Morphological Markers of Oncogenic Transformation in Respiratory Tract Epithelial Cells

C. A. Heckman and A. C. Olson

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

ABSTRACT

The purpose of the present studies was to determine whether changes in cell shape and microvillar density accompanied oncogenic transformation of rat respiratory tract epithelial cells. Two cell lines which became oncogenic during in vitro culture (1000 W and 165 S) were studied. In relatively late passages, but not in early passages, the lines produced keratinizing squamous cell carcinomas when tested in syngeneic hosts. Small colonies, predominantly of clonal origin, were obtained at early and late times after initiation of the lines into in vitro culture. Scanning electron microscopic studies showed that preoncogenic and oncogenic populations differed with respect to the shapes of cells within colonies. Differences in cell shape were further analyzed by estimation of the height and the ratio of length to width for 20 cells sampled from each colony. Each cell was assigned to one of nine classes of cell shape. The frequency with which spindle-shaped cells were observed in colonies increased 3-fold with oncogenic transformation of the 1000 W and 165 S lines. The frequency did not increase during in vitro culture of a third highly oncogenic cell line, BP 3-0. The frequency of observation of spindle-shaped cells in the 1000 W line was not decreased by in vivo growth and rederivation. In fact, the tumor-derived subline, 1000 WT, had a 5-fold greater frequency of expression than did an early passage of the 1000 W line. The number of colonies in which this cell shape was observed also increased 5-fold and came to include nearly one-half of the colonies analyzed. Therefore, expression of spindle shape became prevalent in clonal subpopulations of the line. In early passages of the 1000 W and 165 S lines, most spindle-shaped cells were found at the edges of colonies. This observation suggested that the spindle shape was assumed in response to forces generated during colony expansion. In general, the 1000 W line, which was more oncogenic than the 165 S line, also showed more pronounced morphological alterations. The prevalence of ruffles was well correlated with oncogenicity in the 1000 W line. However, the cell lines differed with respect to the density of microvilli at the cell surface, and this feature did not seem well correlated with oncogenicity. The results suggested that cytoskeletal and/or adhesive mechanisms implicated in shape maintenance were altered in parallel with oncogenic transformation of epithelial cells originating from the respiratory tract.

INTRODUCTION

Previous comparisons of normal and transformed cells in vitro have shown that certain morphological features are correlated with oncogenic transformation. In light microscopic studies, decreased cytoplasmic spreading on substrata was characteristic of neoplastic rodent embryo fibroblast lines compared with normal cell lines (5, 7, 10, 11). Transformation-related changes in cell shape have also been identified by scanning electron microscopy in rodent liver cells (2, 15) and embryonic fibroblasts (4, 17, 30). In chick embryo fibroblasts infected with a temperature-sensitive Rous sarcoma virus, changes in cell shape occurred rapidly after a shift to the permissive temperature (3, 16).

In addition, scanning electron microscopic studies of normal and transformed fibroblasts have provided evidence for increased surface activity in transformed cells, in the form of prominent microvilli (4, 17, 23) or ruffles (3). In comparisons of normal and oncogenically transformed rat liver cells, the latter also exhibited increased degrees of surface activity (2, 15). However, a recent comparison of tumorigenic and nontumorigenic cell lines derived from a single clone of mouse embryo cells showed no consistent alterations in surface features, although greater variability of surface morphology was typical of the tumorigenic lines (30). In a similar study of tumorigenic and nontumorigenic rat fibroblasts in vitro, no universal pattern of surface morphology was found to be associated with tumorigenicity (6). In general, disturbances of cell shape appeared to be more characteristic of oncogenically transformed cells than was changes in surface architecture.

It is important to determine whether morphological alterations are correlated with oncogenic transformation in cells from the lining epithelia, which constitute the major sites of cancer incidence in humans. In an experimental model of respiratory carcinogenesis, cell lines derived from carcinogen-exposed respiratory airway epithelium showed anchorage independence and tumorigenicity after varying lengths of time in culture (19). The characteristics of populations similar in origin but differing in tumorigenicity have been studied in cells from sequential passages of these lines. To accomplish the present objectives, we have compared cell shape and microvillar density in cells from populations derived before and after the time at which anchorage independence and tumorigenicity were expressed. Since cell shape and surface morphology vary considerably among cells of the lining epithelium in vivo (1, 14, 25), significant morphological variability was expected in the cells studied. Therefore, the approach taken was to assess the variability of shape and surface features in cells from small colonies, predominantly of clonal derivation. By this approach, a range of variability could be defined for preoncogenic populations; thus, the appearance of new characteristics or changing frequencies of expression of existing characteristics could be detected.

