Cross-Linked Envelope-related Markers for Squamous Differentiation in Human Lung Cancer Cell Lines

Mark L. Levitt,3 Adi F. Gazdar, Herbert K. Oie, Hildegarde Schuller, and Scott M. Thacher

ABSTRACT

Lung carcinoma cell lines were analyzed in culture and in nude mouse xenografts for both morphological appearance and expression of specific proteins that participate in cross-linked envelope formation during normal squamous cell terminal differentiation. Cross-linked envelope formation, induced by artificial influx of millimolar Ca2+ into the cultured cells, was an exclusive trait of squamous, adenocarcinomas, and mucoepidermoid carcinomas. Small cell lung carcinoma and nonsquamous non-small cell lung carcinoma lines, such as adenocarcinoma and large cell carcinoma, were uniformly negative for cross-linked envelope formation. Involucrin, which is incorporated into the cross-linked envelope by the enzyme transglutaminase, was expressed at highest levels in squamous tumors, but generally at the non-squamous non-small cell lung carcinoma lines also expressed comparable amounts. On the other hand, transglutaminase activity was consistently higher in squamous as opposed to nonsquamous lines, so that in cell culture, a clear contrast between the groups could be observed. A M195,000 protein that is incorporated into cultured human epidermal cell cross-linked envelopes was also observed in some but not all of the squamous lines. Two forms of transglutaminase are expressed in cultured keratinocytes. One of them, tissue transglutaminase, was expressed in the majority of squamous cell lines even though it is not a normal product of squamous differentiation in vivo. Keratinocyte transglutaminase, which is distinct from the tissue form and is normally present during terminal differentiation in squamous epithelia, was measurably present in only one of the six squamous cell lines tested. In nude mouse xenografts, keratinocyte transglutaminase, localized immuno-histochemically with a biotinylated mouse monoclonal antibody, was again present only in a minority of the squamous lines whereas involucrin was expressed in all. In contrast to involucrin, keratinocyte transglutaminase is not an obligatory component of squamous differentiation in the pulmonary carcinoma cell lines tested. Its expression may be of value in further refining their classification.

INTRODUCTION

Lung cancers can be divided into two broad groups: SCLC,2 which demonstrate neuroendocrine differentiation, and NSCLC, comprising all other subtypes. The NSCLC include SCC, adenocarcinomas, and large cell carcinomas as well as cancers of mixed morphology including mucoepidermoid or adenocarcinomas. Expression of specialized biochemical features of normal epithelial cells, such as specific keratins (1-3) and protein components of the desmosome (4), have been used to characterize NSCLCs. Coexpression of traits normally restricted to either squamous or secretory epithelia occur simultaneously in as many as 50% of tumors analyzed (3). In contrast, regions of mucoid and epidermoid differentiation coexist in normal or metaplastic airway epithelia but they rarely intermingle (5, 6). More precise molecular definition of the heterogeneous nature of lung carcinomas is essential to understanding their basic biology.

Squamous cells undergo a programmed cell death during which a cross-linked envelope (or marginal band) is formed that is resistant to boiling in the presence of SDS and reducing agent. The chemical resistance of the CLE is conferred by the presence of ε-(γ-glutamyl)lysine interprotein cross-links (7). Both a keratinocyte transglutaminase, which catalyzes formation of the ε-(γ-glutamyl)lysine bond, and involucrin, a transglutaminase substrate, have been purified from cultured NHEKs and been shown to be expressed in the later stages of differentiation in both epidermis and other squamous epithelia (7-15). Simon, Ma, and coworkers have also described a M195,000 protein from cultured NHEKs that is incorporated into CLEs and is expressed in keratinizing epithelia (16, 17).

Although CLE formation is normally concomitant with terminal differentiation and metabolic death of the cell, living cells in NHEK culture can also be induced to form a cross-linked envelope structure by the forced entry of millimolar concentrations of calcium ion, an essential transglutaminase cofactor (7). Both CLE formation and involucrin expression have been used to analyze the degree of squamous character in neoplastic tissue or transformed cells in culture, including those from lung (2, 9, 10, 18, 19).

