Structural Anomalies of Highly Malignant Respiratory Tract Epithelial Cells

R. L. Manger and C. A. Heckman

ABSTRACT

These studies were designed to determine whether cytostructural changes were related to malignancy and the loss of growth control in epithelial cells. Three highly malignant cell lines were derived from transplantable carcinomas of the respiratory tract and compared with three respiratory tract epithelial lines of negligible malignancy. Keratin cytoskeletons were visualized by indirect immunofluorescence staining, and sample photomicrographs representing each line were prepared. Observers naïve to the immunofluorescent images were asked to classify the encoded photomicrographs into six groups, corresponding to the cell lines, and then into two major classes, each containing three of the original groups. We recorded and analyzed the criteria used in making the classifications to identify the features common to the highly malignant lines. These included the nonuniform spacing of cells in the field of view, the cell shape, and the presence of nonfluorescent areas in the lamellar cytoplasm. The criteria that were not useful for discriminating between images from the malignant lines and those of negligible malignancy were the cell size, the prominence and structure of the filaments and their integration into desmosomes, and the appearance of the nucleus. Since the nonuniformity of keratin distribution in the periphery of the malignant cells suggested a structural anomaly, the cell lines were also examined by scanning electron microscopy. Unlike cells from the lines of negligible malignancy, cells from two of the highly malignant lines showed thickening in the subterminal portions of the lamellar cytoplasm. The results suggested that specific architectural changes at the cellular level might be linked to the process of epithelial transformation and tumor progression.

INTRODUCTION

Because many of the behavioral characteristics of cells are mediated by their cytoskeletal structure, a number of investigators have compared the cytoskeletal organization of normal and oncogenically transformed cells. Of the 3 major cytoskeletal units found in cells, actin microfilaments, intermediate filaments, and microtubules, all have been found to be altered in one or more types of transformed cells in comparison to their normal counterparts (3, 7, 25, 28, 30, 36). However, one of the major drawbacks in using the transformation of cultured cells as a model system for cancer is that these systems mainly consist of fibroblasts whereas the majority of clinical cancers originate in the epithelium (29).

Cytoskeletal alterations have been observed in carcinoma cells in vivo, most notably as an increase in the appearance of microfilaments in the invasive edge of malignant tumors (9, 10, 21, 24). Cytoskeletal constituents were also altered in either target cells or noninvolved cells from humans with hereditary syndromes that include a predisposition to develop cancer (11, 20, 35). It is only recently that model systems appropriate for investigating these changes by cell biological and biochemical approaches have been developed. In one such model, normal and neoplastic mouse mammary gland cells in vitro showed no difference in the organization of microtubules or actin-containing filaments (1). Subsequently, in a detailed study of 13 cultured human mammary carcinoma cell lines, Brinkley et al. (4) found that 4 lines had a pattern of microtubule organization similar to that of normal mammary epithelial cells (type I) and 9 had at least some cells with a diffuse pattern of organization (type II). Thus, some of the lines had deviations from the normal structural phenotype. In a comparison of the actin microfilaments of cultured primary hepatocytes and hepatoma cells, the structures appeared less prominent in the hepatoma cells (26). A bladder epithelial cell line, oncogenically transformed in vitro by benzo(a)pyrene treatment, acquired vimentin filaments during subsequent growth in culture (34). Perhaps the most thoroughly characterized model system, however, consists of spontaneously transformed mouse keratinocytes. Cells from one transformed line were found to acquire vimentin filaments upon transformation (8), but a later study comparing 12 tumorigenic lines with normal cells did not confirm the appearance of vimentin filaments (39).

