Comparison of Surface Material, Cytoplasmic Filaments, and Intercellular Junctions from Untransformed and Two Mouse Sarcoma Virus-transformed Cell Lines

Gerald B. Dermer, John Lue,1 and Harry B. Neustein

Department of Pathology, Hospital of the Good Samaritan Medical Center, Los Angeles, California 90017 [G. B. D.], and Department of Pathology, Children's Hospital of Los Angeles and University of Southern California School of Medicine, Los Angeles, California [J. L., H. B. N.] 90033

SUMMARY

Surface material, cytoplasmic filaments, and intercellular junctions of an untransformed normal rat kidney (NRK) cell line were compared to those of NRK cells transformed by a nonproductive mouse sarcoma virus and to NRK cells transformed by productive mouse sarcoma virus. Surface material was visualized by ruthenium red (RR) or by staining glycol methacrylate sections with acidic phosphotungstic acid. Normal and transformed cells were examined in subconfluent and confluent cultures.

In subconfluent cultures where normal and transformed cells were dividing at equal rates, there was no difference in surface coats. With RR, there was a continuous, thin electron-dense layer at external surfaces of all cells while the staining of this layer by phosphotungstic acid was spotty. Differences in surface material were observed in confluent cultures, where untransformed cells were contact inhibited and did not increase in number while transformed cells continued to divide. Both lines of transformed cells exhibited surface coats similar to those observed in subconfluent cultures, even where cells were closely apposed, while the NRK cells exhibited considerably more extracellular RR-positive material, particularly at regions of cell contact. This material appeared to be involved in cell adhesion.

In confluent cultures, the peripheral cytoplasm of NRK cells had many filaments which were aggregated into bundles and were most abundant near regions of cell-to-cell apposition. The filaments attached to material beneath plasma membranes at sites of intercellular junctions. Transformed cells were clearly deficient in cytoplasmic filaments and intercellular junctions.

In this system, RR-positive material at cell surfaces and the presence of intercellular junctions with associated cytoplasmic filaments may have a role in the regulation of cell growth and multiplication.

INTRODUCTION

Chemical analyses of surface membranes from some lines of OVT2 cells in culture have shown that there are lower amounts of certain sugars (6–8, 17, 18, 20, 35–37, 44, 47) within surface glycoproteins and glycolipids when compared to surfaces of untransformed cells in culture. These data indicate that virus transformation may result in the synthesis of incomplete surface polymers. It was suggested that these differences may be produced by changes in the activities of glycosyltransferases (10, 17, 32) within the cells. Electron microscopic data obtained from human breast biopsies by one of us (12) tend to support these findings.

On the other hand, light and electron microscopic examination of surface coats stained by iron (9), RR (29, 33, 43, 46), or PTA (33) of lines of OVT and normal cells in culture have either shown no differences (46) or increased layers at the surfaces of transformed cells. Cell coats measured by ellipsometry (28) have supported these data.

In order to increase the available data about the morphological changes that may occur at surfaces of transformed cells, we studied the staining of cell surface material by RR or PTA of an untransformed rat kidney cell line in culture and 2 of its virus-transformed counterparts. Normal cells were examined in growing (subconfluent) and nongrowing (confluent) cultures because the apparently contradictory conclusions of histochemists and biochemists regarding normal and transformed surface membranes may in part be due to harvesting cells at different rates of multiplication (16, 20) and cell density (18). Growing transformed cells were also examined in subconfluent and confluent cultures. Since surface material may be lost from cells when they were removed from their substratum (5), cells were fixed and embedded in situ within Petri dishes. In situ embedding is also necessary for maintaining intercellular relationships (29). The abundance of peripheral cytoplasmic filaments within normal and transformed cells and intercellular junctions were also studied because they have been implicated in the regulation of cell movement and growth (30, 31).

MATERIALS AND METHODS

NRK cells which resemble fibroblasts (14) and cells from this line transformed by a B-7 nonproductive mouse sarcoma virus (23) or by a C-7 productive mouse sarcoma virus (22) were grown on 60-mm glass Petri dishes. The cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 25,000 units of penicillin and streptomycin per ml and 200 mM L-glutamine. NRK cells had reached the 47th passage in vitro, B-7 transformed cells the
208th passage and C-7 transformed cells the 185th passage. After plating $3 \times 10^5$ cells per Petri dish, the cells became confluent after 3 days. Normal and transformed cells were examined in subconfluent and confluent cultures.

