

## DISTRIBUTION OF FETAL BOVINE SERUM FIBRONECTIN AND ENDOGENOUS RAT CELL FIBRONECTIN IN EXTRACELLULAR MATRIX

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### ABSTRACT

Normal rat kidney cells were cultured in medium supplemented with normal fetal bovine serum (FBS) or FBS depleted of fibronectin. The cell surface fibronectin of these cultures was visualized by indirect immunofluorescence using species-specific antisera for either rat fibronectin or bovine fibronectin. Anti-rat fibronectin revealed fibrillar structures on the cells grown in either normal medium or fibronectin-depleted medium. Anti-bovine fibronectin revealed similar fibrillar networks, but only on the cells grown in medium containing bovine fibronectin. Staining in each case was abolished by absorption with the homologous antigen. It appears that exogenous fibronectin was incorporated into the same structures as endogenous fibronectin. This finding suggests that circulating fibronectin may serve as a building block for the assembly of extracellular matrix, possibly by cells which are incapable of synthesizing it.

**KEY WORDS** fibronectin · fetal bovine serum · pericellular matrix · species-specific antiserum · immunofluorescence

Fibronectin, a 450,000-dalton glycoprotein, is present in a soluble form in plasma or sera of all vertebrate species that have been studied and in growth media of cells cultured *in vitro*. It is found in an insoluble form on the surface of normal cells and in the surrounding extracellular matrix (for review and references see reference 16).

The basis for the solubility of fibronectin in blood and in culture media as opposed to its insolubility in extracellular matrix is not known. There is evidence that the plasma and cell-derived fibronectins, although very similar (17), are not completely identical. Cellular fibronectin has been found to be slightly slower in SDS polyacrylamide gel electrophoresis (16) and more efficient in altering the morphology of transformed cells toward normal (20) than plasma fibronectin.

Fibronectin in cell layers is present in fibrillar structures which can be visualized in immunofluorescence (9, 18). Fibronectin extracted from cell layers of cultured fibroblasts when added to cul-

tures of transformed cells becomes reconstituted into a fibrillar matrix (1, 19). Because of the obvious importance of the adhesive fibronectin matrix to the behavior of cells, it would be important to understand the processes involved in insolubilization of fibronectin into the matrix form. As a step toward this end, we decided to study whether the circulating form of fibronectin could be converted into the matrix form.

Making use of species-specific anti-fibronectin sera, we observed that fibronectin from fetal bovine serum (FBS) can be found in typical fibrillar structures on the surface of rat cells cultured in the presence of such serum. This shows that the process that results in the incorporation of fibronectin into extracellular matrix is, at least partly, independent of whatever chemical differences might exist between the cellular and plasma forms.

### MATERIALS AND METHODS

#### *Cells and Culture Conditions*

Normal rat kidney (NRK, reference 3) cells were cultured on glass cover slips in Leighton tubes (Bellco Glass, Inc., Vineland, N. J.) in Eagle's minimal essential

medium supplemented with 10% heat-inactivated FBS (Flow Laboratories, Inc., Inglewood, Calif., and Grand Island Biological Co., Grand Island, N. Y.) containing fibronectin or depleted of it.

### Removal of Fibronectin from Serum

Fibronectin was removed from FBS by treatment of the serum with gelatin (Sigma Chemical Co., St. Louis, Mo.) coupled to Sepharose 4B (4) (Pharmacia Fine Chemicals, Uppsala, Sweden), followed by treatment with Sepharose to which antibodies to bovine fibronectin had been coupled. This treatment removed >98% of fibronectin as detected by radioimmunoassay (14).

### Immunofluorescence

Immunofluorescence was performed at room temperature following the procedures of Linder et al. (8). The antibodies used for the staining were prepared as follows: Antisera were raised in rabbits against rat and bovine fibronectin purified using gelatin-Sepharose (13). The initial absorption of the antisera with normal serum depleted of fibronectin has also been described (14). The antisera were absorbed further to make them species-specific. Anti-rat fibronectin was depleted of antibodies cross-reactive with bovine fibronectin by treatment with bovine fibronectin coupled to Sepharose. Antibodies to rat fibronectin were then isolated by absorption and elution from rat fibronectin coupled to Sepharose. Antibodies to bovine fibronectin were prepared similarly by absorption with rat fibronectin-Sepharose and elution from bovine fibronectin-Sepharose. The resulting antibodies were species-specific when tested in immunodiffusion and in radioimmunoassay. The antibody preparations used to stain cells showed no binding of <sup>125</sup>I-labeled heterologous fibronectin, while binding of the homologous antigen was still detectable at dilution 10<sup>-5</sup>.

Gammaglobulin fraction from pooled normal rabbit serum and rabbit anti-bovine alpha-fetoprotein (6) were used as controls. Antibodies to rat and bovine fibronectin were absorbed with Sepharose-coupled rat and bovine fibronectins, respectively, and used as further negative controls.