The results of the above studies on cytostructural alterations in epithelial model systems frequently indicated no changes that could be correlated with transformation. However, previous data from this laboratory suggested that such alterations could be peculiarly difficult to detect in epithelial cells. Studies on respiratory tract epithelial cells which became oncogenically transformed in vitro showed that the process was accompanied by changes in the shapes of cells in colonies (14). Although the changes were more subtle than those typical of viral transformation in fibroblasts (12, 18), they suggested the possibility that cytoskeletal integrity or intercellular adhesion was also altered in a similar way during transformation of epithelial cells. To determine whether these structural anomalies could be detected and studied at the cellular level and to see whether there were any anomalies common to transformed epithelial cells, we derived highly malignant cell lines from rat respiratory tract tissues. Since each of these lines produced tumors in 50% of the animals at a tumor cell dose of 100 cells or less, it was clear that populations sampled by microscopical methods would contain one or more malignant stem cells. This report deals with the architecture of keratins in cells from the highly malignant lines as compared to that in lines of lesser or negligible malignancy and describes structural features common to the highly malignant lines.
Derivation of Cell Lines. All cell and tumor lines were derived from respiratory airway tissues of specific-pathogen-free, inbred F344 rats. The highly malignant cell lines were derived from in vitro-transplantable carcinoma lines, which had been serially transplanted i.m. in isogenic hosts as described previously (16). The MCA-1 line was derived after induction of the primary tumor by intratracheal injections of 3-methylcholanthrene. The B2-1 cell line was cultured from the BP, serially transplanted tumor as described previously (16, 31). The BP3-O line was derived similarly from a tumor originally obtained after administration of benzo[α]pyrene to a heterotopic tracheal transplant (14).

Of the cell lines of lesser or negligible tumorigenicity, one was derived from 7,12-dimethylbenz(a)anthracene-treated heterotopic tracheal transplants and 2 from tracheal organ cultures treated with 12-O-tetradecanoylphorbol-13-acetate (22, 33). These were designated 165S, 2C1, and 4C9, respectively. The cells were maintained as described in previous publications (14, 22, 31, 33).

Tumorigenicity Testing. All of the cell lines were tested for tumorigenicity in irradiated isogenic host animals. The cells were injected in parallel into immune competent and irradiated hosts in order to determine whether immune suppression had a marked effect on their tumorigenicity. F344 rats 5 to 9 weeks of age were given 600 rads of whole-body X-irradiation produced by a GE Maxitron device fitted with a 3-mm aluminum filter. The animals were given injections in the thigh of varying doses of cells 2 days after irradiation. The animals were palpated weekly and those with progressively growing tumors were considered positive. The relative tumorigenicity was determined based on the number of tumor-bearing animals at times up to 22 weeks after the injection of known numbers of cultured cells. The longest latent period observed for any of the lines tested was 14 weeks. The cultured lines considered highly malignant were all found to produce invasive, keratinizing squamous cell carcinomas (16). All formed metastases in the mediastinal lymph nodes following injection in the thigh muscle. In addition, the B2-1 cell line also metastasized to the lung.

Cell lines showing little or no evidence of tumorigenicity after injection of 1000 cells were tested in athymic nude mice to further define their tumorigenicity. Cells from each line were injected into 4 weanling mice at doses of 5 x 10⁶ and 2 x 10⁷. The animals were retained for at least 24 weeks with monthly palpations.

Preparation and Classification of Immunofluorescence Data. At passage levels similar to those subjected to tumorigenicity testing, cells were removed from liquid nitrogen, thawed, and subcultured at least twice before use in experiments. All cell lines were maintained in Waymouth’s medium (Grand Island Biological Co.) containing 10% fetal bovine serum (Reheis Chemical Co.) and added amino acids, putrescine, sodium pyruvate, insulin, and hydrocortisone (13). The medium also included 50 μg streptomycin per ml and 50 units penicillin per ml.

For indirect immunofluorescence studies, cells from confluent cultures were transferred into dishes containing acid-cleaved 12-mm round coverslips. The plating density was 1.2 x 10⁶ cells/100-mm dish. The coverslips were removed 2 days later, rinsed for 5 min in PBS, pH 7.2, at room temperature, and immersed in absolute methanol (Reheis Chemical Co.) and added amino acids, putrescine, sodium pyruvate, insulin, and hydrocortisone (13). The medium also included 50 μg streptomycin per ml and 50 units penicillin per ml.

Highly malignant cell lines: tumorigenicity in tests at low doses in syngeneic hosts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Host irradiation</th>
<th>No. of tumor-bearing animals after injection of various cell doses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 x 10⁴</td>
</tr>
<tr>
<td>B2-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T36</td>
<td>−</td>
<td>2/5</td>
</tr>
<tr>
<td>T37</td>
<td>+</td>
<td>ND⁵</td>
</tr>
<tr>
<td>BP3-O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T21</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>MCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>−</td>
<td>1/5</td>
</tr>
<tr>
<td>T21</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

The abbreviation used is: PBS, phosphate-buffered saline.