Before fixation, the cultures were washed for 5 min with minimal essential medium. Cells were then fixed within the Petri dishes with a 2.0% glutaraldehyde: cacodylate buffer solution (pH 7.3) for 1 hr. Postfixation was carried out with a 1.0% OsO₄ in cacodylate buffer solution for 3 hr. RR was added to both fixatives at a concentration of 50 mg/100 ml (29). The cells were rapidly dehydrated in increasing concentrations of acetone and embedded in situ with vestopal W. Other identical cultures were fixed only in a 2.0% glutaraldehyde: cacodylate buffer solution for 1 hr and embedded in situ with GMA (25). After polymerization, the Petri dishes were broken and the vestopal or GMA layers containing the cells were detached from the glass after a brief immersion in hot water. Thin sections were examined in a Hitachi 85 or Siemens Elmiskop 1A electron microscope. Some sections of vestopal embedded cells were not stained; others were stained with uranyl acetate and lead citrate. GMA sections were stained with 3.0% PTA at a pH of 1.5 for 15 min (11) or with 1.0% PTA in 1.0 N HC1 for 5 min at a pH of 0.3 (40). After staining with PTA, the grids were rinsed briefly in water and air dried.

RESULTS

In the light microscope, each transformed cell is composed of small rounded cells which grow into multilayered colonies. The original untransformed NRK cells are large and flat and do not grow over one another.

Subconfluent Cultures. Subconfluent cultures generally contain isolated cells but cells in contact are occasionally seen. Under these conditions, NRK and transformed cells were growing at equal rates. Cells transformed by B-7 virus (B-7 cells) do not produce virus particles. Viruses are seen budding from surfaces of C-7 transformed cells (C-7 cells) and outside cells in the medium. The surfaces of B-7 and C-7 cells exhibit many long, slender microvilli (Fig. 1). Similar microvilli can be found at the surfaces of NRK cells, but more characteristically, surface projections are shorter and thicker (Fig. 2). The external surfaces of all 3 lines of cells exhibit a regular RR-positive layer about 100 Å thick (Figs. 1 to 3). RR is also found within surface membrane invaginations and membrane-bound vesicles near cell surfaces.

The staining of cell surfaces by PTA is different from that of RR. When GMA sections are exposed to PTA, stain is deposited as small spots or lines, often at external surfaces of microvilli. Most surfaces of every cell do not react with PTA. NRK cells (Fig. 4) or the transformed cells stain in the same manner. Three % PTA at a pH of 1.5 or 1.0% PTA in 1.0 N HCl reacts similarly with cell surfaces.

Confluent Cultures. In confluent cultures of NRK or OVT cells, most of the cells appear close to each other. Adjacent OVT cells are closest to each other either where microvilli of neighboring cells interdigitate (Fig. 5) or where adjacent surface membranes roughly parallel each other (Fig. 6). At these regions as well as at surfaces where cells are farther apart, RR-positive layers are identical and have the same appearance as in subconfluent cultures. Cytoplasm adjacent to regions of nearest cell-to-cell contact occasionally contain parallel sheets of filaments (Fig. 6) which are about 80 Å in diameter. Most frequently these regions lack filaments. C-7 cells, as in subconfluent cultures, show budding viruses or extracellular virus particles (Fig. 7). The surfaces of the viruses stain with RR.

When GMA sections of confluent cultures of B-7 or C-7 cells are exposed to PTA, staining is similar to that seen in subconfluent cultures. Small spots or lines of stain are present at external surfaces of plasma membranes (Fig. 8). Most surfaces, however, do not stain with PTA. Striking changes in cell contacts, quantity of cytoplasmic filaments, and surface material are found in confluent cultures of untransformed NRK cells. Adherent (intermediate) junctions (Fig. 9) are prominent in confluent cultures of untransformed cells but rare in confluent cultures of transformed cells. At these junctions there is a condensation of dense fibrillar material at the cytoplasmic surfaces of the apposed plasma membranes of adjacent cells. Bundles of parallel filaments (Fig. 9) about 80 Å in diameter appear to attach to the dense condensations beneath plasma membranes (Figs. 9 and 10). As indicated above, cell processes of adjacent transformed cells are deficient in filaments. At an adherens junction, the apposed plasma membranes are separated by a 50- to 350-Å cleft of extracellular space. These extracellular spaces contain RR-positive material (Fig. 10) which is present as layers on the surfaces of apposed plasma membranes. Often, strands of RR-positive material can be seen to form bridges between the surfaces layers of adjacent cells or stained material may fill the entire width of the cleft.

