

Cellular Characterization and Successful Transfection of Serially Subcultured Normal Human Esophageal Keratinocytes

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Background: In vitro cell culture models can provide unique insights into squamous epithelial proliferation, differentiation, and neoplastic transformation. Cultures of human esophageal keratinocytes could be advantageous for the study of these processes. **Methods:** Normal human esophageal keratinocytes were cultivated on 3T3 fibroblast feeder layers in vitro and expanded through four serial subcultivations. Confluent tertiary cultures were analyzed by morphological and immunohistochemical techniques to define their basic properties. The ability to transiently transfect cultured esophageal epithelium was tested using a Rous sarcoma virus-luciferase reporter gene by the calcium phosphate and lipofection methods. **Results:** Postconfluent cultures displayed a predominantly basal cell phenotype with limited stratification, widespread expression of keratins 5 and 14, and production of attachment specialization proteins such as $\alpha 6 \beta 4$ integrin and collagen VII. Terminal differentiation markers (involucrin and transglutaminase) were prematurely expressed. The cells expressed growth factors important in proliferation and differentiation, such as transforming growth factor- β and interleukin-1 β . Tertiary cultures were successfully transiently transfected with a Rous sarcoma virus-luciferase reporter gene construct. **Conclusion:** Normal human esophageal cells can be serially passaged through extended numbers of cell generations and transfected by standard methods. This in vitro system may be useful in the study of fundamental cellular processes governing proliferation and differentiation in the esophageal epithelium. *J. Cell. Physiol.* 177:274–281, 1998. © 1998 Wiley-Liss, Inc.

Esophageal squamous cell carcinoma occurs worldwide and has been linked epidemiologically with tobacco smoking, consumption of alcohol, and exposure to N-nitroso compounds (nitrosamines), as well as dietary deficiencies [1,2]. Molecular studies of esophageal squamous carcinomas have suggested that a number of genetic events are required for malignant transformation, including activation of oncogenes and inactivation of tumor suppressor genes. In particular, oncogenes cyclin D1, epidermal growth factor receptor, and tumor suppressor genes p53 and p16 have been implicated in this process [3]. To date, studies of the genetic basis of esophageal cancer have been limited largely to analysis of established tumors. However, insights into the underlying molecular mechanisms of esophageal epithelial proliferation have been greatly enhanced by the establishment of a transgenic mouse model in which the cyclin D1 oncogene has been targeted to the esophageal epithelium with an Epstein-Barr virus promoter in transgenic mice [4,5].

An in vitro model of normal human esophageal epithelium would greatly enhance the ability to study the basic biological mechanisms regulating proliferation and differentiation in a controlled, prospective fashion. Cultures of esophageal keratinocytes could be used to study gene expression, the effects of mitogens and mutagens on growth proliferation and differentiation, and potentially even the identification of stem cells. Normal human epidermal keratinocytes are known to be easily propagated in vitro when grown on feeder layers of 3T3

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fibroblasts [6,7], and normal human esophageal cells have also been reported to grow in culture under similar conditions, albeit more slowly [8–10]. In this study, we established cultures of esophageal keratinocytes, expanded them through multiple serial subcultivations, and analyzed the cultured cells by morphological, immunohistochemical, and molecular techniques to define the basic properties of the resultant epithelial cell sheets, including their expression of cytokeratins, differentiation antigens, anchorage specialization proteins, and autoregulatory growth factors. The ability to transiently transfect cultured esophageal epithelium prepared by this method was then tested.

MATERIALS AND METHODS

Cell culture

Segments of normal human esophagus were harvested within 5–6 h post mortem and confirmed to be free of infection, malignancy, or inflammation by microscopic examination and pertinent information from the medical record. The tissue was acquired with the approval of the Subcommittee on Human Studies of the Committee on Research of the Institutional Review Board and the Tissue Committee of the Pathology Department. Samples of the native mucosa were snap frozen for use as controls for immunohistochemistry. The remainder of the esophageal mucosa from each sample was immediately stripped from the muscularis propria and incubated in Dispase® 2.5 mg/ml (Boehringer Mannheim, Indianapolis, IN) for 2 h to separate the epithelium from the lamina propria. The epithelium was minced and incubated in a mixture of 0.05% trypsin-0.02% EDTA for 30 min. The supernatant was collected and centrifuged at 1,300 rpm for 10 min. All recovered cells were immediately plated onto feeder layers of sublethally irradiated 3T3-J2 mouse fibroblasts in 35 mm Petri dishes at a density of about 3.0×10^5 cells/mm² and cultivated according to the method of Rheinwald and Green [6]. On primary culture and during all subsequent passages, care was taken not to hold the disaggregated cells in suspension for more than 30 min, since it is known that loss of stratum

contact initiates terminal differentiation in normal keratinocytes [7].

