SCANNING ELECTRON MICROSCOPY OF IN VITRO CHEMICALLY TRANSFORMED MOUSE EMBRYO CELLS

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ABSTRACT

A cloned nontumorigenic control cell line of C3H mouse embryo cells (C3H/10T1/2CL8) and two cell lines derived from it by treatment in vitro with 7,12-dimethylbenz(a)anthracene (DMBA) or 3-methylcholanthrene (MCA) were studied by scanning electron microscopy. Confluent control cells were polygonal in shape and extensively flattened with smooth surfaces. Both in vitro transformants were pleomorphic to fusiform in shape, thicker than the control cells, and lacked contact inhibition. Microvilli of variable length and small marginal ruffles were characteristic surface alterations of the MCA-transformed cells, while blebs and numerous cytoplasmic strands extending between cells were typical of the DMBA transformant. Inoculation of the DMBA-transformed cells into C3H mice and re-establishment of cells from one of the subsequent fibrosarcomas in culture revealed an increased number of microvilli on the surface of the cells and an alteration in growth pattern. Other surface characteristics remained the same. A possible relationship between surface topography and outer membrane glycolipids is discussed.

Alterations of the surface membrane of cells may be a crucial change associated with malignancy (17). Previous studies have indicated that membrane glycoproteins (25), glycolipids (8, 15, 19), and lectin-binding sites (3, 7, 18) are altered after in vitro chemical or viral transformation of cells. Furthermore, many of the transformed cells are capable of growth in soft agar, are insensitive to contact inhibition of growth, and are tumorigenic in the appropriate host. Porter et al. have employed the high-resolution capabilities of scanning electron microscopy (SEM) to study cell topography in virally and spontaneously derived transformants of BALB/3T3 cells (31). All the transformants possessed a morphology different from that of the parent cell line, with increased numbers of surface structures in the form of microvilli, blebs, and ruffles observed in the transformed cells. However, only a limited number of viral and spontaneous transformants have been investigated for changes in surface morphology, and no previous studies have employed SEM to study the surface morphology of in vitro chemically transformed cells.

Chemical transformation of cells in culture has been achieved in several laboratories (16), and it is now possible to study the effect of chemical transformation on membrane characteristics and cell topography. Reznikoff et al. (33, 34) established a cloned line of C3H mouse embryo cells which are sensitive to post confluence inhibition of division, have a low spontaneous transformation frequency, and are malignantly transformable by several carcinogenic chemicals. Thus, this cell system has the potential of being used as an in vitro carcinogenicity assay based on the altered morphology and loss of contact sensitivity of the transformed cells (4, 20). In the present study, the scanning electron microscope was used to characterize in detail the surface topography in control

TABLE I

Properties of the Control and Transformed Mouse Embryo Cell Lines

Cell lines	Total passages in culture*	Plating efficiency	Doubling time	Saturation density (cells/100-mm dish × 10^{-6})	Tumor production
		%	h		
C3H/10T1/2CL8	32	16	19.2	3.4	—
DMBA-TCL1	33 (6)	42	19.2	11.6	+‡
MCA-TCL15	35 (10)	42	23.5	16.7	+§
DMBA-tumor	35 (8)	29	26.4	6.3	ND||

* The control cell line, C3H/10T1/2CL8, is 12 passages from cloning. The number in parentheses indicates the number of passages since isolation of the transformed foci.
‡ Reference 20.
§ Reference 34.
|| Not determined.

MATERIALS AND METHODS

The cloned control mouse embryo cells and the transformants derived from them were grown in Petri dishes in Eagle's basal medium plus 10% heat-inactivated fetal calf serum (both from Grand Island Biological Co., Grand Island, N.Y.) in humidified incubators at 37°C with an atmosphere of 5% CO_2 in air without antibiotics or fungizone. Mycoplasma tests conducted by the PPLO (pleuropneumonia-like organisms) Testing Service of Microbiological Associates (Bethesda, Md.) were negative. The C3H/10T1/2CL8 cell line and the tumorigenic MCA-TCL15 cell line were supplied by Dr. C. Heidelberger (33, 34).

