EXTERNAL FIBRONECTIN OF CULTURED HUMAN FIBROBLASTS IS PREDOMINANTLY A MATRIX PROTEIN

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ABSTRACT

The distribution of a major glycoprotein (fibronectin) of human fibroblast cultures was studied in immunoelectron microscopy with peroxidase- or ferritin-labeled antibodies.

External fibronectin was visualized in pericellular structures, in some areas on the growth substratum, and to a lesser degree in close association with the upper and lower surface membranes of the cell. The pericellular fibronectin-containing structures consisted of amorphous or vaguely fibrillar material forming strands or patches, 50–500 nm in diameter; the structures appeared to mediate distant cell-to-cell and cell-to-substrate contacts. When in close association with the plasma membrane, fibronectin markers were seen as discrete patches. The exact relationship between this form of fibronectin and the plasma membrane, however, remained open. Filamentous material was commonly seen in the cortical cytoplasm under patches of membrane-associated fibronectin.

The distribution that we observed is consistent with the proposed roles of fibronectin in cell interactions with neighboring structures and with its presence in vivo as an extracellular glycoprotein in connective tissue matrix and basal laminae.

KEY WORDS basement membrane · cell surface · connective tissue · fibroblast · immunoelectron · intercellular

Fibronectin refers to a glycoprotein of high molecular weight detected in vertebrates as immunologically cross-reactive forms in plasma (known as cold-insoluble globulin) and tissues (32). Immunofluorescence studies have shown fibronectin to be present in primitive mesenchymal and loose connective tissues, in blood vessel walls, and characteristically in various basal laminae in chickens (19) and mice (36).

Fibronectin is a major external protein of cultured fibroblasts, and has been studied under the names of fibroblast surface antigen (28), large external transformation sensitive protein (11), cell surface protein (37), and others (8, 10, 27). The expression and localization of this protein in cultures of fibroblastic cells have been studied by radioactive-labeling techniques including lactoperoxidase (11) and galactose oxidase catalyzed reactions (8), by immunofluorescence staining and scanning electron microscopy (30, 35). In virus-transformed fibroblasts, fibronectin cannot be detected by surface-labeling techniques although such cells synthesize it (33). The great current interest in fibronectin stems from the finding that loss of external fibronectin in transformation represents the largest known difference in polypeptide composition between normal and transformed cells (31).

It has been previously shown by immunofluorescence and radioimmunoassay that fibronectin is also found intracellularly and is secreted or shed in large amounts in cultures of human fibroblasts and astroglial cells (34). Cellular fibronectin has been shown to be in disulfide-bonded dimeric and polymeric forms containing 220,000 dalton subunits (13, 15), and it moves in immunoelectrophoresis as a β-globulin. The plasma form (dimer of 200,000 dalton subunits) has a sedimentation coefficient of 12–14S and contains 5% carbohydrate (21–23). The cellular and plasma forms are both substrates for plasma transglutaminase (factor XIII; [16, 23]).

External fibronectin has a nonrandom fibrillar distribution (30, 35), is very sensitive to exogenous proteolytic cleavage (12), and is a major receptor for lectins (4, 7). Marciani and Bader (20) reported that surface-iodinated fibronectin was not retained in plasma membrane preparations unless membranes were fixed with $ZnCl_2$. A proportion of external fibronectin (cell surface protein) was recovered from chick embryo fibroblasts with 1 M urea (37). These findings suggest that fibronectin is not a conventional intrinsic membrane protein.

We have studied fibronectin in cultures of human fibroblasts by transmission electron microscopy with immunoperoxidase and immunoferritin techniques. The cultures were stained *in situ* to gain information on the relationship of external fibronectin to neighboring cells, to growth substratum, and to associated extracellular material.

MATERIALS AND METHODS

Cell Cultures

Human adult (ES) and embryonic skin (HES-L) fibroblasts, of locally established strains, were studied between the 10th and 25th passages. WI-38 human embryonic lung fibroblasts were obtained from the American Type Culture Collection, Rockville, Md. Cells were grown on 100-mm plastic Petri dishes at 37°C in Eagle's basal medium (diploid), supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. Subcultures were made twice weekly in a 1:2 ratio using 0.25% trypsin and 0.02% EDTA in Hanks' buffered saline to disperse the cells. The stock cultures were tested biweekly for mycoplasma (29), with negative results. For the experiments, cells from confluent cultures were seeded in a 1:2 ratio onto 50-mm diameter (20 cm^2) Petri dishes containing round, 18-mm glass cover slips or 25-mm plastic cover slips (Lux Scientific Corp., Newbury Park, Calif.) in 5 ml of medium.

Antifibronectin Serum

Rabbit antiserum was raised against human plasma fibronectin purified according to a published procedure (23) modified so that fibrinogen was removed by heat precipitation (56°C, 3 min) instead of clotting. The specificity of the rabbit antifibronectin serum is indicated by the following observations: (*a*) the antiserum formed in immunodiffusion against human plasma or against human fibroblast extracts only a single precipitation arc. (*b*) Immunofluorescent staining (see below) of cultured human fibroblasts resulted in fibrillar surface-associated and patchy perinuclear cytoplasmic staining that could be blocked completely by prior incubation of antiserum with purified fibronectin. (*c*) No such staining was observed when normal rabbit serum or rabbit antisera to a variety of other human plasma proteins were substituted for rabbit antifibronectin. The above-described controls (*a*) and (*b*) have been shown in Stenman et al. (30), who also documented the purity of the antigen by polyacrylamide gel electrophoresis with the presence of sodium dodecyl sulfate. (*d*) A single major polypeptide, with apparent molecular weight of 220,000 daltons, was precipitated by the antifibronectin rabbit serum from both cell extracts and medium of metabolically radiolabeled cultures of human fibroblasts (Fig. 1). Further specificity controls concerning immunoperoxidase and immunoferritin procedures are described in Results.

Immunofluorescence

The cultured cells on cover slips were washed three times with phosphate-buffered saline (PBS) and fixed with 3.5% formaldehyde (20 min, 20°C). Fibronectin was stained by the indirect method using rabbit antiserum prepared against human plasma fibronectin and commercially obtained anti-rabbit IgG sheep immunoglobulin (IgG) conjugated with fluorescein isothiocyanate (The Wellcome Foundation, Ltd., The Wellcome Research Laboratories, Beckenham, England). Slides were examined by epifluorescence with a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N. J.). For specificity controls of the antifibronectin antiserum, see above. Antiferritin sheep immunoglobulin (IgG) conjugated with fluorescein isothiocyanate was a gift from Dr. P. Biberfelder, National Bacteriological Laboratory, Stockholm, Sweden.