Isolated cells were grown in a 3:1 mixture of Dulbecco's modified Eagles medium (DMEM): Ham's F12 medium (JHR Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 60 units/ml penicillin (ICN Biomedicals, Costa Mesa, CA), 0.04 mg/ml streptomycin (ICN Biomedicals), 0.2 µg/ml hydrocortisone (Calbiochem, La Jolla, CA), 10^{-10} M cholera toxin (ICN Biomedicals), 5 mg/ml transferrin, 2×10^{-6} M triiodo-L-thyronine (Calbiochem), 5 µg/ml insulin (Sigma, St. Louis, MO), and 10 ng/ml human recombinant epidermal growth factor (UBI, Waltham, MA). The medium was changed every 3–4 days. After 10 days, multiple small colonies of 6–10 cells/clone were present. At this time, all remaining 3T3 cells were removed using EDTA (0.02%), and the growing keratinocytes were released to single-cell suspension with trypsin (0.05%)-EDTA (0.02%) solution and subcultivated under the same conditions. When the cells of the second passage cultures reached 90% confluence, the keratinocytes were again disaggregated and passaged to tertiary cultures at a density of 4×10^5 cells per 60 mm Petri dish. The process was repeated to produce quaternary cultures. For the purpose of immunohistochemical analysis, some tertiary cultures were cultivated for 2 days postconfluency. The postconfluent keratinocyte sheets were detached from the culture dish using Dispase®, embedded in glycerol mounting medium, and snap frozen in liquid nitrogen.

Immunohistochemistry

Frozen sections of both the fresh-frozen keratinocyte sheets and the fresh-frozen native esophagus were cut at a thickness of 5 µm, picked up on glass slides coated with 1% 3-aminopropyl-triethoxysilane (Sigma), fixed in acetone, and air dried. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min. The sections were stained using an avidin-biotin peroxidase complex method with the appropriate Vectastain kits (Vector Laboratories, Inc., Burlingame, CA). All primary antibodies (see Table 1) were incubated in a humid chamber at room temperature for a minimum of 1.5 h. The appropriate secondary antibody was applied for 30 min followed by a 45-min incubation with the avidin-biotin peroxidase complex. The slides were developed using aminoethylcarbazole (Sigma) as the chromogen and counterstained with hematoxylin.

Transient transfection with the Rous sarcoma virus (RSV)-luciferase reporter gene

Esophageal keratinocytes were transfected by the calcium phosphate and lipofection methods. Transient transfection of the plasmid DNA in cultured cells was done by the calcium phosphate method [11] using a CaHPO₄ transfection kit (5' → 3', Inc., Boulder, CO). Cells were plated at a density of 1×10^6 cells/35 mm well and transfected 24 h later with 2 µg of the RSV-luciferase reporter plasmid in a 250 µl of solution consisting of 125 mM CaCl₂, 25 mM Hepes buffer, pH 7.05, 0.75 mM Na₂HPO₄, 5 mM KCl, 140 mM NaCl, and 6 mM glucose. After an 8-h incubation, cells were washed with PBS (BioWhittaker, Walkerville, MD) and fed

Abbreviations

| | |
|------|------------------------------------|
| DMEM | Dulbecco's modified Eagle's medium |
| K1 | keratin no. 1 |
| K4 | keratin no. 4 |
| K5 | keratin no. 5 |
| K6 | keratin no. 6 |
| K10 | keratin no. 10 |
| K13 | keratin no. 13 |
| K14 | keratin no. 14 |
| K16 | keratin no. 16 |
| K17 | keratin no. 17 |
| K18 | keratin no. 18 |
| K19 | keratin no. 19 |
| TGF | transforming growth factor |
| IL | interleukin |
| PBS | phosphate-buffered saline |
| RSV | Rous sarcoma virus |
| mc | monoclonal |
| pc | polyclonal |