The transformation assay was carried out by seeding 500 C3H/10T1/2CL8 cells in 5 ml medium into 60-mm Petri dishes (20). 24 h after seeding, 7,12-dimethylbenz(a)anthracene (DMBA)[1] dissolved in dimethylsulfoxide was added to a final concentration of 1 µg/ml. The duration of exposure was 24 h, and thereafter the medium was changed once weekly for 6 wk when either the dishes were fixed and stained with Giemsa's (20) or transformed foci were isolated by the ring isolation technique (32). Control cultures were treated in the same manner, except that only dimethylsulfoxide was added. One of the transformants isolated (DMBA-TCL1) pro-

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[1] Abbreviations used in this paper: DMBA, 7,12-dimethylbenz(a)anthracene: C3H/10T1/2CL8, cloned nontumorigenic control cell line derived from C3H mouse embryo; DMBA-TCL1, cloned tumorigenic cell line derived from C3H/10T1/2CL8 by in vitro treatment with DMBA; MCA, 3-methylcholanthrene; MCA-TCL15, cloned tumorigenic cell line derived from C3H/10T1/2CL8 by in vitro treatment with MCA.

duced a tumor within 6 wk in each of three C3H mice after subcutaneous inoculation of 1.5 × 10^6 cells per animal (20). The DMBA tumor cell line was established by readapting cells from one of the resultant tumors to culture. Stock cell lines were maintained in liquid nitrogen and used within five passages of thawing.

For studies of exponentially growing cultures and confluent cultures, 1,000 cells or 500,000 cells, respectively, in 10 ml medium were seeded in 100-mm Petri dishes. In both cases, the medium was changed on day 3 and day 6 and the cells were fixed for SEM observation on day 8. The procedures for determining plating efficiency and growth rate of the cells have been previously described (19). Saturation densities were measured by counting the number of cells in the confluent cultures on day 8.

Cell cultures for scanning electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 1 h. Postfixation for 1 h in 1% buffered osmium tetroxide was followed by dehydration in ethanol. Cells were then passed through mixtures of absolute ethanol and Freon 113 to absolute Freon, and were dried by the critical-point method (2) with Freon 13. The dry specimens were lightly coated with evaporated carbon and gold and mounted on stubs with silver paint. An ETEC Autoscan operating at 20 kV was used.

OBSERVATIONS

Characteristics of the cell lines pertinent to the current investigation are presented in Table I. The total passages in culture for all the cell lines are similar and therefore should not be a factor in the comparison of these cells. The transformants had a higher plating efficiency and an equal or slightly slower generation time than the C3H/10T1/2CL8 cell line. The saturation densities and tumorigenicity of the cell lines, two important criteria for

malignant transformation, are also shown in Table I. The saturation densities of the DMBA and 3-methylcholanthrene (MCA) transformants were, respectively, three and five times that of the C3H/10T1/2CL8 cell line.

Grossly, the C3H/10T1/2CL8 cultures appear as uniform thin layers (Fig. 1 a). The DMBA-treated cultures develop foci (Fig. 1 b) varying in size and morphology. Fig. 2 illustrates the edge of one colony. While a dense mat of cells forms the center of the colony, cells toward the edge are more spindle shaped and grow in cordlike arrangements which appear to invade the surrounding nontransformed, contact-inhibited cells. The transformed cells produced fibrosarcomas (Fig. 3) in vivo, while the C3H/10T1/2CL8 cell line was not tumorigenic (Table I). When the cells from one of these DMBA tumors were readapted to culture, they grew to a lower saturation density than the original transformant. These cells tend to grow on other cells rather than the substrate and do not cover the surface of the Petri dish, even when cultured for several weeks.

C3H/10T1/2CL8 Cells

Fig. 4 depicts a confluent population of C3H/10T1/2CL8 cells. These cells are extremely thin and polygonal with smooth surfaces. Occasionally, a few marginal ruffles and small microvilli are present. In their flattened form and growth pattern these cells resemble epithelial cells. Intercellular spaces may appear somewhat distended due to cell shrinkage during dehydration and critical point drying. Although in exponentially growing populations thickening and rounding up occur with concomitant accumulation of numerous microvilli as cells go into division (Fig. 5), epithelioid characteristics are evident in interphase cells. The smoothness of the interphase cell surface in confluent cultures is interrupted only by small pits and stubby microvilli (Fig. 6). Observation of the confluent cell cultures at angles of 70°–90° incident to the beam allows examination of the cells from the side and confirms that they grow in monolayer sheets without overlapping or piling up (Fig. 7).

