

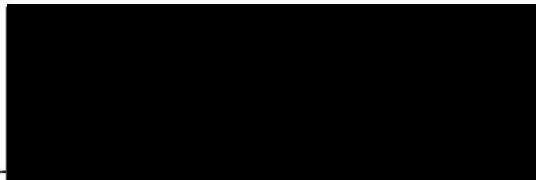
FIBRILLIN, A NEW PROTEIN OF THE EXTRACELLULAR MATRIX, IS A  
STRUCTURAL CONSTITUENT OF MICROFIBRILS

by

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The work presented in this thesis consists of three manuscripts and an appendix. All of the experiments were performed by Lynn Y. Sakai, after she was enrolled in the School of Medicine, with the following exceptions: all electron micrographs were provided by Douglas R. Keene (Shriners Hospital for Crippled Children, Portland, Oregon) of materials which he prepared using antibodies provided by Lynn Sakai, Drs. Hans Peter Bachinger and Nicholas P. Morris (Shriners Hospital for Crippled Children, Portland, Oregon) provided some of the materials (chicks, type IV procollagen) and reagents (collagenase) used in these studies, Drs. Eva Engvall and Erkki Ruoslahti provided some of the materials (vitronectin) and reagents (type VI collagen monoclonal antibody, vitronectin monoclonal antibody) used in these studies, Eileen Roux provided technical assistance in growing mass cultures of fibroblasts to obtain the starting materials for the work described in the appendix and in pasting up the immunofluorescence figures, and Glen Corson provided technical assistance in dissecting some of the chick aortae. The less tangible intellectual debts are many, and these are acknowledged. The first manuscript was published in 1986; the second will be submitted to the Journal of Biological Chemistry in 1988; and the third is the first draft of data which is being prepared for publication.

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## ABSTRACT

Using specific monoclonal antibodies, a new protein of the extracellular matrix has been isolated from the medium of human fibroblast cell cultures. This new protein, fibrillin, is a very large ( $M_r$  350 k) noncollagenous glycoprotein which contains intrachain disulfide bonds. When localized by immunoelectron microscopy in skin, fibrillin appears to be periodically arrayed in structures called "microfibrils." All microfibrils morphologically similar to the elastic fiber microfibrils and oxytalan fibrils in skin can be identified with monoclonal anti-fibrillin labeling. Based on these observations, fibrillin was proposed as a constitutive structural element of these microfibrils. One epitope recognized by a monoclonal anti-fibrillin has been well conserved, allowing studies of the tissue form of fibrillin in embryonic chick aortae. Pulse-chase experiments demonstrate that fibrillin rapidly forms intermolecular disulfide crosslinks which help to stabilize fibrillin in the connective tissue matrix. In sequential extraction experiments, fibrillin is the only large cysteine containing protein which is selectively released from the tissue by disulfide reducing agent. These experiments confirmed earlier data suggesting that microfibrils are resistant to extraction except by disulfide bond reducing agent and supports the hypothesis that fibrillin is a constitutive structural element of microfibrils. Tissue distribution

studies comparing fibrillin and a multifunctional blood protein, vitronectin, which has been reported to localize to microfibrils, suggest as well that fibrillin is a principal structural element while vitronectin may be a biologically important protein with a specific affinity for microfibrils.

## INTRODUCTION

It is presently clear that the connective tissue, or the extracellular matrix, as it is also called, is more than an amorphous glue whose purpose is to hold the cells together. Collagen, whose name is derived from the Greek meaning "to produce glue," is the most abundant protein in the mammalian body. Today the collagen family of proteins includes at least twelve different types. These different types of collagen, each containing three chains folded together into the collagen triple helix, are the products of one gene (three identical chains), two genes (two identical chains together with a different chain), or three genes (three different chains). In addition to collagen, there are various proteoglycans and glycoproteins which can be found in the extracellular matrix.

What functions do these various extracellular matrix proteins perform in the connective tissue? The first answer I would give is that the function of a true "connective tissue" protein should be primarily structural. It should be to hold cells together and to provide the different structural frameworks in which cells are organized into tissues. The connective tissue, far from being an amorphous glue, is composed of a variety of structural elements that can be seen through the electron microscope. There are many thick banded fibers of varying diameter and with varying degrees of orientation, ranging from the highly ordered arrays of fibers with uniform diameter in the corneal stroma (a special "skin" which must be transparent) and the extremely long and continuously straight fibers of the tendon to the fibers in

skin which have different diameters and a much less ordered appearance. There are thinner fibrils and filaments which are not banded, and there are fibrils which are unique to particular locations in the body. There are thick fibers with large dense centers surrounded by small diameter fibrils. There are densely staining regions (basement membranes) which, although they do not look fibrous, serve structural functions, separating epithelial cells from their underlying connective tissue stroma. In addition, there are connective tissue proteins, like the proteoglycan aggregates of cartilage, which cannot be visualized as distinct structures, but which nevertheless perform important structural functions.