The keratinocyte transglutaminase is immunologically and chromatographically distinct from the more widely distributed tissue transglutaminase (11, 15, 20). Certain squamous cell carcinomas and strains of NHEK in culture express both forms of the enzyme, but their properties are clearly different; while tissue transglutaminase is found in the cytoplasm of these cultured cells, keratinocyte transglutaminase is located predominantly in cell particulates in detergent-extractable form. Unlike keratinocyte transglutaminase, tissue transglutaminase expression is not linked to keratinocyte terminal differentiation and it has been assumed that it is not normally required for CLE formation (11, 19, 21).

In this study, using an extensive selection of pulmonary carcinoma cell lines, we examine the relationship between squamous morphological character, cross-linked envelope formation, and cellular content of involucrin, transglutaminase, and M195,000 protein. Involucrin is found not only in squamous lines, but in a significant percentage of nonsquamous, non-envelope-forming cell lines, consistent with an earlier study (9). Surprisingly, keratinocyte transglutaminase is expressed in only a minority of the squamous, cross-linked envelope forming lines while the remainder contain tissue transglutaminase. This apparent defect in the normal expression of squamous cell character is analyzed both in culture and in nude mouse xenografts.

MATERIALS AND METHODS

Cell Cultures. All of the SCLC lines as well as some of the NSCLC cell lines and methods for their establishment and maintenance have been previously described (22, 23). The histological diagnoses of the
tumors from which the cell lines were derived were confirmed by one of the authors (A. F. G.) using standard light microscopic pathological criteria on hematoxylin & eosin-stained sections of surgical biopsies. Cell lines were analyzed by light microscopy of nude mouse xenografts derived from the cell lines and/or electron microscopy of the cell lines or xenografts. Expression of bundles of intermediate filaments (keratin) frequently terminating on well-formed abundant desmosomes (Fig. 1) sufficed to classify the line as an NSCLC line with squamous features. Prior to study, all cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO). NCI-H1072 is a squamous lung-derived tumor which is maintained exclusively by passage as nude mouse xenograft.

Cross-Linked Envelope Formation. The percentage of cells capable of forming cross-linked envelopes was determined as previously reported (7). Cultured cells, if nonadherent, were triturated while adherent cells were disaggregated at 37°C by incubation in 0.025% trypsin in phosphate buffered saline containing 0.25 mM EDTA and 0.05% glucose until single cell suspensions were obtained (about 20–40 min). These cells were washed twice in 5% calf serum to remove and inactivate the trypsin. DNAse I (5 µg/ml, 10 min incubation on ice) was included in (GIBCO). NCI-H1072 is a squamous lung-derived tumor which is maintained exclusively by passage as nude mouse xenograft.

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RESULTS

The pulmonary carcinoma cell lines used in this study had been extensively passaged and therefore it was necessary to classify them histologically based on either culture or nude mouse xenograft rather than original surgical sample (Table 1). None of the SCLC-derived cell lines or xenografts had squamous characteristics. In contrast, eight of the NSCLC tumor-derived cell lines had morphological evidence of squamous differentiation at the light microscopic or ultrastructural level.

<table>
<thead>
<tr>
<th>Tumors with squamous features</th>
<th>Moderately differentiated</th>
<th>Poorly differentiated</th>
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<th>Mucocoeplaroid</th>
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* Bronchioloalveolar features.
Some examples are shown in Fig. 1. In four cases the squamous character had not been recognized in the resected tumor specimen from the patient. For example, we had originally classified NCI-H226 as a mesothelioma (2); in Fig. 1F, abundant keratin bundles are shown in the corresponding cell culture.