MATERIALS AND METHODS

Cell Culture. All of the cell lines used were derived from specific-pathogen-free, inbred female Fischer 344 rats. Two

1 Research sponsored by the Division of Biomedical and Environmental Research, United States Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

2 To whom requests for reprints should be addressed.

Received October 12, 1978; accepted March 21, 1979.
cell lines which became tumorigenic during in vitro culture, 165 S and 1000 W, were derived from 7,12-dimethylbenz[a]anthracene-treated rat tracheal epithelium as described previously (18, 19). The question of whether alterations were reversed by in vivo growth of a cell line was studied by derivation of a subline. 1000 WT, from a tumor obtained by injection of 1000 W cells into a host animal (see “Tumorigenicity Testing”). For comparison with the 1000 WT cell line, a line of marked oncogenicity (BP 3-0) was derived from a transplantable squamous cell carcinoma which had been induced in the rat trachea by benzo(a)pyrene administration (21). A cell pool was obtained by trypsin digestion of tumors (31) in the fifth in vivo passage.

Cells were stored at liquid nitrogen temperatures and then thawed, cultured, and subcultured at least once before use. We obtained samples at selected passage intervals by cloning the cells as described previously (19). 1000 W and 165 S cells were plated in Waymouth’s medium supplemented with 10% fetal bovine serum, amino acids, and sodium pyruvate with or without 0.1 mg insulin and 0.1 mg hydrocortisone per ml of medium (18). The dishes were maintained for 1 to 2 weeks at 37° in humidified 5% carbon dioxide in air. The 1000 W passage 6 and passage 17 cells and 1000 WT passage 9 cells were plated at 1 x 10^6 cells/60-mm dish. Colony-forming efficiencies of 1000 W passage 6 cells and 1000 WT passage 9 cells were found to be 22 and 47% under these conditions. Duplicate dishes from these treatments were fixed 24 hr after the cells were plated for determination of the percentage of single cells plated. In the 1000 W line, 69% of the cells plated singly, and nearly all of the remaining cells plated as doublets or were binucleate. In the 1000 WT subline, 95% of the cells plated singly. The 165 S passage 10 and passage 56 cells were plated at 4 x 10^5 and 4 x 10^6 cells/dish, respectively; the colony-forming efficiencies of the cells at these densities were 7 and 12%, respectively.

The BP 3-0 line was cultured in Waymouth’s medium which contained 10% fetal bovine serum and 0.1 mg insulin and 0.1 mg hydrocortisone per ml of medium (18). Clonal populations were obtained from this line by plating of cells at passages 1 and 21 at a density of 2 x 10^4 and 3 x 10^4 cells/dish, respectively. The colony-forming efficiencies of the cells were 0.7 and 6%, respectively.

Tumorigenicity Testing. Cell lines were chosen for analysis on the basis of their oncogenicity in in vivo tests. Cells of the 1000 W and 165 S lines were tested by i.m. injection of 5 x 10^6 or 1 x 10^7 cells into syngeneic immunosuppressed hosts, with subsequent observation for 10 months. The 165 S line was oncogenic at passage 32 in only one of 4 injection sites, with an in vivo latent period of 255 days. The 1000 W line was oncogenic at passage 25, in 4 of 4 injection sites, with in vivo latent periods of 36 to 50 days (21). Cells of the BP 3-0 line were tested by i.m. injection of cells into immunocompetent hosts, followed by observation for 2 months. The BP 3-0 passage 21 cells formed tumors in at least 3 of 5 hosts at doses of 1 x 10^6 to 1 x 10^7 cells with latent periods of 21 to 42 days. All 3 cell lines formed keratinizing squamous cell carcinomas.

Comparisons among cell lines were facilitated by calculation of a quantitative parameter, L, based on tumor incidence and latent periods of tumor development as previously described (28). L, the reciprocal of the harmonic mean of latent periods for tumors obtained by inoculation of 5 x 10^6 cells, values were 303, 50, and 9 days for the cell lines 165 S, 1000 W, and BP 3-0, respectively.

