Aberrant Expression of Keratin Proteins and Cross-Linked Envelopes in Human Esophageal Carcinomas

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ABSTRACT

When compared to normal esophageal epithelium, marked alterations in keratin protein and cross-linked envelope expression were found in human esophageal carcinomas. Examination of the pattern of keratin proteins extracted from either several primary esophageal tumors or carcinomas xenotransplanted in nude mice revealed a dramatic reduction in the amount of keratin protein, especially in the M, 52,000 to 61,000 range. In seven of eight of the primary tumors, the major M, 52,000 and M, 61,000 esophageal keratins were not detected, and the remaining tumor exhibited a marked reduction in these two keratins. The major M, 57,000 and minor M, 59,000 esophageal keratins were found in varying but reduced amounts in the different tumors. The major M, 57,000 keratin seemed to be the most conserved keratin of this intermediate-molecular-weight keratin class (M, 52,000 to 61,000). In contrast, the lower-molecular-weight keratins (M, 46,000 to 50,500) were usually conserved in the carcinoma cells and were present at levels approximating that of the nontransformed counterpart. The minor M, 37,000 and 44,000 keratins from normal esophageal epithelium were retained in the tumor cells but often in reduced amounts. The expression of another differentiated function, cross-linked envelopes, in the carcinoma cells varied from unimpaired to severely restricted capacity to form envelopes.

In conclusion, specific alterations in keratin protein and cross-linked envelope expression were found in human esophageal carcinomas.

INTRODUCTION

Human esophageal epithelium is a noncornified, stratified, squamous epithelium. Similar to other stratified, squamous epithelia (for review, see Ref. 17), it contains abundant keratin organized as bundles of tonofilaments within the cytoplasm of the cell (5, 13, 26). However, the spectrum of keratins synthesized by human esophageal epithelium is distinctive and serves as a tissue and cell-type specific marker (5). These cells also exhibit another marker characteristic of stratified, squamous epithelium, involucrin (4). Involucrin is one of the precursor proteins of the cross-linked envelope found in the stratum corneum of epidermis (16, 17, 24, 25, 33, 34) and has been demonstrated in all stratified, squamous epithelia, whether or not they possess a stratum corneum (4). The cross-linked envelope has been used as a marker for studying the development of the keratinocyte during embryonic life (3) and represents a sensitive indicator of squamous differentiation.

Because of large variations in incidence even within the same country (11, 12, 15, 19, 41, 43, 45), environmental and nutritional factors, as well as cultural habits, have been purported to play an important role in human esophageal carcinogenesis (12, 15, 18, 19, 23, 29, 39, 40, 44, 45). Progression to the neoplastic state, especially well characterized in epidermis, is frequently associated with defects in the normal program of terminal differentiation (6, 13, 21, 26, 27, 30, 32, 37, 42, 46). In the present study, we examined human esophageal tumors for alterations in either keratin protein or cross-linked envelope expression which might be characteristic of the malignant state. While human esophageal carcinomas exhibited consistent alterations in keratin expression, the tumors displayed a variable capacity to undergo envelope formation.

MATERIALS AND METHODS

Tissue Procurement. Human esophageal carcinomas (tumor type, squamous cell carcinomas) and samples of corresponding nontumorous "histologically normal" esophagi were obtained at the time of surgery. Human esophageal tumors, xenotransplanted in athymic nude mice, were obtained at the time of autopsy. Additional human esophagi were obtained at "immediate autopsy" (i.e., within 1 hr of death of the donor). Newborn human foreskins were collected at circumcision. Tissues were maintained in L-15 medium at 4°C prior to processing.

Radiolabeling of Keratin Proteins In Vivo. Small explants of human skin and esophagus and esophageal tumors (minced) were labeled with [35S]methionine (specific activity, >600 Ci/mmol; Amersham/Searle Corp.) as described previously (5). In the case of normal tissue (epidermis and esophagus), the epithelium was detached from the underlying stroma by heating at 65°C for 5 min (5).

Extraction of Keratin Proteins from Human Epidermis, Esophagus, and Esophageal Tumors. Human epidermis, esophageal epithelium, and esophageal tumors were thoroughly homogenized and repeatedly extracted with 20 mM Tris-HCl, pH 7.4. The insoluble material, containing mainly keratin proteins, was pelleted by centrifugation and solubilized in 20 mM Tris-HCl, pH 7.4, containing 2% SDS2 and 10 mM dithiothreitol, as described in detail elsewhere (5). Inclusion of a protease inhibitor (phenylmethylsulfonyl fluoride) did not alter the pattern of keratin proteins.

Immunoprecipitation. Keratin proteins were selectively immunoprecipitated with keratin antisera (35) from [35S]methionine-labeled keratin-enriched cell extracts by a modification (5) of the method of Kessler (20).