TABLE 1. Summary of antibodies used in immunohistochemical analysis of native esophageal mucosa and cultured human esophageal cells

| Antigen | Antibody source | Dilution | Antibody class ¹ |
|------------------------------------|---|----------|-----------------------------|
| K1 | Birgit Lane, M.D. University of Dundee | 1:5 | mc mouse IgM |
| K10 | Birgit Lane, M.D. University of Dundee | 1:75 | mc mouse IgG |
| K10/13 | Immunotech Westbrook, ME | Neat | mc mouse IgG |
| K14 | Birgit Lane, M.D. University of Dundee | 1:50 | mc mouse IgG |
| K16 | Birgit Lane, M.D. University of Dundee | 1:10 | mc mouse IgG |
| K17 | Dako Carpinartia, CA | 1:50 | mc mouse IgG |
| K18 | Progen Heidelberg, Germany | Neat | mc mouse IgG |
| K19 | Dako Carpinartia, CA | 1:50 | mc mouse IgG |
| IL-1 β | Genzyme Cambridge, MA | 1:100 | pc rabbit IgG |
| TGF β 1 | Collaborative Biomedical Products Bedford, MA | 1:100 | pc Turkey IgG |
| TGF β 2 and TGF β 3 | Santa Cruz Bio Technology, Inc. Santa Cruz, CA | 1:50 | pc rabbit IgG |
| TGF α | Oncogene Science, Inc. Cambridge, MA | 1:20 | mc mouse IgG |
| α 6 β 4 integrin | Becton Dickinson San Jose, CA | 1:300 | mc mouse IgG |
| Involucrin | Biomedical Technologies, Inc. Stoughton, MA | 1:10 | pc rabbit IgG |
| Collagen VII | Serotec Ltd. Indianapolis, IN | Neat | mc mouse IgG |
| Filaggrin | Biomedical Technologies, Inc. Stoughton, MA | 1:300 | mc mouse IgG |
| Transglutaminase | Biomedical Technologies, Inc. Stoughton, MA | Neat | mc mouse IgG |

¹mc = monoclonal; pc = polyclonal.

with fresh DMEM (Sigma). Transfections were carried out three times. All cells were harvested for the luciferase assay at 48 h posttransfection.

Lipofection of the esophageal keratinocytes was performed with Transfectam[®] (Promega, Madison, WI). Cells were plated in the same fashion as described for the calcium phosphate transfection method. Plasmid DNA (2 μ g) and the transfection reagent were each added to 50 μ l of 150 mM NaCl solution, vortexed, mixed together, and after 10 min, added to the esophageal keratinocytes. Cells were harvested for the luciferase assay 48 h posttransfection. Transfections were carried out three times.

Luciferase assay

Cells were washed twice with PBS, lysed in 200 μ l of 1 \times cell culture lysis reagent (Promega), and 40 μ l of the lysate was mixed with 100 μ l of luciferase assay reagent consisting of 20 mM Tricine, 1.07 mM MgCO₃, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 530 μ M ATP, and 470 μ M luciferin. The luciferase activities were measured with a monolight luminometer (Analytical Luminescence Laboratory, San Diego, CA). Transfection efficiency was normalized by quantitatively measuring the human growth hormone secreted by transfected cells according to the manufacturer's directions as provided in the HGH-TGES 100T kit (Nichols Institute, San Juan Cap-

istrano, CA). The variation in luciferase activity among the three separate transfections carried out was not greater than 15%.

RESULTS

Esophageal keratinocyte growth in culture

Successful recovery of viable esophageal epithelial cells from autopsy specimens was dependent on collection of the tissue within 6 h after death. The plating efficiency in primary cultures was low, with only about 1% of the cells forming colonies. On subcultivation, however, the plating efficiency of the cells increased about 8- to 10-fold. The growth curve is depicted in Figure 1. On each passage, the cells that attached to the surface of the culture dish and underwent cell division were morphologically consistent with basal cells, having a small size, cuboidal shape, and a high nuclear-to-cytoplasmic ratio. In tertiary cultures maintained for 48 h postconfluence, the cells had begun to stratify and the proportion of differentiating suprabasal cells relative to basal cells had increased. Nevertheless, stratification of the submerged cultures remained limited within the time frame of the study. The resultant cell sheets were no more than three cell layers deep at maximum thickness.

Immunolocalization studies

Analysis of cultured esophageal keratinocytes by immunohistochemistry revealed expression of cytoke-

