# Concomitant Loss of Cell Surface Fibronectin and Laminin from Transformed Rat Kidney Cells

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ABSTRACT Both fibronectin and laminin were found by immunofluorescence as a matrix at the surface of normal rat kidney cells. These matrices were absent from the surface of virally transformed rat kidney cells. Soluble fibronectin and laminin were detected in the culture media of the transformed as well as the normal cells. Culture supernates of the transformed cells contained even more fibronectin than the supernates of the normal cells while laminin was present in similar amounts in both culture media. This shows that the loss of fibronectin and laminin from the surface of the transformed cells is caused by failure of the cells to deposit these proteins into an insoluble matrix and not caused by inadequate production. Fibronectins isolated from culture media of the normal and transformed cells were similar in SDS polyacrylamide gel electrophoresis. Laminin isolated from culture media by affinity chromatography on heparin-Sepharose followed by immunoprecipitation was composed of three main polypeptides, one with a molecular weight of 400,000 and two with a molecular weight close to 200,000 in both cell types. Fibronectins from both cell types were equally active in promoting cell attachment. Rat fibronectin from transformed cell cultures was found by immunofluorescence to be incorporated into the matrix of normal human fibroblasts grown in the presence of such fibronectin. The transformed cells, like the normal cells, when applied to culture dishes coated with fibronectin, readily attached and spread on the substratum, requiring approximately the same amount of fibronectin as the normal cells. On the basis of these results it seems that the failure of the transformed cells to incorporate fibronectin into an insoluble cell surface matrix is not a consequence of a demonstrable change in the functional characteristics of the fibronectin molecule or in the ability of the cells to interact with fibronectin. It may depend on as yet unidentified interactions at the cell surface. Similar interactions may be needed for the deposition of laminin into the matrix, because laminin was also absent from the surface of transformed cells, despite its being synthesized by these cells.

Fibronectin and laminin are two noncollagenous components of extracellular matrix and basement membranes (6, 8, 20, 34, 35).

Fibronectin, a large cell surface glycoprotein of fibroblasts and other normal cells, is usually absent or found in reduced amounts on the surface of transformed cells (for reviews see references 15, 26, 37, and 40). Some transformed cells are capable of producing fibronectin in somewhat reduced amounts, as compared to normal cells, but fail to incorporate it into a cell surface matrix (38). The defect that leads to failure to retain fibronectin at the cell surface is not known, but it can be overcome by treating the cells with glucocorticoids, sodium butyrate, and cAMP (11, 14, 21). The defect is of considerable importance because fibronectin, by mediating cell attachment, profoundly affects the morphology and motility of cells (1, 16, 22, 39), and the lack of fibronectin at the cell surface may correlate with the degree of tumorigenicity and metastatic capacity of malignant cells (4, 32). It has been suggested that the failure of transformed cells to retain fibronectin at the cell surface could be caused by a defect in the fibronectin molecule itself (37) or in the cell surface receptor for fibronectin (18).

Laminin is a recently described glycoprotein isolated from a basement membrane-forming mouse tumor (35). Laminin consists of at least two polypeptides with molecular weights of ~400,000 and 200,000. Extracellular matrix of endodermal cells contains polypeptides with similar properties (6), and these are immunologically similar to laminin (29, 35). Laminin is chemically and immunologically distinct from fibronectin



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(35). The function of laminin is unknown, but it is present in basement membranes and has been suggested to play a role in the differentiation of kidney tubules (8).

We report here that cultures of transformed rat kidney cells contain both rat fibronectin and rat laminin in amounts comparable to or higher than those of their normal counterpart cells, but the transformed cells fail to incorporate these proteins into an insoluble matrix at the cell surface as do the normal cells.

## MATERIALS AND METHODS

Normal rat kideny cells (NRK), subline 9 (7, 17); the transformed rat kidney cell line (TRK) 1255 B-7 which was derived from NRK transformed by Kirsten sarcoma virus (17); and human skin diploid fibroblasts, obtained from Dr. Ingeburg Goetz, City of Hope National Medical Center, were grown under similar conditions in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 g/ml streptomycin, and 2 mM glutamine (Flow Laboratories, Ingelwood, Calif.). The bovine serum used was depleted of fibronectin in some experiments by treatment with gelatin Sepharose and anti-bovine fibronectin-Sepharose as described (13). Cultures were free of mycoplasma as tested by the method of Russell et al. (28).