Understanding of this first "architectural" function of connective tissue proteins has been recently expanded through the use of highly specific monoclonal antibody probes in immunoelectron microscopy. Previously, investigations of the banding patterns of thick fibers in the tissue and of the banding patterns of segment long spacing (SLS) crystallites, artificial collagen quaternary structures, led to the conclusion that collagen forms these ordered fibers, based upon the location of basic and acidic residues along the molecules which are in quarter-staggered array (Kühn, 1982). However, since types I, II, and III form SLS which can fit the 67nm period of cross-striations found on tissue fibers, it was not known which collagen(s) contributed to the fibers found in specific tissues. Specific anti-collagen antibodies, which could be used to immunolocalize the different types of collagen, have been extremely difficult to prepare due to the very poor immunogenicity of collagen and to crossreacting antibodies contained in the unpurified serum (Timpl, 1982). Therefore, the production of

"monospecific" anti-collagen antisera required extensive cross adsorption of the antiserum on affinity columns constructed from the different collagen types, to remove antibodies directed at determinants, or possibly contaminants, shared by the other collagens, and then final adsorption and elution from an affinity column of the desired collagen specificity (Timpl, 1982). Even this stringent procedure yielded antibodies which produced contradictory findings. The invention of the hybridoma technology has allowed the production of a number of well-defined, anti-collagen antibodies which have contributed to our knowledge of the tissue distribution and location of specific collagen types.

With R. E. Burgeson and D. W. Hollister, I produced the first monoclonal antibodies specific for human collagens (Hollister, et. al., 1982; Sakai, et. al., 1982). These were an anti-type II collagen and an anti-type IV collagen antibody, which were useful in staining the matrix of human cartilage (anti-II) and all human basement membranes (anti-IV). However, the tissue distributions of these two collagens were known from tissue extraction studies, and our data only confirmed the immunolocalization studies performed with polyclonal antisera. Nevertheless, these reagents have served as useful controls in subsequent tissue distribution studies, and the anti-type IV monoclonal antibody helped to demonstrate, by rotary shadowing antibody-type IV collagen complexes, the location of a particular peptide, containing the epitope, in tetrameric type IV collagen (Dieringer, et. al., 1985). The type IV antibody was also useful in suggesting that a large collagenous fragment, obtained from pepsin digests of human amnion, was not type IV collagen, but a new collagen, type VII (Bentz, et. al., 1983).

Monoclonal anti-collagen probes have been most useful in rapidly immunolocalizing the new collagens. Monoclonal antibodies specific for type VII collagen, for example, have demonstrated a unique tissue distribution. Immunofluorescence revealed that type VII was restricted to the basement membrane zone of only certain tissues, like skin (the dermal-epidermal junction was positive, but not the basement membrane zones of capillaries, nerves, or fat cells), amnion, cornea (Bowman's membrane was positive, while Descemet's membrane was not), and the epithelial basement membrane zones of trachea, bladder, and vagina, but not the basement membrane regions of kidney, liver, lung, intestine, and stomach (Figure 1) (Sakai, et. al., 1986a). In these special regions, there are structures called "anchoring fibrils," which in some fortuitous electron micrographs, can appear to be banded. This pattern of bands seemed similar to that of type VII SLS crystallites (Burgeson, et. al., 1985), so it was hypothesized that type VII might be a component of anchoring fibrils. Immunoelectron microscopy with monoclonal anti-VII demonstrated the specific localization of type VII to anchoring fibrils (Sakai, et. al., 1986a) and described a newly visualized "network" of anchoring fibrils, extending below the dermal-epidermal basement membrane and presumably helping to secure the basement membrane region to the underlying stroma (Keene, et. al., 1987).

Monoclonal antibodies specific for human type VI collagen have also been prepared (Hessle and Engvall, 1984). Using these antibodies, type VI collagen, once called "intima" collagen because it was first extracted from the intimal layer of blood vessels, has since been shown by immunofluorescence to be present in almost all tissues.

