Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2746–2750, May 1985
Cell Biology

Incorporation of cellular and plasma fibronectins into smooth muscle cell extracellular matrix *in vitro*

(polypeptides/monoclonal antibody/differentiation)

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Communicated by Charles P. Bean, December 5, 1985

ABSTRACT **Fibronectins isolated from the conditioned medium produced by cultures of undifferentiated (monolayer) and differentiated (nodular) swine vascular smooth muscle cells are similar but not identical. In general, the nodular-cell fibronectin has a smaller molecular mass than monolayer-cell fibronectin and appears to lack the COOH-terminal interchain disulfide linkage. We studied the incorporation of cellular and plasma fibronectins into the cell layer. Smooth muscle cells bound 2.5 times more monolayer-cell fibronectin than nodular-cell fibronectin. Polypeptide fragments of human plasma fibronectin were used as a model system to investigate fibronectin incorporation into the cell layer. Only intact molecules were incorporated into the cell layer and subsequently organized into fibers. Polypeptide fragments of molecular mass 205 kDa and 185 kDa were not incorporated even though they retained the collagen-, cell-, and heparin-binding regions. Incorporation appears to require an activity associated with either the NH_2-terminal or COOH-terminal domains. We propose that fibronectin activity is lost during differentiation of smooth muscle cells.**

Fibronectin affects cell adhesion, motility, and differentiation *in vitro* (1–5). For example, both chondrocyte and smooth muscle cell differentiation is inhibited by exogenous fibronectin (6, 7) and cultures of differentiating mesenchymal cells display either an alteration in fibronectin distribution (8–13) or a loss of fibronectin from the cell layer (14). Differentiation of swine smooth muscle cells in culture is accompanied by a morphological transition from a substrate-attached cell monolayer to multicellular nodules (15) and we have established that the fibronectin synthesized by the undifferentiated cells in monolayer culture (FN-M) is functionally distinct from the fibronectin synthesized by the differentiated cells in nodular culture (FN-N) (16). Thus, the fibronectin isolated from monolayer cultures is active as an inhibitor of differentiation while the fibronectin produced by nodular cells is inactive. Further, fibronectin isolated from human plasma inhibits differentiation and the inhibitory activity is lost from human plasma fibronectin after it is incubated in conditioned medium from nodular-cell cultures.

Since it was reasonable to assume that only cell-bound fibronectin was active in inhibiting cell differentiation, we assessed the ability of cells to bind either FN-M or FN-N. The structural requirements for fibronectin incorporation into the cell layer were also evaluated using trypsin-derived fragments of human plasma fibronectin. An interpretation of our results could be that either the COOH-terminal or NH_2-terminal region of the fibronectin molecule is required for the binding and incorporation of fibronectin into the extracellular matrix.

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MATERIALS AND METHODS

Cell Culture and Metabolic Labeling. Primary cultures of vascular smooth muscle cells were initiated from segments of swine thoracic aorta and cells were used at passage levels 3–8 (7). Conditioned medium was prepared from confluent monolayer and nodular cell cultures grown in roller bottles containing medium 199/5% fetal bovine serum (Flow Laboratories). Metabolically labeled conditioned medium was prepared by incubating confluent cultures in Dulbecco's modified Eagle's medium supplemented with nonessential amino acids/penicillin (250 units/ml)/streptomycin (1.67 mg/ml)/L-glutamine (300 μg/ml)/[^{35}S]methionine (25 μCi/ml; 1 μCi = 37 GBq; New England Nuclear) for 20 hr. Labeled medium was harvested into 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$), centrifuged, and used without further storage.