Confluent cultures of NRK cells are also commonly joined by another kind of cell attachment that does not seem to have been previously described (Fig. 11). Long strands of RR-positive material are found outside of adherens junctions within extracellular spaces. They are particularly prominent near ends of adjacent cells and are made up of a meshwork of fibrils, from 200 to 250 Å in diameter (Fig. 12), which are oriented parallel to each other or in different directions. These strands connect surfaces of adjacent cells. Material of this kind is occasionally seen in subconfluent cultures of NRK cells where cells are in contact but never in any cultures of transformed cells. These strands are only weakly stained by PTA.

Away from regions of cell attachment, the surfaces of NRK cells display the same kind of RR layers as seen at all surfaces in subconfluent cultures or at surfaces of transformed cells in confluent cultures. These layers are regular and about 100 Å in width. PTA staining of the surfaces of confluent cultures of NRK cells is minimal.

DISCUSSION

In our system, the regulation of cell multiplication of untransformed cells at high cell densities seems to be linked to the amount of cell surface RR-positive material and to the presence of intercellular junctions with associated cytoplasmic filaments. First, surface layers stained by RR are identical in cultures of growing cells whether they are untransformed or...
transformed by a productive or nonproductive oncogenic virus. Transformed B-7 or C-7 cells are actively growing in subconfluent and confluent cultures while untransformed NRK cells are growing only in subconfluent cultures. In these cultures, RR layers at all surfaces are regular and about 100 Å thick. In contrast, confluent NRK cells which are contact inhibited exhibit increased amounts of extracellular RR-stained material, at the ends of adjacent cells. This material appears to be involved in the strong mutual adhesivity of normal confluent cells and its absence in confluent cultures of transformed cells might reflect the weak adhesion between these cells (15). The RR-stained material is continuous with the surface coat material of plasma membranes and forms strands which bridge the extracellular space between neighboring cells. The strands appear to be composed of a meshwork of fibrils 200 to 250 Å in diameter.

Similar RR-stained fibrils have been found associated with synovial collagen (34) and were thought to be composed mainly of hyaluronates. Extracellular spaces of confluent cultures of normal 3T3 cells also have abundant strands of fibrillar material (30) which sometimes appeared attached to surface coat material. Little fibrillar material was seen in subconfluent cultures and none was found in cultures of SV40-transformed 3T3 cells (30). The observation that these strands are intensely stained by RR indicates they contain acidic mucosubstances (26) and that mechanical forces may exist at these sites (27). Thus our data support the view that complex carbohydrates are involved in intercellular adhesion (38, 42) and that there is a significant increase in the sugar content of cells when they cease to divide (20).

The results also agree with the chemical analyses of surface membranes from various lines of OVT and normal cells in culture. These data have shown transformed cell surfaces to be deficient in various components (6–8, 17, 18, 20, 35–37, 44, 47). However, these studies have measured levels of sialoglycoproteins and glycolipids and sialic acid. The decreased amounts of surface sialic acid (7, 17, 18, 35–37, 44, 47) that have been found by chemical analysis in cultures of virally transformed fibroblasts of different origins is not reflected in our cultures of fibroblast-like cells by differences in the PTA staining of untransformed and transformed cell surfaces. The reason for this discrepancy is not known, but it is possible that in the NRK line terminal sugar residues of glycoproteins and glycolipids are not primarily sialic acid. The reduction in PTA staining we have observed at surfaces of normal cells in vivo (12, 41) which are intensely stained by PTA. Moreover, the decreased amounts of surface sialic acid (7, 17, 18, 35–37, 44, 47) that have been found by chemical analysis in cultures of virally transformed fibroblasts of different origins is not reflected in our cultures of fibroblast-like cells by differences in the PTA staining of untransformed and transformed cell surfaces. The reason for this discrepancy is not known, but it is possible that in the NRK line terminal sugar residues of glycoproteins and glycolipids are not primarily sialic acid. The reduction in PTA staining we have observed at surfaces of neoplastic human breast epithelium (12) indicates some malignant cells in vivo are also deficient in surface components.