Scanning Electron Microscopy and Data Processing. Cultures dishes containing colonies of cells were processed by a standard method in which small discs were removed from the dishes, dried at the critical point, and examined by scanning electron microscopy (20). The primary analysis was carried out on coded discs analyzed in a blind study. For 8 randomly selected colonies from each dish, the colony diameter was measured, and 10 cells each from the colony edge and from its interior were sampled. Each cell was viewed at x6000 and assigned numerical indices for 2-dimensional shape, i.e., length and width, and, for the third dimension, height. Stringent criteria for 2-dimensional shape were established, so that cells with ratios of long to short axes of less than 2:1 were assigned an index of 1. Cells with ratios of 2 to 3:1 were assigned an index of 2; cells with ratios greater than 3:1 were assigned an index of 3 (see tables). One of 3 possible categories (flat, domed, or rounded) was assigned for height estimation. The microvillar density of each cell viewed was also estimated by comparison of the surface of the cell with standard electron micrographs which showed microvillar densities of 1.8, 3.3, 5.3, and 7.5 microvilli per sq μm of surface area. Depending on which standard the cell most nearly resembled, a numerical index of 1, 2, 3, or 4 was assigned; cells with no microvilli were assigned a 0 index, as described previously (30).

The data were processed by consideration of each possible combination of parameters (i.e., 5 categories of microvillar density, 3 categories of shape, and 3 categories of height) as a member of a multicomponent matrix. The number of cells in each member of the matrix was tabulated by means of a PDP 11/40 computer for individual clones and for all of the cells analyzed within each population sample (8 clones). Modification of individual parameters in populations from the cell lines was determined from separately plotted distributions for microvillar density, 2-dimensional shape, and height.

Light Microscopic Studies. The possibility that morphological alterations were correlated with differences in the number of cells within colonies or the areas occupied by colonies was studied in early passages of the 1000 W and 1000 WT cell lines. Cultures were prepared for light microscopic examination as described previously (12).

RESULTS

Initial Morphological Observations. The range of variability of cell shape and surface features within cell lines was assessed at various passages by growth of small colonies from cells plated at low density. Initially, the overall morphology in colonies from the 1000 W and 1000 WT cell lines was studied. In most colonies from an early passage of the 1000 W line, the cells were highly spread and closely adherent to adjacent cells (Fig. 1a). In a later passage, the cells were typically more domed; occasionally, clones contained a number of flattened spindle-shaped cells, which could overlap adjacent cells (Fig. 1b). Because it was possible that these alterations could be reversed by returning the cells to an in vivo environment, clonal populations were also sampled from the 1000 WT subline of the 1000 W line at an early time after its derivation. If the time of in vitro maintenance were a major determinant of cellular
morphology, clones from this subline would be expected to resemble those sampled from an early passage of the 1000 W cell line. In a few clones, the cells were highly spread and closely adherent to the adjacent cells, similar to those from an early passage of the 1000 W line (cf. Fig. 1, a and c). At the other extreme, some clones from the 1000 WT line contained many spindle-shaped or flattened spindle-shaped cells. Even in clones which contained cells of nonepithelioid shapes, however, a few cells were still highly spread and polygonal (Fig. 1d).

Compared with cells from the 1000 W line, 165 S cells usually appeared more domed and less adhesive to adjacent cells. When colonies from an early passage were examined, most cells appeared slightly elongated and domed, although highly spread cells were also seen (Fig. 2a). Colonies from a later passage contained fewer highly spread cells and more cells which were elongated or which formed long cytoplasmic extensions (Fig. 2b). However, the morphological distinctions between the early and late passages of this line were less pronounced than those between early and late passages of the 1000 W cell line.

It was possible that some or all of the morphological alterations observed in these 2 cell lines were strictly related to sequential subculture and therefore could be expected to occur even in cell lines which were already oncogenic at the time when in vitro growth was initiated. To determine which alterations were likely to be independent of the acquisition of oncogenicity during in vitro culture, we studied the tumor-derived BP 3-0 line at early and late passages. Colonies derived from passage 1 usually had highly spread, squamous cells at their center. Near or at the edges of the colonies, the cells were frequently elongated in a direction perpendicular to that of colony expansion (Fig. 3a). In colonies from a later passage (passage 21), most cells were domed even in the central areas of the colonies. Cells which were cylindrical or formed long, spindle-shaped extensions of cytoplasm were frequently found in both the central and peripheral areas of these colonies (Fig. 3b).

Semiquantitative Analysis of Cell Shape. The increased numbers of domed and spindle-shaped cells during in vitro culture of the 1000 W and 165 S cell lines suggested that, as the lines became anchorage independent and tumorigenic, the cells underwent structural alterations which permitted certain shapes to be assumed more readily than in early passages. This possibility was investigated quantitatively by categorizing cells of clonal populations from early and late passages, as detailed in "Materials and Methods." Since there were 3 possible categories of 2-dimensional shape and 3 possible categories of height, processing of the primary data placed each cell in one of 9 possible shape classes (Figs. 1 to 3).