DMBA-TCL1 Cells

The DMBA-TCL1 cells show a different growth pattern in exponential cultures (Fig. 8) and in confluent cultures (Fig. 9) than do the parent C3H/10T1/2CL8 cells (Figs. 4 and 5). In exponential growth the transformant cells are more pleomorphic and tend to pile up rather than grow over the substrate (Fig. 8). A profusion of slender strands 1 μm or less in diameter extends between adjacent or nearby cells over distances of up to 125 μm. Microvilli and small blebs are also in greater abundance on the surface of the transformants (Fig. 8). At confluence (Fig. 9), the transformed cells are more pleomorphic and densely packed than the control cells. Slender strands, tiny pits, blebs, and microvilli are consistently observed on the cell surfaces in the confluent cultures (Fig. 10). Loss of contact inhibition in the DMBA transformant can be demonstrated by an "on edge" view achieved by a high tilt angle. This shows that the cultures contain several layers of cells (Fig. 11), a fact not easily detected by en face observation of confluent cultures, and have a growth pattern strikingly different from that of the confluent C3H/10T1/2CL8 cells (Fig. 7).

DMBA Tumor Cells

Inoculation of the DMBA-TLC1 cells into C3H mice produces fibrosarcomas. When re-established in tissue culture, the tumor cells assume elongate, fusiform, or pleomorphic shapes during logarithmic growth, typical of growing fibroblasts (Fig. 12). Cell surfaces are covered with microvilli; some surfaces display nodular elements of varying sizes, and a few have small marginal ruffles. Long, thin cytoplasmic processes extend across wide intercellular spaces to make contact with the nearest cells, forming loose fascicles of the cells and their processes. The inclination of the cells to grow on their neighbors results in the development of cellular cords forming a meshlike reticulum. Although this growth characteristic remains as the density of the culture increases, the cell morphology is altered. In dense cultures, the cells grow in patches several cell layers thick, overlying one another in random fashion (Fig. 13). There is no evidence of confluence even after several weeks in culture. The cells become flatter, broader, and more polygonal as the cultures become denser (Fig. 13). Microvilli, blebs, and marginal ruffles remain on the cell surface. The filamentous strands extending between adjacent cells are characteristic of DMBA tumor cells (Fig. 14). Similar strands are also present in the DMBA-TCL1 cells (Fig. 10).
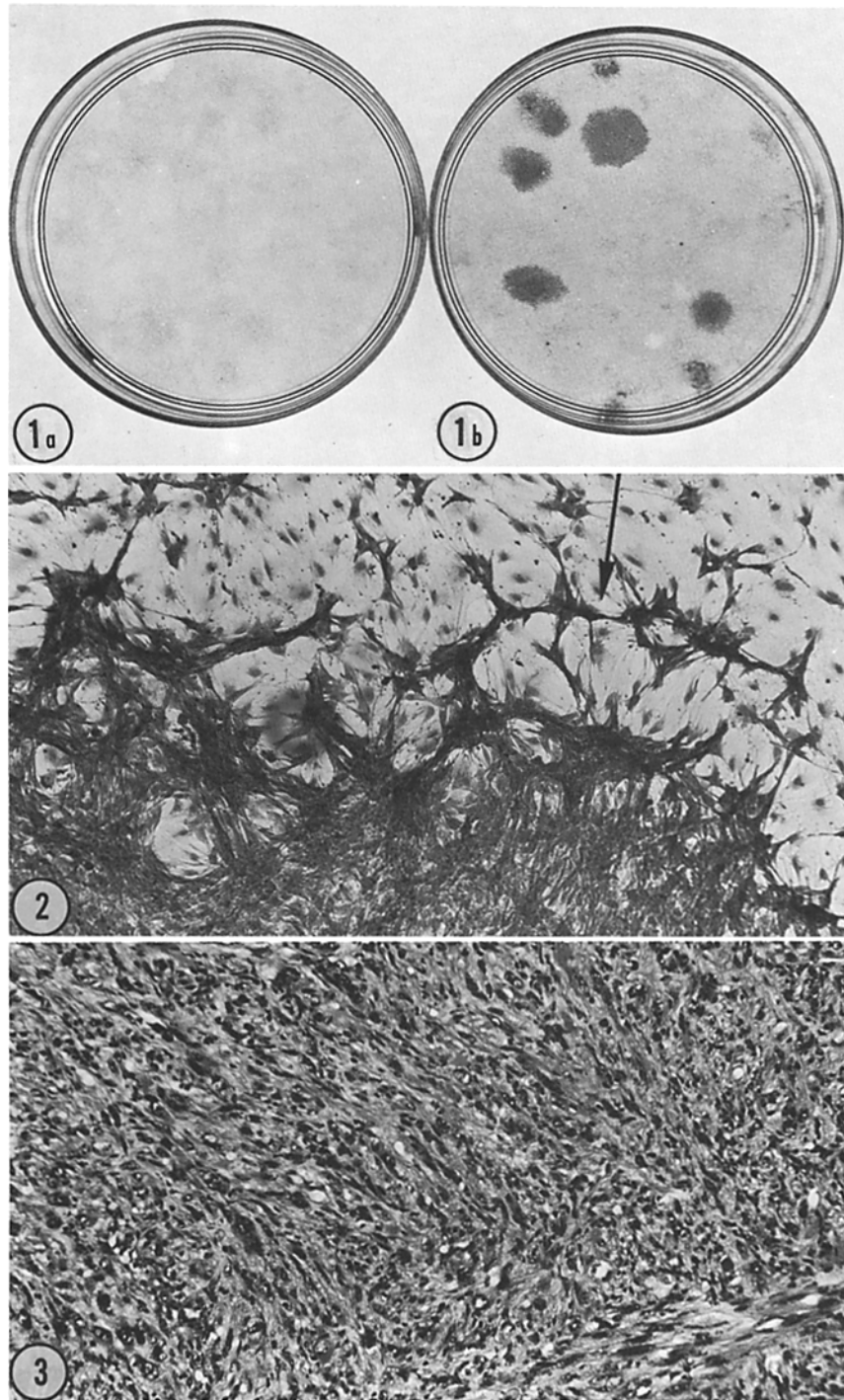
FIGURE 1 (a) C3H/10T1/2CL8 cells grow as a uniform monolayer. (b) Treatment in vitro of the C3H/10T1/2CL8 cells with DMBA induces development of foci of varying sizes and shapes.