Immunoperoxidase Conjugate

Horseradish peroxidase-conjugated goat IgG against rabbit IgG (Miles-Yeda, Rehovot, Israel) was used diluted 1:30 in Dulbecco's phosphate-buffered saline containing 0.2% bovine serum albumin.

Immunoferritin Conjugate

Anti-rabbit IgG serum was raised in a sheep, and the sheep IgG was isolated. Horse spleen ferritin (~50 mg/ml, six times crystallized, Pentex Biochemical, Kan-

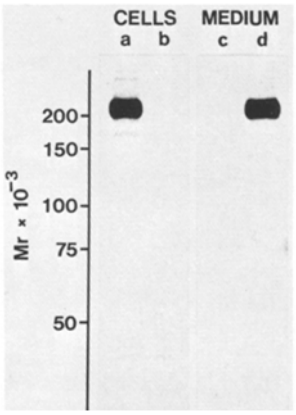


FIGURE 1 Electrophoresis of [^{35}S]methionine-labeled proteins immunoprecipitated by antifibronectin rabbit serum or normal rabbit serum from cultures of human skin fibroblasts to demonstrate specificity of the antiserum. Fibroblast culture was labeled for 3 h with [^{35}S]methionine, and proteins were precipitated from medium and cell extracts by a double antibody technique, described in detail elsewhere (25), briefly as follows. The cell layers were dissolved in Tris-buffered saline containing 0.5% sodium deoxycholate and protease inhibitors. The cell extract and medium were clarified by centrifugation at 15,000 *g* for 60 min. Antifibronectin rabbit serum (*a*, *d*) or normal rabbit serum (*b*, *c*) was added, and after incubation for 2 h, the antigen-antibody complexes were precipitated using sheep anti-rabbit gammaglobulin. The immunoprecipitates were washed and collected by centrifugation, dissolved in a buffer containing sodium dodecyl sulfate and mercaptoethanol, and analyzed by polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate (1), followed by scintillation autoradiography (18). Size standards included plasma fibronectin, 2×10^5 daltons; α_2-macroglobulin, 1.6×10^5 daltons; phosphorylase a, 9.3×10^4 daltons; serum albumin, 6.8×10^4 daltons; and ovalbumin, 4.3×10^4 daltons, all labeled with [^{14}C]formaldehyde by the method of Rice and Means (26).

kakee, Ill.) was diluted 1:5 in PBS and sedimented three times by ultracentrifugation at 100,000 *g* for 3 h. In the conjugation, the "two-step glutaraldehyde high concentration" procedure of Kishida et al. (17) was followed. Purified glutaraldehyde ($A_{235}/A_{280} = 0.3$) was obtained from Leiras, Turku, Finland. The conjugate, separated on a Sepharose 6B column, was used at an $OD_{440} = 1.5$ with about 1 mg of ferritin per milliliter. In immunodiffusion, the preparation reacted against normal rabbit serum and strongly against sheep antiferritin serum, kindly provided by Dr. Astrid Fagraeus of the National Bacteriological Laboratory, Stockholm, Sweden.

Immunoperoxidase Staining

Cultured cells on cover slips were prefixed as follows. The cells were briefly rinsed three times with warm (37°C) Dulbecco's phosphate-buffered saline and fixed for 30 min with the following mixture: 0.5% glutaraldehyde and 1% paraformaldehyde with 2 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2, temperature 37°C. After 10-min fixation, the cells in fixative were cooled to ±0°C. The cover slips were then washed with ice-cold PBS containing 0.02 M lysine-HCl, pH 7.2, to block free aldehyde groups.

The fixed cells on cover slips were treated in a moist chamber at 4°C for 30 min with a 1:40 dilution of antifibronectin serum (100 μl/cover slip), and washed three times for 10 min with a large volume of PBS with magnetic stirring. The washed cover slips were exposed to the conjugate for 30 min and washed three more times. The cover slips were then refixed with a mixture of 2% glutaraldehyde and 4% paraformaldehyde with 2 mM $CaCl_2$ in cacodylate buffer, pH 7.2, for 30 min at 4°C, followed by three washes with 0.05 M Tris-HCl buffer, pH 7.6, at room temperature. The peroxidase-catalyzed staining reaction was performed with the following mixture: 0.05% diaminobenzidine-HCl (Fluka A.G., Buchs, Switzerland) and 0.001% H_2O_2, 0.1 M sucrose, in 0.05 M Tris-HCl buffer, pH 7.6. After 10-min staining at room temperature, the cover slips were briefly rinsed three times with Tris-buffered sucrose and stored at 4°C in this solution till further processing.

Immunoferritin Staining

After fixation, treatment with antifibronectin serum and washing as described above, the cells on cover slips were treated with the ferritin conjugate at 4°C for 30 min and washed. The cells were then refixed, rinsed, and stored in PBS at 4°C.

Light and Electron Microscopy

For light microscopy, peroxidase-stained cells on glass cover slips were dehydrated in a graded series of ethanol, treated with xylol, and mounted on object glasses with Diatex (AB Wilh. Becker, Stockholm, Sweden). A Zeiss Universal microscope with phase-contrast optics was used.

For electron microscopy, the peroxidase- or ferritin-stained cells on plastic cover slips were postfixed with 1.5% OsO_4 in 0.1 M phosphate buffer, pH 7.2, for 2 h at room temperature, and dehydrated with ethanol. When indicated, block-staining with 2% uranyl acetate in 94% ethanol was used. The cover slips were mounted in Epon 812. After two days polymerization, the Epon blocks were sectioned vertically against the cell layer. The thin sections (~80 nm in thickness), with or without uranyl acetate and lead citrate staining, were observed and photographed in a JEM 100 B electron microscope at 80 kV.