Isolation and Purification of Fibronectins. Cellular fibronectins were isolated from serum-free conditioned medium by gelatin agarose or heparin agarose affinity chromatography (17–19). Heparin and gelatin binding components (see Fig. 1) were isolated from confluent or nodular cell cultures by mixing 1.2 ml of metabolically labeled conditioned medium with 100 μl of affinity resin for 30 min. The resins were subsequently washed five times with 1 ml of P_i/NaCl containing 1 mM ($PhMeSO_2F$), and once with 0.5 M urea. The resins were collected and boiled for 3 min in 100 mM Tris Cl, pH 6.8/2% $NaDodSO_4$/20% glycerol/0.04% bromphenol blue (2× sample buffer). In some cases, 5% 2-mercaptoethanol was added as a reducing agent. Isolated fibronectins were dialyzed against medium 199 containing 1 mM $PhMeSO_2F$.

Cellular fibronectin concentrations were computed by absorbance at 278 nM (20) and by slab gel electrophoresis. For the latter method, an LKB laser scanning densitometer was used to compare labeled and stained FN-M and FN-N with a concentration (250 ng–5 μg per track) gradient of human plasma fibronectin. Radioactivity was determined by liquid scintillation spectrometry of purified fibronectin (before gel electrophoresis) and of fibronectin slices taken from the slab gel. Specific activities of FN-M and FN-N were calculated from the resulting data.

Human plasma fibronectin was purified from a cryoprecipitate supplemented with 1 mM $PhMeSO_2F$ and 1% Trasylol, by passage through Sepharose 4B resin followed by affinity purification on gelatin-agarose. Gelatin-binding material was eluted with 4 M urea and further purified by heparin-

Abbreviations: FN-N, fibronectin isolated from nodular-cell conditioned medium; FN-M, fibronectin isolated from monolayer-cell conditioned medium; HBSS, Hanks' balanced saline solution; P_i/NaCl, phosphate-buffered saline; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

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agarose affinity chromatography. A 200-kDa fragment (205 kDa/185 kDa doublet) of plasma fibronectin was generated by limited digestion of 40 mg of intact fibronectin with trypsin at 1 μg/ml at 22°C for 30 min. The reaction was stopped by the addition of soybean trypsin inhibitor at 40 μg/ml and 1 mM $PhMeSO_2F$. The digest was fractionated by gelatin affinity chromatography. Gelatin-binding fragments were further fractionated by heparin-affinity chromatography. The 200-kDa fragment was stored at 1 mg/ml. The 120-kDa fragment, kindly provided by Erkki Ruoslahti, contained the cell binding site but was not bound by either gelatin or heparin (21, 22). Bovine plasma fibronectin was similarly isolated from citrated cow blood.

Polyacrylamide Gel Electrophoresis. The slab gel electrophoresis system described by Laemmli (23) was used. The $NaDodSO_4$ gels were 7.5% and 5% polyacrylamide in the separating and stacking gels, respectively. Gels saturated with dimethyl sulfoxide/2,5-diphenyloxazole were autoradiographed on Kodak X-Omat film at −70°C (24). Fibronectin bands were identified by using either monoclonal or polyclonal antibodies to detect antigens transferred to nitrocellulose paper (25).

Immunological Methods. Monoclonal antibodies (IgG fraction of mouse ascites fluid) directed against fibronectin were produced and characterized using published methods (26–28). An enzyme-linked immunoadsorbance assay (ELISA) developed for connective tissue components was used to evaluate antibody specificity (19, 29, 30). Fibronectins were diluted to 40 μg/400 μl in Voller's buffer (30) and subsequently diluted at 1:2 in 96-well flat-bottomed microtiter plates (Dynatech, Alexandria, VA). Fibronectin was adsorbed for 16 hr at 4°C to yield a protein concentration range from 11 ng to 20 μg. After washing with 0.05% Tween 20 in P_i/NaCl the wells were incubated for 60 min with antibody OLD/04.11.041 (human specific) or YNG/03.47.116 (cross-reactive). After extensive washing the wells were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG at 1:500 (Cappel Laboratories, Cochranville, PA) for 60 min. Color was developed using *o*-phenylenediamine (0.1 mg/ml) and 30% H_2O_2 (0.1 μl/ml). Absorbance was evaluated at 492 nM and a background absorbance of 0.08 OD units was subtracted from each value.