Immunoelectron microscopy with these antibodies has localized type VI collagen, in fibroblast cell cultures, to fine filaments possessing a 100nm periodicity (Bruns, et. al., 1986). With the same monoclonal antibodies, we have localized type VI collagen, in human tissues, to small (3-5nm) diameter filaments which are distinct from other larger diameter microfibrils (Sakai, Keene, and Engvall, 1986b).

Type VIII collagen was originally described as a product of endothelial cells in culture (Sage, Pritzl, and Bornstein, 1980) and was later characterized as a fragment isolated from pepsin digests of bovine corneal Descemet's membrane (Kapoor, Bornstein, and Sage, 1986). Its tissue distribution has been unknown for some years. In collaboration with Helene Sage and Paul Bornstein, I have recently made a monoclonal antibody which specifically recognizes the pepsin fragment of type VIII collagen and have performed studies of its distribution. Type VIII collagen appears to have a very restricted tissue distribution: it is present in Descemet's membrane, in the sclera, in the meninges of the brain and optic nerve, in fine fibrils in the spinal cord, and in all perichondrium, but it is not present in blood vessels, skin, kidney, liver, muscle, lung, or ligament (Figures 2-4) (Kapoor, et. al., manuscript submitted for publication).

These are examples of immunofluorescence and immunoelectron microscopy with monoclonal antibody probes helping to identify the protein components of special connective tissue structural elements. In addition, there has been much recent progress made in our understanding of the structure of the common, abundant collagen fiber with the 67nm banding pattern. With monoclonal antibodies specific for chick types I and V collagen, Linsenmayer and his collaborators have shown that the

uniform and highly ordered collagen fibers in the chick corneal stroma are "heterotypic," that is, the same fibril contains both types I and V collagen (Fitch, et. al., 1984; Birk, et. al., 1988). Previous work which localized, by immunoelectron microscopy using a cross-adsorbed polyclonal antiserum, type III collagen to small diameter fibers (Fleischmajer, et. al., 1980) has led to the supposition that small "reticular" collagen fibers are composed of type III collagen, while the large diameter collagen fibers are type I. After producing a monoclonal antibody specific for a helical epitope on human type III collagen, we discovered that immunoelectron microscopy with this probe revealed labeling of all collagen fibers, with large and small diameters, in human skin, demonstrating that these fibers, too, are probably heterotypic (Keene, et. al., 1987).

Of course, the immunolocalization of proteins to particular structures is only part of the story. Evidence that a protein is a component of a particular structure, and evidence for what its actual structural function might be, is also based upon much biochemical data, examples of which I have only briefly alluded to. In this regard, monoclonal antibodies have also proved to be useful reagents for asking biochemical questions. For example, by characterizing type VII collagen epitopes recognized by different monoclonal antibodies, a large noncollagenous terminal domain of type VII, which may be extremely important in the potential interactions of type VII collagen with proteins of the basement membrane and of the basal surface of the epithelial cell, has been described (Lunstrum, et. al., 1986; Lunstrum, et. al., 1987).

This has been the background in which this thesis is set: a

growing appreciation for the complexity of the connective tissue, a primary interest in the proteins which form structural elements in the connective tissue, and an understanding that these structural elements may be composed of single proteins or multiple proteins. In addition, my intention has been to exploit the hybridoma technology to help solve the central problem we, in connective tissue biochemistry, must face at some time in our careers: how can we study connective tissue structural elements when they are designed to be insoluble? There is no simple solution to this problem, as I can certainly say, but the hybridoma technology has made a considerable contribution to the specific project which is the subject of this thesis.

To round out this general discussion of connective tissue, and because this issue is raised in the third manuscript of this thesis, I should also add that there is a growing body of literature which suggests that extracellular matrix proteins may have biological functions which are independent of any purely structural function. There are data that the extracellular matrix affects cell shape and polarity; it affects the migration of embryonic cells and the outgrowth of neurites and tumor cell invasion; it affects cell adhesion, cell differentiation, and cell proliferation. Indeed, cell surface "receptors" for extracellular matrix proteins have been identified, implying that there are important signals which must be communicated from the matrix to the cell. Viewed from the perspective of modern society, the relationship between the individual (cell) and the environment (matrix) is intimate and complex: the individual acts on the environment, and the environment definitely shapes the individual. Documenting the details of this relationship is going to be a very long

and complex process. For now, I am content to try to specify and characterize the proteins which play some part in the architecture of the connective tissue.