A comparison of populations from early and late passages of the 1000 W line showed that the percentage of cells observed in various classes changed over the time of culture. The data shown in Table 1 indicate that 2 types of alterations distinguished late-passage 1000 W cells; they appeared to assume flattened shapes (Classes 1 and 2) less readily and spindle shapes (Classes 5 and 8) more readily than cells from an early passage. It was important to determine whether these altered frequencies of expression could be reversed experimentally. When cells from the 1000 WT subline were compared with those from the 1000 W line, they resembled the early 1000 W populations in the proportions of cells having domed and rounded polygonal shapes (Classes 4 and 7). The distribution of 1000 WT cells into various classes of spindle-like shapes (Classes 5, 6, 8, and 9) was not significantly different from that observed in the late passage of 1000 W. For Classes 5 and 8, the frequency of expression was significantly higher than that in an early passage of the 1000 W line. Since the frequency of transitions observed in these 2 cell lines were likely to be independent of the acquisition of oncogenicity.

Table 1

<table>
<thead>
<tr>
<th>Shape class</th>
<th>1000 W</th>
<th>1000 WT</th>
<th>165 S</th>
<th>BP 3-0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>220 days</td>
<td>363 days</td>
<td>220 days</td>
<td>581 days</td>
</tr>
<tr>
<td>Flat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1* (Class 1)</td>
<td>49.8 ± 2.5</td>
<td>36.7 ± 2.3</td>
<td>42.1 ± 4.2</td>
<td>5.0 ± 2.8</td>
</tr>
<tr>
<td>2 (Class 2)</td>
<td>9.8 ± 2.7</td>
<td>7.8 ± 2.1</td>
<td>5.7 ± 0.9</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>3 (Class 3)</td>
<td>0.2 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Domed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Class 4)</td>
<td>30.1 ± 2.3</td>
<td>33.3 ± 3.5</td>
<td>31.6 ± 1.9</td>
<td>66.1 ± 3.4</td>
</tr>
<tr>
<td>2 (Class 5)</td>
<td>4.9 ± 1.2</td>
<td>10.3 ± 2.2</td>
<td>12.4 ± 2.1</td>
<td>11.9 ± 3.8</td>
</tr>
<tr>
<td>3 (Class 6)</td>
<td>0</td>
<td>0</td>
<td>0.5 ± 0.3</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Rounded</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Class 7)</td>
<td>4.7 ± 0.9</td>
<td>9.6 ± 1.6</td>
<td>4.6 ± 0.5</td>
<td>13.4 ± 2.4</td>
</tr>
<tr>
<td>2 (Class 8)</td>
<td>0.5 ± 0.2</td>
<td>1.7 ± 0.9</td>
<td>2.5 ± 0.6</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>3 (Class 9)</td>
<td>0</td>
<td>0.6 ± 0.5</td>
<td>0.6 ± 0.6</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>

*Index describing ratio of long to short axis.

\(p = 0.10\)

\(p = 0.05\)

\(p = 0.20\)

\(p = 0.01\)

\(p = 0.20\)
expression of these phenotypes was not reversed by redenervation of the cell line; these shape classes were considered nonrevertant.

The shapes of 165 S cells were analyzed by a protocol identical to that used for the 1000 W line (Table 1). The distribution of 165 S cells in the domed polygonal (Class 4) and flattened spindle-shaped (Class 5) classes was relatively high, confirming the visual impression that 165 S cells were more domed and elongated than 1000 W cells. A comparison of early and late passages from the 165 S line showed similar percentages of cells in most classes. However, the percentage of spindle-shaped cells (Class 8) was significantly higher in the late passage, and the percentage of extremely elongated spindle-like cells (Class 9) increased 6-fold. Our observations suggested that time-dependent alterations were more pronounced in 1000 W populations than in 165 S populations. Statistically significant alterations occurred in more cell shape classes in the 1000 W than in the 165 S line. The 165 S line had been maintained in continuous culture for longer than the 1000 W line before the late passages were sampled, so that the extent to which morphological expression was altered did not depend solely on the time elapsed since initiation of the cell lines. However, it was possible that similar alterations would occur as a result of continuous culture of any cell line, regardless of its oncogenicity. Therefore, the morphological characteristics of cells in small colonies were determined for a cell line of marked tumorigenicity, as estimated by its L values and by the number of cells required for 50% tumor take. Comparisons of colonies from early (passage 1) and late (passage 21) passages of the BP 3-0 line showed that phenotypic drift occurred with respect to the polygonal shapes, but the representation of spindle-shaped cells in the population decreased slightly, rather than increasing.

