A SCANNING ELECTRON MICROSCOPE STUDY OF SURFACE FEATURES OF VIRAL AND SPONTANEOUS TRANSFORMANTS OF MOUSE BALB/3T3 CELLS

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ABSTRACT

Cells of the mouse line Balb/3T3 as well as three virus-induced transformants and two spontaneous transformants grown in vitro have been studied for their topography by scanning electron microscopy. The parent cell in confluent culture closely resembles an endothelial cell in its form and in the structure of its association with adjacent cells. The tumorigenic transformants produced by SV40, murine sarcoma virus, or polyoma viruses are fusiform to pleomorphic and distinctly different from the cell of origin. They show relatively smooth surfaces except for blebs and marginal microvilli. Perhaps most surprising is the similarity they bear to one another. This is made the more singular by the very different form shown by the tumorigenic transformants of spontaneous origin. One of these, S2-4, possesses a thickened rather than the lamellar form of the parent A31 cell and is covered by long microvilli and many spherical blebs. The other, TuT3, more closely resembles the cell of origin but shows extensive ruffling at its margins. All transformants grow without evidence of contact inhibition.

The significance of the surface morphologies and the factors influencing cell form are discussed.

INTRODUCTION

The mouse cell line, Balb/3T3 (1), which can be kept under continuous culture, has been extensively studied because the cells are strongly contact inhibited and can be transformed by any one of several agents including oncogenic DNA- and RNA-containing viruses (2), radiation, and chemical carcinogens (3). They also spontaneously transform in cell culture and the transformants are recognizable by their changes in shape and pattern of growth, features observable by light microscopy. The transformed cells acquire the ability to grow under a variety of conditions that restrict the growth of the "normal" Balb/3T3 cells (4). It is reasonable to suppose that additional characteristics of these transformants, including the distribution of surface antigens, might be evident in higher resolution images and that such images might help understand their altered growth properties and their acquired tumorigenicity. Toward this end we have sought to take advantage of some recent improvements in scanning electron microscopy (SEM) and specimen preparation techniques (5, 6).

A few interesting observations have emerged.
First, the parent strain (Balb/3T3), generally thought to be a fibroblast cell, appears to be epithelioid for it grows in sheets of closely knit cells. Then it has been observed that transformants of the Balb/3T3 show characteristic surface abnormalities that are relatively constant for any one strain through successive generations of culturing. Of the strains examined, the spontaneous transformed variant, S2-4, shows the most extreme departures from Balb/3T3. Though they retain in their growth some expression of the epithelial character of the parent line, the S2-4 cells are thicker, show structurally complex surfaces, and tend to pile up in their proliferation. Viral transformation by either DNA or RNA tumor viruses yields spindle-shaped, fibroblast-like cells showing no evidence of contact inhibition. Only in minor surface features are they different from one another.

**Materials and Methods**

Six different cell lines were examined: the “normal” Balb/3T3, clone A31 (3T3-A31); an SV40-transformed subclone derived from it (SV-T2); a mouse sarcoma virus transformant of A31 (K-A31); a mouse polyoma virus transformant (Py-3T3-4a); an in vitro-selected spontaneously transformed variant (S2-4); and an in vivo-selected spontaneously transformed (TuT3).

Approximately 2 X 10⁴ cells were plated onto 22 X 22-mm coverslips in 30-mm plastic petri dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.). All cells were grown in Dulbecco’s modified Eagle’s medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml fungizone) (GIBCO), at 37°C in a high humidity incubator under an atmosphere of 5% CO₂-95% air. Fresh media was added to the dishes after every 72-h period and at no time was the media composition altered. A minimum of 25 cultures of each cell type was examined at intervals between 2 and 11 days on a Reichert inverted microscope using phase optics; the final of these examinations immediately preceded fixation for electron microscopy. Because variations in cell morphology were almost never seen during the increase in density of the cultures, comparative electron microscopy was consistently performed on cultures immediately before confluency was reached. At this time cell division was still evident, the cells were in contact and any responses to cell contact were in effect.