RESULTS

Tumorigenicity Determinations. To obtain populations of cells with defined neoplastic potential, we tested 10 cell lines derived from the rat respiratory tract epithelium for in vivo tumorigenicity. This number included 4 lines from transplantable squamous cell carcinomas and 6 lines derived after in vivo or in vitro carcinogen treatment of the epithelium. We defined as highly malignant those cell lines that produced tumors in 50% or more of the syngeneic hosts at a dose of 100 cells (Table 1). Lines showing little or no evidence of tumorigenicity when injected into syngeneic hosts at a dose of 1 x 10⁵ cells were retested at high doses in athymic nude mice. Those lines that produced tumors in 50% or fewer of these animals at doses approximating 1 x 10⁶ cells (Table 2) were considered to be of negligible tumorigenicity. Thus, populations of cells differing in tumorigenicity by at least an order of magnitude were available for matrix comparisons.
The 6 cell lines used in these studies could be distinguished from one another on the basis of their phase-microscopic appearance (Fig. 1). However, highly malignant lines (Fig. 1, A to C) did not appear to differ from the lines of negligible malignancy (Fig. 1, D to F) in any features consistently seen at this level. Cells from all of the lines stained positively with anti-K, keratin antibody (Fig. 2), confirming their epithelial origin. That the filaments studied were composed of keratin was indicated by the absence of any fluorescence in a filamentous pattern in control samples treated with nonimmune serum rather than anti-K, keratin antibody (Fig. 2), and a third identified similar images as “footpads.” The terms referred to a “fan-shaped arrangement on the perimeter of cells,” while a third identified similar images as “shadowy ends” to describe an uneven pattern of fluorescence in the lamellar cytoplasm of cells from the B2-1 and BP3-0 lines. A second observer referred to a “fan-shaped arrangement on the perimeter of cells,” while a third identified similar images as “footpads.” The terms were all used to describe cells which showed fluorescence at the extreme edge, producing a mottled appearance in the

**Structural Anomalies of Respiratory Tract Epithelial Cells**

Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of tumor-bearing animals after injection of various cell doses</th>
<th>5 x 10^6</th>
<th>2 x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C1</td>
<td></td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>T13</td>
<td></td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>T23</td>
<td></td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>4C9</td>
<td></td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>T16</td>
<td></td>
<td>2/2</td>
<td>1/2</td>
</tr>
<tr>
<td>T30</td>
<td></td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>165S</td>
<td></td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>T32</td>
<td></td>
<td>0/2</td>
<td>1/2</td>
</tr>
</tbody>
</table>

* MCA7, the most immunogenic of the highly malignant lines, formed tumors in 2 of 2 host animals at a dose of 1 x 10^2 cells.

**Light-Microscopic Observations and Observer-based Classifications.** The 6 cell lines used in these studies could be distinguished from one another on the basis of their phase-microscopic appearance (Fig. 1). However, highly malignant lines (Fig. 1, A to C) did not appear to differ from the lines of negligible malignancy (Fig. 1, D to F) in any features consistently seen at this level. Cells from all of the lines stained positively with anti-K, keratin antibody (Fig. 2), confirming their epithelial origin. That the filaments studied were composed of keratin was indicated by the absence of any fluorescence in a filamentous pattern in control samples treated with nonimmune serum rather than anti-K, keratin antibody (Fig. 2, D and H). Control preparations treated with buffer rather than antiseraum lacked noticeable fluorescence (data not shown).

Preliminary examination of immunofluorescence images from 2 lines of negligible malignancy and 2 highly malignant lines suggested that cells from the highly malignant lines might be distinguished from less malignant cells by their structural features. To test this hypothesis, we asked individuals who were unfamiliar with the immunofluorescence images to classify photomicrographs sampled from each of the 6 lines into 6 groups of equal size. In a second-level exercise, they were asked to combine the groups into 2 major classes, each containing 3 of the original groups and to describe the criteria they used for discriminating among the groups and classes. The individuals made up their own criteria for classification.