Antibody specificity was also evaluated after fibronectins were transferred electrophoretically from the 7.5% polyacrylamide slab gels to nitrocellulose paper. After transfer for 90 min at 100 V, the nitrocellulose paper was incubated with 5% bovine serum albumin in 10 mM Tris Cl/150 mM NaCl for 60 min at 37°C. The paper was first washed and incubated with primary mouse antibody in P_i/NaCl for 3 hr, then washed extensively with approximately 2 liters of 20 mM Tris Cl, pH 7.4/0.5 M NaCl/0.5% Nonidet P-40 buffer. A second antibody (horseradish peroxidase-conjugated goat anti-mouse IgG; Cappel Laboratories, 1:650) was added and incubated for an additional 90 min. After further washing for 16 hr in the Tris/saline/Nonidet P-40 buffer, the paper was incubated for 60 sec with diaminobenzidine at 0.5 mg/ml (Sigma) and 0.1% H_2O_2 in P_i/NaCl. The transfer of protein to nitrocellulose paper was monitored using Coomassie blue to stain a duplicate transfer.

Immunofluorescence Microscopy. Cells were grown to prenodular confluency on glass coverslips and the cell layer was washed extensively with Hanks' balanced saline solution (HBSS; GIBCO) before incubation for 18 hr with human plasma fibronectin or fibronectin fragment at 50 μg/ml. The cell layer was washed, fixed in 3% paraformaldehyde for 3 min at 0°C, and incubated with primary antibody (mouse IgG) for 30 min. The cell layer was again washed and incubated with rhodamine isothiocyanate-conjugated goat anti-mouse IgG at 1:20 (Cappel Laboratories). The coverslips were mounted on slides and photographed with an Olympus BH2 microscope on Kodak Tri-X film at an exposure of 2 min.

Iodination of Fibronectins. Purified intact human plasma fibronectin and 200-kDa fragments (200 μg) were iodinated by incubation for 60 sec in 0.25 M $KHPO_4$ (pH 7.6)/2 mM $PhMeSO_2F$/chloramine-T, 50 μg/ml, containing 1 mCi (1 Ci = 37 GBq) of $Na^{125}I$. The mixture was reduced with $Na_2S_2O_5$ and diluted with buffer containing bovine serum albumin at 10 mg/ml and 1% Trasylol. Iodinated products were recovered after purification through Dowex 1 × 8 ion exchange resin followed by gelatin-agarose affinity chromatography. The specific activity of recovered material was 420 μCi/mg. For binding studies, the cells were washed five times with HBSS and incubated with iodinated fibronectin (2×10^6 cpm/ml) in medium 199 containing bovine serum albumin at 1 mg/ml. At 0, 1, and 4 hr, the cell layer from three cultures was washed five times in HBSS and solubilized directly into 100 μl of 2× sample buffer and 5% 2-mercaptoethanol and the radioactivity was determined.

RESULTS

Several differences between fibronectins isolated from the conditioned medium of monolayer cell and nodular cell cultures were noted after gel electrophoresis (Fig. 1). Although both fibronectins migrated slower than a myosin (200 kDa) marker, the average molecular mass of gelatin-purified and reduced FN-M (track B) was about 20–30 kDa larger than that of similarly purified FN-N (track C). In the absence of reducing agent, gelatin-purified FN-M migrated as a single band with an approximate molecular mass of 450 kDa (track D). In contrast, gelatin-purified FN-N migrated as two components with approximate molecular masses of 220 and 450 kDa (track F). Approximately 35% of the radioactivity asso-