In preparation for electron microscopy, the cells were fixed at 37°C for 20 min with 3% glutaraldehyde buffered with 0.05 M cacodylate, pH 7.2, and 0.5 X Puck’s saline G (GIBCO). After a brief wash in 1 X saline G (37°C) they were postfixed for 15 min at room temperature with 1% osmium tetroxide buffered with 0.2 M cacodylate buffer, pH 7.2, and dehydrated rapidly in acetone. The fine structure of the cell surfaces was preserved against drying artifact by taking the specimens through the critical point of liquid CO₂ as described by T. F. Anderson (7) using a Sorvall critical point dryer (5) (Ivan Sorvall, Inc., Newtown, Conn.). The cells were then coated with carbon followed by a thin film of gold (100 Å) in a vacuum evaporator. The preparations were viewed and photographed with a Cambridge S-4 stereoscan electron microscope operated at 20 kV. The SEM images of the fixed cells correlated positively with those of the living cells as viewed in the light microscope.

**Observations on Cells**

Fig. 1 depicts a population of A31 cells which is clearly confluent. Where a space is evident it is most probably a product of shrinkage resulting from acetone dehydration. The habit of growth as well as the generally thin, flattened form of the cells, is characteristic of squamous epithelial cells and leaves one with the impression that the original line may have come from either the endothelial cells or pericytes of small blood vessels (8). Small microvilli, approximately 0.1 µm in diameter and of variable length, are distributed along the cell margins and sparsely over the cell center. At their edges where the cells are in contact, the microvilli and more slender filopodia tend to reach across the intervening gap (Fig. 2) as they do in endothelia observed in situ (9-12). Infrequently, there are also small ruffles at the cell margins. Apart from these features the surfaces show nothing remarkable; they are, in fact, comparatively unadorned as might be expected of cell surfaces designed to face the circulating blood.

The SV40-transformed Balb/3T3 cells are strikingly different (Fig. 3). From the endothelial form shown in Fig. 1 they are transformed to spindle shapes and elongate, pleomorphic forms more typical of growing fibroblasts. They show in their growth no evidence of confluency or of contact inhibition of cell division. The cells seem quite independent, cross each other in a random fashion, and obviously grow in several layers.
FIGURE 1  BALB/c3T3 (clone A31) epithelioid sheet. Numerous filopodia at the cell margins mark the perimeter of each cell. Microvilli on the surface tend to concentrate over the cell center. X 1,000.

FIGURE 2  High magnification view of interdigitation of the cellular extensions (filopodia) of adjacent A31 cells. Many of these resemble microvilli in size and shape. X 7,100.
Figure 3  SV40 virus transformant of the A31 line. The cells have lost their contact inhibition and have adopted an elongated, fibroblast-like form. Note the numerous blebs (arrows) but general absence of microvilli on the surfaces. Cellular extensions appear at the cell margins (asterisks). $\times$ 1,000.

Figure 4  K-A31 cells transformed by murine sarcoma virus, an RNA tumor virus. These cells are also elongate and lack contact inhibition. They are distinguished from the SV40 virus transformants by an increase in surface modifications. Small microvilli and blebs of varying size appear on the surfaces as well as fine cytoplasmic extensions which interconnect many of the cells (asterisks). $\times$ 1,000.
Except for a few filopodia at the cell margins, the surfaces are free of microvilli. Blebs, which vary in size from 1.0 to 5.0 µm, are the only surface structures seen with any frequency.

Cells of the K-A31 line (murine sarcoma virus-transformed) show several features in common with the SV40-transformed cells (Fig. 4). The cells are similar in overall form and seem similarly unaffected by contact. They pile up with the same abandon. They differ slightly from the SV-T2 cells and parent A31 cells in having more surface adornments. These are mostly in the nature of small blebs such as are found in other transformants and a few very short microvilli such as occur on the surfaces of the A31 cells. Evident also are a number of slender strands which, at uniform diameters of less than 0.1 µm, extend away from and between cells over distances of 10-15 µm. The nature of these is not clear.

Cells of the polyoma virus-transformed strain (Py-ST3-4a) repeat again in their appearance many features of the other two viral transformants (Fig. 5). The majority of the cells adopt forms characteristic of fibroblasts. Their surfaces are extraordinarily smooth except for a scattering of small microvilli and some ruffles at the ends of the pseudopodia. The cells overlie one another in a random fashion as though unresponsive to contact with other cells. One feature unique to this cell line is a tendency of the cells to fuse and form giant multinucleated units.