FIGURE 2 Cells at the edge of a DMBA-transformed colony are spindle shaped and grow in cordlike arrangements (arrow). Cells in the center form a multilayered dense mat. × 45.

FIGURE 3 Fibrosarcomas are produced by the inoculation of DMBA-TCL1 cells into C3H mice. × 115.
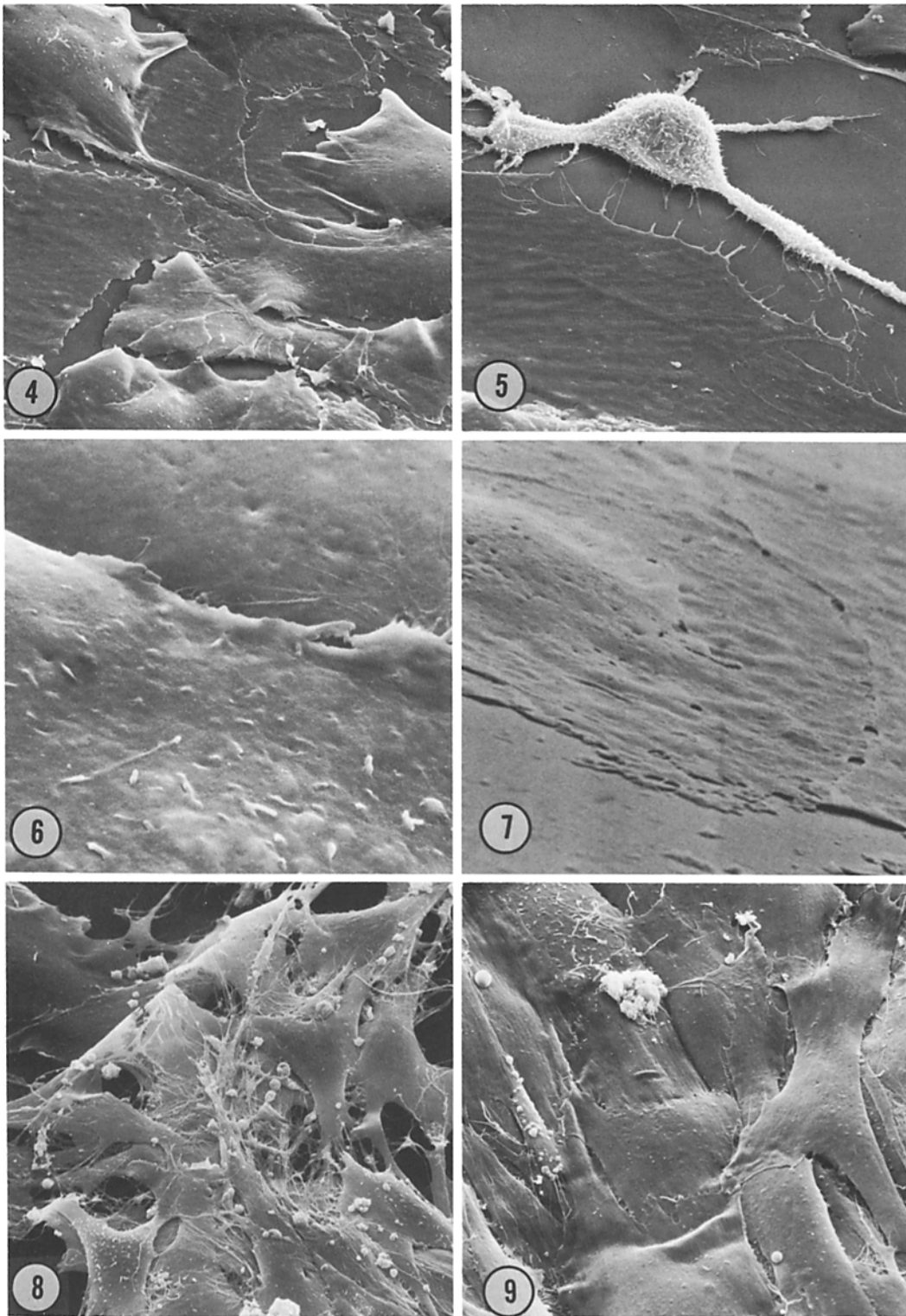
MCA-TCL15 Cells