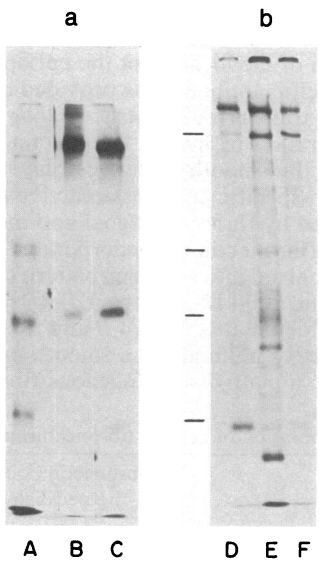


FIG. 1. Gel electrophoresis patterns of fibronectins from smooth muscle cell-conditioned medium. Fibronectins were isolated from metabolically labeled conditioned medium by gelatin (tracks B, C, D, and F) or heparin (track E) affinity chromatography. (*a*) Reduced preparations of FN-M (track B) and FN-N (track C). (*b*) Nonreduced preparations of FN-M (track D) and FN-N (tracks E and F). The bands of molecular mass greater than 200 kDa have been immunologically identified as fibronectin. The identities of all of the lower molecular mass bands have not been established; however, the two bands at 38–43 kDa are neither derived from fibronectin nor antigenically similar to fibronectin (31). The molecular mass standards in track A, myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), and ovalbumin (46 kDa), are also indicated by hash marks in *b*.

ciated with the fibronectin-containing bands of FN-N was present in the 220-kDa band (track F). Approximately 10% of the fibronectin remained at the top of the stacking gel. Evidence that FN-N retained heparin binding activity is presented in track E. The fibronectins (220 kDa, 450 kDa, and top of the stacking gel) from heparin- (track E) and gelatin- (track F) purified samples were distributed similarly in the gel.

Binding of FN-M and FN-N to the prenodular confluent cell layer was examined using metabolically labeled fibronectins isolated by gelatin and heparin affinity chromatography. The specific activities of the two fibronectins were virtually identical. During an incubation period of 15–240 min about 2–3 times more FN-M than FN-N was bound to the cell layers (Table 1).

A 200-kDa fragment was generated by trypsin digestion of intact human plasma fibronectin. During the early time of digestion a 25- to 30-kDa heparin-binding fragment was released. The fragment probably represents the NH_2-terminal domain (32–35). More than 80% of the fibronectin remaining after 30–45 min of digestion was present as 205-kDa and 185-kDa fragments. These were purified by sequential gelatin- and heparin-affinity chromatography and were the only digestion products that bound to both ligands. Cleavage at the COOH terminus was established using nonreduced preparations (data not shown). The 200-kDa doublet retained gelatin, cell, and heparin binding activity.

In order to test the binding of human plasma fibronectin to swine smooth muscle cells by indirect immunofluorescence, the specificity of the primary mouse anti-human IgG was first evaluated by an ELISA (Fig. 2) Monoclonal antibody OLD/04.11.04 was specific for human fibronectin (human specific) and recognized an epitope on intact fibronectin as well as on the 200-kDa and 120-kDa fragments. Antibody YNG/03.47.116 crossreacted with human, bovine, and swine fibronectins (cross-reactive). It also recognized an epitope on the 120-kDa fragment (data not shown). Additional confirmation of the location of the epitope recognized by the human-specific antibody was provided by antigens electrophoretically transferred to nitrocellulose paper (Fig. 3).

The incorporation of human fibronectin and fibronectin fragments into the smooth muscle cell layer was visualized with the human-specific and cross-reactive mouse monoclonal IgG followed by fluorescent goat anti-mouse IgG. Intact human plasma fibronectin was incorporated into the cell layer and was organized into a fibrous pattern (Fig. 4A). In contrast, neither the 200-kDa nor the 120-kDa fragments were detected in the cell matrix (Fig. 4 *B* and *C*). After treatment with cross-reactive antibody, the smooth muscle cells were seen to contain a matrix of endogenous fibronectin derived either from cell synthesis or from the serum in the culture medium (Fig. 4 *D–F*).

