Keratin Expression in Normal Esophageal Epithelium and Squamous Cell Carcinoma of the Esophagus

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ABSTRACT

The 8-nm keratin filament is a major component of the cytoskeleton of epithelial cells and epithelial-derived cancers (carcinomas). Recently, it has been shown that the pattern of keratins produced by an esophageal epithelial cell undergoes change upon malignant transformation. In order to evaluate the potential importance of these differences in providing improved diagnostic techniques for pathology, we have investigated the consistency of the patterns of keratins expressed in normal esophageal epithelium, squamous cell carcinoma (SQCC) of the esophagus, and cultured esophageal epithelial cells. In six patients, the keratin pattern expressed by SQCC of the esophagus and corresponding normal esophageal epithelium was consistently different as judged by immunoblot analysis of electrophoretically separated protein extracts. Whereas the SQCCs typically expressed major keratins with molecular weights of 58,000, 56,000, 50,000, and 46,000, the normal esophageal epithelium produced two major keratins with molecular weights of 58,000 and 52,000 and a minor keratin with a molecular weight of 56,000. When normal esophageal epithelial cells were grown in tissue culture, their keratin pattern changed, and keratins with molecular weights of 58,000, 56,000, 52,000, 50,000, 46,000, and 40,000 were expressed. Although some minor variations in keratin patterns were seen, the major differences in keratin pattern expressed by normal esophageal epithelial tissue, SQCC of the esophagus, and cultured esophageal cells were consistent and reproducible.

INTRODUCTION

There will be an estimated 9000 new cases of esophageal cancer in the United States in 1984. Although this cancer accounts for only 1.5% of all cancers, it will be responsible for 8600 deaths (33). The most common symptom, dysphagia, develops when the primary tumor is already an advanced lesion and has compromised one-half to two-thirds of the esophageal lumen. There are no “early warning” symptoms or signs. In addition, 90% of patients are found to have metastatic disease at autopsy (31). Patients with esophageal achalasia are at a higher risk than the general population for developing esophageal carcinoma (39). Despite advances in surgery and radiation therapy, the 5-year survival for esophageal cancer continues to be less than 10% in most series (5, 37). Similarly, the objective response to chemotherapeutic agents such as cis-platinum and bleomycin is only in the order of 20% or less, with responses lasting only 2 to 3 months (31). Patients die of pulmonary aspiration or inanition following recurrent locoregional disease or metastasis.

Squamous cell carcinoma accounts for 90% of esophageal cancers. SQCCs of the esophagus is graded according to the degree of histological differentiation. The well-differentiated and moderately differentiated tumors display morphological features generally characteristic of normal epithelial differentiation (25). In SQCCs, these features include intercellular bridge formation and keratinization manifested by dyskeratosis and squamous pearl formation. Biochemical alterations that accompany changes in morphology are just beginning to be explored in this disease. Such molecular differences could be useful in the early detection of SQCC of the esophagus.

Since keratins are a major constituent of the esophageal epithelium, they were chosen as a focal point for investigating the possible biochemical changes that may arise during the transition from the stratified squamous epithelial lining of the esophageal lumen to SQCC of the esophagus. The human keratins are a family of about 20 related proteins (M, 40,000 to 70,000) that polymerize to form 8- to 10-nm-diameter cytoplasmic filaments in most if not all epithelial cells (for a review, see Ref. 20). Usually, only 2 to 6 keratins are expressed by an individual cell. Variability in expression of different keratins is related to species, cell type, state of cellular differentiation, and extracellular factors. Keratins continue to be expressed in epithelial-derived cancers (carcinomas) (21, 29, 41).

Several laboratories have recently described alterations in the pattern of keratin expression in human esophageal epithelium when compared to SQCC of the esophagus (1, 2, 21). In this report, we further biochemically define the consistency of these alterations. We relate our findings to the potential of a biochemical approach to cancer detection of a cancer that expresses a different keratin pattern than its tissue of origin.

MATERIALS AND METHODS

Tissue Collection. Six samples of esophageal carcinoma (histologically confirmed as SQCC) and 6 samples of adjacent normal esophageal epithelium from 6 different patients were obtained from resected surgical specimens immediately after operation. Four samples of normal esophageal epithelium were obtained from surgical specimens of patients operated for reasons other than esophageal carcinoma. Three samples were obtained at autopsy.