We asked 8 individuals, only 4 of whom had prior experience in microscopy, to carry out the exercise. The extent to which the observers confused any one cell line with another was indicative of the similarity between the 2 lines. The MCA7 cells were identified correctly as a single group by 5 of the 8 observers, and when misclassified, were grouped with samples from the BP3-0 or B2-1 lines. Only 2 of the observers recognized all samples from the BP3-0 line as belonging to a single group, the others classifying some samples with those from the B2-1, 4C9, or MCA7 lines. B2-1 cells were recognized as a single group by 2 observers and misclassified by the others into any one of the remaining 5 groups. The 4C9 line was identified as a group by 3 of the observers, the rest combining samples with representatives of all the other lines except MCA7.

All of the observers confused the 2C1 and 165S lines with one another. In a few cases, samples from the 165S line were grouped with any one of the other lines, except MCA7. Samples of 2C1 cells were occasionally grouped with those from the 4C9, B2-1, and BP3-0 lines. The number of observers who classified the majority, 6 to 10, of the samples from a single cell line into a single group could be used as an index of the "uniqueness" of the lines. When this analysis was done, we found that observers identified the MCA7, BP3-0, and 4C9 lines more readily as groups than the other lines (Chart 1). We noted that those individuals having the least experience with microscopy had the greatest difficulty in grouping together the samples taken from a single cell line.

**Features Used for Classification of Immunofluorescence Images.** In analyzing the results of the second-level classification challenge, in which the observers combined the 6 groups established in the first sorting operation into 2 major classes, we found that 7 of the 8 observers placed the highly malignant lines MCA7 and BP3-0 into one class and the lines of negligible malignancy, 2C1 and 165S, into the other. One-half of the observers placed all 3 highly malignant lines in one class, and all 3 lines of negligible malignancy in the other. The remainder combined highly malignant MCA7 and BP3-0 lines with the 4C9 group. One observer combined the MCA7, B2-1, and 165S lines into a single class. This was an unusual classification, and the criteria for making this discrimination were analyzed. They appeared to involve the pattern of integration of the keratin filaments into the desmosomes joining the cells. Examples of the more typical classification patterns obtained on decoding the photomicrographs are shown in Table 3.

The criteria used by the observers who formed a single class from the groups containing highly malignant lines were of interest. We thought a compilation of these criteria would define some of the features common to malignant cells. One of the observers used the term "shadowy ends" to describe an uneven pattern of fluorescence in the lamellar cytoplasm of cells from the B2-1 and BP3-0 lines. A second observer referred to a "fan-shaped arrangement on the perimeter of cells," while a third identified similar images as "footpads." The terms were all used to describe cells which showed fluorescence at the extreme edge, producing a mottled appearance in the

**Table 2**

| Cell lines of negligible malignancy: tumorigenicity in tests at high doses in immune deficient animals |
|---------------------------------------------------------------|------------------|
| Number of tumor-bearing animals after injection of various cell doses | 5 x 10^6 | 2 x 10^6 |
| 2C1 | T13 | T23 |
| 4C9 | T16 | T30 |
| 165S | T32 |

* MCA7, the most immunogenic of the highly malignant lines, formed tumors in 2 of 2 host animals at a dose of 1 x 10^2 cells.

**Chart 1.** The number of observers who classified the majority of samples from a cell line into one group was an index of how readily the line was recognized. The "uniqueness" of lines correlated poorly with their malignancy.
images (Fig. 3). Another observer described a more even distribution of fluorescence within cells from the 3 lines of negligible malignancy, terming this "material more evenly distributed throughout the field." Additional criteria that were used to combine the groups of highly malignant cells were the irregularity in cell shape and the tendency for overlapping to occur among the cells. These criteria were deduced from phrases used by observers who described the combined cells as being "out of shape," "pulled apart (not round)," and "overlapping and stretching over each other." We compared the immunofluorescence images with the criteria used by observers who classified the highly malignant lines together. The MCA cells seldom exhibited the uneven peripheral fluorescence seen in the other highly malignant lines but showed considerable overlapping of the cells and pronounced distortion of their shape (Fig. 2A). The B2-1 and BP3-0 cells frequently showed bright peripheral fluorescence at the edges of the cytoplasm next to less intensely stained areas (Figs. 2, B and C, and 3). These features were especially obvious in B2-1 cells, which were referred to by one observer as being "very, very shadowy."