RESULTS

Distribution of Surface-Associated Fibronectin at Light Microscope Level

The typical fibrillar distribution of surface fibronectin seen by indirect immunofluorescence (Fig. 2*a*) corresponded largely to fibrillar structures seen in phase-contrast microscopy (Fig. 2*b*) and was also found to be typical of the pattern seen by immunoperoxidase (Fig. 3*a*) and by immunoferritin-immunofluorescence staining (Fig. 4*a*). The figures also show the developing pattern of surface-associated fibronectin, from sparse 1-day-old cultures (Figs. 2*a* and 3*a*) to dense 3-day-old cultures (Fig. 4*a*), in which an extensive network of stained fibronectin is seen.

The specificity of the antifibronectin antibodies and the surface localization of the staining reactions have been described in Materials and Methods.

Figs. 3*b* and 4*b* are controls for the immunoperoxidase and immunoferritin staining patterns, respectively.

Electron Microscope Immunoperoxidase Results

Immunoperoxidase staining of surface-associated fibronectin was used to extend the light microscope observations to the ultrastructural level, as the method permitted examination of the cells by both light and electron microscopy. The specificity of the immunoperoxidase electron microscope staining was tested by control experiments. When the antifibronectin rabbit serum was blocked with purified fibronectin (Fig. 5) or substituted with normal rabbit serum, no dense staining was seen. When, instead of fibronectin, human albumin was used to "block" the antiserum or when untreated antifibronectin serum was used, heavy precipitates were seen (Figs. 6–8). The densely stained material formed distinct patches or longer continuous strands along cell surfaces. These corresponded well to the fibrillar pattern of stained fibronectin in light microscopy.

Most of the fibronectin that was visualized by immunoperoxidase staining was seen as material not attached to cell surface membrane, particularly in old dense cultures. The only ultrastructural details seen in such fibronectin-containing material were vague extracellular filamentous structures; specifically, no membrane was seen. The ultrastructure was better discerned in similar material in stained control specimens not obscured by peroxidase reaction products. Some stain was found adjacent to plasma membrane often at sites of cell-to-cell contact (Fig. 7) or cell-to-substrate contact (not shown), or in noncontacting areas of plasma membrane (Fig. 6). Peroxidase stain was not found at sites of direct membrane-membrane or membrane-substrate contacts (see Fig. 7). In contrast, distant contacts (e.g. over 50 nm) appeared to be mediated by patches (Fig. 7) or strands (Fig. 8) of fibronectin-containing material. The latter were seen especially in older dense cultures. When present adjacent to cell surface membrane, peroxidase-labeled fibronectin was often accompanied by submembranous filamentous structures (Fig. 6). Because of the intrinsic limitations in the resolution of the peroxidase staining method, unequivocal correlation could not be established.

Immunoferritin Results

In indirect immunoferritin electron microscopy, fibronectin was detected (*a*) in extracellular structures where fibronectin was not closely associated with cell surface membrane, and (*b*) closely associated with the membrane. Only occasional or no ferritin particles were seen in control experiments in which the antifibronectin antiserum was blocked by purified fibronectin (Fig. 9) or the antiserum was substituted with normal rabbit serum. The specificity of the immunoferritin-labeling pattern was also tested in a three-step immunofluorescence staining procedure where formaldehyde-fixed fibroblasts were sequentially exposed to anti-human fibronectin rabbit serum, then to the conjugate (anti-rabbit IgG-ferritin), and finally to fluorescein conjugated antiferritin sheep IgG. A fibronectin staining pattern (Fig. 4*a*) indistinguishable from that obtained by ordinary immunofluorescence staining was seen. When, in this three-step procedure, antifibronectin serum was substituted with normal rabbit serum (Fig. 4*b*), no staining was seen, indicating that the conjugate did not appreciably bind to the fixed cells nonspecifically.

The extracellularly located fibronectin was commonly found in patchy (Figs. 10, and 11) or strandlike structures (Figs. 12, 13, and 17), 50–500 nm in diameter. These structures were present on both the upper and lower sides of the cells and were more abundant in older dense cultures. The structures were composed of amorphous or