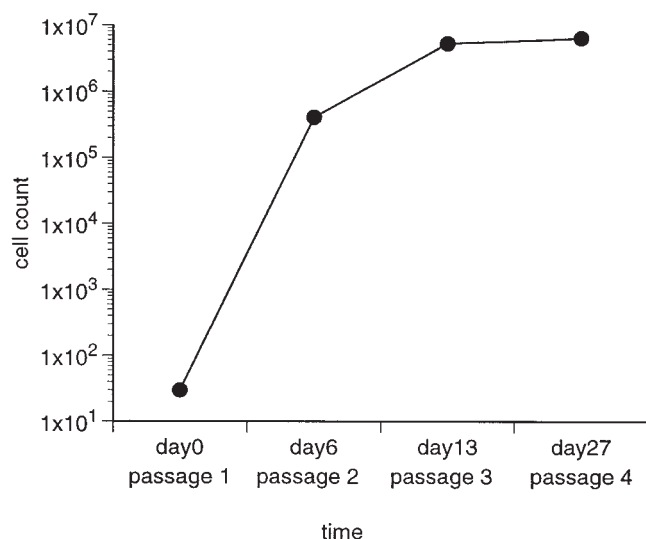


Fig. 1. Growth curve of cultured primary esophageal cells. The cell count (logarithmic y-axis) of serially subcultivated esophageal cells is depicted as a function of time (days) and passage.

tins characteristic of basal keratinocytes and of hyperproliferating epithelium. Specifically, keratin no. 14 (K14), the acidic (type I) keratin that pairs with the basic (type II) keratin no. 5 (K5) to form the major cytoskeletal intermediate filaments of the basal cells of all stratified squamous epithelia [12,13], was strongly and uniformly expressed by basal cells within the cultured esophageal cell sheets and the native esophageal mucosa (Fig. 2a,b). Northern blot analysis of cultured cells showed high expression of K5 and K14 mRNA (data not shown), confirming the immunolocalization results on a molecular level. Keratin 16 (K16), a type I cytokeratin expressed almost exclusively by rapidly proliferating keratinocytes [13,14], was expressed by suprabasal cells throughout the cultures and, focally, by scattered single cells in the mid portion of the native mucosa. Keratin 17 (K17), another keratin characteristic of the hyperproliferative epithelium, was strongly expressed by both basal and suprabasal cells throughout the cultures but was not expressed by the native mucosa (Fig. 2c,d).

Keratin 19 (K19) has widespread epithelial tissue distribution [15,16] but is characteristically expressed in vivo by the basal cells of adult nonkeratinizing squamous epithelia, the basal cells of fetal skin, cutaneous Merkel cells, the stem cells of the outer root sheath of hair follicles, and slow-cycling cells at the tips of rete ridges in glabrous skin and palatal mucosa that are believed to be stem cells [13,16]. We found that K19 was strongly and uniformly expressed throughout the cultured esophageal cell sheets and the basal cells of the native mucosa. In addition, inappropriate expression of keratin 18 (K18), a cytokeratin usually expressed in vivo only within simple (nonstratified) epithelia [13], embryonic epidermis [17], epidermal Merkel cells, and some metastatic squamous cell carcinomas of skin [14], but also commonly inappropriately

expressed by cutaneous keratinocytes in culture [18], was observed within the esophageal epithelial cultures as well. The native esophageal mucosa showed no expression of K18.

Keratin 13 (K13), the type II keratin that pairs with keratin 4 (K4) to form the major cytostructural components of the differentiating suprabasal cells in nonkeratinizing squamous epithelia, was appropriately expressed in the native esophageal mucosa but was not expressed in the cultured epithelium. No staining for keratin 1 (K1) or its pair keratin 10 (K10), cytokeratins expressed in the suprabasal layers of both keratinizing (e.g., epidermal) and parakeratinizing (e.g., hard palatal and gingival) squamous epithelia but not in nonkeratinizing (e.g., esophageal and soft palatal) epithelia, was seen in either cultured or native esophageal epithelium.

Involucrin and transglutaminase, proteins involved in the formation of the cornified envelope of differentiated keratinocytes and characteristically expressed by the cells within the superficial layers of normal stratified squamous epithelia, both keratinizing and nonkeratinizing [19], were expressed prematurely in all suprabasal layers of the cultured esophageal cell sheets. These proteins, markers of keratinocyte terminal differentiation in stratified squamous epithelia, were expressed in the appropriate location (i.e., the outer third of the epithelium) in the native mucosa. Filaggrin, a marker of granular cell differentiation in keratinizing squamous epithelium [13,19], was not observed in cultures of esophageal cells and was weakly expressed by the native esophageal mucosa.

Structural components of hemidesmosomes and anchoring fibrils, characteristically expressed by esophageal basal cells, were demonstrated immunohistochemically in the cultured esophageal basal cells. The integrin heterodimer, $\alpha 6 \beta 4$, a component of hemidesmosomes [20], was strongly expressed by the basal cells of the cultured cell sheets (Fig. 3a,b). The expression of this protein and the strong adherence of the cultured cells to the floor of the culture vessel suggest that cultured esophageal epithelial cells, like cultured epidermal keratinocytes, form hemidesmosomes along the attachment face of their plasma membranes [21]. Type VII collagen, the major structural protein of anchoring fibrils, was also found to be expressed by the cultured esophageal cells (Fig. 3c,d).