Cross-Linked Envelope Formation. In this and a prior study of cross-linked envelope formation in squamous pulmonary carcinoma cell lines (2), no preexisting envelopes were found to have arisen in living cultures. Unlike normal cultured human epidermal or bronchial keratinocytes, which spontaneously form denaturation-resistant squames in the absence of membrane perturbation (29, 30), treatment with 100 μg/ml A23187 was necessary to cause cross-linking. All of the tumor cell lines showing squamous differentiation formed cross-linked envelopes, with the exception of NCI-H520 (a very poorly differentiated line with only rare keratinizing cells in the tumor and xenograft). Envelope formation varied from 1.25 to 55%. Cells lacking squamous features failed to form cross-linked envelopes (Fig. 2).

The underlying biochemistry of cross-linked envelope formation did not appear to be the same in each line. The effect of 0.8 M NaCl on cross-linked envelope formation was compared to that of calcium ionophore in three cases (Table 2). The ionophore-dependent values show some difference with Fig. 2, reflecting changes in confluency or other parameters affecting cell status. Two of the lines tested, NCI-H596 and NCI-H292, gave essentially equivalent results by the two methods, as is also reported for cultured NHEKs (7). In the case of...
Fig. 2. Cross-linked envelope formation in lung cancer cell lines. Percentage of detergent-resistant envelopes was determined by comparison with cell count prior to induction of envelope formation by ionophore A23187. Results are the percentage envelope formation under optimal conditions. Noninduced controls were all negative.

Table 2 Envelope competence of cultured human lung tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>100 µg/ml A23187</th>
<th>0.8 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H596</td>
<td>8%</td>
<td>12 ± 7.1% (9)*</td>
</tr>
<tr>
<td>NCI-H292</td>
<td>71%</td>
<td>35 ± 6.4% (5)</td>
</tr>
<tr>
<td>NCI-H322</td>
<td>26 ± 7% (2)</td>
<td>2 ± 1.5% (2)</td>
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*Percentage of cells forming detergent-resistant structures ± standard deviation (number of assays).

the line NCI-H322, however, the percentage of envelopes induced was 10-fold greater in the presence of ionophore. It was also observed that envelopes of all cell lines lacked the phase density or highly refractile nature that is observed in envelopes which have formed spontaneously or following Ca2+-permeabilization in cultured NHEKs.

Involucrin Content and Transglutaminase Activity. Involucrin levels in the cell lines were compared by immunoblotting equal quantities of cell cytoplasmic protein (Fig. 3). All of the squamous lines, with the exception of NCI-H520, expressed definite, readily measurable amounts of involucrin. Four nonsquamous NSCLC lines were clearly positive also and expressed quantities of cell cytoplasmic protein (Fig. 3). All of the squamous NSCLC lines were clearly positive also and expressed quantities of cell cytoplasmic protein (Fig. 3).

Table 3 Determination of involucrin levels in NCI cell lines. Cytosolic extracts (40 µg of protein) were electrophoresed in 10% polyacrylamide SDS gel and transferred to nitrocellulose. The blots were incubated with a primary rabbit anti-human involucrin antiserum, followed by a goat anti-rabbit biotinylated secondary antibody, and detected by an avidin-biotinylated peroxidase complex. Lanes 1–3, squamous cell lines; lanes 4–6, nonsquamous NSCLC; lanes 7–9, SCLC.

Fig. 3. Immunoblot determination of involucrin levels in NCI cell lines. Cytosolic extracts (40 µg of protein) were electrophoresed in 10% polyacrylamide SDS gel and transferred to nitrocellulose. The blots were incubated with a primary rabbit anti-human involucrin antiserum, followed by a goat anti-rabbit biotinylated secondary antibody, and detected by an avidin-biotinylated peroxidase complex. Lanes 1–3, squamous cell lines; lanes 4–6, nonsquamous NSCLC; lanes 7–9, SCLC.

Fig. 4. Total cellular transglutaminase activity is compared similarly (see Fig. 6C). The results demonstrate that the cell lacked keratinocyte transglutaminase or “Peak I” activity (11) to an estimated lower limit of detection of 2% of total cell activity based on visual inspection of the figure.