### Radioactive Labeling of Cellular Proteins

Confluent cell cultures were kept for 16 h in MEM lacking methionine (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.), supplemented with 10% dialyzed serum, and penicillin, streptomycin and glutamine as described above. The cultures were then refed with the same medium containing 5 $\mu$Ci/ml [$^{35}$S]methionine (New England Nuclear, Boston, Mass.) and maintained for another 24 h.

### Isolation of fibronectin and laminin

Fibronectin was purified from rat plasma and from culture media of NRK and TRK cells using affinity chromatography of gelatin-Sepharose (10).

Laminin was isolated from the extracellular matrix of a mouse endodermal cell line, PF HR-9, as described (6, 29). Affinity chromatography on heparin-Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) was used to isolate fractions enriched in laminin from culture media of NRK and TRK cells as described elsewhere (30). Briefly, 200 ml of culture medium previously depleted of fibronectin by treatment with gelatin-Sepharose was passed through a 40-ml heparin-Sepharose column equilibrated with 0.05 M Tris-HCl, buffer pH 7.0. After the column was washed with the Tris-HCl buffer, the bound proteins were eluted with 0.4 M NaCl in this same buffer.

### Antisera

Rabbit anti-rat fibronectin was prepared as described (27). Antibodies to laminin were prepared by immunizing rabbits with laminin extracted from PF HR-9 extracellular matrix (29). Goat anti-laminin (35) was a gift from Dr. G. R. Martin, The National Institutes of Health.

### Quantitation of Fibronectin and Laminin

Fibronectin was quantitated by radioimmunoassay (27). To quantitate fibronectin in cell layers, the cell layers were washed with phosphate-buffered saline (PBS) and then extracted with 4.0 M urea, in 0.01 M Tris-HCl, pH 7.2. Laminin was quantitated by enzyme-linked immunosorbent assay, ELISA (9). Briefly, wells in nontreated microtitration plates (Flow Laboratories) were coated with 100 $\mu$l of a 0.1 $\mu$g/ml solution of purified laminin. Rabbit antiserum to laminin, diluted 1:50,000, was incubated overnight in such wells in the presence of various amounts of purified laminin or of unknown samples. After washing, enzyme-labeled antibodies to rabbit immunoglobulin were added to all wells and incubated for 5 h. The amount of enzyme bound to the wells was measured colorimetrically and provided an indirect measure of the amount of antigen present.

### Immunofluorescence

Fibronectin and laminin were detected in cell monolayers by indirect immunofluorescence (13). Cells were cultured on glass cover slips and fixed on those using 3% paraformaldehyde. Rabbit or goat serum was diluted 1:40 and incubated for 30 min at room temperature with the fixed cells. After washing, fluorescein-labeled goat anti-rabbit IgG or rabbit anti-goat IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) diluted 1:40 was used for detection of bound antibodies. The cells were then washed, mounted in glycerol, and viewed under an Olympus IM microscope equiped with an epi-illuminator.

### Immunoprecipitation

Proteins were isolated from labeled culture media and heparin-Sepharose eluates by incubation with antiserum and protein A Sepharose (Pharmacia Fine Chemicals). 1 ml of radioactive sample was incubated with 20 $\mu$l of antiserum or control serum and 100 $\mu$l of a 50% suspension of protein A Sepharose for 3–5 h at room temperature with gentle rocking. After washing of the beads four times with 0.9% saline containing 0.05% Tween 20 (Atlas Chemical Industries, Inc., Wilmington, Del.), bound material was eluted with 2% SDS.

### Electrophoretic Analyses

Polyacrylamide gel electrophoresis in the presence of SDS with or without 2-mercaptoethanol was carried out according to Laemmli (19) using 5 or 7% acrylamide. Molecular weight markers, including ferritin (440,000 daltons unreduced, 220,000 reduced) and catalase (60,000 daltons reduced) were obtained from Pharmacia Fine Chemicals.

### Fluorography

Fluorography was carried out at − 70°C using "Enhance" (New England Nuclear).