Growth factors important in proliferation and differentiation, such as interleukin (IL)-1 β and all three isoforms of transforming growth factor (TGF)- β , were expressed by the cultured esophageal cells. These growth factors are also known to be expressed by cutaneous keratinocytes in culture [22]. TGF- β 1 is known to induce the expression of hyperproliferative keratins (keratin 6 [K6]/K16) by epidermal keratinocytes [23] and is likely to be involved in the autoregulatory control of expression of these keratins in vitro. No expression of TGF- α by esophageal cells was detected by immunostaining, however. In this regard, the cultured esophageal keratinocytes differed from cultured epidermal keratinocytes [24], suggesting that site-specific differences in cytokine expression profiles may exist among stratified squamous epithelia.

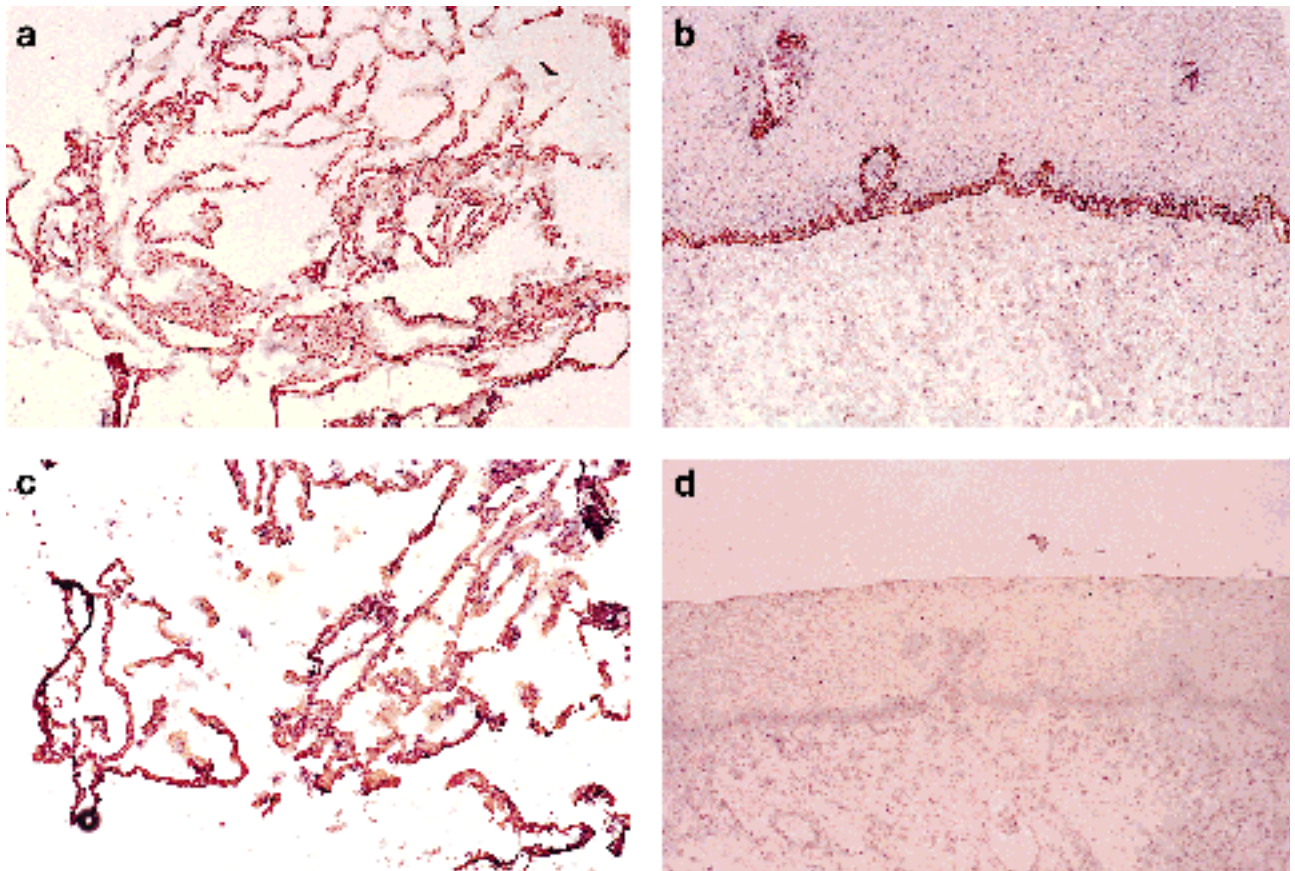


Fig. 2. Immunolocalization of K14 and K17 in cultured esophageal cells and normal esophageal mucosa. Keratin 14, a cytostructural protein characteristic of basal cells of stratified squamous epithelia, is strongly expressed by (a) cultured esophageal keratinocytes and

(b) the basal cells of the normal esophagus from which they were grown. K17, a keratin characteristic of hyperproliferating keratinocytes, is strongly expressed by (c) cultured esophageal keratinocytes but is not expressed by (d) the epithelium of the normal esophagus.