In agreement with observations from a fibroblast line (31) adherens junctions are common in confluent cultures of untransformed NRK cells but rare in confluent cultures of OVT cells. Specialized cell attachment sites such as these junctions probably represent another component of the morphological basis for the greater adhesivity of normal cells in culture (15).

Cytoplasmic filaments 70 Å in diameter (α filaments) (30, 31) have been found to be abundant in confluent, untransformed and revertant 3T3 cultures, particularly near adherent junctions. These filaments are much less prominent in confluent cultures of SV40-transformed cells. The data from our system coincides with these observations. These filaments appear to insert on plasma membranes via dense plaques (1) which in our lines are mostly at sites of adherens junctions. Thus we would agree with those conclusions (31) which suggest that the regulation of α filaments is also important in the complex regulation of cell movement and growth.
Although the B-7 virus is defective, in that viruses are not produced in cells infected and transformed by this virus, this defect does not prevent the changes in cell features we have described. Therefore, virus replication appears controlled by different factors than those which produce cellular changes in surface material, cytoplasmic filaments and intercellular junctions.

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**Fig. 1.** Cultures of subconfluent, transformed cells exhibit long slender microvilli. All cell surfaces are covered by a thin RR-positive layer. × 36,000.

**Fig. 2.** Subconfluent cultures of untransformed NRK cells generally exhibit shorter and thicker surface projections than in transformed cultures. However, RR-stained surface layers appear identical to those in subconfluent, transformed cultures. × 36,000.

**Fig. 3.** At high magnification, RR-positive surface layers in untransformed or transformed subconfluent cultures appear regular and about 100 Å thick. × 108,000.

**Fig. 4.** PTA is deposited as small spots (arrows) on GMA sections at the surfaces of subconfluent cultures of untransformed NRK cells. × 36,000.

**Fig. 5.** A region of close cell-to-cell apposition in a confluent culture of B-7 transformed cells. Surfaces of interdigitating microvilli of each adjacent cell exhibit thin RR-stained layers. × 36,000.

**Fig. 6.** A region of close cell-to-cell apposition (between the 2 arrows) in a confluent culture of transformed cells. RR layers at these sites are identical to surface layers where cells are farther apart or to surface layers seen in all subconfluent cultures. Filaments (f) are present in 1 cell process. Section stained with uranyl acetate and lead citrate, × 27,500.

**Fig. 7.** An extracellular virus particle is seen in confluent cultures of C-7 transformed cells. Its surface is stained by RR (arrow). The surface of the adjacent microvillus exhibits an RR-stained layer about 100 Å thick. Section stained with uranyl acetate and lead citrate, × 108,000.

**Fig. 8.** Surfaces of transformed cells within confluent cultures exhibit a spotty reaction with PTA (arrows), × 36,000.

**Fig. 9.** A region of cell-to-cell contact in a confluent culture of untransformed NRK cells. Adherens junctions (arrows) and cytoplasmic filaments (f) are prominent. Section stained with uranyl acetate and lead citrate, × 36,000.

**Fig. 10.** Adherens junction at high magnification in a confluent culture of untransformed NRK cells. Adjacent cell processes (A and B) contain parallel filaments which attach to the dense condensations beneath plasma membranes. The extracellular space of the junction contains RR-positive material which fills the cleft between cells (at double arrows) or forms a bridge between adjacent cells (at single arrow). Section stained with uranyl acetate and lead citrate, × 108,000.

**Fig. 11.** Strands (arrows) of extracellular RR-stained material connect cell processes (A and B) of adjacent cells in confluent cultures of untransformed NRK cells. Section stained with uranyl acetate and lead citrate, × 11,000.

**Fig. 12.** The strands of extracellular RR-stained material which connect surfaces of adjacent untransformed NRK cells (A and B) are composed of fibrils about 250 Å in diameter oriented in different directions. Small round structures are fibrils cut in cross-section. × 36,000.
Comparisons of Untransformed and Transformed Cells

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