Cell Culture. One sample of esophageal epithelium was minced finely and prepared for culture according to the method of Rheinwald (27).

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Cells were cultured in the presence of lethally mitomycin C-treated mouse 3T3-fibroblasts in a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's Medium F-12 (40), supplemented with 10% fetal calf serum, hydrocortisone (0.4 μg/ml), 0.1 μm cholera toxin, 20 μm triiodothyronine, human transferrin (5 μg/ml), insulin (5 μg/ml) (3, 19), and epidermal growth factor (4 ng/ml) (30). The medium was changed every 3 to 4 days.

**Extraction of Keratins.** Esophageal epithelium was separated from underlying connective tissue by sharp and blunt dissection. Esophageal epithelium and cancer samples were each cut into 3- x 3-mm pieces, placed in liquid N2, and ground into a powder. The powder was suspended in 5 ml of T6:E6 buffer containing 0.1% phenylmethylsulfonyl fluoride (Sigma) and homogenized (10). The homogenate was centrifuged at 12,000 × g for 10 min at 4°. The pellet was washed 5 times in T6:E6 buffer. Water-insoluble proteins were resuspended in 8 μ l urea:10% 2-mercaptoethanol with the aid of sonication, followed by incubation at 37°.

For extraction of keratins from cultured esophageal epithelial cells, the primary culture and first and second subcultures were harvested when 75% confluent. The 3T3 fibroblasts were selectively removed using EDTA, and the remaining cells were scraped into 5 ml of T6:E6 buffer. The procedure for isolation of the keratins was identical to that described above.

**Antibody Specificity.** Antibodies specific for (a) keratins of cultured human epidermal cells (M, 46,000 to 58,000) and (b) the gel-purified M, 40,000 keratin of SCC-15, a cell line of SQCC of the tongue (29), were raised in New Zealand White rabbits. A mixture of antisera was used for all immunoblots analyses. This mixture showed cross-reactivity with all keratins of M, 40,000 to 58,000, with slightly stronger reactivity to the M, 50,000 and 56,000 keratins.

**Immunoblot Analysis (Western Blot).** Proteins were resolved by sodium dodecyl sulfate:polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose paper (4). The paper was incubated at 37° for 2 hr in a solution of 5% bovine serum albumin (Fraction V; Sigma), 0.9% NaCl solution (saline) containing a 1:100 dilution of antisera against epidermal keratins, and gel-purified M, 40,000 keratin. The paper was then thoroughly washed in a saline:10 μm Tris:0.1% Triton X-100 solution. The keratins were identified by indirect labeling of the bound antibody with 125I-labeled Staphylococcus aureus Protein A (Amersham). Radiolabel was detected by autoradiography.

**Electrophoresis.** Keratins were separated using 8.5% sodium dodecyl sulfate:polyacrylamide slab gel electrophoresis according to the procedure of Laemmli (18). Gels that were not subjected to immunoblot analysis were stained with a solution of 0.1% Coomassie blue, 50% methanol, and 10% acetic acid and then destained with a solution of 5% methanol and 10% acetic acid.

**Immunofluorescence.** Esophageal epithelial cells were grown on double-chamber Lab-Tek slides (Miles). The cultured cells and 5-μm sections of esophageal tissue and SQCC of the esophagus were fixed in methanol (~20°) for 5 min. This was followed by incubation at 37° for 30 min with specific rabbit antiserum against human epidermal keratin (1:50 dilution) and then by incubation at 37° for 20 min with fluorescence-conjugated goat anti-rabbit IgG (Miles; 1:20 dilution).

**RESULTS**

Previously, we demonstrated that an antiserum prepared against total keratins from cultured cells showed general cross-reactivity with all the keratins of cultured basal epidermal cells and of differentiating epidermis (12, 17). When this antiserum was incubated with normal human esophageal tissue, it reacted strongly with all of the cells of the epithelial layer, but not with cells of the subepithelial connective tissue (Fig. 1a). With the use of this antikeratin antiserum, the invasive cells of an SQCC of the esophagus could also be easily distinguished from the surrounding smooth muscle and connective tissue (Fig. 1b). When cells were cultured in vitro from normal esophageal epithelium, they continued to express keratins, as seen by the strong immunofluorescence of a cultured esophageal colony (Fig. 1c). The surrounding fibroblast feeder layer showed no immunoreactivity with the anti-keratin antiserum. In the cultured cells, the filamentous network of the 8-nm keratin fibers was readily apparent, further clarifying the specificity of this antiserum in esophageal epithelial cells.