Transfection of esophageal keratinocytes in culture

Tertiary cultures of esophageal keratinocytes were transiently transfected with the RSV-luciferase reporter gene by the calcium phosphate and the lipofection methods. Luciferase activity was standardized to human growth hormone activity to insure transfection efficiency. RSV-promoter activity, indicating successful transfection, was observed using either method (Fig. 4).

DISCUSSION

The esophageal mucosa is a nonkeratinizing squamous epithelium. Parakeratosis or keratinization, when it occurs in the esophagus, is usually a marker of epithelial injury and represents a transient, reactive aberration in mucosal differentiation. Morphological and ultrastructural studies permit the classification of the esophageal epithelium into three layers: 1) a basal layer composed of small, undifferentiated cuboidal cells that include the proliferating (stem cell) compartment; 2) an intermediate or prickle layer of large, glycogen-filled cells; and 3) a superficial layer of flat cells that are terminally differentiated and glycogen depleted.

The cells of the suprabasal layers are irreversibly committed to terminal differentiation. As the cells migrate from the basal to the superficial layers, they become progressively more differentiated and ultimately slough from the surface; the entire epithelial population is continually renewed by this mechanism. Within the basal layer, regulatory controls exist that balance commitment to differentiation and outward migration of progeny generated from stem cells vs. the maintenance of substratum adhesion and the potential for further cell division. One of the most characteristic features of squamous mucosal dysplasia (intraepithelial neoplasia) is the loss of this equilibrium with the accumulation of undifferentiated basaloid cells. The pathological diagnosis of neoplastic transformation of any squamous mucosa may be made based on this feature alone. Culture models of human squamous cell carcinoma using cell lines have also shown that defective terminal differentiation is a consistent characteristic of neoplastic squamous cells [25].

The study of the normal biology of esophageal squamous epithelial cells, as well as that of their pathobiology, has been hampered by the dearth of in vitro mod-