The criteria used by those observers who classified the 4C9 line with the highly malignant MCA, and BP3-0 lines were quite different from those discussed above. These individuals relied on such features as the sharpness, dimensions, and arrangement of the keratin filaments, the appearance of the nucleus, the cell size, and extent of contact among the cells. One observer described this combined group of cells as having "more pointed and sharp' filaments. A second observer characterized the group of cells as being diminished in "size and number of contacts." The architecture of the keratin cytoskeleton was similar for 2 lines of negligible malignancy, 2C1 and 165S. These cells contained fine filaments that were often radially arranged in the cytoplasm and that rarely appeared to aggregate (Fig. 2, F and G). Cells of the 4C9 line differed in that the filaments appeared to aggregate readily into prominent thick fibrils and the shapes of the cells were somewhat irregular (Fig. 2E). In the second classification, we were unable to note any difference between observers depending on their experience with light microscopy.

In the second classification exercise, the observers generated 2 major classes from the 6 formed in the original sorting task (Table 3). Of the 10 samples representing a cell line, the proportion placed in each class depended largely on the extent of confusion among the samples as they were classified in the first exercise. In considering the sorting operations of the observers, we arbitrarily designated the class containing the cells most readily discriminated, the MCA cells, as Class I and determined how often representatives of each line were placed into this class. Class I was found to include the following proportions of samples: 100% of MCA; 84% of BP3-0; 56% of B2-1; 35% of 4C9; 10% of 165S; and 1% of 2C1 (Chart 2). Thus, the average observer perceived the MCA-BP3-0 cell lines and the 165S-2C1 lines as phenotypic extremes and placed them in different major classes, regardless of the extent to which they confused samples within these groups in the initial classification exercise.

Scanning Electron-Microscopic Observations. The pattern of keratin localization in the lamellar cytoplasm of malignant cells showed keratin-poor or diffusely stained areas adjacent to the intensely fluorescent margin of the cell, suggesting that the areas represented structural features, such as ruffles. When the cells were examined by scanning electron microscopy, the only structural feature that appeared to correlate with the keratin-poor areas were thickenings in the lamellar cytoplasm of BP3-0 and MCA cells. Many of these cells were characterized by lamellar cytoplasm which tapered off abruptly at the cell edge (Fig. 4, A and B). However, thickenings, ruffles, blebs, and other features were not seen in B2-1 cells, which also displayed discontinuities in the fluorescent images. Cells from lines of negligible malignancy had lamellar regions that were flat on the culture surface (Fig. 4, C and D).

DISCUSSION

Changes in cell shape have been found universally in models for the physical, chemical, and viral transformation of mesenchymal cells. These changes, as seen by phase microscopy, are typically described in terms of reduced lamellar spreading.
or more rounded or spindle-shaped cells. Particularly for the transformation of mesenchymal cells by viruses, the cellular basis of shape changes is thought to relate to the production of proteins with tyrosine-specific protein kinase activities. It is clear that the transforming activity of 4 viruses is due to proteins with this activity: Rous sarcoma virus (6); Abelson murine leukemia virus (38); Snyder-Theilin feline sarcoma virus (2); and Y73 avian sarcoma virus (19). Cell shape could be modified in various ways by the elevation of kinase activity, since these kinases phosphorylate certain cytoskeletal proteins. In Rous sarcoma virus-transformed cells, the tyrosine residues of vinculin were phosphorylated most heavily, with myosin heavy chains, α-actinin, filamin, and vimentin undergoing phosphorylation to trace levels (32).

The extent to which these changes in mesenchymal cells serve as a model for transformation of epithelial cells, the common targets of clinically relevant carcinogenic events, was unknown. As pointed out in the "Introduction," changes in cytoskeletal organization were less prevalent in epithelial than in mesenchymal models for transformation. Comparisons between established tumorigenic and nontumorigenic cell lines failed to show consistent differences in cell shape. Lines derived from liver showed such differences (27) whereas those of mammary gland origin did not (1, 5, 37). However, subtle modifications in cell shape were seen in lines that became tumorigenic over the course of in vitro culture (14, 17). A major problem in these comparisons has been that the epithelial models available for comparisons have been few and poorly characterized in relation to models for mesenchymal cells.