To explore the nature of the keratins synthesized by these cells, water-insoluble proteins were first extracted from a sample of esophageal tissue and then separated electrophoretically. The pattern of proteins produced by this sample is shown in Fig. 2a, Lane 1. Major bands with molecular weights of 58,000 and 52,000 and a minor band with a molecular weight of 56,000 can be seen. The pattern of proteins changed significantly when epithelial cells were cultured in vitro (Fig. 2a, Lane 2). The M, 52,000 band diminished, and the M, 56,000 band increased in intensity. In addition, prominent new bands (M, 50,000 and 46,000) appeared. With successive subculturing, the M, 40,000 band became more pronounced (Fig. 2a, Lanes 3 and 4). Interestingly, this pattern was very similar to that produced by an SQCC of the esophagus (Fig. 2a, Lane 5), which in turn was similar to the pattern produced by a cultured SQCC of the tongue (Fig. 2a, Lane 6) (41).

The protein bands observed in Fig. 2a cross-reacted strongly with the antisera against keratins of cultured human epidermal cells (Fig. 2b). The pattern of keratins seen by immunoblot analysis was very similar to that visualized by Coomassie blue staining, with the exception that the M, 50,000 and 56,000 keratins cross-reacted somewhat more strongly with the antisera than did the other keratins. Since this method was more specific and more sensitive for the keratins, it was chosen for subsequent analyses.

To determine the consistency of the patterns of keratins produced by esophageal SQCCs and normal esophageal epithelium, we examined the keratins synthesized by 6 samples of carcinoma and the corresponding normal epithelium obtained from 6 different patients (Fig. 3). Five of 6 samples of SQCC of the esophagus produced keratins of M, 58,000, 56,000, 50,000, and 46,000. In one sample (Fig. 3, Lane 5C), the M, 58,000 keratin band was weak or absent. In many of the tumor samples, additional keratins could be seen. Three carcinomas produced a keratin with a molecular weight of 52,000 (Fig. 3, Lanes 2C, 3C, and 4C). One expressed a minor keratin with a molecular weight of 40,000 (Fig. 3, Lane 4C). Two other minor bands with molecular weights of 42,000 (Fig. 3, Lanes 2C) and 44,000 (Fig. 3, Lanes 1C and 6C) showed cross-reactivity with the anti-keratin antiserum.

Of the 6 esophageal SQCCs, 4 were histologically graded as moderately differentiated, and 2 were poorly differentiated. We did not observe a trend in the variation of keratin pattern between the moderately and poorly differentiated SQCCs of the esophagus.

All 6 samples of corresponding normal esophageal tissue expressed 3 keratins with molecular weights of 58,000, 56,000, and 52,000. Four of the 6 samples produce low levels of the M, 50,000 keratin characteristic of the tumors, but none of these biopsies expressed detectable levels of the smaller M, 40,000 to 46,000 keratins. Thus, in all of the samples where the SQCC of
the esophagus could be compared with the normal esophageal epithelial tissue from the same patient, the keratin pattern was markedly different. Although all of the major differences in keratin patterns between tumor and normal tissue were constant, minor variations in these patterns were clearly present.

To further explore the frequency of occurrence of these minor differences, we investigated the keratin patterns of 7 additional samples of normal esophageal tissue (Fig. 4). These samples all expressed the triplet of keratins with molecular weights of 58,000, 56,000, and 52,000 that seemed to be characteristic of normal esophageal tissue. However, 3 of these new samples showed an additional keratin with a molecular weight of 50,000 (Fig. 4, Lanes 4 to 6). Its strong immunoreactivity gave no doubt as to its presence in these tissue samples, although the actual level of M, 50,000 keratin here was less than that of the M, 52,000 keratin. Only one sample showed a keratin with a molecular weight of 46,000 (Fig. 4, Lane 5; patient with hepatic cirrhosis), and this band was extremely weak in relation to other keratins expressed. (Compare same lane, lighter exposure at right.) Another atypical keratin pattern included an M, 40,000 band (Fig. 4, Lane 6), although the tissue was also unusual in that it came from a 24-hr-old neonate who had died of congenital anomalies. In summary, variations in the normal esophageal keratin pattern could be identified, but these differences were largely of a minor nature.