It was possible that the increased representation of spindle-like shapes in the cultured populations was due to the presence of a few colonies in which the shape was drastically altered from normal, such as those shown in Fig. 1 b and d. Therefore, the prevalence of various shape classes in the populations was tabulated on a colony-by-colony basis (Table 2). The number of colonies in which a particular class was represented changed in only a few instances. The distribution in the class of rounded polygonal shape (Class 7) on a colony-by-colony basis was completely reversed by redenervation of the 1000 W line. The number of colonies in which one or more flattened spindle-shaped (Class 5) or spindle-shaped (Class 8) cells were found increased 1.5- and 3-fold, respectively, between the early and late passages of the 1000 W line. The number of colonies containing extremely elongated spindle-like cells (Class 9) also increased. When the late passage of 1000 W was compared with the 1000 WT subline, the latter had similar or significantly greater numbers of colonies which contained cells in these spindle-shaped classes.

When early and late passages of the 165 S line were compared, the number of colonies which contained spindle-like cells (Classes 8 and 9) increased approximately 2.5- and 4-fold, respectively. No other significant changes were observed. In a similar comparison of early and late BP 3-0 populations, the number of colonies containing the nonrevertant shape classes (Classes 5 and 8) did not change.

The colony-by-colony analysis suggested that the acquisition of tumorigenicity by cell lines was accompanied by an increase in the proportion of clonal populations which contained cells of spindle-like shape. The lack of uniformity of this phenotypic expression within clonal populations was to be expected, since spindle shapes were not representative of the normal range of epithelioid morphologies. In our initial morphological studies, spindle-shaped cells often seemed localized at the edges of colonies, where they were stretched out along an axis perpendicular to the direction in which the colony was expanding. As a test of whether spindle-shaped cells were preferentially lo-

### Table 2

**Fraction of colonies which contained one or more cells in various shape classes**

Colonies derived from 7 populations of defined oncogenic potential were analyzed. Populations obtained from the 1000 W, 1000 WT, and BP 3-0 cell lines at the designated number of days in culture were sampled 4 times as detailed in “Materials and Methods”; populations from the 165 S cell line were sampled 3 times.

<table>
<thead>
<tr>
<th>Shape class</th>
<th>1000 W 220 days</th>
<th>363 days</th>
<th>1000 WT (63 days)</th>
<th>165 S 220 days</th>
<th>581 days</th>
<th>9 days</th>
<th>126 days</th>
<th>BP 3-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1* (Class 1)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.50 ± 0.22d</td>
<td>0.33 ± 0.18</td>
<td>0.94 ± 0.04</td>
<td>0.97 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>2 (Class 2)</td>
<td>0.78 ± 0.08</td>
<td>0.72 ± 0.08</td>
<td>0.69 ± 0.06</td>
<td>0.04 ± 0.04</td>
<td>0.13 ± 0.07</td>
<td>0.75 ± 0.07c</td>
<td>0.47 ± 0.09f</td>
<td></td>
</tr>
<tr>
<td>3 (Class 3)</td>
<td>0.03 ± 0.03</td>
<td>0</td>
<td>0</td>
<td>0.03 ± 0.3</td>
<td>0</td>
<td>0.03 ± 0.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Domed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Class 4)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2 (Class 5)</td>
<td>0.59 ± 0.09a</td>
<td>0.91 ± 0.06e</td>
<td>0.88 ± 0.09c</td>
<td>0.83 ± 0.11</td>
<td>0.96 ± 0.04</td>
<td>0.88 ± 0.05</td>
<td>0.88 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>3 (Class 6)</td>
<td>0</td>
<td>0</td>
<td>0.06 ± 0.04</td>
<td>0.04 ± 0.04</td>
<td>0.08 ± 0.08</td>
<td>0.06 ± 0.04*</td>
<td>0*</td>
<td></td>
</tr>
<tr>
<td>Rounded</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Class 7)</td>
<td>0.59 ± 0.13b</td>
<td>0.84 ± 0.03f</td>
<td>0.59 ± 0.06b</td>
<td>0.88 ± 0.07</td>
<td>0.88 ± 0.07</td>
<td>0.44 ± 0.11d</td>
<td>0.81 ± 0.06d</td>
<td></td>
</tr>
<tr>
<td>2 (Class 8)</td>
<td>0.09 ± 0.03a</td>
<td>0.25 ± 0.09f</td>
<td>0.44 ± 0.06g</td>
<td>0.29 ± 0.11c</td>
<td>0.67 ± 0.11c</td>
<td>0.47 ± 0.06</td>
<td>0.56 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>3 (Class 9)</td>
<td>0</td>
<td>0.13 ± 0.09</td>
<td>0.03 ± 0.03</td>
<td>0.13 ± 0.07*</td>
<td>0.54 ± 0.23e</td>
<td>0.19 ± 0.08*</td>
<td>0.06 ± 0.04*</td>
<td></td>
</tr>
</tbody>
</table>

* Index describing ratio of long to short axis.