Both the NCI-H226 and NCI-H322 lines also contained proteins immunoreactive to the CUB 7401 antibody at the molecular weight of tissue transglutaminase. These data give further evidence that the process of cross-linked envelope formation in the NCI-H596, NCI-H226, and NCI-H322 cell lines is not entirely normal since it relies on the tissue but not the keratinocyte form of transglutaminase.

Fig. 7 demonstrates, however, that several of the lines described in Table 1, all of which are capable of forming cross-linked envelopes, express a Mr 195,000 protein which is incorporated into NHEK cross-linked envelopes induced by high salt in culture (16, 17). The Mr 195,000 protein was detected by the antibody AE11 on immunoblots of cell particulate material, and relative intensity of staining was NCI-H322, NCI-H226 > NCI-H596, NCI-H157. Staining appeared to be completely absent in the line NCI-H226.

Expression of Keratinocyte Transglutaminase in Nude Mouse Xenografts of Squamous Lines. Of the cultured cell lines described, several could be successfully grown as nude mouse xenografts. Frozen sections of these tumors, as well as the tumor NCI-H1072, which was not available in cell culture, were stained for both involucrin and keratinocyte transglutaminase. The antibody B.C1, directed to keratinocyte transglutaminase, was purified and biotinylated to eliminate the background caused by second antibody detection of endogenous mouse IgG.
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Table 4 Immunoprecipitation of keratinocyte transglutaminase in lung carcinoma line NCI-H157

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Transglutaminase activity in immunoprecipitate</th>
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<tbody>
<tr>
<td>B.C1</td>
<td>4,136</td>
</tr>
<tr>
<td>B.D4</td>
<td>2,391</td>
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<tr>
<td>Control*</td>
<td>309</td>
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</table>

* [3H]Putrescine incorporation into dimethylated casein (average of two assays). Activity of transglutaminase added to assay is 6,500 cpm.

Fig. 4. Quantitation of involucrin in NCI cell lines according to densitometry tracings of immunoblots.

Fig. 5. Transglutaminase activity in NCI cell lines. Total cpm were corrected by subtraction of buffer blank values prior to calculation of specific activity. Results are given as the mean of triplicates. Coefficients of variation for lines with greater than 100 cpm/µg protein ranged from 3 to 41%, with the range never falling below this specific activity.

In Fig. 8, the NCI-H1072 tumor xenograft shows expression of both involucrin and keratinocyte transglutaminase. Staining for both proteins was located within and adjacent to regions of keratin pearl formation and did not usually extend to the stromal boundary. Both antigens presented irregular patterns also noted in involucrin staining of squamous cell carcinomas of skin and lung (9, 18).

The NCI-H596 xenograft also reacted to antiinvolucrin antibodies; in addition, B.C1-biotin stained similar regions where differentiation appeared to be most advanced (Fig. 9). This is in direct contrast to the behavior of NCI-H596 in culture, where it lacks keratinocyte transglutaminase activity. The NCI-H322 cell line was also positive for involucrin but, unlike NCI-H596, was negative for keratinocyte transglutaminase (Fig. 10). The NCI-H292 cell line was also negative for immunohistological determination of keratinocyte transglutaminase; an unexplained high background in this tumor when stained by normal rabbit serum prevented a clear demonstration of the presence of involucrin in the xenograft although it is clearly detectable in the culture cell. Cell line NCI-H157 was not available as a nude mouse xenograft for these experiments.

DISCUSSION

Of the pulmonary carcinoma cell lines examined in this study, those of squamous morphological character have a unique capability to form cross-linked envelopes in culture. Consistent with this observation, the squamous cell lines also expressed...
transglutaminase activity, which is essential for CLE formation, and involucrin, a constituent of CLEs in normal squamous epithelia. Several nonsquamous NSCLC lines also expressed involucrin at levels comparable to squamous lines, but they did not form CLEs or express substantial transglutaminase activity. SCLC lines were completely negative for all three markers.