DISCUSSION

The reproducible triplet of 3 keratins with molecular weights of 58,000, 56,000, and 52,000 defines normal human esophageal epithelium and seems to be a pattern characteristic of this epithelium. These results are in good agreement with the previous findings of Banks-Schlegel and Harris (1) and Moll et al. (20). Although the molecular weight values for the 3 keratins vary among laboratories, the reports are most likely identical, and the 3 keratins have been assigned numbers of 4 (M, 58,000), 6 (M, 56,000), and 13 (M, 52,000) according to the catalogue system of Moll et al. (20).

Whereas Keratins 4 and 6 remain predominant in tissue samples of SQCC of the esophagus, Keratin 13 is frequently present in reduced, if detectable, levels (this paper; Refs. 2 and 21). In contrast, the levels of M, 46,000 (No. 17) and M, 50,000 (Nos. 14 and 15) keratins increase substantially in the carcinoma. Of 11 samples of normal esophageal tissue, only one (Fig. 4, Lane 4) showed relatively high levels of Keratins 14 and 15, and none produced large amounts of Keratin 17. Thus, these changes in keratin synthesis seem to be consistent biochemical differences between the normal epithelium and SQCC of the esophagus.

Our finding that the levels of M, 50,000 and M, 46,000 keratins are usually elevated in esophageal carcinomas is in good agreement with that of Moll et al. (21), who reported an increase in the level of Keratin 14 (M, 50,000) for one type of SQCC of the esophagus and an increase in Keratin 15 (M, 50,000) for another. Consistent with our results, both types of SQCC of the esophagus produced large amounts of Keratin 17 (M, 46,000). These observations differ from those of Banks-Schlegel and Harris (2), who reported no change in keratins with molecular weights of 46,000 to 50,500 as judged by anti-keratin immunoprecipitation of proteins extracted from normal and tumor tissues. It is possible, however, that the distortion of the electrophoretic migration of the keratins caused by the large amounts of immunoglobulin (M, 50,000) in the precipitates described in that study masked these differences in keratin levels.

Whereas the pattern of keratins produced by normal esophageal epithelium differs considerably from that of other human epithelia, e.g., epidermis, tongue, and bronchus, the pattern produced by SQCC of the esophagus is largely indistinguishable from that produced by SQCCs arising in other organs (Refs. 21 and 23; Footnote 6). The disappearance of differences in keratin patterns also takes place in cells cultured from various rabbit and human epithelia (7, 40). In the present study, we have shown that 4 major keratins (M, 46,000, 50,000, 56,000, and 58,000) are produced by cultured human esophageal cells. This pattern is very similar to that produced by cultured human epidermal, corneal, and conjunctival cells (35) and supports similar findings recently reported by Banks-Schlegel and Harris (1) and Wu et al. (40).

We have shown that this in vitro pattern appears shortly after placing the esophageal cells in culture and is maintained through successive subculturings. One interesting change in this keratin pattern is the appearance of the M, 40,000 keratin which increases steadily through several rounds of subculturing (Fig. 2A, Lanes 2 to 4). Wu et al. (40) have also identified this keratin in cultured human esophageal cells. It is present in large amounts in some normal epithelia (20, 36) and in some other cultured cells (11, 35, 40). It has also been found in a number of different carcinomas (21, 23, 41). Its role in the cytoskeleton remains unknown.

The changes in keratin pattern observed for cultured esophageal cells are likely to be transient. Previously, it was shown that, although epithelial cells from different sources develop an indistinguishable pattern of keratin expression in vitro, they reacquire their distinctive keratin patterns when the cultured cells are injected s.c. into athymic mice (7). Recently, it has been shown that the level of vitamin A in the culture medium can have a profound influence on the pattern of keratins produced by an epithelial cell, thereby providing a possible basis for this observation (11).