### Cell Attachment Assay

A modification of the method of Grinnell et al. (12) was used to assess the attachment of cells to fibronectin (25). Nontreated microtitration plates (Flow Laboratories) were coated with fibronectin, laminin, and other proteins by incubating 0.1 ml containing 0.3 to 30 $\mu$g/ml of protein in PBS, pH. 7.2 in the wells for 2 h at 37°C. Monolayer cultures of NRK and TRK cells were dispersed by treatment with 0.1 mg/ml trypsin (type III; Sigma Chemical Co., St. Louis, Mo.) in PBS. The detached cells were washed three times with 0.5 mg/ml soybean trypsin inhibitor (GIBCO) in PBS, and $10^4$ cells were added to protein-coated microtiter wells. The cells were allowed to attach for a period of 1 h at 37°C. Unattached cells were removed by washing the wells with PBS. The attached cells were then fixed with 3% paraformaldehyde, stained with a 1% solution of toluidine blue, and counted with an Artek cell counter (Dynateck Laboratories, Inc., Dynatech Corp., Alexandria, Va.). Under optimal conditions an average of 70–80% of the cells added to each well remained attached after washing.

### Incorporation of Rat Fibronectin into Human Cell Matrix

Normal human fibroblasts were plated at a low density on glass coverslips in fresh media. When the cells were attached the medium was replaced with 24-h conditioned medium from TRK cultures that had been filtered with a 0.22-$\mu$m filter (Millipore Corp., Bedford, Mass.). Such medium was replaced daily for three consecutive days, after which the cells were prepared for immunofluorescence.

## RESULTS

### Fibronectin and Laminin in NRK and TRK cells

Immunofluorescent staining of NRK cells for fibronectin revealed dense fibrillar structures on both upper and lower surfaces of the cells (Fig. 1 *a*). Such structures were not detectable on the TRK cells (Fig. 1 *c*), although some diffuse staining could be observed. The immunofluorescent staining of NRK cells for laminin also revealed a fibrillar pattern, but this was less intense and required a higher magnification for precise localization. The distribution was different from the fibronectin staining in that laminin was limited to the same focal plane as the cells (Fig. 2 *a*). The TRK cells lacked a similar staining pattern. Some fluorescence that appeared intracellular was detected in occasional cells, but no matrix was present. (Fig. 2 *b*). Identical staining patterns on NRK cells were obtained with

rabbit anti-serum to endodermal cell laminin prepared in our laboratory and the goat anti-laminin serum. The immunofluorescent stainings could be inhibited by absorption of the anti-fibronectin or anti-laminin with fibronectin or laminin, respectively. Controls with nonimmune rabbit and goat sera gave no staining.

The conditioned media from TRK cells contained more rat fibronectin than comparable conditioned culture media from NRK cells as measured by radioimmunoassay (Table I). About 5% of the fibronectin in cultures of NRK cells was associated with the cell layer. In TRK cells this was only ~0.4% and probably represents mostly intracellular fibronectin. Laminin was also detected in the conditioned media of TRK and NRK cells; 72-h culture medium of both cell types contained ~2.5 $\mu g/10^6$ cells as measured by ELISA.

The identity of the fibronectin and laminin detected in the culture media was confirmed by immunospecific isolation of [$^{35}$S]methionine-labeled proteins followed by electrophoresis in SDS polyacrylamide gels and fluorography. The material isolated using anti-fibronectin and protein A-Sepharose showed a major band at the same position as the reference plasma fibronectin. This was the case with the unreduced fibronectins (Fig. 3, lanes *a* and *b*) as well as with the reduced fibronectins (Fig. 3, lanes *c* and *d*). Under unreduced conditions, both the dimer and some subunit size fibronectin were seen.

Laminin in the culture media was first enriched by fractionation on heparin-Sepharose and then isolated using rabbit antiserum and protein A-Sepharose. The material thus isolated from both cell types contained a polypeptide with an apparent molecular weight of ~400,000 and a doublet of polypeptides of ~200,000 daltons (Fig. 4). These peptides migrated to the same position as mouse laminin (35) and it has been shown previously that the 200,000-dalton component of mouse endodermal cell laminin gives a double hand in SDS gel electrophoresis (29). No gel bands were detected in samples where normal rabbit serum was used instead of antilaminin. A fourth major band in NRK-derived material and some minor bands in TRK-derived material were detected with antisera raised against both laminin from mouse endodermal cells and mouse tumor laminin (35). These were not studied further but could represent degradation products of laminin.

