Topography of Nonneoplastic and Neoplastic Cells of Common Origin

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SUMMARY

The possibility that neoplastic transformation may characteristically alter cell surface morphology prompted a comparison by scanning electron microscopy of nonneoplastic and tumorigenic cell lines from a single clone of mouse embryo cells. Among those studied by scanning electron microscopy, six lines of this clone proved nonneoplastic, and nine others underwent neoplastic transformation in culture, as evidenced by tumor production in vivo.

Combined cinephotomicrography and scanning electron microscopy allowed the determination of postmitotic time and topography of individual cells without perturbing the cells or detectably altering their surface morphology; no pattern of morphological change as a function of postmitotic time was evident in either nonneoplastic or neoplastic cell populations.

Accordingly, these cell populations could be compared under their usual conditions of attached asynchronous growth despite differences in proliferation rates. Cells of the neoplastic lines were characteristically less spread, and some lines displayed greater morphological variability than was evident among cells of nonneoplastic lines. However, most cells in all nine neoplastic lines and all six nonneoplastic lines were smooth surfaced. Thus, the exaggerated incidence of microvilli, ruffles, or blebs reported for established tumor-derived lines and most morphologically transformed lines did not prove a reliable criterion of neoplastic state for these cell lines of common origin grown under the same culture conditions.

INTRODUCTION

Cell surface morphology has been considered an index of the neoplastic state of cells in culture (9, 25, 36, 41, 42). Previous studies have generally contrasted the appearance of cells from established tumor-derived lines with presumed counterparts in primary culture, or have contrasted the appearance of morphologically transformed, presumably neoplastic cells with nontransformed controls. However, factors other than neoplastic state (e.g., period in culture, cell type, treatment with oncogenic agents, etc.) could determine cell surface morphology. A more rigorous comparison is possible with a recently developed clone of mouse embryo cells in which some derivative lines have remained nonneoplastic and others have undergone "spontaneous" neoplastic transformation evidenced by their growth as malignant tumors in vivo (33). Thus, the usefulness of surface features as criteria of neoplastic transformation can be evaluated in lines of common origin under the same culture conditions.

In order to compare nonneoplastic and neoplastic cell populations, which grow asynchronously at dissimilar proliferation rates, it was first necessary to establish whether these cells undergo predictable changes in surface morphology as they progress through the intermitotic period (27, 38). By combining cinephotomicrography with SEM, the postmitotic time and surface morphology of the same individual cells could be determined without disturbing cell attachment (35) and growth by synchronization procedures. Since cell surface morphology is extremely sensitive to environmental perturbations (37), cells were studied under their usual culture conditions of attached asynchronous growth. Thus, any consistent differences in surface morphology between these well-documented nonneoplastic and tumorigenic lines of common origin could be identified and considered indicative of their neoplastic state in culture (40).

MATERIALS AND METHODS

Clonal Origin of Cells. A cell line initiated from a mince of 12-day C3Hf/HeN mouse embryos was cloned twice as described previously (33). These cells were grown at 37.5° in stoppered T-15 Pyrex flasks in 3 ml antibiotic-free NCTC 135 medium with 10% fetal bovine serum (Flow Laboratories, Rockville, Md.) (12), and some cultures were transferred temporarily from this medium to NCTC 135 with 10% horse serum (Flow Laboratories, Rockville, Md.) (Chart 1) in an attempt to accelerate spontaneous neoplastic transformation (11). At intervals, cells suspended in medium were frozen and preserved in liquid nitrogen (13), and many lines used in the present study were derived from the frozen stocks.

Assay for Neoplastic Transformation. Cells were harvested mechanically and inoculated i.m. into X-irradiated syngeneic hosts (31). Most mice received 1 to 2 × 10⁶ cells
each, and mice in the larger groups each received $10^5$, $10^6$, or $10^7$ cells. Mice that failed to develop tumors were observed for 9 to 12 months. All tumors were diagnosed as sarcomas, and a representative tumor from each neoplastic line was carried for 2 additional transplant generations in nonirradiated mice. In 1 case, cells explanted from a tumor produced by cells of line 7943 were cultured for 9 passages and then examined.

Cells in a given sample were considered nonneoplastic when subsequent implants of that line or its parent produced no tumors (Chart 1, Films 2, 5, 7, and 8). In addition, the cells in Films 3, 4, and 6 were considered nonneoplastic since those lines (8206 and 8407) did not show cytological criteria of neoplastic change (4) at the times these films were made, and cells of line 8407 were not killed by activated macrophages (21). Cells in Films 1, 14, and 15 were also considered nonneoplastic, although cells of that line (8408) injected 3 months after filming produced 1 tumor with a latent period of 267 days.