The pattern of keratins produced by SQCCs may also be influenced by the level of vitamin A in the tissue. In some SQCCs, altered sensitivity to the vitamin has been observed and may cause changes in keratin expression (17). Although extracellular factors most certainly play a role in altering the pattern of keratins produced by SQCCs, additional factors might contribute to permanent changes in keratin expression in the cancers (28). It is quite possible, for instance, that the altered program of differentiation which occurs during the development of the malignant state leads to changes in keratin expression. Alternatively, the changes in keratin synthesis may arise from the malignant transformation of a rare population of esophageal epithelial cells that produce a pattern of keratins different from most of the cells of the intact tissue.

It seems quite remarkable that, despite the many factors known to influence keratin gene expression, consistent differences exist between the pattern of keratins produced by SQCC of the esophagus and normal esophageal epithelium. Since the esophagus is accessible to endoscopic biopsy and cytological brushing, it may be possible to take advantage of these biochem-
cancers. Techniques such as measurement of DNA content (22) and scanning electron microscopy (14) have already been used to investigate and diagnose precancerous and early malignant lesions of the esophagus in humans. Mass screening programs in areas of high risk have resulted in earlier detection of esophageal carcinoma and possibly improved survival (42). The use of polyclonal antibody gel electrophoresis and monoclonal antibodies in identifying specific keratins characteristic of various malignant neoplasms has become increasingly valuable (13, 20, 21, 23, 24, 32). These diagnostic and investigational methods have thus far been utilized primarily for tumors that are clinically apparent or symptomatic.

The development of monoclonal antibodies that recognize distinct keratin patterns, cell types, and states of cellular differentiation has been described (6, 9, 26, 36, 38). As we learn more about the amino acid sequence differences between the many different keratins (8, 15, 16, 34), it should be possible to generate antibodies specific for each keratin, even if it shares a high degree of homology with other keratins. It seems within the grasp of existing technology to develop an antibody that would distinguish esophageal epithelium, dysplastic epithelium, and SQCC of the esophagus based on their differences in keratin expression. Such an antibody may be useful in generating a biochemical method of cancer detection which, when used in conjunction with standard pathological techniques, may result in earlier detection of SQCC of the esophagus.

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REFERENCES

Fig. 1. Anti-keratin immunofluorescent staining of normal esophageal epithelium (a), SQCC of the esophagus (b), and cultured esophageal epithelial cells (c). se, subepithelial tissue; ct, connective tissue; E, esophageal epithelium; C, SQCC of the esophagus. Note that only the epithelial tissue, the epithelial-derived cancer, and the cultured esophageal colony show immunoreactivity with the anti-keratin antisera. Fibroblasts, connective tissue, and smooth muscle tissue show no reactivity. Note also the fine cytoplasmic keratin intermediate filament network in the cultured esophageal epithelial cells (c). a, x 420; b, x 420; c, x 840.

Fig. 2. a, electrophoretic separation of keratins. Water-insoluble proteins were separated by electrophoresis through an 8.5% polyacrylamide gel, and the protein bands were visualized by staining with Coomassie blue. Lane 1, esophageal epithelial tissue; Lane 2, primary culture of esophageal epithelial cells; Lane 3, first subculture; Lane 4, second subculture; Lane 5, SQCC of the esophagus; Lane 6, SCC-15 (a cultured cell line of SQCC of the tongue). A, actin. b, immunoblot analysis of keratin patterns using anti-keratin antibodies prepared against cultured human epidermal cells. Lanes are as in Fig. 2a.
Fig. 3. Immunoblot analysis of keratin patterns of normal esophageal epithelial tissue (E) and SQCC of the esophagus (C) from 6 patients, labeled 1 to 6. Tumor grade: moderately differentiated (MD) and poorly differentiated (PD) SQCC of the esophagus.

Fig. 4. Immunoblot analysis of keratin patterns of esophageal epithelial tissue from an additional 7 patients, Lanes 1 to 7. Note the presence of an M, 46,000 band in Lane 5. This esophageal tissue was obtained from a patient who died of hepatic cirrhosis. A shorter exposure time of the bands in Lane 5 is shown on the right. Note that with a shorter exposure time, the M, 46,000 band is no longer visible. The keratin pattern in Lane 6 was from esophageal epithelial tissue of a neonate who died of congenital anomalies.
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