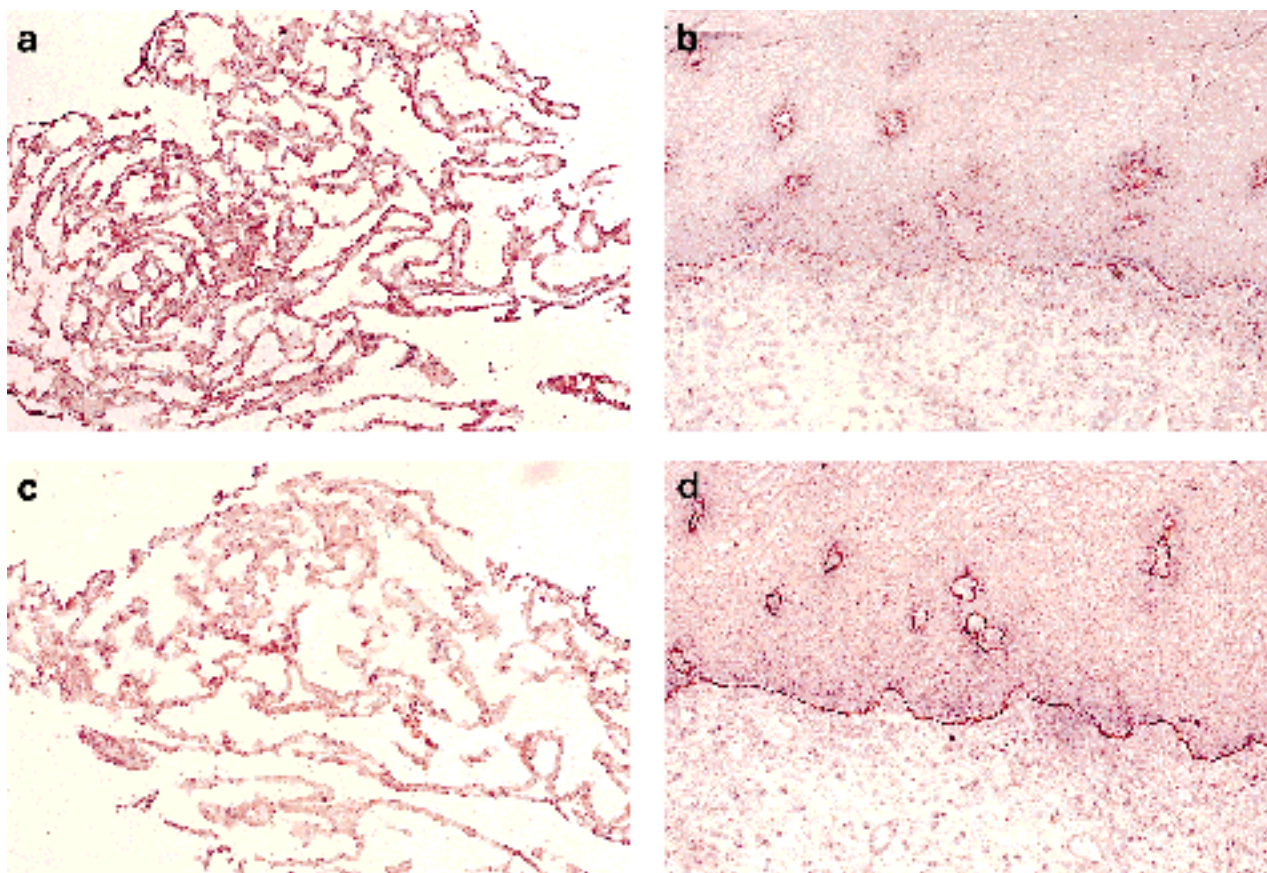


Fig. 3. Immunohistochemical staining for basal cell attachment specialization proteins, $\alpha 6 \beta 4$ integrin and collagen VII, by cultured esophageal cells and normal esophageal mucosa. The hemidesmosomal component, $\alpha 6 \beta 4$ integrin, is strongly expressed by (a) cultured esopha-

geal keratinocytes and (b) the basal cells of the normal esophagus from which they were grown. Collagen VII, the major structural component of anchoring fibrils, is also expressed by both (c) cultured esophageal keratinocytes and (d) the basal cells of the normal esophagus.

els. Previous attempts at long-term culture of human esophageal epithelium have been limited to explant cultures of either rabbit [26], rat [27], or human esophageal mucosa [28–30]. Because of the marked species differences, even among inbred strains, with respect to susceptibility of specific epithelial systems to neoplastic transformation, it might be expected that the usefulness of animal mucosal explant models for the study of human esophageal proliferation and transformation would be limited. Although some use has been made of human esophageal mucosal explant models to study the effects of carcinogenic factors like ionizing radiation and cytotoxic drugs [31–33], the labor-intensive nature of the method and the difficulty in standardizing the techniques and quantifying the results have limited its acceptance. Furthermore, in explant models, the potentially modulating effects of the associated esophageal mucosal stroma cannot be eliminated and may represent a significant variable when attempting to study the impact of growth-promoting or transforming factors on the epithelium. The use of pure epithelial cultures for the study of mutagenesis would be biologically advantageous in this regard. Technically, a cell culture would provide other important advantages over ex-

plant systems as well. In particular, the ability to store frozen disaggregated esophageal cells from donor specimens without significant loss of viability or colony-forming efficiency [8], to greatly expand the numbers of normal cells from a single donor to provide genetically identical epithelia for comparative studies, and to easily harvest the cultivated epithelium for quantitative analyses after experimental manipulation represent significant benefits. Furthermore, the appropriateness of cultured epithelia as models for the study of mutagenesis is underscored by investigational experience using cutaneous keratinocytes for this purpose. Prior studies have shown that 1) premalignant and malignant human squamous cells can be identified in culture by objective criteria; 2) human epidermal keratinocytes in culture are susceptible to the same carcinogenic influences as they are in vivo; and 3) in vitro methods exist for evaluating premalignant characteristics of initiated or partially transformed epidermal keratinocytes [34].