** Mean ± S.E.

* p = 0.10.

* p = 0.05.

* p = 0.20.

* p = 0.02.

* p = 0.01.
located at the edges of colonies, the ratio of "edge" cells to total cells was calculated for each class (Table 3). Since the ratio was equivalent to the frequency with which cells were observed at the colony edge, a ratio of 1.00 indicated that all cells in a particular class were found at the edge, while a ratio of 0.50 indicated a random spatial distribution, i.e., no preference for either the edge or interior of the colony. In the early passages of both the 1000 W and 165 S cell lines, all or nearly all the spindle-shaped cells (Class 8) were found at the edges of colonies. In later passages of these lines, spindle-shaped cells were found in the interior with a higher frequency than in early passages. However, due to the increase in absolute numbers of cells in this shape class in the later passages, the absolute numbers of spindle-shaped cells at the edges of colonies also increased. [The absolute numbers may be derived by multiplication of the percentage of cells falling into any shape class (Table 1) by the frequency with which cells in the class appeared at the colony edge (Table 3).] The trend toward random spatial distribution of the spindle-shaped cells was not reversed by derivation of the 1000 WT subline. Other alterations in site preference were of marginal statistical significance or occurred in the polygonal classes. In early and late passages of the BP 3-0 cell line, spindle-shaped cells had a near-random distribution with respect to the colony edge and interior.

Control Determinations. The finding that spindle-shaped cells were, in some cases, preferentially located at the edges of colonies suggested that this shape was assumed in response to forces exerted during outward expansion of the colonies. Such factors as the absolute area and rate of growth of the colonies might also have determined whether cells would assume spindle shapes. These possibilities were assessed by comparing populations from early passages of the 1000 W and 1000 WT lines with respect to cell number and colony diameter.

The density of cells within colonies of equivalent diameter varied considerably (Chart 1), but colonies of similar size from the early 1000 W and 1000 WT lines contained similar numbers of cells. To determine whether colony size was a critical factor in the appearance of spindle-shaped cells, we compared colonies of similar diameters from the 2 lines (i.e., those which fell within the same range of diameter measurements (Chart 1, A to B' on the abscissa)). The number of spindle-shaped cells in these samples averaged 0.6 and 2.8%, respectively, values which fell within the range defined by standard errors of the means for the entire populations sampled from the lines (Table 1). In a second analysis, we determined whether colonies with similar growth rates from the 2 lines contained similar numbers of spindle-shaped cells. Colonies selected from the 1000 W and 1000 WT lines following 2 weeks of in vitro growth were analyzed. Their average diameters were 670 ± 60 μm (S.D.) (8 colonies) and 670 ± 80 μm (7 colonies), respectively. However, the percentages of spindle-shaped cells observed were 0 and 3.6%, respectively, values which fell near the means for the populations analyzed (Table 1). Semiquantitative Analysis of Cell Surface Features. Detailed observations on the surface features of individual cells were made at the same time as observations on cell shape. Blebs were hardly ever seen on surfaces of cells from any of the lines used. Ruffles were found on the surfaces of many 1000 W cells, but only on cells at the edges of colonies. Approximately 10% of the 1000 W passage 6 colonies, 50% of the 1000 W passage 17 colonies, and 30% of the 1000 WT passage 9 colonies contained some cells with ruffles. Cells of the 165 S line never appeared to form ruffles at their surfaces. Approximately 20% of the colonies in the BP 3-0 line from both early and late passages contained cells with ruffles.

The question of whether characteristic changes in the density

### Table 3

<table>
<thead>
<tr>
<th>Shape class</th>
<th>Ratio of edge to total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>220 days</td>
</tr>
<tr>
<td>Flat 1* (Class 1)</td>
<td>0.45 ± 0.02abc</td>
</tr>
<tr>
<td>2 (Class 2)</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td>3 (Class 3)</td>
<td>NC</td>
</tr>
<tr>
<td>Domed 1 (Class 4)</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td>2 (Class 5)</td>
<td>0.55 ± 0.15</td>
</tr>
<tr>
<td>3 (Class 6)</td>
<td>0</td>
</tr>
<tr>
<td>Rounded 1 (Class 7)</td>
<td>0.50 ± 0.09</td>
</tr>
<tr>
<td>2 (Class 8)</td>
<td>1.00abc</td>
</tr>
<tr>
<td>3 (Class 9)</td>
<td>NC</td>
</tr>
</tbody>
</table>