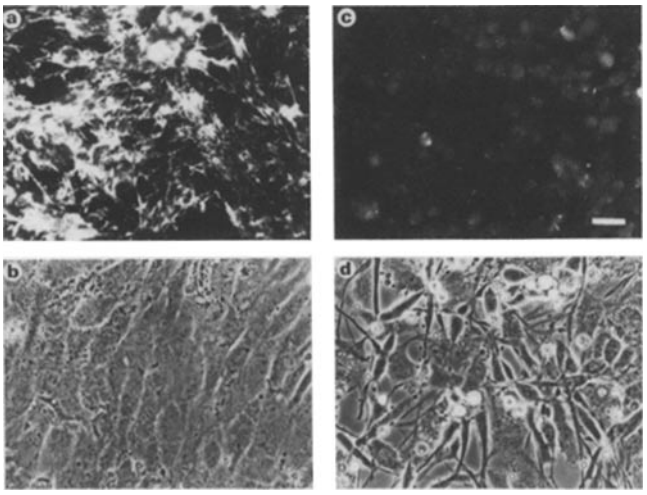


FIGURE 1 Immunofluorescent staining of NRK (*a*) and TRK (*c*) cells with antifibronectin, and phase-contrast micrographs of NRK (*b*) and TRK (*d*) cells. Bar, 50 $\mu$m.

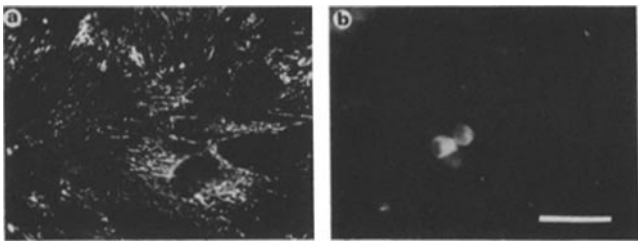


FIGURE 2 Immunofluorescent staining of NRK (*a*) and TRK (*b*) cells with antilaminin. Bar, 50 $\mu$m.

TABLE I

*Concentration of Fibronectin in Culture Media and Cell Layer of Rat Kidney Cells*

| Samples* | NRK cells | TRK cells |
|---|---|---|
| | $\mu g/10^6$ cells | |
| Cell layer | 1.05 ± 0.17 | 0.37 ± 0.09 |
| Media | 20.5 ± 6.1 | 82.8 ± 28.5 |

* Fresh media were added to subconfluent cultures and samples were taken 24 h later.
Cell numbers were determined by counting duplicate cultures. Mean and standard deviation of five determinations from independent cultures.

## Cell Attachment

To test the ability of the NRK and TRK cell fibronectins to promote cell attachment, NRK and TRK cells were grown in media which had been depleted of bovine fibronectin, and the cell-derived fibronectin was isolated by affinity chromatogra-

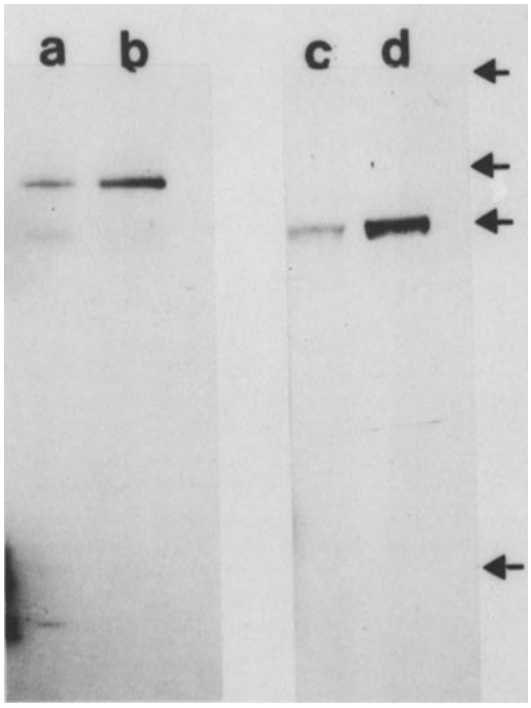


FIGURE 3 Isolation of [$^{35}$S]methionine-labeled fibronectin from culture media with anti-rat fibronectin and protein A Sepharose. The isolates were analyzed on a 5% SDS polyacrylamide slab gel under unreduced (lanes *a* and *b*) or reduced (Lanes *c* and *d*) conditions and visualized by fluorography. Fibronectins from NRK cells (*a* and *c*) and from TRK cells (*b* and *d*). Arrows (from top to bottom) indicate the position of top of gel; unreduced rat plasma fibronectin and unreduced ferritin, which comigrate; reduced rat plasma fibronectin and reduced ferritin; and reduced catalase.