To see whether changes in cytoarchitecture were prevalent among epithelial cells, we evaluated the tumorigenic potential of cell lines derived in the course of previous studies (14, 16, 22, 31, 33). Having defined 3 lines of marginal tumorigenicity and 3 highly tumorigenic lines, we compared them with respect to the cytoarchitecture of the keratins, the predominant constituent of the cytoskeleton in these cells. Interestingly, human observers found similarities among the highly malignant cell lines, based on the visualization of their keratin cytoskeletons. The observers who were able to group cells from the malignant lines together relied on the irregularity of cell shape, the tendency for cells to be grouped together in the field or to overlap, and a pattern of bright fluorescence at the cell edge. Other criteria, such as cell size and the dimensions or arrangement of keratin fibrils within the cells, did not appear of value for classifying together the groups of cells with similar biological potential.

These results indicate that there are certain structural features of highly malignant cells which provide recognizable signals to human observers. Given the complexity of human visual psychology, these signals are not expected to provide us with new "markers" for tumorigenic potential. However, they are important in that they suggest these cells share common biophysical and/or biochemical properties and indicate the kinds of features that must be studied further at the cellular level. For example, the cellular basis of the terminal distribution of keratins in highly malignant cells should be elucidated. Other studies at this level should be directed to answering such questions as: What mechanism is used by cells to achieve a uniform spacing relative to one another? What cytostructural constituents mediate alterations of cell shape in epithelial cells?

The present results suggest that cytoarchitectural changes are common in transformed epithelial cells, but that they may be more subtle than observed in the more familiar mesenchymal cell models for transformation. Such changes in the more commonly studied model systems have recently been attributed to the phosphorylation of tyrosine residues in cytoskeletal proteins, particularly vinculin (15, 32). Perturbing the structure of this protein, which is concentrated at sites of actin-membrane interaction, could alter the properties of the attachment plaques that hold the cell surface in close apposition to the substratum. The major unanswered question is how this perturbation could affect the properties of cells so as to make them tumorigenic. For epithelial cells, it is unlikely that adhesive functions are mediated by the same proteins and that the same molecular mechanism would be implicated in the architectural changes accompanying transformation. Thus, the finding of such architectural changes in independently derived, highly malignant epithelial cell lines suggests that the modulation of cytoarchitecture is itself an important aspect of cellular transformation. Whether these modulations, like those of mesenchymal cells, depend on the perturbation of intracellular cytoskeletal structures remains to be determined. Since the sorting patterns of human observers suggested that the actual dimensions and organization of keratin filaments were poorly correlated with the malignant potential of cells, we suspect that more minor constituents of the cytoskeleton are likely to give rise to the observed alterations. One of the most compelling questions raised by these studies is whether there are behavioral alterations on the part of transformed cells based on the structural anomalies seen in these and in the better-understood mesenchymal model systems. Such behavioral changes are currently under investigation in this laboratory, as they would offer a rational pretext for the observed prevalence of architectural changes in transformation.

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REFERENCES


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Fig. 1. Phase micrographs of highly malignant cell lines (A to C) and lines of lesser malignancy (D to F). A, MCA; B, B2-1; C, BP3-0; D, 4C9; E, 2C1; F, 165S. x 100.
Fig. 2. Keratin cytoskeletons of highly malignant cell lines (A to C) and lines of lesser malignancy (E to G). A, MCA; B, B2-1, showing sidewise aggregation of filaments (arrow); C, BP3-0, showing the prominent peripheral fluorescence characteristic of malignant cells; D, BP3-0, nonimmune serum control; E, 4C9, showing some aggregation of filaments; F, 2C1; G, 165S, showing a relatively uniform distribution of filaments subcellularly; H, 165S, nonimmune serum control. × 750.
Fig. 3. Lamellar cytoplasm of a cell from a highly malignant line showing discontinuous areas of immunofluorescence. × 1700.

Fig. 4. Scanning electron micrographs of cells from highly malignant cell lines (A and B) and lines of negligible malignancy (C and D). A, MCA-; B, BP3-0; C, 4C8; D, 165S. Cells from the highly malignant lines frequently showed thickening in the lamellar cytoplasm (arrows). The lamellar cytoplasm of the other cell lines contained no thickened areas but occasionally showed ruffles at the extreme edge. × 1000.
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