* Index describing ratio of long to short axis.
bc p = 0.10.
cd p = 0.05.
df p = 0.20.
NC, not calculated due to availability of too few (1 or 2) observations.
a,b p = 0.01.
a,b,c p = 0.10.
of surface features accompanied the development of oncogenicity in respiratory tract epithelial cell lines was studied by estimation of the density of microvilli on cell surfaces. Microvillar density data are shown in Chart 2. In colonies derived from both early and late passages of the 1000 W line, individual cells were quite variable in the density of microvilli on their surfaces. In many cases, cells with no microvilli and cells which were densely covered with microvilli were found in the same colony. On the average, the density of microvilli increased in the late passage of the line but decreased again in the 1000 WT subline (cf. Chart 2, a to c).

In colonies from an early passage of the 165 S cell line, cells had uniformly high densities of microvilli, while cells from a later passage showed more variability, with some cells appearing in categories of few (Category 1) or no (Category 0) microvilli (cf. Chart 2, d and e). In colonies from the BP 3-0 line, there was great variability among cells within a single colony as well as from colony to colony. The average microvillar density of cells from the BP 3-0 cell line decreased between the early and late passages (cf. Chart 2, f and g).

**DISCUSSION**

The results of the present studies suggested that changes in cell shape specifically accompanied oncogenic transformation in cell lines derived from the respiratory tract epithelium. The number of cells which assumed spindle shapes in colonies increased with time in 2 cell lines which became oncogenic during in vitro culture, but not in a cell line which was highly oncogenic when first initiated into culture. In further experiments, the presence of spindle-shaped cells in colonies was shown to be stable, in that their presence was not reversed by a period of in vivo growth of the 1000 W line. In early passages of the 1000 W and 165 S lines, spindle-shaped cells (Class 8) appeared almost exclusively at the edges of colonies. The finding that few cells in early passages of these lines assumed spindle-like shapes suggested that these cells were relatively resistant to deformation by forces of the magnitude generated during colony expansion. If the frequency of expression of the spindle shape and its site specificity in colonies were correlated with the oncogenicity of cell lines, rather than with trivial effects of in vitro culture conditions, then tumor-derived sublines should resemble late passages of the cell lines in these respects. This was indeed the case for the 1000 WT subline. Of the nonrevertant shape classes, the spindle shape (Class 8) was a more extreme departure from typical epithelioid shapes than the flattened spindle shape (Class 5). In the early and late passages of the 1000 W line, approximately 5 and 15%, respectively, of all cells found at the colony edge were of the latter class. A similar analysis of the 1000 WT subline indicated that 19% of the cells at the colony edge were of this shape class. The increased frequency of Class 5 cells at the periphery suggested that the forces generated at the edges of colonies were capable of deforming only a small fraction of the cells from early populations but a substantial fraction of the cells from late and tumor-derived populations.

It should be emphasized that the altered frequencies of
expression of spindle shape in the 1000 W and 165 S cell lines could not be accounted for by a primary increase in either the thickness or the degree of elongation of the cells. A comparison of the late passage of 1000 W with the 1000 WT subline suggested that increases in thickness in the polygonal classes (Classes 1, 4, and 7) were partially reversed by growth of the line in vivo. The trend toward increased thickness of polygonal cells was considered common to cultured cell lines but unrelated to the oncogenicity of the lines. This conclusion was supported by the observation that the numbers of cells in these classes increased significantly in the late passage analyzed from the BP 3-0 line, even though the line was highly oncogenic when first initiated into in vitro culture.

The trends toward increased thickness of cells during in vitro culture were of interest in light of recent results showing that changes in cell thickness were tightly coupled to DNA synthesis (8, 9). The work by Folkman and Moscona (9) showed that the flattest cells had the highest [%H]thymidine labeling indices. Although late populations derived from the 1000 W and BP 3-0 lines showed greater average thickness of cells and faster doubling times than the early populations, these characteristics may not be interdependent. However, flat cells observed in cultured epithelial cell lines included highly specialized squamous cells, so that the proportions of these cells in the populations could reflect differences in specialization as well as in growth potential.