Cells transformed in vitro by treatment with MCA display epithelioid characteristics similar to those of cells transformed with DMBA. Morphologically, the cells are flattened, thicker than the parent C3H/10T1/2CL8 cells, broad, and polygonal to pleomorphic in shape both in cultures of logarithmic growth (Fig. 15) and in cultures of high density (Fig. 16). The cells grow on other cells in a crisscross fashion (Fig. 16). Small marginal ruffles are more common in these cells than in the DMBA-TCL1 cells (Fig. 17). Blebs and slender strands are infrequent. Microvilli of variable lengths and orientations and small pits or indentations interrupt the generally smooth surface (Fig. 17). The growth of MCA-transformed cells on top of one another occasionally results in piled up foci (Fig. 18). The cells surrounding the mound resemble those of the dense, multilayered MCA-TCL15 cultures, i.e., they are broad, flattened, thick cells (Fig. 19 *a*), whereas the cells composing the mound itself are more rounded or fusiform in shape and have a greater number of microvilli (Fig. 19 *b*).

DISCUSSION

The morphology that cells adopt in culture depends on a variety of factors. Cells in sparse cultures are frequently thicker than those in dense cultures, reflecting a relationship between morphology and cell contact (31), and stage of the cell cycle (12, 26, 30). Variations in the composition of the culture medium, such as changes in pH (27, 28, 37), addition of dibutyryl cyclic adenosine monophosphate to the medium (27), treatment with trypsin (9, 11, 36), and use of fresh or conditioned medium (37) can also affect cell morphology in vitro. In the present study the C3H/10T1/2CL8 cultures and the transformed cell lines were treated in an identical manner so that the effect of such variables would be negligible when comparing their surface features.

Distinctive changes in cell morphology and growth characteristics occur in the in vitro chemically transformed cells. The cells are more pleomorphic or fusiform in shape and their surfaces display increased numbers of microstructures in the form of microvilli, blebs, and ruffles. Similar changes have been noted in virally and spontaneously transformed cells (31). Despite these general similarities in the transformed cell lines, specific differences in surface morphology are associated with the individual transformants. Microvilli of variable length and small marginal ruffles are characteristic alterations of the MCA-TCL15 cell surface whereas an increased number of blebs and numerous cytoplasmic strands extending between cells are typical of the DMBA-TCL1 cells.

Inoculation of the DMBA-TCL1 cells into C3H mice and re-establishment of cells from one of the subsequent tumors in culture results in more extensive alteration of cell shape (compare Figs. 9 and 13) and a moderate increase in the number of microvilli (compare Figs. 10 and 14). The basis of these changes is not known, but Glick et al. (13) have suggested a correlation between the surface membrane glycopeptides and the expression of malignancy after passage of in vitro chemically transformed hamster embryo cells in vivo. Certain glycopeptides were detected in significant amounts

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Figure 4 Confluent C3H/10T1/2CL8 cells are polygonal and extensively flattened with smooth surfaces. × 600.

Figure 5 C3H/10T1/2CL8 cells in exponential growth. Interphase cells are similar to those in confluent cultures. Numerous microvilli appear as cells round up for division. × 640.

Figure 6 The surface of C3H/10T1/2CL8 cells is relatively unadorned. Only small pits and stubby microvilli are present. × 4,000.

Figure 7 Side view of C3H/10T1/2CL8 cells in confluent culture demonstrates their monolayer growth pattern. × 1,200.