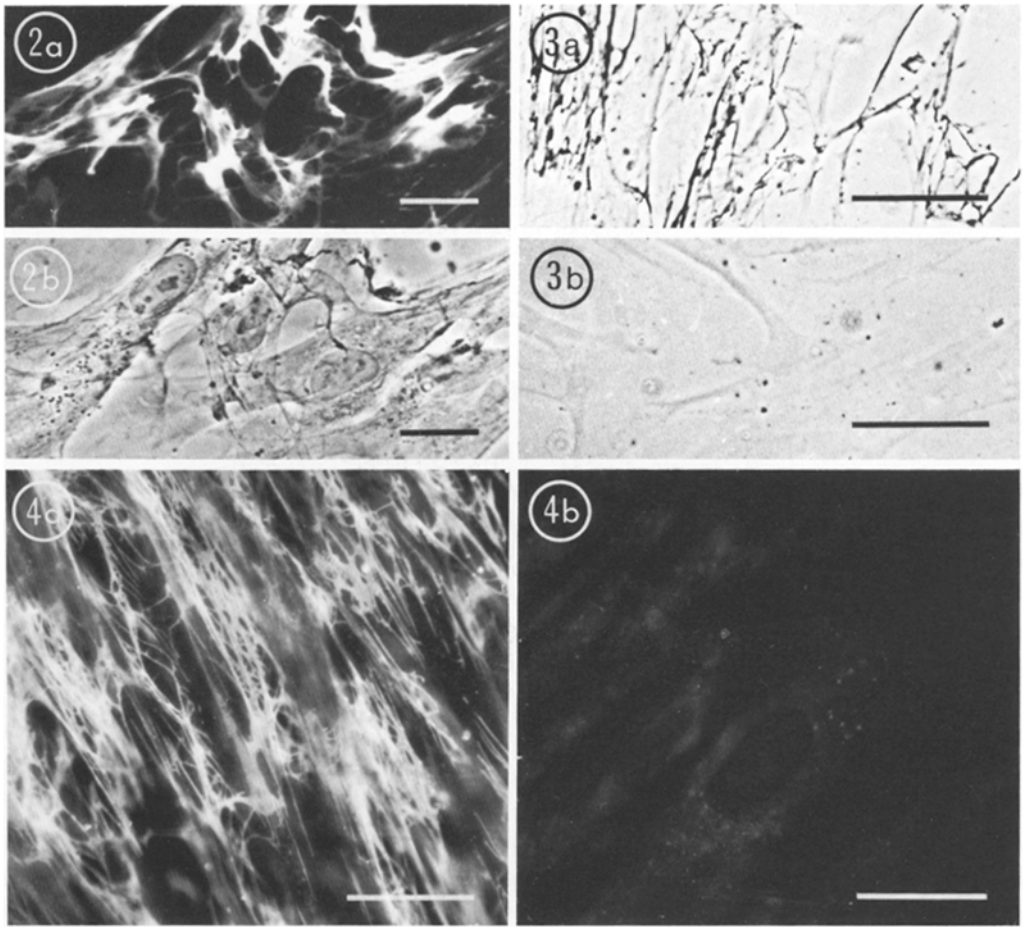


FIGURE 2 Immunofluorescence fibronectin staining of cultured human embryonic skin fibroblasts. 1-day culture was fixed with formaldehyde and indirectly stained using anti-human fibronectin rabbit serum and fluorescein conjugated sheep IgG against rabbit IgG. Immunofluorescence micrograph (Fig. 2*a*) and phase-contrast micrograph of the same field (Fig. 2*b*). The external fibronectin fluorescence appears as a network corresponding largely to fibrillar structures on, below, or between the cells. Bar, 20 μm. × 500.

FIGURE 3 Immunoperoxidase staining of fibronectin. The cell layers are faintly visualized in phase-contrast micrographs with minimal contrast (decentered condensor lense). The cultures were identical to that of Fig. 2, but were fixed with a mixture of glutaraldehyde and paraformaldehyde. After fixation, the culture was treated with antifibronectin rabbit serum (3*a*) or normal rabbit serum (3*b*), then with peroxidase-conjugated goat IgG against rabbit IgG. The culture was refixed with glutaraldehyde and paraformaldehyde, and subsequently treated with peroxidase substrates. Fig. 3*a* shows a darkly stained fibronectin pattern comparable to that seen by indirect immunofluorescence (see Fig. 2*a*). In Fig. 3*b*, no staining is seen. Bar, 40 μm. × 400.

FIGURE 4 Indirect fibronectin-ferritin-fluorescence staining of human embryonic skin fibroblasts 3 days after subculture. The culture was fixed with formaldehyde, treated with antifibronectin rabbit serum (Fig. 4*a*) or with normal rabbit serum (Fig. 4*b*), then with ferritin-conjugated sheep IgG against rabbit IgG, and finally with fluorescein-conjugated antiferritin IgG. Fig. 4*a* In this dense 3-day-old culture, with cells in a parallel array, fibronectin is visualized as an abundant network of interconnecting fibrillar fluorescence. Fig. 4*b* Lack of immunofluorescence demonstrates the specificity of the antifibronectin antibodies and shows that neither the ferritin conjugate nor the fluorescein conjugate bind nonspecifically to cell cultures to a significant degree. Bars, 20 μm. × 830.

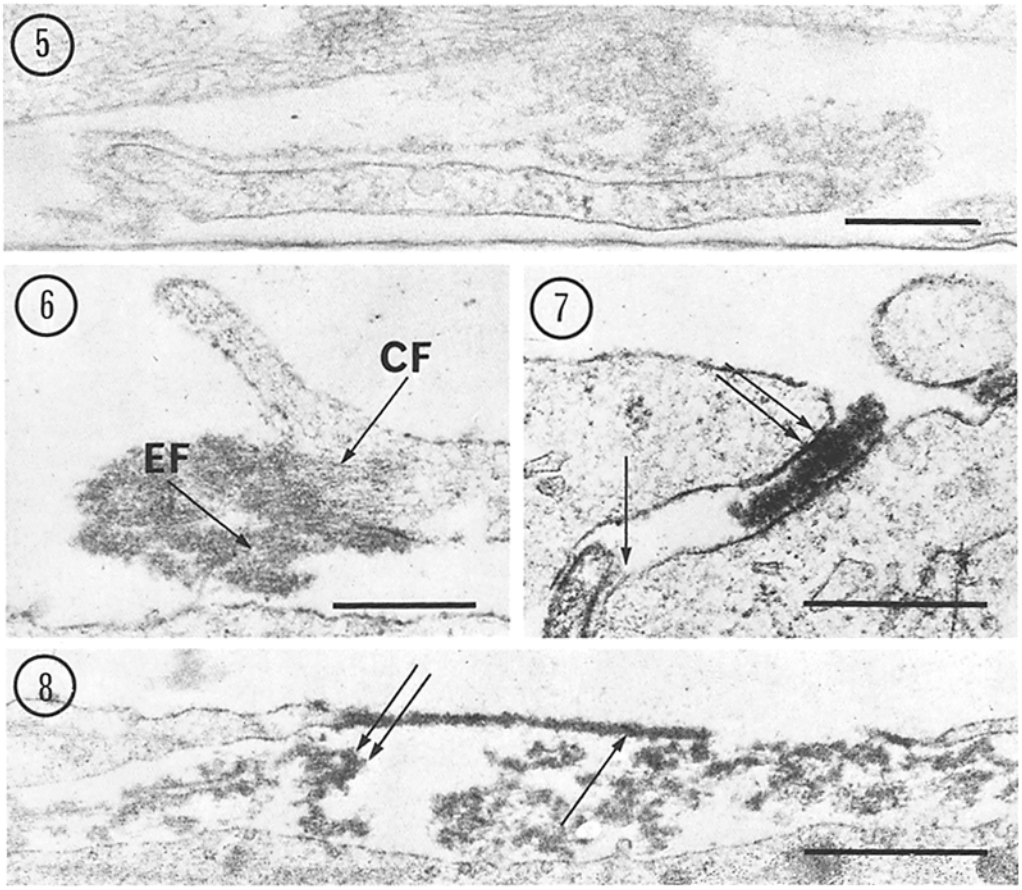


FIGURE 5 Immunoperoxidase electron micrograph of stained control fibroblasts 2 days after subculture, showing no staining. The culture was fixed with glutaraldehyde and paraformaldehyde (as in Fig. 3), exposed to antifibronectin rabbit serum pretreated with purified plasma fibronectin, then to peroxidase-conjugated anti-rabbit IgG, refixed, and treated with peroxidase substrates. After postfixation with OsO_4 and block-staining with uranyl acetate, 60-nm thin sections were prepared and observed without poststaining. To prepare the blocked antifibronectin serum, 100 μg of purified human plasma fibronectin was added to 1 ml of diluted (1:20) antifibronectin serum. The mixture was kept first for 60 min at room temperature and then overnight at 4°C, and then was clarified by centrifugation for 60 min at 10,000 *g* at 4°C. Bar, 200 nm. × 95,000.