For immunofluorescence, the cells were fixed in 3.5% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 30 min, washed with PBS, then incubated for 30 min with rabbit antibodies to fibronectin or the control antibodies. The cover slips were washed extensively with PBS and then incubated for 30 min with purified goat antibodies to rabbit IgG conjugated with fluorescein isothiocyanate (Sigma Chemical Co.). The cover slips were washed again and mounted in buffered glycerol (pH 7.4) on microscope slides and examined and photographed for fluorescence under a Zeiss Universal microscope with an epi-illuminator (× 320).

## RESULTS

### Fibronectin-Depleted Culture Medium

Fibronectin as determined by radioimmunoassay was only partially removed from FBS by repeated treatment with an excess of gelatin-Sepharose. Satisfactory results were obtained with a combination of gelatin-Sepharose and anti-fibronectin-Sepharose treatments (Table I). The medium contained a residual 0.02 µg/ml of bovine fibronectin which was <1% of the original concentration. No substantial change was observed in the growth pattern of cells grown in medium depleted of fibronectin.

### Species-Specific Immunofluorescent Staining of Fibronectin in Cell Layer

Cell surface fibronectin was visualized by indirect immunofluorescent staining of cultured cells using species-specific antisera. As expected, anti-rat fibronectin revealed fibrillar structures in cultures of NRK cells (Fig. 1 a). A similar but somewhat weaker staining was obtained with anti-bovine fibronectin when the cells were grown in the presence of FBS containing bovine fibronectin (Fig. 1 c). Cells grown in the medium depleted of bovine fibronectin showed unchanged staining with anti-rat fibronectin (Fig. 1 e) but no staining with anti-bovine fibronectin (Fig. 1 g). The staining with each antiserum was abolished by absorption with the homologous antigen (Fig. 1 b, f, d, and h). Normal rabbit gammaglobulin gave no staining with cells grown in either medium. As a

TABLE I  
Bovine Fibronectin Concentration of Untreated FBS, FBS Extracted with Gelatin-Sepharose, and FBS extracted with Gelatin-Sepharose and Anti-Bovine Fibronectin-Sepharose

|  | Sample |             |
|--|--------|-------------|
|  | No.    | Concn µg/ml |
| FBS*   | 1      | 25.0        |
|  | 2      | 27.5        |
|  | 3      | 30.5        |
|  | 4      | 24.7        |
| FBS extracted with gelatin-Sepharose                                       | 1      | 9.0         |
|  | 2      | 11.2        |
|  | 3      | 9.2         |
|  | 4      | 8.2         |
| FBS extracted with gelatin-Sepharose and anti-bovine fibronectin-Sepharose | 1      | 0.170       |
|  | 2      | 0.190       |
|  | 3      | 0.260       |

\* Four different lots from two suppliers.