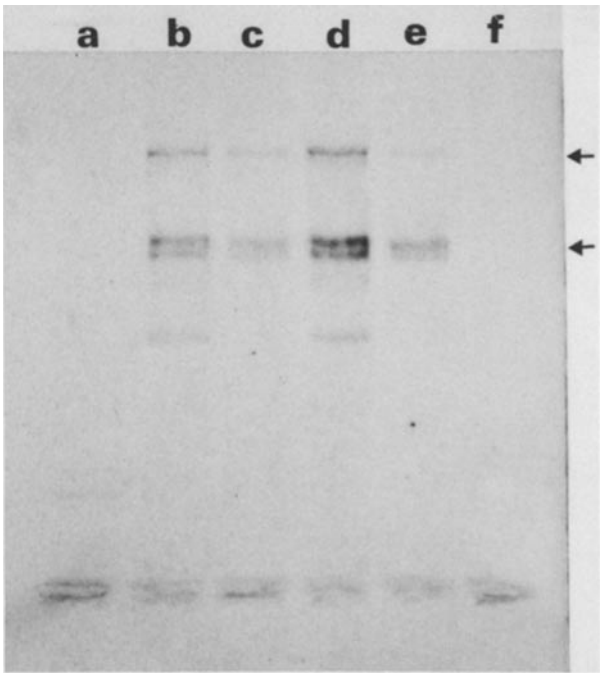


FIGURE 4 Isolation of [$^{35}$S]methionine-labeled laminin from the heparin-Sepharose bound fraction of culture media with antilaminin. The precipitates were analyzed on a 5% polyacrylamide slab gel electrophoresis under reduced conditions and visualized by fluorography. NRK medium incubated with normal rabbit sera (*a*) and two different rabbit antilaminin sera (*b* and *d*). TRK medium incubated with normal rabbit sera (*f*) and the two antilaminin sera (*c* and *e*). The positions of the 400,000- (upper arrow) and 200,000- (lower arrow) dalton polypeptides of mouse endodermal cell laminin used as a marker are shown.


FIGURE 5 A 7% SDS polyacrylamide slab gel electrophoresis under reduced conditions of fibronectin isolated from culture media and rat plasma. The proteins were visualized by staining with Coomassie Brilliant Blue. Rat plasma fibronectin (*a* and *d*); NRK fibronectin (*b*); TRK fibronectin (*c*).

phy on gelatin-Sepharose. SDS polyacrylamide gel electrophoresis showed one major and one minor band in fibronectin from NRK cells, while fibronectin from TRK cells was more heterogeneous (Fig. 5). This was a consistent finding in three independent isolations. It could be caused by increased proteolysis in the transformed cultures during the 3 d of culture used to accumulate enough fibronectin in the medium for isolation.

The cell attachment-promoting activity of fibronectins from the NRK and TRK cultures was similar when tested with NRK cells (Fig. 6 *a*). The attachment of TRK cells was also promoted by both fibronectins (Fig. 6 *b*). The promotion of attachment was of a magnitude similar to that of NRK cells. There was no significant difference in the morphology of the TRK and NRK cells spread on fibronectins from these two cell types (Fig. 7). Laminin isolated from endodermal cells did not promote the attachment of either cell type in this assay (not shown).

To determine whether the fibronectin from TRK cells could become incorporated into extracellular matrix, normal human fibroblasts were cultured in the presence of TRK fibronectin, then stained with species-specific anti-rat fibronectin. Human fibroblasts cultured in the conditioned media from TRK cells were positive for rat fibronectin. (Fig. 8). When the same antiserum was used to stain human cells grown in the presence of conditioned media from human fibroblasts, no staining was detected.

## DISCUSSION

In contrast to some other transformed cells (2, 38), the TRK cells, a highly malignant cell line (3), accumulates more fibronectin than their normal counterpart. Yet, we found that, under normal culture conditions, little of this fibronectin is incorporated into an extracellular matrix of these cells as seen by immunofluorescent staining.