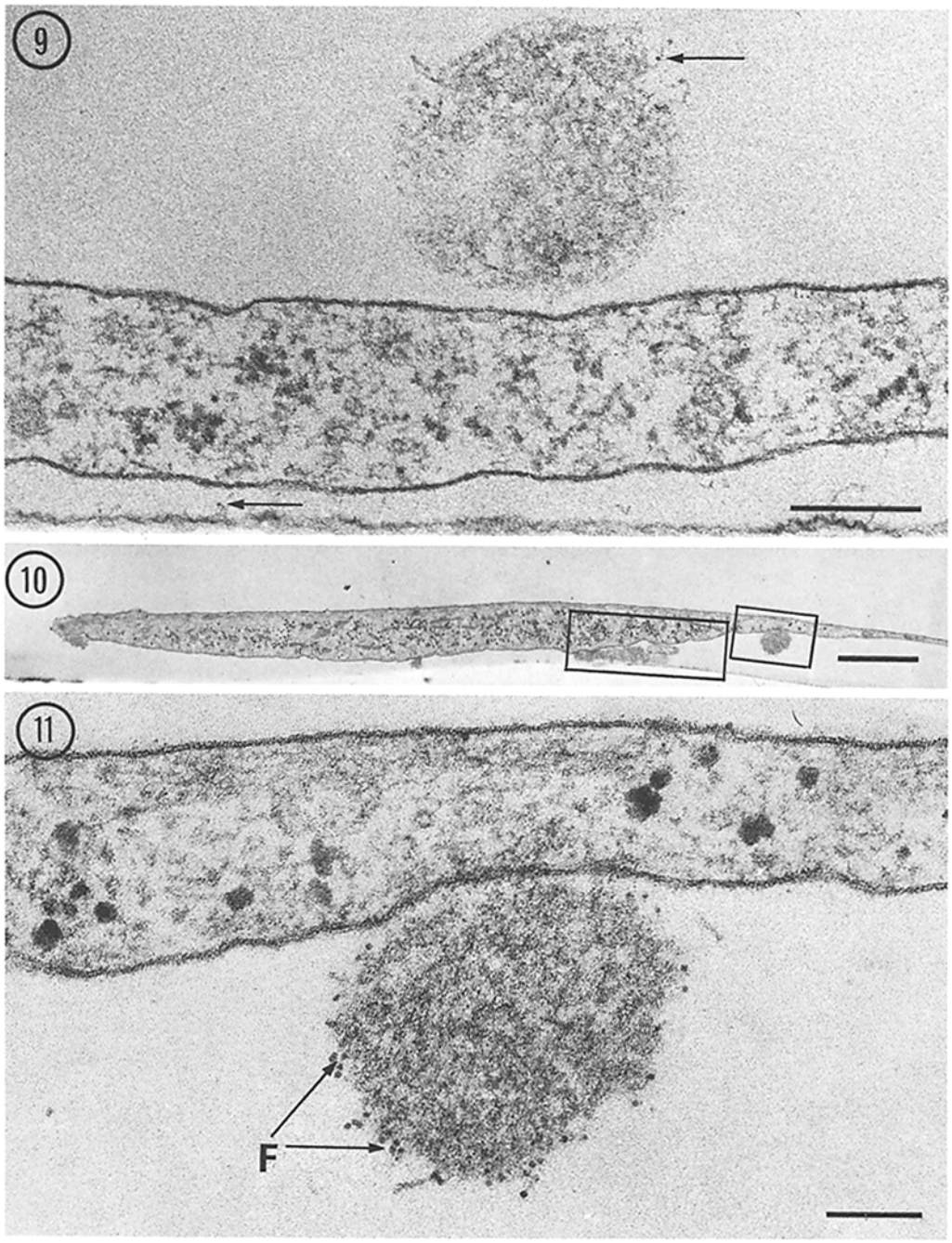
FIGURE 6 Peroxidase-stained fibroblasts 6 days after subculture. The fixed cells were treated with antifibronectin rabbit serum and peroxidase-conjugated goat IgG against rabbit IgG. Stain is seen in extracellular material in association with cell surface. Vague extracellular filamentous (*EF*) structures but no membranes can be discerned within the material. Abundant cytoplasmic filaments (*CF*) are found adjacent. The extracellular stained material does not mediate contact with the underlying cell surface in this section. Bar, 500 nm. × 37,000.

FIGURE 7 Immunoperoxidase-stained fibroblasts 1 day after subculture. A patch of stained extracellular material (double arrow) is found between two neighboring cells. The material is closely associated with the plasma membranes conceivably forming a distant contact between the cells. A closer contact (arrow) is also seen, without peroxidase reaction product. Bar, 400 nm. × 60,000.

FIGURE 8 Peroxidase-stained dense culture of fibroblasts 6 days after subculture. Patches of stained extracellular material (double arrow) are seen between overlapping cells. Fibronectin-containing extracellular material (arrow) in the form of a 50–100-nm thick bridge extends between two cells. Bar, 1 μm. × 24,000.

vaguely filamentous material. Ferritin particles were rarely encountered inside the material, presumably due to steric hindrance. Not uncommonly, ferritin markers were detected on similar extracellular material associated with the growth substratum (surface of the plastic cover slip).

A part of fibronectin labeled with ferritin was in close association with cell surface membrane. The proportion of such membrane-associated fibronectin was higher in young sparse cultures. The distance between the ferritin particles and the periphery of the membrane triple layer varied;



at several sites the ferritin markers were aligned at a distance of less than 50 nm (Fig. 14). Other features characteristic of the membrane-associated form were a highly uneven distribution and the presence of electron-dense material seen in high magnification as a thin layer parallel to the membrane. The distance between the ferritin particles and this layer was 25 nm or less. In many sections the membrane-associated, fibronectin-containing material extended peripherally to form thicker structures, thus representing a continuum of fibronectin from close to the membrane to far away from it (Figs. 15, and 17). The membrane-associated material was located on both the upper and the lower cell surfaces, and was predominantly detected in young cultures. In sites where ferritin was seen closely associated with the plasma membrane, condensations of submembranous filaments were often but not always present (Fig. 16).