Table 1. Incorporation of cellular fibronectin into the cell layer

Time, min	Fibronectin bound, ng per dish	
	FN-M	FN-N
0	—	—
15	0.03	0.03
30	0.10	0.02
60	0.08	0.03
120	0.18	0.07
240	0.34	0.14

Attached confluent prenodular cultures were washed and then incubated at 37°C with 5.6 ng (1×10^5 cpm) of [^{35}S]methionine-labeled fibronectins (FN-M or FN-N) for the times indicated. The cell layers were then washed five times with HBSS and scraped into 100 μl of 2× sample buffer containing 5% 2-mercaptoethanol and 2% $NaDodSO_4$. Solubilized material was assayed for incorporated radioactivity. The specific activities of the purified fibronectins were as follows: FN-M = 16.0 μCi/μg and FN-N = 16.1 μCi/μg.

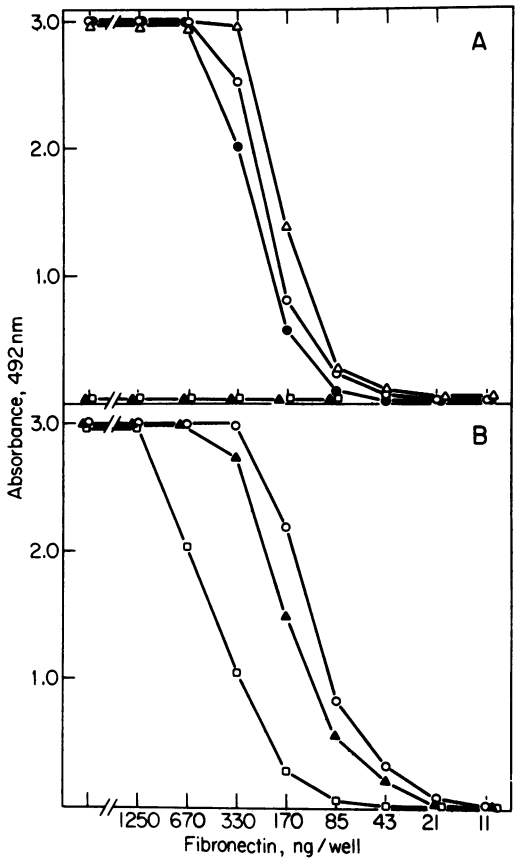
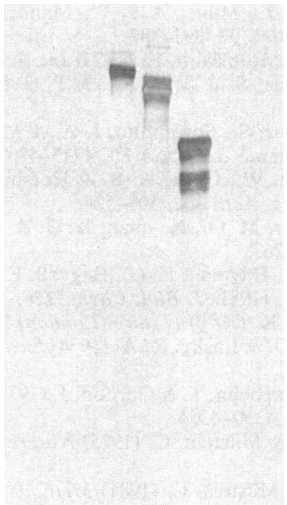


FIG. 2. ELISA analysis of antifibronectin monoclonal antibodies. Fibronectins were adsorbed to microtiter plates and assayed as described in *Methods*. (*A*) Response to human-specific antibody. (*B*) Response to cross-reacting antibody. The fibronectins were intact human plasma fibronectin (○), the 200-kDa fragment of human plasma fibronectin (●), the 120-kDa fragment of human plasma fibronectin (△), intact bovine plasma fibronectin (▲), and swine cellular fibronectin (□).

The binding of intact human plasma fibronectin and the 200-kDa fragment derived from it was quantitatively evaluated using iodinated fibronectins. The confluent prenodular smooth muscle cells bound up to 40 ng of intact fibronectin. In contrast, less than 3 ng of the 200-kDa fragment was bound (Fig. 5).

DISCUSSION

By examination of the electrophoretic mobility of the fibronectins, FN-N and FN-M, we could show that a significant fraction (35%) of FN-N lacked the COOH-terminal interchain disulfide linkage although it retained gelatin and heparin affinity. The remaining FN-N ran on the gels in positions expected for dimeric or multimeric fibronectin. Whether those molecules were also cleaved at the COOH terminus and aggregated through some an alternative disulfide linkage (36–38) was not established. However, the fact that FN-N, on the average, migrated faster than FN-M when both were reduced (Fig. 1) suggested that most of the FN-N molecules were similarily modified. Reduced FN-N appeared to co-migrate with the upper band of the plasma fibronectin doublet and had a mass greater than that of the 205-kDa fragment derived from plasma fibronectin (data not presented). Whether the FN-N and the 205-kDa fragment were similarly processed was not established. Cellular fibronectins are more heavily glycosylated than plasma fibronectins and gen-