The defective incorporation of fibronectin into the matrix of TRK cells obviously is not caused by the presence of reduced amounts of fibronectin in such cultures. Our results also exclude some other possibilities as the cause of the lack of cell surface fibronectin in these cells. The lack does not seem to be caused by an alteration in the fibronectin produced by the TRK cells. The fibronectin has both a collagen-binding site and a cell binding site, as it can be isolated on gelatin-Sepharose and performs in a cell attachment assay equally well as fibronectin from NRK media, and it can also become incorporated into the extracellular matrix of normal cells.

Our results also make it unlikely that a loss of cell surface receptors for fibronectin in TRK cells would be the reason for the lack of cell surface fibronectin, as we found that the attachment and spreading of TRK cells to a surface coated with fibronectin was unimpaired.

The TRK cells also lacked a cell surface laminin matrix. There is a striking parallel between the expression of laminin and that of fibronectin in the NRK and TRK cells. In the NRK cells, these proteins were found both at the cell surface and in the culture medium, whereas in the TRK cells they were

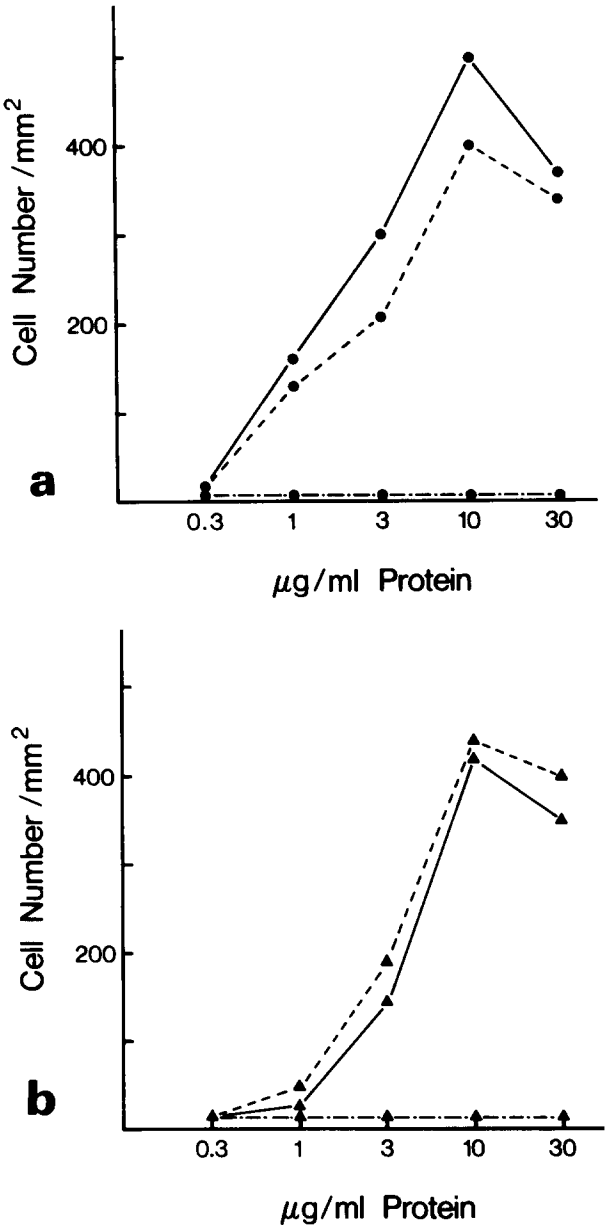


FIGURE 6 Cell attachment-promoting activity of fibronectin from NRK and TRK cells. Microtiter wells were coated with various concentrations of NRK fibronectin (---), TRK fibronectin (—), or BSA (-·-·-), and the number of NRK cells (*a*) or TRK cells (*b*) attached to such wells after 1 h was determined.

found almost exclusively in the medium.