Contact areas, either between neighboring cells (Fig. 17) or between cell and substrate (Figs. 12, 15, and 18), were preferential locations of fibronectin-containing material. At sites where immediate membrane contact was present, ferritin was not seen (Figs. 16, and 19). More distant contacts appeared to be very commonly mediated by a layer of material rich in ferritin-fibronectin (Figs. 10, 12, 15, 17, and 18). At sites of cell-to-cell or cell-to-substrate contact, irrespective of whether ferritin-markers were present or not, numerous cytoplasmic cortical filaments were often found (Figs. 15, 16, and 19). Submembranous vesicles were commonly seen in areas of fibronectin-containing material (Figs. 13–15, and 17). Fibronectin could not be detected inside these vesicles.

DISCUSSION

The present study indicates that in cultured human fibroblasts external fibronectin is present (*a*) in association with plasma membranes, and (*b*) in extracellular structures. Both immunoperoxidase and immunoferritin electron microscopy gave the same result. Membrane-associated fibronectin had an uneven distribution and it represented a minor portion of total external fibronectin, especially in older dense cultures. The membrane-associated form of fibronectin was best discerned by immunoferritin staining, which, unlike the immunoperoxidase method, allowed accurate topographical localization. Increased density of the cytoplasmic submembranous filaments was often but not always seen beneath membrane-associated fibronectin. In perpendicularly sectioned membrane sites, the distance between the ferritin and the outer edge of the membrane triple layer ranged between 20 and 50 nm. An electron-dense zone, possibly representing fibronectin molecules, was detected between ferritin and the membrane, less than 25 nm from the ferritin. Whether the membrane-associated fibronectin molecules interact with lipid bilayer themselves or via receptors remains to be determined.

The extracellular structural form of fibronectin was visualized as patchy or strandlike material (50–500 nm in diameter) with no visible membranous elements. Both the absolute and relative amounts of this form of fibronectin appeared to

FIGURE 9 Human fibroblast control stained by the immunoferritin method. The cell culture was fixed with a mixture of glutaraldehyde and paraformaldehyde, treated with blocked antifibronectin serum (see Fig. 5), and then with ferritin-conjugated sheep immunoglobulin against rabbit IgG. The culture was then refixed with glutaraldehyde and paraformaldehyde, postfixed with OsO_4, and processed for sectioning. The samples were block-stained with uranyl acetate, and the sections were poststained with uranyl acetate and lead citrate. Patch of extracellular material is seen above a peripheral part of the cell. Only occasional ferritin particles (arrows) are bound to the material or elsewhere. Bar, 200 nm. × 95,000.

FIGURE 10 Immunoferritin-stained fibroblast 1 day after subculture. The fixed culture was treated with rabbit anti-human fibronectin serum, and processed further as in Fig. 9. Patches of extracellular material are seen below the cell, some of which attach the cell to the growth substratum. Greater magnifications of the material in framed insets are seen in Figs. 11 and 12. Bar, 1 μm. × 11,000.

FIGURE 11 Inset from Fig. 9 showing a rounded patch, ~400 nm in diameter, of extracellular material. Ferritin markers (*F*) circumscribe the patch. No ferritin is seen in the gap between the material and the cell surface membrane, indicating steric hindrance as a possible cause of the absence of ferritin within the material. Bar, 100 nm. × 130,000.

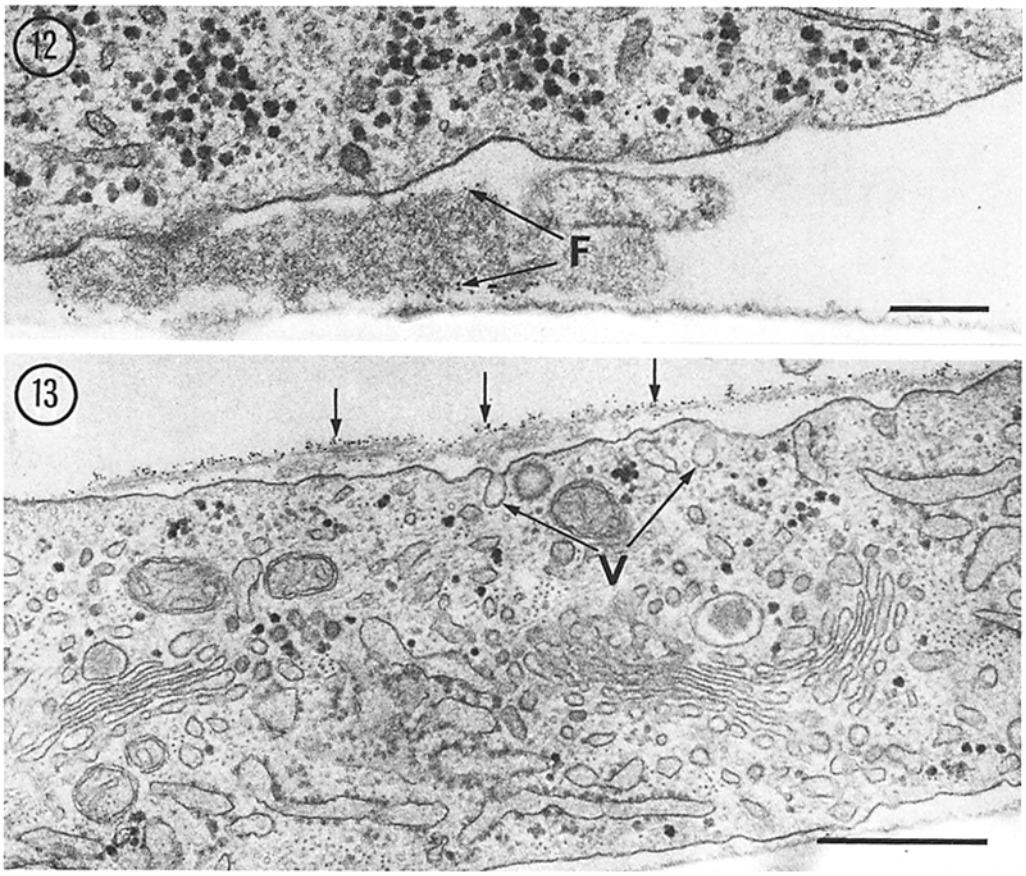


FIGURE 12 Inset from Fig. 9 showing ferritin-staining extracellular material. The material is seen as a 100–200-nm thick "pillow" between the lower cell surface and the growth substratum, and is in contact with the cell outer membrane as well as with the substratum. Ferritin markers (*F*) are attached to the material. No ferritin is distinguished within the material itself. Bar, 200 nm. × 65,000.