A B C D E F

FIG. 3. Localization of the epitope recognized by the human-specific antibody. Fibronectins were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose paper. The paper was stained with human-specific antibody followed by horseradish peroxidase-conjugated goat anti-mouse IgG. The fibronectins used were bovine plasma fibronectin, 10 μg (track A); swine cellular fibronectin, 5 μg and 10 μg (tracks B and C); intact human plasma fibronectin, 3 μg (track D); the 200-kDa fragment, 3 μg (track E); and the 120-kDa fragment, 5 μg (track F). Track F also shows degradation products of the 120-kDa fragment that contain the recognition site for the human-specific antibody.

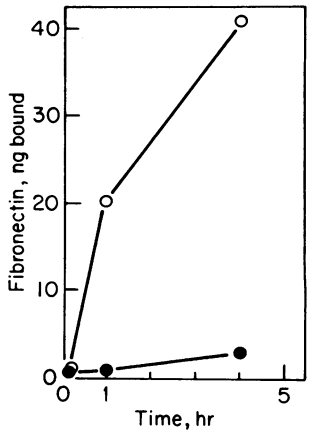


FIG. 5. Binding of iodinated fibronectin or the 200-kDa fragment to cell layers. Attached confluent prenodular cultures were incubated with intact fibronectin (2×10^6 cpm/ml) (○) or the 200-kDa fragment (2×10^6 cpm/ml) (●) for the times indicated. Nonspecific binding of 3–4 ng was subtracted from each value.

erally exhibit larger molecular masses (4, 5).

Conditioned medium contains gelatin- and heparin-binding components other than fibronectin (Fig. 1). The gelatin-binding component of about 75 kDa (tracks B and C) did not bind heparin and may be similar to previously identified gelatin-binding proteins (39, 40). The components at 43 kDa (track D) and 38 kDa (track E) were not immunologically related to fibronectin (31, 52).

Both of the monoclonal antibodies used for immunofluorescence recognized epitopes in a 120-kDa fragment of human plasma fibronectin. The 120-kDa fragment has been shown to lack gelatin- and heparin-binding activity but to be active in cell adhesion (21). Other mapping studies placed the target epitope at between 60 kDa and 100 kDa from the gelatin binding domain (unpublished data). The 205-kDa fragment has been shown previously to retain a COOH-terminal fibrin binding domain that was lacking in the 185-kDa fragment (33, 35, 41, 42). Because neither the 205-kDa nor the 185-kDa fragments were bound by the cells we conclude that the COOH-terminal fibrin binding domain was perhaps necessary, but not sufficient, for binding.

The binding of fibronectin to cells is a complex process that appears to be receptor mediated (43, 44). Further, the receptors involved in cell attachment may be distinct from those that bind soluble fibronectin to cells (45, 46). Fibronectin in solution must be rendered insoluble before cell binding can be detected (47). It has been proposed that insolubility may be facilitated either by fibronectin self-aggregation through interactions of free sulfhydryl groups (32, 37) or by adsorption to a substrate (48). Our results suggest that for