Cells were considered neoplastic when tumors developed from implants made prior to sampling (Chart 1, Films 11 to 13, 16 to 21). Cells in Films 9 and 10 were also considered neoplastic because they derived from line 7943, which produced a sarcoma when assayed 7 days later.

Cinephotomicrography and Film Analysis. For filming, cells were trypsinized [2 ml 0.02% Versene (Microbiological Associates, Bethesda, Md.) followed by 0.5 ml trypsin-Versene solution (18)], suspended in NCTC 135 with 10% fetal bovine serum, and inoculated (3 ml containing $1.5 \times 10^5$ cells) into a modified T-15 flask (14). Each flask contained a 9- x 9-mm acid-cleaned (8) No. 1 coverslip (Bellco Glass, Inc., Vineland, N. J.) bearing an asymmetrical scribe (39). Cultures were gassed with 10% CO$_2$ in air to adjust the pH to 7.3, sealed with a silicone stopper, and filmed in an incubator room at 37.5°C.

Cells were photographed for 48 hr at $\times 80$ with phase-contrast optics [usually with a $\times 10$ (0.25 numerical aperture) achromatic objective (Zeiss Winkel)] in green light (550-nm interference filter) at 1 or 2 frames/min on 16-mm Kodak SO410 film (Eastman Kodak, Rochester, N. Y.). Films were developed in a Kodak Recordak Prostar microfilm processor with Prostar developer.

Films were examined at $\times 26$ on a Model C11 Vanguard viewer with M16C head (Vanguard Instrument Corp., Melville, N. Y.). Individual cells in the last frame before fixation were followed as the film was run backward to determine the period between mitosis and fixation. A photographic enlargement of this last frame was later used to identify the same cell in the SEM preparation. By maximum likelihood methods (24), Weibull density functions were fitted to data comprising intermitotic times for 60 nonneoplastic and 45 neoplastic cells and postmitotic times for an additional 101 nonneoplastic and 29 neoplastic cells (Chart 2). The maximum absolute deviation between the observed distribution of the intermitotic times (obtained by life table methods) and the estimated Weibull distribution function was 0.071 for nonneoplastic and 0.087 for neoplastic lines. The fit of the estimated distribution functions is adequate to approximate from a given postmitotic time the progress of that cell through the mitotic cycle.

FBS Fetal Bovine Serum (10%)  
HS Horse Serum (10%)  
- One Mouse without Tumor  
- One Mouse with Tumor  
- Freeze-thaw of Cells  
- Film Number

Cell Line

7914
8206
8164
8407
8726
8322
7943
8475
7915
8165
8406
8727
8713
7944
8712
8476

Chart 1. Genealogical relationships of clones and derivative lines with the results of in vivo assays for neoplastic transformation.

- , mice receiving implants of cells that failed to grow as tumors; ■, mice that developed sarcomas from the implants. The area of each block is proportional to the number of mice inoculated.

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Most mammalian cells grown at 37° have a variable G1 period but relatively constant periods of S (7 hr), G2 (4 hr), and M (1 hr) (20). On the basis of these values and the estimated Weibull distributions, the conditional probability that a cell is in a particular stage can be calculated for each postmitotic time. These conditional probabilities are plotted as functions of time for nonneoplastic and neoplastic lines (Charts 3 and 4).

**RESULTS**

Cells from 19 cultures of 6 nonneoplastic lines and 26 cultures of 9 neoplastic lines, all descendants from the same original clone and grown under the same culture conditions, were examined by SEM; of these, 10 cultures of nonneoplastic and 11 of neoplastic lines had been filmed. In general, the surface morphology of these cells was remarkably uniform. Most cells in both nonneoplastic and neoplastic lines were smooth-surfaced (Figs. 1 to 5). Microvilli, and to a lesser extent ruffles and blebs, were present on some cells in each culture of both nonneoplastic (Fig. 6) and neoplastic lines. Neoplastic lines 7943 and 8727 were ex-
geneous, including some cells with numerous microvilli, blebs, or ruffles (Fig. 7).

A more detailed comparison of the incidence of microvilli confirmed that cells of the neoplastic lines were not characteristically more villous (Table 1). In fact, 85% of the cells lacked microvilli, compared with 79% of cells in the nonneoplastic lines. A few extremely villous cells (Category 4) were found exclusively in the neoplastic lines, but they constituted only 1% of the cells examined, and they occurred in only 2 of the 5 lines. Cells with many microvilli (Category 3), although absent from the sample in Table 1, did appear in nonneoplastic lines (Fig. 6).