Polyacrylamide Gel Electrophoresis. Keratin proteins were resolved electrophoretically on 8.5% gels [acrylamide:bisacrylamide (30:0.8, w/w)] according to the method of Laemmli (22), except that 10% (v/v) glycerol was included in the separation gel. Gels were stained in a solution of 0.25% Coomassie blue in 50% trichloroacetic acid and destained in a solution of 20% methanol and 7.5% glacial acetic acid. Gels with labeled keratin proteins were fixed overnight in a solution of 30% methanol and 7.5% glacial acetic acid, fluorographed with En3Hance (New England Nuclear, Boston, MA), and exposed to Kodak X-Omat AR5 film.

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The abbreviations used are: SDS, sodium dodecyl sulfate; SCC, squamous cell carcinoma.
Cross-Linked Envelopes. Trypsin-disaggregated cells (3 x 10^6 to 10^8) from either normal-appearing human esophageal epithelium or esophageal tumors were pelleted by centrifugation and resuspended in 1.5 ml of Liebowitz L-15 medium (Grand Island Biological Co., Grand Island, NY). Calcium ionophore (X-537A) (34) was added to a final concentration of 50 nmol/ml to induce cross-linked envelope formation. Following incubation at 37°C for 3.5 hr, the cells were pelleted by centrifugation, resuspended in phosphate-buffered saline, and counted to obtain a total cell count. Next, SDS and dithiothreitol were added to a final concentration of 2% and 10 mM, respectively, to dissolve all cells not possessing envelopes and to solubilize the cellular contents of cells possessing envelopes. The number of cross-linked envelopes was then counted using a hemocytometer chamber, and the percentage of total cells possessing cross-linked envelopes was calculated.

RESULTS

Classification of the Human Esophageal Carcinomas. Histological diagnosis of the esophageal tumors was made independently by 2 pathologists (Dr. Richard Schlegel, National Cancer Institute NIH, Bethesda, MD, and Dr. Jonathan Said, Cedars-Sinai Medical Center, Los Angeles, CA), according to conventional histological criteria (31), specifically, the degree of anaplasia, keratinization, keratin pearl formation, and intercellular bridges. Tumors were classified as well-differentiated SCC if they showed orderly stratification, obvious intercellular bridging, and keratinization with pearl formation. When the bulk of the tumor appeared "undifferentiated" and overt keratinization and/or intercellular bridges were only present focally or were discerned with difficulty throughout the specimen, the tumor was classified as a poorly differentiated SCC. Moderately differentiated SCC exhibited intermediate features, characteristically displaying overt keratinization throughout the bulk of the tumor in hematoxylin and eosin-stained sections. While adenocarcinomas of the esophagus can be found, none of the tumors in this series was classified as an adenocarcinoma. To facilitate analysis of the data, histological grading of the tumors is shown in the respective figures and table.

Alterations in Keratin Protein Expression in Human Esophageal Carcinomas. Keratin-enriched protein fractions were extracted, as described in the "Materials and Methods," from 8 primary human esophageal carcinomas and normal esophageal epithelium obtained from the same individual at the time of surgery. Representative examples of the patterns of keratin-enriched proteins isolated from either human esophageal carcinoma or normal esophageal epithelium obtained from the same individual at the time of surgery are shown in Fig. 2. All of the tumors displayed a marked reduction in proteins in the M, 37,000 to 61,000 region of the gel, characteristic of human esophageal keratin proteins (5). Immunoprecipitation of [35S]methionine-labeled cell extracts with keratin antiserum supported their identity as keratins (Fig. 2). All 8 primary esophageal tumors exhibited either a marked reduction (1 of 8 cases) or nondetectable amounts (7 of 8 cases) of the major M, 52,000 and 61,000 human esophageal keratins. The major M, 57,000 and minor M, 59,000 esophageal keratins were found in varying but usually reduced amounts in the different tumors. The major M, 57,000 keratin appeared to be the most conserved keratin of the intermediate keratin class (M, 52,000 to 61,000) found in the tumor cells. In contrast, M, 46,000 to 50,500 keratins were always conserved in the malignant esophageal cells and were present at levels approximating that of the normal-appearing epithelium. While only the M, 48,000 and 50,500 keratins were resolved on the gel shown in Fig. 2, better resolution of these proteins on another gel (Fig. 2, right) revealed the presence of an additional M, 49,500 keratin protein. These findings are consistent with the data from Coomassie blue-stained protein gels demonstrating the presence of all 3 keratins in extracts of both normal and carcinoma cells. The minor M, 37,000 and 44,000 esophageal keratins were retained in the tumor cells but frequently in reduced amounts. Although the sampling was small, the amount of keratin appeared to correlate with the degree of tumor differentiation.

