublished data.
Fig. 3. Immunofluorescent staining of normal human esophageal epithelial cells and esophageal carcinoma cell lines using keratin antiserum. Cultured normal human esophageal epithelial cells (CHES) and esophageal carcinoma cell lines HCE-3, -4, -5, -6, -7 (NM), and -8 were stained with antiserum to whole keratin purified from human stratum corneum using the technique of indirect immunofluorescence as described in “Materials and Methods.” In general, the esophageal carcinoma cells were less intensely stained and were not characterized by a predominantly perinuclear staining pattern as compared with normal esophageal keratinocytes. Note that in cell line HCE-8 even the spindle-shaped cells reacted strongly with keratin antiserum, attesting to their epithelial nature. The pattern of keratin staining was found to vary markedly among the different carcinoma cell lines, exhibiting a fine lacey network in some and a thicker, more cobwebby appearance in others.
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