A small number of previous reports by Banks-Schlegel and coworkers [8–10] have documented successful cultivation and subcultivation (number of passages not stated) of human esophageal keratinocytes

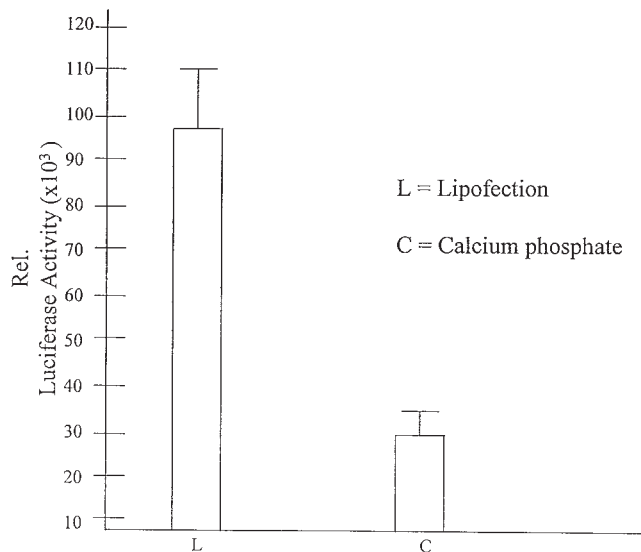


Fig. 4. Transient transfection of cultured esophageal cells with the RSV promoter-luciferase reporter gene. The RSV-luciferase plasmid (2 μ g) was cotransfected with the human growth hormone gene (2 μ g). Luciferase activity was assayed after 48 h and standardized to human growth hormone activity. Activities represent the mean \pm SD.

using either serum-supplemented medium and feeder layers, such as used in this investigation, or hormone-supplemented, serum-free medium in the absence of feeder layers. The studies showed that human esophageal cells have a doubling time of approximately 32 h whether cocultivated with 3T3 fibroblasts or grown in serum-free medium without feeder layers. However, in the absence of feeder cells, the cultured cells fail to stratify, do not form cross-linked envelopes as readily, and cannot be subcultivated [8]. The importance of using subconfluent cells for passaging was also emphasized by demonstrating a progressive decrease in colony-forming efficiency, proportional to the time the cultures are left at confluence [8]. However, in this series of studies, characterization of cultivated esophageal epithelia was very limited and the potential of the system for the study of esophageal carcinogenesis was not pursued. Although protein extraction studies by Banks-Schlegel showed that both esophageal and epidermal keratinocytes exhibit some evidence of tissue-specific expression of keratins *in vitro*, topographic distribution of keratins within these epithelia was not investigated [9]. Studies of protein distribution were limited to the immunolocalization of involucrin in various stratified squamous epithelia and their cultured counterparts, but they showed that involucrin synthesis begins prematurely in all keratinocyte cultures, regardless of their epithelial origins [10]. That involucrin synthesis is prematurely expressed by esophageal epithelium in culture was confirmed and expanded in the present study. The same phenomenon occurred with other differentiation antigens as well.

In our structural and functional analyses of esophageal cells grown on feeder layers *in vitro*, we observed few features analogous to those of the native esopha-

geal mucosa. The findings reflected the relative immaturity of the cells in culture and selection for cells of the basal type. The widespread expression of K5 and K14 and the lack of expression of K13 confirmed the predominant basal cell phenotype in the cultures. The expression of K16 and K17 coincided with the hyperproliferative state of the cells. By histological analysis as well, differentiation of the cultured esophageal cell sheets was judged to be incomplete, based both on the limited degree of stratification present and the lack of a planate surface layer. However, these findings were not unexpected. The histological immaturity of the cultured esophageal epithelium and the other observed deviations from normal differentiation, such as the premature suprabasal expression of proteins involved in the production of cornified envelopes, are analogous to aberrations observed in cultures of epidermal keratinocytes. Therefore, they would appear to reflect programmed adaptations of keratinocytes to the culture environment. Furthermore, *in vitro* studies of cutaneous keratinocytes have shown that differentiation of the cultivated cells can be manipulated experimentally by altering the culture environment. Terminal differentiation can be induced by exposing the cultures to air (i.e., raising submerged cultures to the air-liquid interface in the culture vessel) [35,36] or to nonphysiological conditions (e.g., high calcium or vitamin D3 concentrations in the medium or the addition of phorbol esters to the medium) [37]. Moreover, transplantation studies have shown that the aberrations in structural maturity and differentiation programs displayed by cutaneous keratinocytes in culture are transient. All are readily reversible after grafting to viable stromal beds under normal physiological conditions [21,37,38]. Analogously, transplantation studies of cultured esophageal epithelium exposed to carcinogens *in vitro* or transfected with exogenous DNA might be useful for defining permanent vs. reversible alterations in cell regulation induced by these experimental means. In this regard, the results of the transient transfection experiment in this study showing that exogenous plasmid DNA can be successfully transferred to cultured esophageal keratinocytes suggest that the study of gene expression by transfection methods would be possible in this system. Retroviral vectors for transducing oncogenes into esophageal keratinocytes for the purpose of extending their life-span, and possibly immortalization, are currently under investigation.

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