Figure 8 DMBA-TCL1 cells in exponential growth are distinguished from the parent C3H/10T1/2CL8 cells by a greater abundance of blebs of varying sizes and a profusion of slender strands extending across adjacent cells. Cells tend to pile up rather than grow over the substrate. × 720.

Figure 9 DMBA-TCL1 cells in confluent culture are more pleomorphic and densely packed than the parent C3H/10T1/2CL8 cells. × 680.
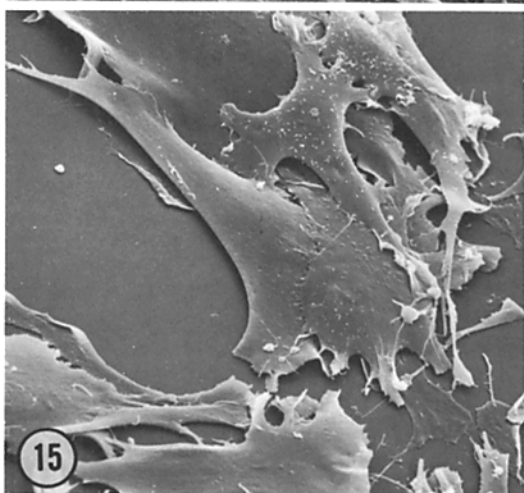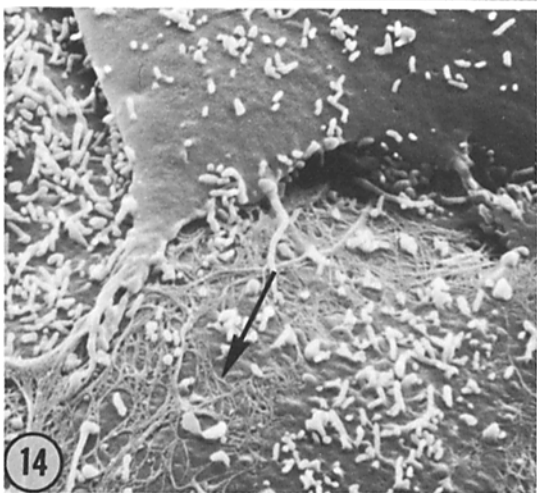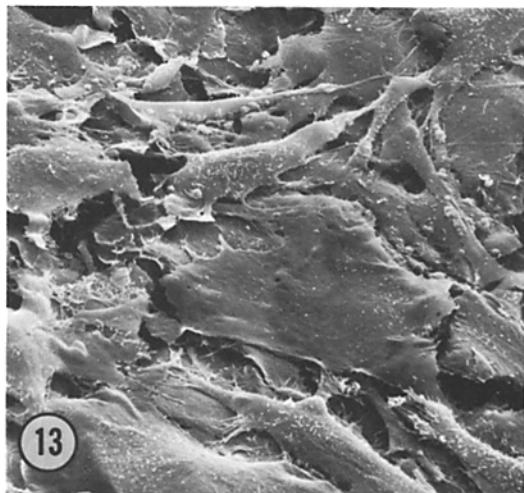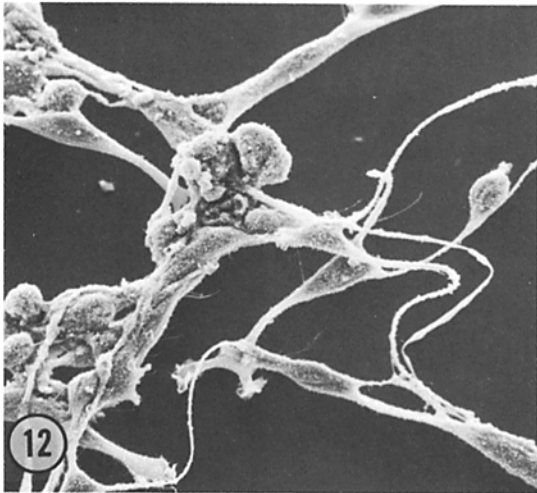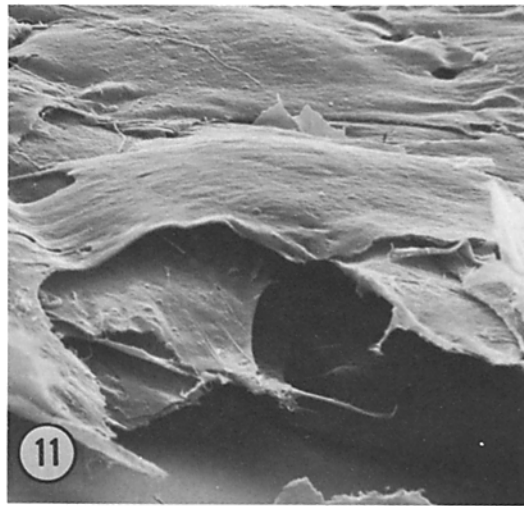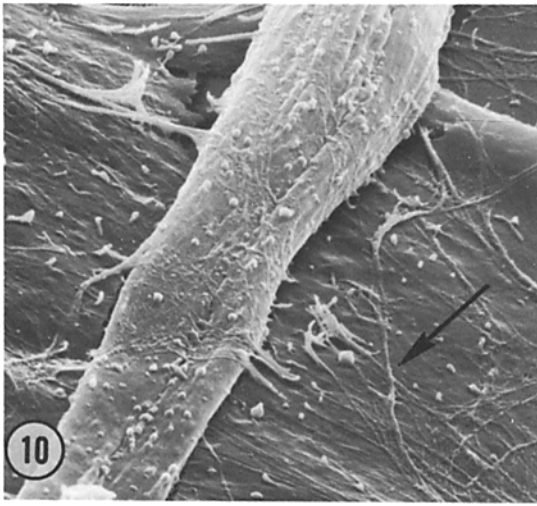
only after passage of the transformed cells in vivo (13).