In an earlier study, both squamous and nonsquamous pulmonary carcinomas were reported to form CLEs (2). The nonsquamous CLE-forming line described was NCI-H226, which is reclassified here as a moderately differentiated squamous cell carcinoma based on xenograft and cultured cell morphology. None of the nonsquamous lines analyzed in this study formed CLEs. The exceptional nonsquamous NSCLC line, NCI-H520, a poorly differentiated SCC with occasional keratinizing cells in the original tumor and in xenograft, did not form CLEs and lacked transglutaminase activity when tested in culture. Involucrin, although present, was at an extremely low concentration. This discrepancy may result from the differing behavior of the cell line in culture and in xenograft tumor. Since seven of eight squamous lines do form CLEs in culture, this property can be considered a very reliable indicator of squamous character.

The cross-linked structures formed in the lung cancer cell lines were considerably more fragile and less robust in appearance than those of cultured NHEK, which in turn are less refractile and uniform in appearance than CLEs extracted from epidermis in vivo (31). Envelope character and chemical composition also depend on depth within the stratum corneum, pathological state (normal or psoriatic) and, in culture, the presence or absence of retinoic acid (31, 32). There were substantial quantitative differences in CLE formation among the NSCLC lines (see Fig. 2). Furthermore, CLE formation was independent of method of stimulation (by Ca²⁺-ionophore or 0.8 M NaCl) in some cell lines but not in others (Table 2), suggesting that the squamous lines differed not only in the quantity of envelope precursors present, but their kind as well.

Involucrin was prominent in squamous cell lines, but was not limited to them. This confirmed an earlier immunohistological analysis of lung tumor biopsies by Said et al., who found involucrin in all squamous cell carcinomas, two of 20 adenocarcinomas, and six of 12 large cell undifferentiated carcinomas analyzed (9). In normal tissue, involucrin is found only in stratified squamous epithelia (8), including regions of pulmonary squamous metaplasia (9). The present studies clearly confirm that involucrin can occur in neoplastic cells which lack any definitive morphological evidence of squamous character.

In contrast to involucrin content, the level of transglutaminase activity clearly demarcates squamous from nonsquamous NSCLC lines (Fig. 5). It appears that a critical amount of transglutaminase activity is needed in order for CLE formation to be successful. At the same time, CLE formation in the squamous lines themselves was poorly correlated with transglutaminase activity on a quantitative basis (compare NCI-H157 and NCI-H125, for example), and this was also true for involucrin content. The content of M, 195,000 envelope precursor protein, which was present in a subset of squamous lines tested, likewise lacked predictive value for CLE formation (Fig. 7) and
appeared to be absent from at least one CLE-forming line. It is likely that there are other squamous cell-specific envelope precursor proteins yet to be identified. For example, neither involucrin nor any comparable cytoplasmic transglutaminase substrate have been identified in cultured keratinocytes of any species except primates, yet rodent keratinocytes nonetheless do form excellent cross-linked envelopes (27, 32-34). Such unidentified cross-linked envelope precursors may have an important influence on CLE formation in the cell lines studied and have better predictive value for squamous morphological character than involucrin or the M, 195,000 protein. The M, 57,000–59,000 cytokeratins were present in pulmonary squamous lines tested in an earlier study (2), but there is no evidence that desmosomal components or keratins are incorporated into the CLE (7, 16, 32).