FIGURE 13 Ferritin-stained fibroblast 1 day after subculture. Ferritin (arrows) is seen attached to a layer of extracellular material, in which filamentous structures can be discerned. The material is located on the upper side of the cell and conceivably forms a strand (compare with Fig. 2) in the cell culture. Also, in this longitudinal section the ferritin molecules are predominantly found in the surface of the material. Cytoplasmic vesicles (*V*) are seen in the cortical area of the cell. Bar, 500 nm. × 45,000.

increase during incubation of the fibroblast cultures. In experiments with surface-iodinated hamster fibroblasts, Graham et al. (9) located most iodinatable 220,000 dalton protein to a dense (ζ = 1.25–1.26) particulate fraction. The extracellular nonmembranous fibronectin visualized on our study represents in all likelihood the same fraction. We consider the pericellular network of fibronectin-containing material of dense fibroblast cultures to represent an "in vitro connective tissue matrix". This finding of extracellular structural fibronectin is compatible with results of in vivo studies, in which fibronectin was found in primitive mesenchymal and loose connective tissues, in walls of blood vessels, and characteristically, in basal laminae (see Introduction).

There are indications that fibronectin may interact with collagenous proteins in the pericellular matrix. At the immunofluorescence level, collagen in the cell layers of cultured fibroblasts has a reticular distribution similar to that of fibronectin (3). Double immunofluorescence studies show that several of the collagenous proteins show extensive, but not complete, codistribution with extracellular fibronectin in vitro.[1] Soluble fibro-

[1] Vaheri, A., M. Kurkinen, V. -P. Lehto, E. Linder, and R. Timpl. Manuscript in preparation.

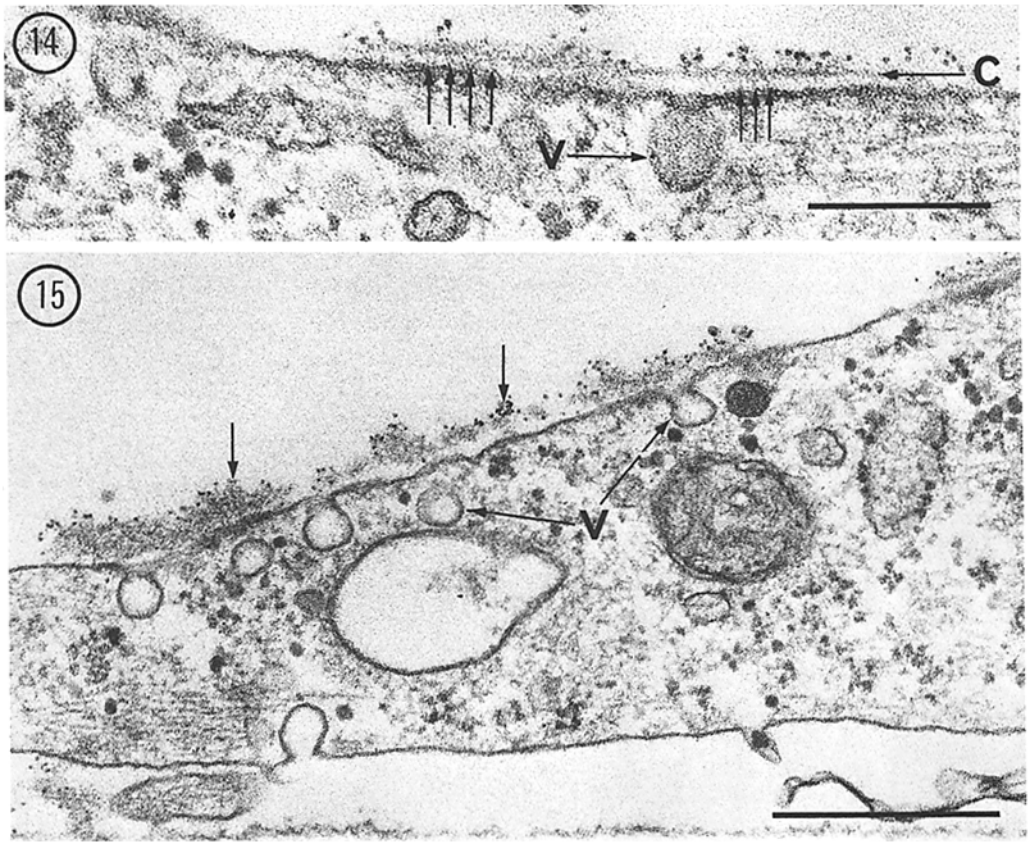


FIGURE 14 Ferritin-stained oblique section of fibroblast surface, as judged by the fusion point of the cortical vesicle (*V*) with the cell outer membrane. Ferritin is seen attached to a thin layer of material in close association with the membrane. Electron-dense material (arrows) can be resolved between the membrane and a central condensation (*C*). The distance between the ferritin particles and the condensation is <25 nm. Bar, 200 nm. × 120,000.

FIGURE 15 Fibroblast, in which ferritin (arrows) indicates the presence of fibronectin in material associated with the upper cell surface membrane. In the lower left-hand corner is a more distant contact between the cell and the substratum. The contact is mediated by material containing ferritin-fibronectin. Cytoplasmic filaments are found adjacent. Several cortical vesicles (*V*), presumably of transport type, are seen below the dorsal fibronectin-containing material. Bar, 400 nm. × 75,000.

nectin has been shown to interact with collagen and gelatin (6, 14). However, double immunoelectron microscopy may be needed to define the accurate mode of organization of fibronectin in relation to collagen in the matrix.