Variable Expression of Cross-Linked Envelopes in Human Esophageal Carcinomas. To analyze for alterations in the pathway of terminal differentiation in human esophageal carcinomas, we induced envelope formation in trypsin-disaggregated cells from normal human esophageal epithelium and esophageal carcinomas with the calcium ionophore, X-537A (34), and then estimated the extent of terminal differentiation by calculating the percentage of cells that formed cross-linked envelopes, structures which are resistant to SDS and reducing agent (38). Compared to normal human esophageal cells, human esophageal carcinoma cells exhibited a variable capacity to form cross-linked envelopes, ranging from 3.1 to approximately 100% of the cells (Table 1). There was some correlation, in general, between envelope-forming capabilities and the degree of tumor differentiation with more differentiated tumors forming more envelopes. Examination of the cross-linked envelopes by phase-contrast microscopy (data not shown) revealed that these structures were similar in size and appearance to the cross-linked envelopes from normal esophageal epithelium. The majority of the tumors examined (6 of 8 cases) displayed a markedly reduced capacity to form envelopes, indicating a defect in pathway of terminal differentiation. However, carcinoma cells from 2 tumors formed cross-linked envelopes at levels comparable to that of normal esophageal epithelium.

Table 1

<table>
<thead>
<tr>
<th>Human esophageal epithelium</th>
<th>HEA208</th>
<th>88.5</th>
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<tbody>
<tr>
<td>HEA(ME94)</td>
<td>73.4</td>
<td></td>
</tr>
<tr>
<td>HEA424</td>
<td>104.0</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Human esophageal carcinomas</th>
<th>HET-ChTr</th>
<th>113.0</th>
<th>MD* WD SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HET-DaAn</td>
<td>103.0</td>
<td>MD SCC</td>
<td></td>
</tr>
<tr>
<td>HET-228</td>
<td>41.4</td>
<td>PD-MD SCC</td>
<td></td>
</tr>
<tr>
<td>HET-TPN55-47</td>
<td>30.0</td>
<td>MD-WD SCC</td>
<td></td>
</tr>
<tr>
<td>HET-ChCa</td>
<td>18.0</td>
<td>MD-SCC</td>
<td></td>
</tr>
<tr>
<td>HET-TPN54-19</td>
<td>17.9</td>
<td>MD-WD SCC</td>
<td></td>
</tr>
<tr>
<td>HET-220</td>
<td>8.4</td>
<td>PD SC</td>
<td></td>
</tr>
<tr>
<td>HET-82-41(NM)</td>
<td>3.1</td>
<td>PD SC</td>
<td></td>
</tr>
</tbody>
</table>

* All tumors were primary esophageal carcinomas except HET-82-41(NM), which was a primary esophageal tumor which had been xenotransplanted into a nude mouse.
* MD, moderately differentiated; WD, well differentiated; PD, poorly differentiated; NM, nude mouse.
DISCUSSION

Human esophageal carcinomas were characterized by distinct alterations in keratin protein expression. Most notably, the major M, 52,000 and 61,000 keratin proteins found in normal esophageal epithelium were either significantly reduced (1 case) or nondetectable (7 cases) in the carcinoma cells. The major M, 57,000 and minor M, 59,000 keratins were present in varying but frequently reduced amounts in all of the esophageal carcinomas; however, of the intermediate-molecular-weight keratins (M, 52,000 to 61,000), the level of expression of the M, 57,000 keratin appeared to be the least affected upon transition to the malignant phenotype. Interestingly, the lower-molecular-weight keratins (M, 48,000 to 50,500) were usually conserved in the malignant cells at levels approximating that of the normal counterpart. Similarly, the minor M, 37,000 and 44,000 esophageal keratins were retained in the tumor cells, although generally in reduced amounts.

Our results confirm and extend the findings of Moll et al. (26, 27), who examined 2 squamous cell carcinomas of the esophagus and reported on the disappearance of major keratins 4 (M, 59,000) and 13 (M, 54,000) in one tumor and 5 (M, 58,000) and 13 (M, 54,000) in a second tumor. Keratins 4, 5, and 13 are most probably identical to the M, 61,000, M, 59,000, and M, 52,000 keratins described in this study, respectively. Hence, while we observed no loss of keratin 5 in any of the tumors examined, we similarly noted either a dramatic reduction (1 case) or nondetectability (7 cases) of the major M, 61,000 and 52,000 keratins (Moll’s keratins 4 and 13). In agreement with Moll et al. (26, 27), the expression of the lower-molecular-weight keratins (M, 46,000 to 50,500) (keratins 14 to 17 in Moll’s study) was maintained in the tumor cells. We have also noted the presence of relatively small amounts of M, 37,000 and M, 44,000 keratins in the esophageal tumor cells and their normal counterpart. We are currently examining the relationship between this M, 44,000 keratin and the M, 40,000 keratin reported by Moll to be present in increased amounts in esophageal carcinomas.