To obtain a rough assessment of the malignancy of the 3 cell lines studied, we have calculated the reciprocal of the harmonic mean of latent periods for tumor formation, L, for each line (see “Materials and Methods”). The BP 3-0 line appeared to be very malignant, since the dose of cells required for 50% tumor induction in host animals was less than 100 cells and the latent period required for tumor formation was short. Although the BP 3-0 line resembled the late passages from the 1000 W and 165 S lines in the percentage of spindle-shaped cells (Table 1), the number of colonies containing such cells (Table 2), and the site specificity shown by the cells in colonies (Table 3), it differed in that the expression of spindle shapes over the time of in vitro maintenance was stable. Therefore, the stability of phenotypic expression over time appeared more important than the absolute incidence of spindle-shaped cells in populations. It is important to note that the changes in deformability suggested by the present studies preceded those related to metastasis (27).

A striking dissimilarity of the present results to previously described morphological correlates of oncogenic transformation (2, 4, 15, 17) is the absence of meaningful changes in the density of microvilli at cell surfaces. Therefore, the value of microvillar density as a morphological correlate of oncogenicity seemed poor in the respiratory tract epithelial models, as might be expected from the variability of normal respiratory tract epithelial cells in vitro with respect to this feature (12). A poor correlation between microvillar density and neoplastic change has also been found previously (30). Ruffles, however, were present on the surfaces of many cells from the most oncogenic lines studied. Their presence may have been related to the cytoskeletal and/or adhesive alterations underlying deformability increases in the cells studied. Finally, blebs and knobs typical of some transformed cells (4, 23, 24, 30) were not seen on the oncogenically transformed cells studied here.

What is the significance of spindle-like shapes in epithelial cells? Our present hypothesis is that they reflect changes in intercellular adhesion or in the deformability of the cells. Deformability may be enhanced through one of several mechanisms, including reduced rigidity of internal cytoskeletal elements. Reordering of cytoskeletal structure has recently been proposed to be a primary factor in the loss of growth control in fibroblasts (24). The importance of adhesion in shape maintenance is indicated by previous studies in which the shapes of single cells were altered experimentally by altering properties of the substrate (9, 13). Changes in adhesive mechanisms have been recognized as important factors in oncogenic transformation (26, 29). The possibilities of adhesive and cytoskeletal changes are under further investigation by direct measures of cell shape (22).

ACKNOWLEDGMENTS

We thank C. D. Farmer for valuable technical assistance, Dr. J. N. Dumont and Dr. Paul Nettesheim for use of scanning electron microscope and animal facilities, Dr. W. H. Olson for advice on statistical tests, and Dr. Katherine K. Sanford and Dr. John S. Cook for helpful comments on the manuscript.

REFERENCES


Fig. 1. Scanning electron micrographs of colonies from the 1000 W and 1000 WT cell lines. Numbers, shape classes. a, colony from passage 6 of the 1000 W line. Most cells appeared to be flat polygonal (Class 1), but some appeared to be domed polygonal (Class 4) or flattened spindle shaped (Class 5). Line C, artifact caused by flexing of the plastic substratum. b, colony from passage 17 of the 1000 W line. While a few cells were classified as flat polygonal (Class 1), most appeared to be domed polygonal (Class 4), flattened spindle shaped (Class 5), rounded polygonal (Class 7), or spindle-shaped (Class 8). Some cells in the colony also overlapped other cells (arrows). c, colony from passage 9 of the 1000 WT subline. While some cells appeared to be flat polygonal (Class 1) or flat elongated (Class 2), most were classified as domed polygonal (Class 4) or flattened spindle shaped (Class 5). d, colony from passage 9 of the 1000 WT subline. Many cells were spindle-shaped (Class 8) or had highly elongated spindle-like (Class 9) shapes. Other cells were flattened spindle shaped (Class 5) or domed polygonal (Class 4). A few cells were also classified as flat polygonal (Class 1). x310.

Fig. 2. Scanning electron micrographs of colonies from the 165 S line. a, colony from passage 10 of the line. The majority of the cells were classified as domed polygonal (Class 4) or flattened spindle shaped (Class 5). b, colony from passage 56 of the line. Most cells fell into Classes 4 and 5, as in a. x430.

Fig. 3. Scanning electron micrographs of colonies from the BP 3-0 line. a, colony from passage 1 of the line. In the interior of the colony, most cells were flat polygonal (Class 1) or flat elongated (Class 2). At the colony edge, cells were frequently domed polygonal (Class 4) or flattened spindle shaped (Class 5). Spindle-shaped cells (Class 8) were also seen. b, colony from passage 21 of the line. While most cells appeared to be domed polygonal (Class 4), flattened spindle-shaped (Class 5) and rounded polygonal (Class 7) cells were also present. x310.
Morphological Markers of Oncogenic Transformation in Respiratory Tract Epithelial Cells

C. A. Heckman and A. C. Olson


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/39/7_Part_1/2390

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.