Spontaneously transformed cells of Balb/3T3, the S2-4 line (Figs. 6, 8a and b), are like the parent cells in their tendency to grow in sheets. Such contacts as are formed seem, however, to be less enduring than between the cells of A31. The individual cells are much thicker than the "normal" equivalent and in this demonstrate the common behavior of malignant cells to round up. Their surfaces are extraordinary. A few individual cells show blebs and such cells are probably in early G1 of the cell cycle (13). Others are characterized by large numbers of microvilli which are about 0.1 µm in diameter and in some instances several micrometers in length. The microvilli are unusual in being curved and bent over, frequently close to the cell surface in a way reminiscent of those which occur at the margins of the A31 cells.
Figure 6  S2-4 spontaneous transformant of A31. The cells deviate substantially from the shape of the normal (A31) cells and show no evidence of contact inhibition. Their surfaces show, compared with A31 cells, a larger number of long microvilli (M), considerable ruffling (arrows), and more blebbing (B). × 1,000.

Figure 7  TuT4 is another spontaneous transformant of the normal A31 cells. These are very different from the S2-4 cells as well as from the viral transformants. They more closely resemble the cell of origin but show greater ruffling activity at their margins and a loss of contact inhibition (arrows). × 1,000.
The population density of microvilli is uniformly high.

Another spontaneous transformant, TuT3, differs strikingly from the S2-4 line. Here the cells spread quite thinly and are relatively smooth like the control A31 cells (Fig. 7). What sets them apart from the others is the prominence of ruffles at their margins and their tendency to separate from other cells and pile up in their growth and proliferation.

Fig. 8a and b shows the surfaces of a few S2-4 cells in stereo. This method of imaging gives a much improved impression of the nature of the several surface features. The blebs are seen as small spheres, in some instances supported on pedicles. Microvilli appear as slender filaments which project vertically from the surface or, more frequently, adopt a more proné relation to the surface.

**DISCUSSION**

The Balb/3T3 line is a continuous line of mouse cells that is nontumorigenic and contact inhibited. It may, as noted above, give rise to transformed, tumorigenic variants that are recognizable different from the parent cells. These differences, heretofore described largely in terms of the interaction of their surface with plant lectins (14, 15) and by light microscopy (16, 17), are now described in greater detail by SEM. While most of the observed changes are not at present interpretable, their visualization at these resolutions makes the study of this valuable cell line and its variants more attractive. Starting with a cell (A31) of known form, one can follow the alterations in form and surface features that accompany the acquisition of tumorigenic properties. When these are explored in greater depth and especially

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*Figure 8a and b* This is a stereo pair of the spontaneous transformant S2-4 which, when fused, offers a greater perspective of the surface modifications. (A folding, cardboard, pocket-size stereoscopic viewer manufactured by Taylor-Merchant Corp., New York, is useful for looking at stereo pairs.) × 2,000.
in relation to changes in the internal structure of the cells (18), the surface expression of transformation may acquire greater meaning. This will become increasingly so as the resolutions of the SEM are used to define the presence and distribution of specific antigens and binding sites on the cell surface.

The clone A31 cells are more epithelioid than fibroblastic. The cells, when confluent, assemble in sheets and show many of the characteristics of endothelial tissues that line the vessels of the peripheral circulation. That this tissue may in fact have been the origin of this cell line is further supported by the studies of Franks and Cooper (8). These authors observe that most lines of cells isolated from embryonic tissues have features in common and are similar in many respects to cells of the vascular endothelium or associated pericytes. This is borne out as well by numerous transmission electron microscope studies of this tissue which show finger-like projections from the luminal surface (microvilli) of the endothelial cells and others of similar form extending across cell junctions (9-12); these correspond to the structures observed at the confluent margins of the A31 cells (Fig. 2).

The forms that cells adopt in vitro doubtless depend on a variety of factors of which a few have been identified. In some instances contact with other cells is involved. For example, CHO cells in confluence, observed during the cell cycle, go through a series of morphological changes which includes during S and G2 a relatively well-spread lamellar form (13). The same cells maintained in a very sparse culture where little or no contact is achieved retain during most of the cycle a humped-up form more characteristic of G1 (19). Thus it appears that cell form is one expression of contact inhibition of growth and movement.