Keratin protein profiles have been shown to change during different stages of terminal differentiation, especially in epidermis (1, 2, 8–10, 14, 28, 36). While it is plausible that the differences in keratin protein patterns between normal esophageal epithelium and esophageal carcinomas (i.e., squamous cell carcinomas) could be attributable to the extent of differentiation, it is likewise possible that the type of keratin pattern which results may be related to the type of tumor being established (in this case a squamous cell carcinoma). In this regard, we have found that squamous cell carcinomas arising from either the esophagus (a stratified, squamous epithelium) or the bronchus (a non-squamous epithelium) possess virtually identical keratin patterns (7).

Analyses of ionophore-induced cross-linked envelopes serve as a sensitive indicator of terminal differentiation. All stratified, squamous epithelia, including esophageal epithelium, have been shown to possess involucrin (4), one of the precursor proteins participating in envelope formation (33, 34). Although human esophageal epithelium does not have a stratum corneum, and therefore does not have cross-linked envelopes on its surface, the majority of cells dissociated from the epithelium possess the capacity to form envelopes and will do so following exposure to calcium ionophore (Table 1; Ref 4). When examining cells dissociated from human esophageal squamous cell carcinomas for their ability to form cross-linked envelopes, we found that these tumor cells displayed a variable but usually reduced capacity to form cross-linked envelopes which correlated largely with the extent of tumor differentiation. However, the fact that the majority of cells from 2 of the tumor cases exhibited an ability to form envelopes which was comparable to that of normal esophageal keratinocytes suggested that transition to the malignant phenotype need not always be associated with a defect in terminal differentiation, as assayed by cross-linked envelope formation. This alteration in the commitment to undergo terminal differentiation exhibited by most tumor cells has previously been demonstrated in several cell lines derived from squamous cell carcinomas of the skin and oral cavity (32). However, since this property was being examined in cell lines, it was possible that selection of a certain cell type may have occurred during establishment of the cell line. These results indicate that most primary squamous cell carcinomas (in this case, of the esophagus) display this defect in the program of terminal differentiation. We speculate that such an alteration in the triggering of terminal differentiation would augment clonal expansion by a malignant cell.

In conclusion, human esophageal carcinomas exhibited specific alterations in keratin protein and cross-linked envelope expression.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Human esophageal carcinomas characterized by alterations in the pattern of keratin-enriched proteins. Keratin-enriched protein fractions were extracted from human esophageal carcinomas (HEC), autopsy specimens of normal human esophageal epithelium from noncancerous patients (HEE), surgical specimens of normal appearing human esophageal epithelium (N), and the corresponding esophageal tumors (squamous cell carcinoma) (T) from 5 different individuals (abscissa, 1 through 5) as described in "Materials and Methods." Histological grading of the tumors indicated one poorly differentiated (5), one moderately differentiated (1), and 3 moderately to well-differentiated (2, 3, and 4) tumors in the series examined here. Samples containing 100 µg of protein were analyzed on 8.5% polyacrylamide gels. Left, position of molecular weight markers (Lane M) run concurrently on the gel. The molecular weight standards were phosphorylase B (M, 92,000), bovine serum albumin (M, 68,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 30,000), and soybean trypsin inhibitor (M, 21,000). Right, the molecular weights (x103) assigned to the keratins of normal human esophageal epithelium. Note that the keratins of human esophageal epithelium (A/), and the corresponding esophageal tumors (squamous cell carcinoma) (T) from 5 different individuals (abscissa, 1 through 5) were very reproducible and showed only minor interindividual variation. Human esophageal epithelium. Note that the autopsy and surgical specimens of human esophageal epithelium exhibited virtually identical keratin patterns. Human esophageal epithelium (A/), and the corresponding esophageal tumors (squamous cell carcinoma) (T) from 5 different individuals (abscissa, 1 through 5) were immunoprecipitated with keratin antiserum, analyzed on 8.5% gels, and subjected to autoradiography as described in "Materials and Methods." The tumor samples used were the same as those characterized in Fig. 1. Left, position of molecular weight markers. Right, molecular weights (x103) assigned to the keratins of the corresponding esophageal epithelium (N) and the corresponding tumor (T) from 5 different individuals (abscissa, 1 through 5). The molecular weights (x103) assigned to the keratins of normal esophageal epithelium and the tumor are shown to the left and right of the gel, respectively. Esophageal tumors were characterized by either a marked reduction (one case) or absence (4 cases) of the major M, 52,000 and 61,000 esophageal keratins. The other keratins were retained in the tumor cells, although sometimes in reduced amounts.
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