Cells of nonneoplastic and neoplastic lines were compared with respect to other surface features by examining enlarged scanning electron micrographs (×400) of each field that had been filmed (Chart 1). The surface morphology of cells that had been filmed was indistinguishable from that of cells located outside the illuminated area and that of cells in replicate cultures not filmed. The incidence of ruffles and blebs did not correlate with neoplastic state (Figs. 2 to 5). On the other hand, cells of most nonneoplastic lines showed less flattening or spreading on the coverslip, compared with cells from nonneoplastic lines. Also, abundant lateral processes, many of which could be retraction fibers (29), occurred more frequently in cultures of neoplastic lines. Cells cultured from one tumor originating from an implant of line 7943 generally resembled cells of the parent culture (cf., Figs. 7 and 8).

These cultures were grown from inocula of the same size for comparable periods; approximately one-half of them were subconfluent at the time of fixation, with cells occurring singly and in discrete groups of variable size, rather than as a continuous sheet. Variations in cell density among these cultures and in different areas of the same coverslip did not greatly influence the appearance and distribution of surface features as described above. The morphology characteristic of a particular line was generally displayed by individual cells as well as by contiguous cells at higher densities, although this observation was not evaluated quantitatively (22).

The nonneoplastic and neoplastic cell populations differed significantly in the distribution of intermitotic times (p < 0.001) (Chart 2). However, this difference in growth rates apparently did not influence surface morphology. Cells of most lines, both nonneoplastic and neoplastic, were generally similar and remarkably uniform within each culture except for a small population of rounded cells. Many of these rounded cells were identified from the cine films as cells in mitosis. Although some cells remained flat throughout mitosis, most were round with numerous microvilli and lateral processes or retraction fibers (38) (Fig. 9); cytoplasmic blebs were noted on many, but not all, cells fixed in anaphase and telophase during filming. Some cells also assumed a rounded form for several hours with no evidence of mitosis and then resumed a typical flattened shape. Dying cells commonly rounded and blebbbed just prior to degeneration; such cells were rare in these films.

Analyses of cine films also yielded precise postmitotic times for many individual cells fixed during interphase. Scanning electron micrographs (×600) of 149 cells from nonneoplastic lines and 97 cells from neoplastic lines were studied in order of increasing postmitotic time. Most cells fixed more than 30 min after mitosis were relatively flat and smooth in both nonneoplastic and neoplastic lines (Figs. 10 to 16). The morphological variations that did occur (especially pronounced in line 7943) did not correlate with postmitotic time. In particular, abundant microvilli, roundness, and blebs did not characterize cells fixed at relatively short (Figs. 10 to 14) or long intervals (Figs. 15 and 16) after mitosis. The surface morphology of these cells did not change predictably as a function of postmitotic time. When nonneoplastic and neoplastic cells were compared at similar stages of the cell cycle, as estimated from the Weibull density distributions (Charts 3 and 4), the results did not differ from those described above. Even in G1 (just after mitosis) and at long postmitotic times (with a high probability of representing G2), cells of nonneoplastic lines were characteristically more spread and more uniform in surface morphology than those of neoplastic lines. The incidence of microvilli, ruffles, and blebs did not reliably distinguish between cells of nonneoplastic and neoplastic lines at any stage in the cell cycle.

**DISCUSSION**

Comparison of nonneoplastic and neoplastic cell lines growing at dissimilar rates requires consideration of the influence of cell cycle on surface morphology. Some previous reports conclude that cells in G1 are distinctively more rounded with a higher incidence of microvilli and blebs than cells in S or G2 (7, 17, 27, 38). Since cell lines with slower growth rates would include a larger proportion of cells in

### Table 1

**Incidence of microvilli on the exposed surfaces of nonneoplastic and neoplastic cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of cultures</th>
<th>No. of cells with microvilli, relative incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8322a</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>8408</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>8476a</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>8713</td>
<td>3</td>
<td>56</td>
</tr>
<tr>
<td>Totals</td>
<td>8</td>
<td>127</td>
</tr>
<tr>
<td>Neoplastic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7943b</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td>8475</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>8712b</td>
<td>4</td>
<td>69</td>
</tr>
<tr>
<td>8726</td>
<td>4</td>
<td>79</td>
</tr>
<tr>
<td>8727b</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>Totals</td>
<td>17</td>
<td>288</td>
</tr>
</tbody>
</table>