There is now some evidence that cyclic AMP is involved in these phenomena. The addition of dibutyryl cyclic AMP to the culture medium induces CHO cells (also strains L-929 [20] and 3T3 [21]) to become highly anisometric and strongly bipolar in morphology (22). Though not identical to the form changes induced by contact inhibition they are similar (20). The response of the cells in terms of growth appears to be identical (21). Electron microscope observations on the microtubules in the treated cells reveals a doubling in population and a change in orientation from random to one that parallels the long axis of the dibutyryl cyclic AMP-affected cells. It seems indeed that in most instances when the form of the cell becomes more anisometric and growth slows or stops the number of tubules increases. Concomitant with these events which are associated with contact inhibition the concentration levels of endogenous cyclic AMP increases (23, 24). One may assume then that cyclic AMP encourages tubule assembly and therefore a shift in the equilibrium between disassembled and assembled tubulin in the direction of assembly. Thus the tubulin involved in completely formed tubules is not available for involvement in the form changes associated with cell motility (25, 26) or even with mitosis. In cases where cell proliferation is active as in transformed cells, cyclic AMP levels are much lower (23). These observations and correlations seem pertinent to an understanding of the behavior and morphological properties of the A31 and transformed cells studied here (27).

The viral transformation by SV40, murine sarcoma, and polyoma is accompanied by striking changes in cell morphology. From their polygonal epithelioid form the A31 cells change to a spindle shape with increased numbers of blebs and filopodia. Similar changes for SV40 have been reported by Perecko et al. from SEM studies (28). The spontaneously transformed lines S2-4 and TuT3 show a very different pattern with retention of the epithelioid morphology but with extensive surface alterations including numerous blebs and microvilli. The progeny, then, of a single cell can be altered by both extrinsic and intrinsic factors. We find it particularly interesting that the morphological changes induced by SV40, the murine sarcoma virus, and the polyoma virus are so very similar. The shape of the cells, the major surface characteristics, and the habit of growth are alike in each. Minor differences in the number of small microvilli, the number of small blebs, and slender filopodia are found, but could merely represent variations associated with phases in the cell cycle. Apparently subtle differences in the cells not recognized by SEM do find expression in the colonial morphology and the density of cells in the colonies (29).
The total loss of the epithelioid form and acquisition of a fusiform shape suggests that the cytoskeleton represented by microtubules may have been restructured. To settle this question the fine structure of these cells will have to be thoroughly examined. It does appear, however, that the form-controlling machinery and the mechanism by which contact inhibits growth are both affected by the confirmed presence of the viral genome for even though microvilli and filopodia are present at their margins, the transformed cells seem not to adhere or display any effect of contact. It is appropriate to note that the concentration of cyclic AMP is reduced in the viral transformants (Py-3T3-4a) (30) and that the morphology of the cells as well as their contact inhibition is reportedly restored to normal by dibutyryl cyclic AMP (21).

These characteristics of the viral transformants are made the more dramatic by the quite different structural abnormalities of the spontaneous transformants. Surface features, microvilli, blebs, and ruffles occur in exaggerated number and form. The surface topography gives the impression of being very irregular and active. The assumption usually made is that these actively growing tumorigenic cells use these various surface extensions to enhance the uptake of metabolites (31)-(33). Whether the surface activity of the spontaneous transformants is characteristic of other or even all tumorigenic cells of this origin is not known, but the evidence available to date from SEM suggests that it is (32)-(34). We do know that spontaneous transformants appear quite dissimilar from one another in their topography and, from yet unpublished observations, that other spontaneous transformants derived from A31 have their own distinctive characteristics and are unlike S24 and TuT3. Since the surface morphology of the viral transformants of A31 do not include extensive production of ruffles and microvilli these cells must be able to accomplish enhanced uptake by some other devices (35, 36). Whether the different tumor viruses bring about transformation through some distinctive surface alteration remains to be determined; that they can induce profound alterations in the form of the A31 cells, possibly through cytoskeletal remouldings and changes in the topography of these cells, is clearly indicated.

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