It is the general consensus that changes in cell morphology and an increase in the numbers and types of surface microstructures have a basis in accompanying chemical and subsurface structural alterations (1, 22, 23, 29, 31, 35), which may be reflected in various cell properties such as mobility of the cell surface, cell-cell contacts, enzyme activity, and cell recognition (24). In this respect, it is of interest that the DMBA-TCL1 cells and MCA-TCL15 cells exhibit alterations in cellular gangliosides (21). There is some evidence that gangliosides, which are believed to be primarily outer membrane components (15), may be involved in some of the biochemical alterations underlying changes in growth properties and surface morphology associated with morphological (10) and malignant transformation (5, 6, 14).

It is not yet known whether oncogenic chemicals and viruses in general evoke similar cellular responses involving surface alterations at some stage in the neoplastic process. Further studies with normal and transformed cells are in progress to clarify the relationships among transformation, cellular morphology, and membrane glycolipid composition.

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FIGURE 10 Slender strands (arrow), tiny blebs, and microvilli are consistent surface features of the DMBA-TCL1 cells. × 4,000.

FIGURE 11 Side view of DMBA-TCL1 cells in dense culture demonstrates multiple cell layer growth pattern. × 1,200.

FIGURE 12 DMBA tumor cells. Inoculation of DMBA-TCL1 cells into C3H mice produced fibrosarcomas. Cells from one of these tumors were re-established in culture. During exponential growth, DMBA tumor cells are pleomorphic to fusiform in shape with numerous microvilli, some blebs, and a few marginal ruffles. The tendency of cells to grow on adjacent cells results in development of cellular cords forming a meshlike reticulum. × 340.

FIGURE 13 In dense cultures, the DMBA tumor cells are fusiform to polygonal in shape and grow over one another in random fashion forming multilayered patches. × 600.

FIGURE 14 Microvilli and blebs are visible on surfaces of DMBA tumor cells. Filamentous strands (arrow) extending over adjacent cells are a distinctive characteristic of these cells. × 4,000.