| No. of microvilli; 1, few microvilli; 2, moderate numbers of microvilli; 3, many microvilli; and 4, entire surface covered with microvilli. |

| These lines have been tested more directly for tumorigenicity; statistical analysis of data from these lines alone does not differ significantly from analysis of the entire sample. |
the variable (20) G, stage, a distinctive morphology associated with G, would complicate comparisons between asynchronous nonneoplastic and neoplastic cell populations. In this study, however, most interphase cells of all lines were smooth and, within 30 min after mitosis, resumed their smooth, relatively flat form indistinguishable from other interphase cells. This pattern is similar to that reported for SV40/3T3 cells (7). In addition, the present study revealed no pattern of topographic change with increasing postmitotic time. These results are consistent with the uniformly flat, smooth appearance of most interphase cells in these asynchronous cultures. Unchanging cell topography through interphase has also been observed in low-density CHO monolayers (30) in both synchronized and asynchronous cultures. Synchronization was deliberately avoided in this study because of the uncertainty concerning the impact of available, effective procedures on cell topography. In any case, our objective was to compare unperturbed nonneoplastic and neoplastic cells under their usual conditions of asynchronous growth.

In recent studies comparing the topography of nonneoplastic and neoplastic cells in culture, characteristic features have been sought that might serve as reliable indices of neoplastic state (38). Among approximately 40 presumed neoplastic lines examined, cells of most lines were described as less spread and flat than normal cells. Increased numbers of microvilli, ruffles, or blebs were noted on cells of 21 long-term lines established from tumors (6, 16, 19, 25, 26, 28, 36) and on cells of 13 lines transformed in culture (5 virus-infected [1, 17, 28, 36], 4 X-irradiated (5), 2 carcinogen-treated (19), and 2 spontaneous [27, 28]), compared with low-passage fibroblasts from normal tissues or with lines BALB/3T3 or BHK21/C13. However, cells of 5 virus-transformed lines reportedly failed to show increased incidence of surface features in comparison with noninfected controls (6, 7, 17, 23, 34); cytopathic effects of viral infection may alter the incidence of microvilli independent of neoplastic transformation (10). None of these reports quantify the incidence of surface features on individual cells or the proportion of cells showing these features, although smooth-surfaced cells are less conspicuous than those with striking surface features and may be underestimated in mixed cell populations. Also, in most of these studies, the nonneoplastic and neoplastic states of cultured cells were not verified by assays for tumor production in suitable hosts. Continuous monitoring of tumorigenicity is essential because nonneoplastic cells frequently undergo spontaneous transformation which is difficult to detect morphologically, and cells cultured from tumor explants could represent nonneoplastic tissue components (32).

In most previous comparisons, the nonneoplastic and neoplastic cell populations differed with respect to cell type, period in culture, and probably other factors, such as proliferation rate, which might significantly influence cell topography and thus complicate interpretation. In addition, most published examples of neoplastic cells were selected as established lines or as morphological transformants. Cells of established lines may not be representative of the neoplastic cells of most tumors, since explants from only a small proportion of tumors (especially of human origin) survive in culture (15). The exaggerated surface features common on cells of established lines could reflect their capacity for long-term growth in culture. Most examples of transformed cells have been selected exclusively on the basis of their unusual morphology and growth pattern in culture. Therefore, the abundant surface features that often appear on their progeny correlate well with abnormal cell form but not necessarily with neoplastic state. The selection of morphological transformants from the general population would never reveal neoplastic cells with more normal form and presumably smoother surfaces. Such smooth-surfaced neoplastic cells may be common, since half the transforming virus-infected lines (38) and all 9 tumorigenic lines in the present study comprise predominantly smooth-surfaced cells.

This study was designed to ensure a more rigorous topographic comparison between nonneoplastic and neoplastic cells. These lines of clonal origin were grown in parallel under similar culture conditions for comparable periods in the absence of deliberately added viruses or other oncogenic agents that might independently affect cell topography. These lines were assayed repeatedly in vivo for tumorigenic potential as a measure of their nonneoplastic or neoplastic state and were chosen for examination irrespective of their morphology and growth pattern. In this comparison, the cells of neoplastic lines appeared characteristically less spread, but the proportion of smooth-surfaced cells was equivalent to that in nonneoplastic lines. Cells of the neoplastic lines showed greater variability in surface features even among neighboring cells or cells of comparable postmitotic time, possibly reflecting the genetic heterogeneity of aneuploid cell lines. Two of the neoplastic lines showed marked variability; the fact that the same morphological variability occurred among cells cultured from a tumor produced by one of these lines indicated that any or all of the diverse cell features observed, including the predominant smooth surface and flat shape, may characterize tumorigenic cells. Cells with exaggerated surface features are conspicuous when they occur, but they are infrequent or even absent from many neoplastic cell populations described to date, and therefore cannot be considered representative of the neoplastic state.