The molecular mechanism in the formation of the fibronectin matrix may be based on polymerization. External fibronectin has been shown to be a disulfide-bonded dimer and to form larger disulfide-linked aggregates (13, 15). Furthermore, in the presence of activated transglutaminase (blood coagulation factor XIII), surface-labeled fibronectin molecules are cross-linked to high molecular weight complexes (16). We see two possible ways of formation of the extracellular fibronectin network. First, all extracellular "matrix" fibronectin may be derived from membrane-associated protein by apposition of new molecules via the plasma membrane. In support of this possibility, fibronectin associated with plasma membrane was commonly seen to be continuous with pericellular material also containing labeled fibronectin. The extracellular substrate-attached and intercellular fibronectin, found predominantly in strandlike form, could at a given point represent a summary of the past history of cellular movements, analo-

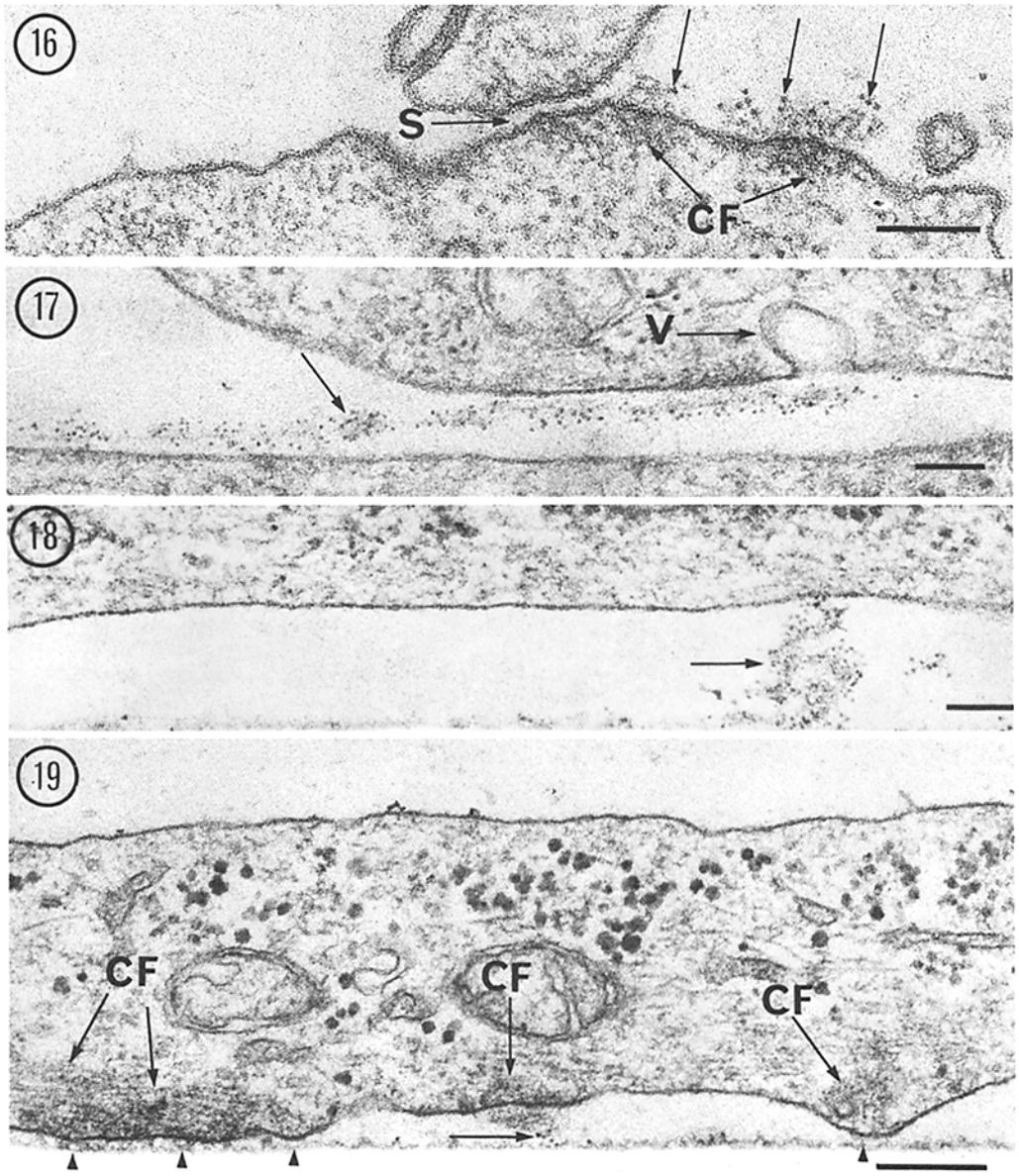


FIGURE 16 Ferritin-stained fibroblast surface. The markers (arrows) are seen closely associated with the cell surface membrane. Notice the neighboring cell-to-cell association with no ferritin particles in the intermembranous space (*S*). Numerous cortical cytoplasmic filaments (*CF*) are seen below the ferritin-stained zone as well as the site of cellular association. Bar, 100 nm. × 140,000.

FIGURE 17 Ferritin-stained neighboring fibroblasts. A strand of ferritin-labeled material (arrow) mediates a more distant type of contact. The intercellular distance, 100 nm, presumably allows access of the marker into the contact space. A cytoplasmic vesicle (*V*) opens at the site of fibronectin-containing material. Bar, 100 nm. × 90,000.

FIGURE 18 Ferritin-labeled material (arrow) associating fibroblast surface with the growth substratum. Bar, 100 nm. × 90,000.

FIGURE 19 Ferritin-stained fibroblast. Bare cell surfaces such as seen here are characteristically devoid of ferritin markers. Many such cell surfaces are seen 1 day after subculture. Intimate contacts (arrow heads) between plasma membrane and the substratum, as a rule, lack ferritin markers. More distant contacts mediated by extracellular material, faintly visible in this field, commonly show ferritin (arrow) as an indication of fibronectin. Cytoplasmic filaments (*CF*) are seen at both types of cell-to-substrate contacts. Bar, 200 nm. × 75,000.

gous to a spider's web or snail's slime pathway. As a second possibility, the soluble fibronectin secreted or shed in large quantities (up to 50 μg per mg total cellular protein in 24 h into the medium by cultured human fibroblasts [24]) could aggregate to form the fibronectin-containing structures, as is thought to be the case in collagen formation (2).

There was no evidence for the presence of fibronectin at sites of intimate membrane-membrane or membrane-substrate contacts. This result can, of course, be due to steric hindrance of the markers. A major finding of both immunoperoxidase and immunoferritin electron microscopy was that fibronectin-containing structures mediated distant cell-to-cell and cell-to-substrate contacts. This observation together with the presence of fibronectin in basal laminae in vivo (19, 35) and the immunofluorescence findings of Chen et al. (5) on contacting cells leads us to conclude that fibronectin probably is a structural protein that functions in cell-matrix and cell-cell interactions.

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