Perhaps the most striking observation was that while all cell lines with in vitro capability to form cross-linked envelope structures had substantial transglutaminase activity, as expected, only one of the lines, NCI-H157, clearly expressed the keratinocyte transglutaminase. Transglutaminase in cell particulates of other cell lines tested did not react to monoclonal antibodies specific to keratinocyte transglutaminase. A detailed examination of cultured NCI-H596 by ion-exchange chromatography also confirmed the absence of keratinocyte transglutaminase. NCI-H596 and other squamous cell lines tested have tissue transglutaminase, a ubiquitous enzyme of liver, red blood cells, macrophages, and other tissues (21, 35, 36). Rubin and Rice have also reported a cultured squamous cell carcinoma of tongue (SCC-4) capable of forming CLEs which expresses tissue transglutaminase (19). Although tissue transglutaminase expression can occur in cultured keratinocytes, it does not appear to be involved in squamous differentiation. It is stimulated by treatment of cultures with retinoids, but CLE formation is simultaneously depressed (21). Studies of keratinocyte transglutaminase subcellular localization (15, 20, 37), on the other hand, strongly support its involvement in normal cornification.

In confirmation of cell culture studies, only a minority of involucrin-positive xenografts stained with antibody to keratinocyte transglutaminase (Table 5). The NCI-H596 xenograft, which is apparently negative in culture, expressed keratinocyte transglutaminase in the nude mouse xenograft. It is uncertain whether this is due to clonal evolution in the nude mouse, that is, expansion of a tiny minority of cells which constitutively express the enzyme, or whether it is a specific response of the cell to a new environment, in analogy to the results of Doran et al. (38) who transplanted cultured rabbit epithelial cells to the nude mouse and observed expression of more highly differentiated character. Two other cell lines, NCI-H292 and NCI-H322, also capable of cross-linked envelope formation in culture, were uniformly negative with respect to staining for keratinocyte transglutaminase by the B.C1 antibody in nude mouse xenograft.

Expression of keratinocyte transglutaminase is a normal part of nonneoplastic squamous differentiation in airway epithelia, and the absence of keratinocyte transglutaminase in CLE-forming carcinoma lines was quite unexpected. In culture, bronchial epithelial cells undergo squamous differentiation and form cross-linked envelopes either spontaneously or as a result of ionophore treatment (30, 39). There is substantial keratinocyte transglutaminase activity in the particulate fraction of cultured normal human bronchial epithelial cells as determined by immunoprecipitation with specific antibodies. A study of hamster tracheal squamous metaplasia has also shown that keratinocyte transglutaminase is expressed only in metaplastic regions but not in normal secretory or ciliated cells (14).

If the loss of keratinocyte transglutaminase expression is a more or less random event which takes place along the cancer cell’s evolution to neoplasia, one would predict no strong correlation between its expression and cellular characteristics conferring malignancy. A contrasting perspective has been suggested by Greaves (40), who has pointed out that specific types of leukemic cells seem to conserve or stabilize well-defined normal cellular types which are expressed only transiently during hematopoietic differentiation. In the case of pulmonary carcinomas, an example of this stabilization may be found in the resemblance between mucoepidermoid and adenosquamous carcinomas and an infrequent mucoid-squamous “dual phenotype” which is observed during vitamin A-deprived tracheal squamous metaplasia in hamster (6). The absence of keratinocyte transglutaminase in CLE-forming cell lines appears to be abnormal when the comparison is made to cells of healthy squamous epithelia. It is also possible, however, that a cell type capable of attenuated CLE formation in the presence of tissue transglutaminase alone does appear briefly during squamous metaplasia or epithelial wound healing.

The data presented here suggest a biochemical grading system for levels of squamous differentiation in pulmonary NSCLC tumors may be useful. The most advanced lines would be those which express both keratinocyte transglutaminase and involucrin.

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3 S. M. Thacher and A. M. Jetten, unpublished observations.
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Fig. 10. Staining of NCI-H322 as xenograft tumor in nude mouse. a, normal rabbit serum (preimmune) (1:250); b, rabbit antiseraum to involucrin (1:250); c, biotinylated B.C1 (2 μg/ml). Staining with antinvolucrin antibodies is positive but staining for keratinocyte transglutaminase is not. Bar = 40 μm.

Table 5

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</table>

* NA, NCI-H1072 was not available in culture.

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