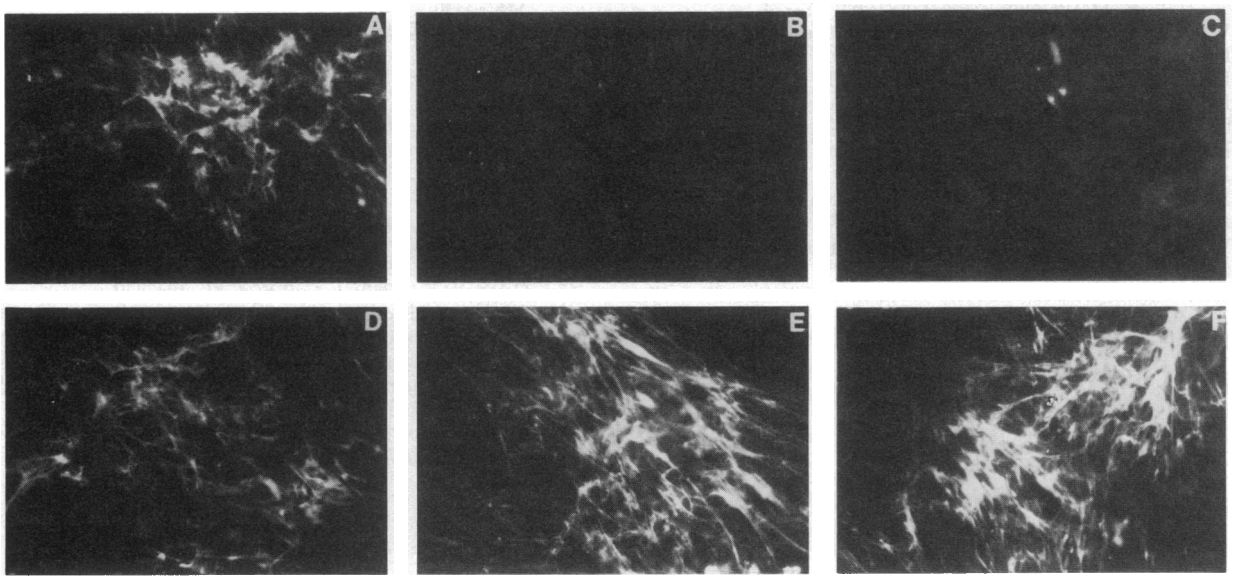


FIG. 4. Fibronectin distribution on smooth muscle cells. Prenodular cultures of smooth muscle cells were incubated for 16 hr with intact human plasma fibronectin (*A* and *D*), the 200-kDa fragment of human plasma fibronectin (*B* and *E*), or the 120-kDa fragment of human plasma fibronectin (*C* and *F*) at 50 μg/ml. (*A–C*) Cultures incubated with human-specific antibody. (*D–F*) Cultures incubated with cross-reacting antibody. The presence of the cell layer was confirmed using light (Nomarski) microscopy (not presented).

fibronectin to interact with the extracellular matrix the molecule must be virtually intact. Even the presence of three of the major active sites, the collagen, cell, and heparin binding domains, was insufficient for cell binding.

The conclusions presented here may be generalized. For example, in the presence of dexamethasone, hepatoma cells synthesize a fibronectin that is about 40% monomeric and lacks cell binding activity (49). Further, there is evidence that monomeric fibronectin is not incorporated into mouse tissue *in vivo* (50). For smooth muscle cell differentiation, we propose a model that is consistent with the known activities of fibronectin and with our experimental observations. Smooth muscle cells *in vivo* are differentiated cells which function to provide elasticity to the artery wall. However, after exposure to plasma mitogens through injury to the endothelium, the smooth muscle cells modulate (51) and become proliferative cells. After the injury is repaired, the cells differentiate and regain smooth muscle cell function. We propose that the tissue culture system mimics those processes. The role of fibronectin (either cellular or plasma) is to guide and anchor the cells during the wound healing process. Once that has been completed, the fibronectin is cleaved to an inactive form that may then either be cleared or remain in place. In support of this hypothesis, we find that the fibronectin associated with differentiated cells has a smaller molecular mass than that of undifferentiated cell fibronectin and appears to lack one or more terminal regions. It would appear, then, that one or both of the terminal regions are required for the formation of fibronectin matrix.

We are grateful to Dr. Erkki Ruoslahti for generously providing the 120-kDa fragment and to Ellen Reich for technical assistance in the preparation of other fibronectin fragments. This work was supported by grants from the National Institute on Aging, National Institutes of Health (AG00697), and the New York Chapter of the American Heart Association.

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