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REFERENCES


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SEM of Neoplastic Cells in Culture

Fig. 1. Phase contrast image of neoplastic mouse fibroblasts (line 7943) from the last cine frame before fixation (a), and SEM image of the same field (b). Note the tetranucleate cell (*). Individual cells that had been followed in the film to determine postmitotic time can thus be identified and examined by SEM. All scale markers represent approximately 10 μm in the horizontal axis at the focal plane. a, × 600; b, × 400.

Fig. 2. Nonneoplastic line 8713, comprising mostly flat, spread, smooth-surfaced cells. Microvilli appear on some cells throughout this culture, including some in contiguous groups. × 1700.

Fig. 3. Nonneoplastic line 8322, comprising mostly less-spread, smooth-surfaced cells with many lateral processes and limited contact among neighboring cells. × 1400.

Fig. 4. Nonneoplastic line 8726, comprising mostly less-spread, smooth-surfaced cells with many lateral processes. Small ruffles appear at the edges of some cells. × 1400.

Fig. 5. Nonneoplastic line 8712, comprising mostly less-spread, smooth-surfaced cells with many lateral processes. Variable cell form is characteristic of this line. × 1300.

Fig. 6. Nonneoplastic line 8322. This cell (*) displays an unusually high incidence of microvilli on its exposed surface. × 1150.

Fig. 7. Nonneoplastic line 7943. Note extreme heterogeneity of cell form in this line with respect to size, shape, degree of flattening, and the prominent microvilli (*), ruffles (r), and blebs (b) on some cells. × 1150.

Fig. 8. Cells from a tumor explant after the 9th passage in culture. These cells display the same diverse forms as cells from nonneoplastic line 7943, which produced the tumor (cf., Fig. 7), indicating that any or all of these cell forms may be tumorigenic. × 1000.

Fig. 9. Nonneoplastic line 7943. This cell (*) was fixed in prophase 3 min after the onset of mitosis (2530 min after a previous mitosis) and is surrounded by numerous lateral processes. A moderate number of scattered microvilli and a small group of several blebs (b) appear on its exposed surface. The cell in the upper right-hand corner was fixed in anaphase 29 min after rounding for mitosis; it possesses some lateral processes and is covered with microvilli but lacks cytoplasmic blebs. × 1150.

Fig. 10. Nonneoplastic line 8475. These cells (*) were fixed 30 min after division, probably in G1 (conditional probabilities from Chart 4: G1, 0.961; S, 0.037; G2, 0); they have already flattened and display few microvilli on their exposed surfaces. A few blebs (b) and ruffles (r) are also present on 1 cell. × 1800.

Fig. 11. Nonneoplastic line 8322. This cell was fixed 40 min after mitosis, probably in G1 (conditional probabilities from Chart 3: G1, 0.938; S, 0.059; G2, 0.003), and has already spread and flattened onto the coverslip. The exposed surface is smooth with very few blebs or microvilli. This cell was moving at the time of fixation (arrow); ruffles occur on the leading edge (lower left), and retraction fibrils persist at the opposite trailing edge. Note the reference scribe on the surface of the glass coverslip (upper left). × 2000.

Fig. 12. Nonneoplastic line 8475. This cell was fixed 200 min after mitosis, probably in G1 (G1, 0.887; S, 0.113; G2, 0). The cell is flat and its exposed surface is extremely smooth. × 1150.

Fig. 13. Nonneoplastic line 8408. This cell (*) was fixed 255 min after mitosis, probably in G1 (G1, 0.868; S, 0.113; G2, 0.019). The cell is flat, spread, and smooth. × 1250.

Fig. 14. Nonneoplastic line 8408. This cell was fixed 428 min after mitosis, probably in G1 (G1, 0.797; S, 0.161; G2, 0.042), and resembles that in Fig. 13, except for the presence of scattered microvilli. × 750.

Fig. 15. Nonneoplastic line 8322. This cell was fixed 3098 min after mitosis, probably in G1 (G1, 0.036; S, 0.303; G2, 0.661) and is spread and flat with a few microvilli on its exposed surface. × 1800.

Fig. 16. Nonneoplastic line 7943. This cell was fixed 2375 min after mitosis, probably in G1 (G1, 0.052; S, 0.332; G2, 0.616), and is extremely flat and smooth. × 1000.
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