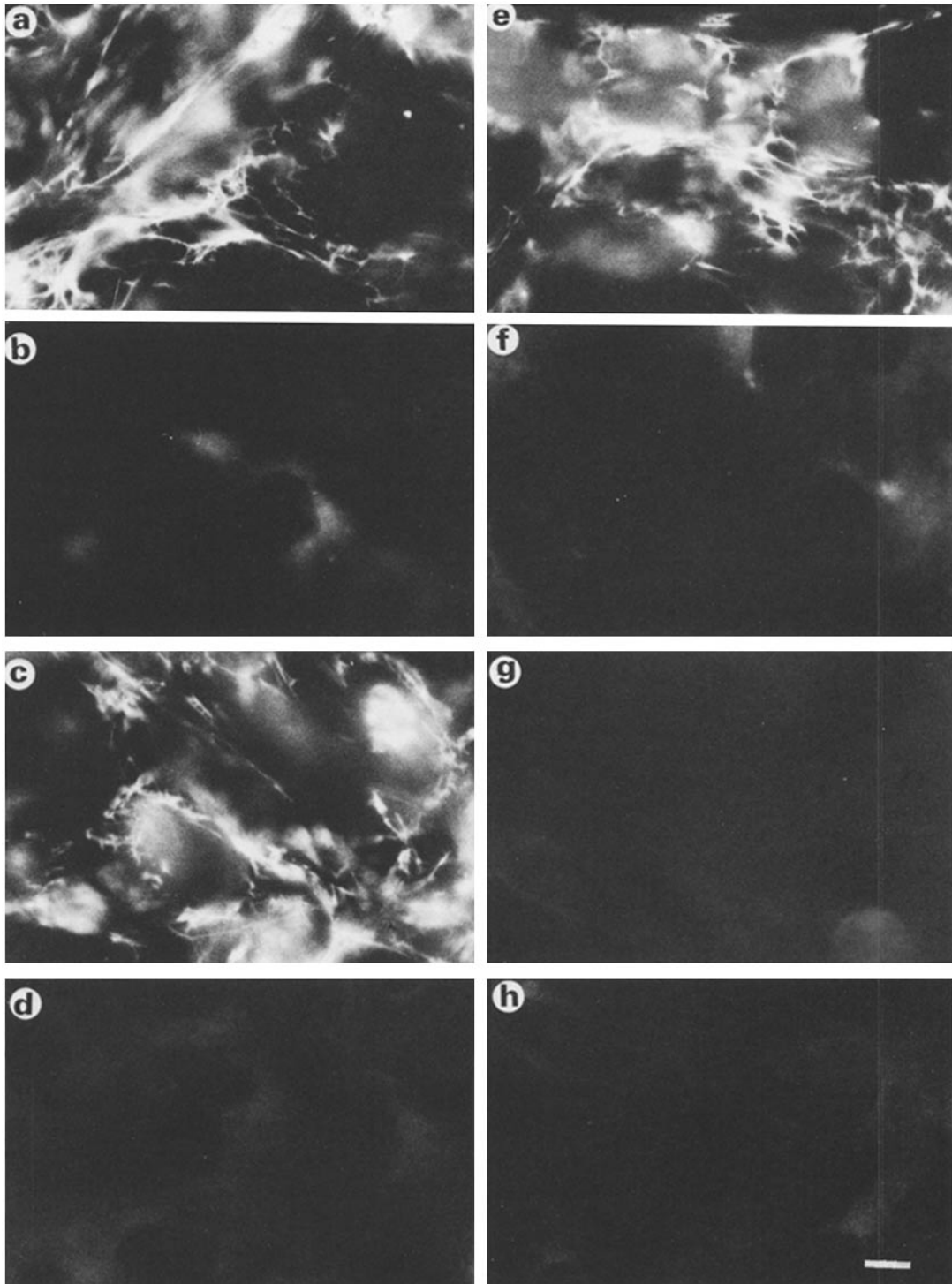


FIGURE 1 Cells in panels *a*, *b*, *c*, and *d* were cultured in medium containing bovine fibronectin; cells in panels *e*, *f*, *g*, and *h* were cultured in fibronectin-deficient medium. The cells were stained as follows: Anti-rat fibronectin (*a* and *e*); anti-rat fibronectin absorbed with rat fibronectin (*b* and *f*); anti-bovine fibronectin (*c* and *g*); anti-bovine fibronectin absorbed with bovine fibronectin (*d* and *h*). Photographic exposure time for panels *a*, *c*, and *e* was 60 s; for panels *b*, *d*, *f*, *g*, and *h*, a 5-min exposure was required to bring out cellular outlines. Bar, 60  $\mu$ m.

TABLE II  
Concentration of Rat and Bovine Fibronectin in the Culture Medium and Cell Layer of Rat Fibroblasts

|   | Spent medium     |                    | Cell layer               |                    |
|---|------------------|--------------------|--------------------------|--------------------|
|   | Rat fibronectin  | Bovine fibronectin | Rat fibronectin          | Bovine fibronectin |
|   | $\mu\text{g/ml}$ |                    | $\mu\text{g/mg protein}$ |                    |
| Cells grown in normal medium                | 5.5              | 3.0                | 4.8                      | 0.5                |
| Cells grown in fibronectin-deficient medium | 3.8              | 0.02               | 4.5                      | <0.1               |

control for the specificity of the uptake of fibronectin by the cell layer, we tried to stain the cells with antibodies to bovine alpha-fetoprotein, another glycoprotein present in FBS (6). No staining was obtained.

#### Radioimmunoassay of Fibronectins in Cell Layer

The presence of bovine fibronectin in the cell layer was corroborated by results obtained with radioimmunoassay. About 10% of the total fibronectin in the cell layer was of bovine origin when the cells were grown in the presence of fetal calf serum containing fibronectin (Table II). The relative amount of bovine fibronectin in the extracellular matrix may be higher than this figure would suggest, since intracellular fibronectin contributes to the rat fibronectin in our assay.

#### DISCUSSION

Our results show that serum fibronectin can be found in the cell layer of normal cultured cells. The similarity of the staining patterns of the fibronectin produced by the cells and that originating from the culture fluid makes it unlikely that this would be due to simple trapping of the FBS fibronectin into the cell layer. Instead, it appears that the serum fibronectin becomes incorporated into the same structures in the extracellular matrix as the fibronectin derived from the cells. This interpretation is supported by the fact that bovine alpha-fetoprotein, another glycoprotein present in FBS, was not found in the cell layer.

The function of the circulating fibronectin, which has a concentration of 300  $\mu\text{g/ml}$  in human plasma (10), is not known. There is evidence that serum fibronectin may function as a nonspecific opsonin (2), which, through its affinity to collagen (4) and fibrin (15), would promote uptake of effete tissue by the reticuloendothelial system. In vitro,

the plasma form of fibronectin mediates cell adhesion and spreading when used to coat collagen or plastic surfaces (5, 7, 11, 12). The present findings suggest that one of the functions of the circulating fibronectin may be to serve as material for the assembly of the extracellular matrix.

A number of different investigators (see reference 20 and references cited therein) have presented arguments pro and con as to the identity of plasma and cellular fibronectins. Our data weigh on the side of similarity because, whatever chemical differences might exist between the fibronectin in plasma and that on the cell surface, they do not seem to influence the incorporation of these fibronectins into the pericellular matrix. This distribution depends on yet unknown interactions at the cell surface.

Our results also raise the problem of contamination of cellular fibronectin preparation with serum-derived fibronectin and suggest that the detection of fibronectin on the cell surface by the commonly used species-nonspecific antisera is not unambiguous evidence for the synthesis of fibronectin by those cells.

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