We have previously shown that soluble fibronectin present in serum can become insolubilized in the matrix of cultured cells (13). This and the present results suggest that whether fibronectin becomes incorporated into a matrix or is released in a soluble form does not depend on structural differences in the fibronectin molecule. Fibronectin interacts with collagen (10), glycosaminoglycans (23, 24, 33), and with cell surfaces to induce cell attachment (16, 18, 22). We have recently shown that laminin shares at least one of these interactions of fibronectin, that with glycosaminoglycans (30). Quantitative and qualitative changes in glycosaminoglycans have been demonstrated to occur in transformation (5, 31, 36). Such changes could result in a concomitant loss of both fibronectin and

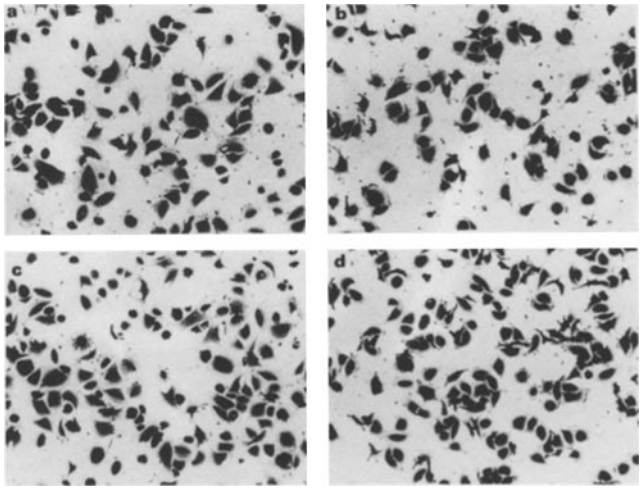


FIGURE 7 Photomicrographs showing attachment and spreading of NRK and TRK cells on plates coated with 10 μg/ml fibronectin. In panels *a*) and *b*) the wells were coated with NRK fibronectin. In panels *c* and *d* TRK fibronectin was used. *a* and *c* show attachment of NRK cells, *b* and *d* of TRK cells. The attached cells were fixed with 3% paraformaldehyde and stained with toluidine blue.

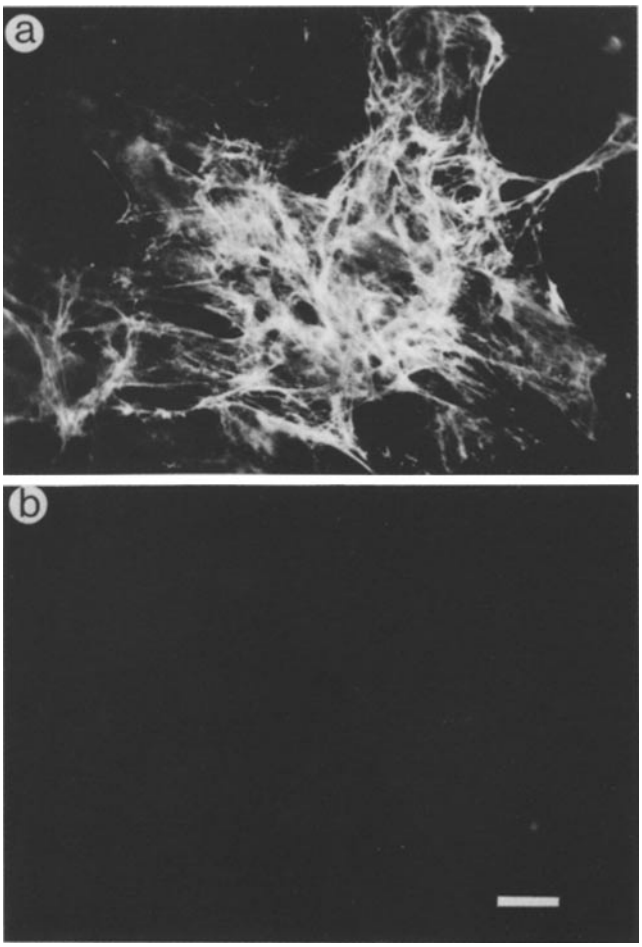


FIGURE 8 Immunofluorescent staining of normal human fibroblasts cultured in the presence of fibronectin-containing culture media from TRK cells (*a*) or from normal human fibroblasts (*b*) and stained for rat fibronectin using a species-specific antiserum. Bar, 50 μm.

laminin at the cell surface because both proteins interact with glycosaminoglycans. We are presently testing this hypothesis.

We thank Dr. George Martin for a gift of laminin and antiserum to laminin, Dr. Ingeburg Goetz for human fibroblast, and Marsha Bell and Aulikki Pekkala for technical assistance.

This work was supported by grant CA 27455 from the National Cancer Institute and AM 26693 from the National Institute for Arthritis and Metabolism, Department of Health and Human Services.

*Received for publication 6 June 1980, and in revised form 15 September 1980.*

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