FIGURE 15 MCA-TCL15 cells in exponential growth are flattened, but thicker and more pleomorphic than the parent C3H/10T1/2CL8 cells. Small marginal ruffles are common in this transformant. × 600.
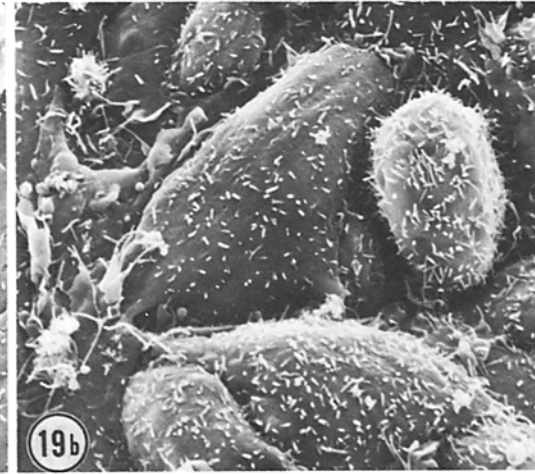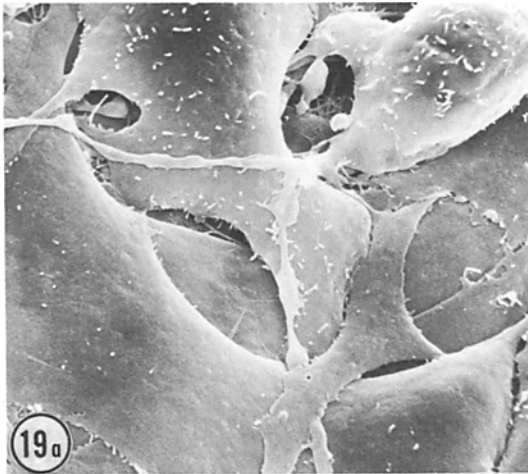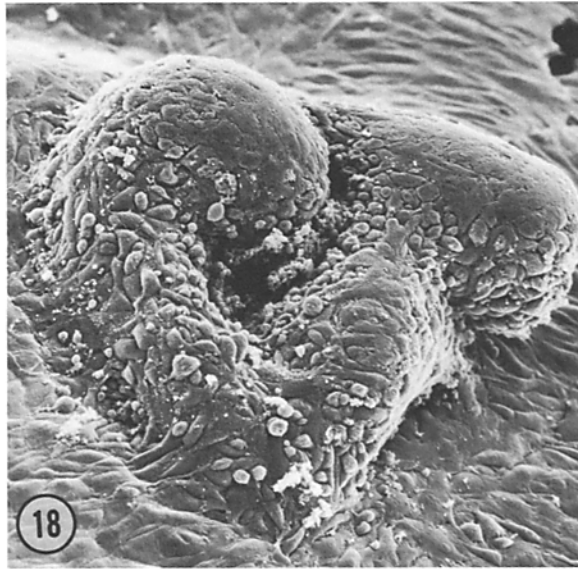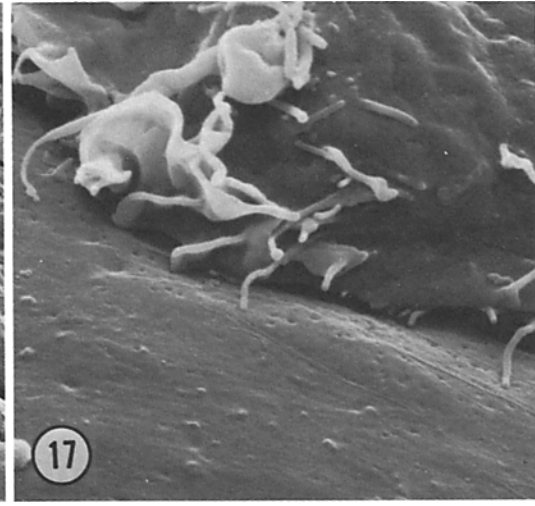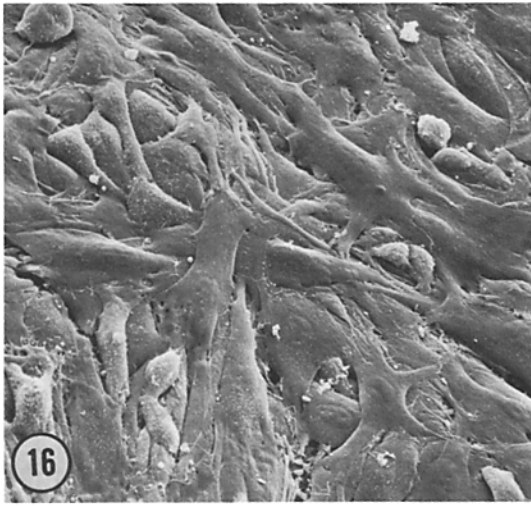
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FIGURE 16   Cell shape of MCA-TCL15 cells in dense cultures deviates considerably from that of the C3H/10T1/2CL8 cells. MCA-TCL15 cells grow on top of one another in a crisscross fashion. × 300.

FIGURE 17   Surfaces of MCA-TCL15 cells show small bumps, pits, and stubby microvilli. Small marginal ruffles are more common in these cells than in DMBA-TCL1 cells. × 4,000.

FIGURE 18   Occasionally the multilayered growth pattern of the MCA-TCL15 cells results in piled up cellular mounds. × 105.

FIGURE 19   (a) MCA-TCL15 cells at the base of the mound are broad, flattened, thick cells characteristic of the dense MCA-TCL15 cultures. × 1,440. (b) Cells composing the mound itself are more pleomorphic and have a greater number of microvilli. × 1,720.

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