#### **SUBJECT OF THE THESIS**

The subject of this thesis had its origin in my work in collagen biochemistry. It began with a mouse which had been immunized with a "pure" sample of type VII collagen, prepared by pepsin digestion of human amnion and purification by differential salt precipitation followed by chromatography on carboxy methyl cellulose (Bentz, et. al., 1983). The mouse made antibodies which recognized type VII collagen (Sakai, et. al., 1986a), and it also made antibodies which yielded an extremely interesting immunofluorescence pattern on human skin, a pattern similar to histochemical stainings of the "oxytalan" and elastic fiber network (Cotta-Pereira, Rodrigo, and Bittencourt-Sampaio, 1976). Because I thought these antibodies might be the first useful tools which could open the door to our understanding of the protein constituents of these fibrils, I selected the hybridomas which gave this immunofluorescence pattern. I intended to use these monoclonal antibodies as (essentially unlimited and uniform) chemical reagents to identify and characterize a unique "fibrillar antigen" of the connective tissue.

The first manuscript, published in the Journal of Cell Biology, volume 103: 2499-2509, in December, 1986, describes the use of monoclonal antibodies to define a new protein, which we named "fibrillin," and to immunolocalize fibrillin to special connective

tissue microfibrils. Because proteins secreted into the medium of cultured cells are not crosslinked into a matrix and because I wanted knowledge of a whole native form of the antigen, I chose to investigate the "fibrillar antigen" by metabolically radiolabeling fibroblast cell culture proteins with various compounds and isolating the antigen by immunoaffinity chromatography. It had been previously reported that the microfibrillar protein(s) could be selectively solubilized by disulfide bond reducing agent (Ross and Bornstein, 1969). However, a subsequent study (Muir, Bornstein and Ross, 1976), which sought to identify a microfibrillar protein from the medium and matrix of monkey arterial smooth muscle cells, based upon its incorporation of radiolabeled cysteine (or cystine), did not succeed in identifying a specific microfibrillar protein. Without the benefit of a specific monoclonal antibody reagent, it had been difficult to identify the microfibrillar protein among all the radioactive cys-labeled proteins in the cell cultures, the most abundant of which is fibronectin and which, for lack of specific reagents, was suggested as the "presumptive subunit" of microfibrils (Muir, Bornstein, and Ross, 1976). It was not enough to know that microfibrils are composed of proteins which are stabilized by disulfide bonds. The monoclonal antibodies were a real breakthrough. Based on the ubiquitous tissue distribution of fibrillin and on its periodic immunolocalization to the microfibril, we hypothesized that fibrillin is a constitutive structural element of connective tissue microfibrils.

The second manuscript addresses that hypothesis in a traditional biochemical way. Fibrillin had been described only as a cell culture product. I wished to know, what is fibrillin like in the tissue? Under

what conditions can it be extracted? How insoluble is it? Is it stabilized in the tissue by disulfide bonds? The data provided in this study supported the hypothesis that fibrillin is a constitutive structural element of connective tissue microfibrils.

The third manuscript presents a new finding, that a well characterized multifunctional blood protein, vitronectin, can be immunolocalized to fibrillin microfibrils. This finding is interesting in itself. However, here it is data that supports the hypothesis that fibrillin is a constitutive structural element of microfibrils by showing additional tissue localization of fibrillin and by demonstrating that vitronectin, while it can be localized by immunoelectron microscopy to microfibrils, does not codistribute with fibrillin. There may be other "microfibrillar proteins", but immunolocalization should not be the only criteria for determining their function as structural.

#### **HISTORY OF EFFORTS TO EXTRACT THE CONNECTIVE TISSUE MICROFIBRIL**

Connective tissue "microfibrils" have been visualized by the electron microscope as 10nm diameter fibrils with "hollow," nonstaining cores and a bead-like appearance. They were first described in proximity to basement membranes (Low, 1961; Low, 1962). They can be found as bundles of microfibrils, or in association with the elastic fiber, surrounding dense amorphous cores with a peripheral mantle of microfibrils. There has been considerable controversy over whether or not elastic fiber microfibrils are the same or different from similar looking microfibrils which appear to lack dense cores (for review, see Cleary and Gibson, 1983). However, without knowledge of the protein

components of these structures, this controversy could not be settled.

Efforts over the last twenty years to extract microfibrillar proteins from tissues have been hampered by the extreme insolubility of these structures. Progress was first made by Ross and Bornstein in 1969 who showed that, while microfibrils were resistant to extensive extraction by 5M guanidine hydrochloride and to digestion by collagenase, they appeared to be solubilized by the disulfide bond reducing agent, dithioerythritol. Their extraction protocol, modified in small ways, has been followed by most investigators seeking to isolate microfibrillar proteins. However, to date, no well-characterized microfibrillar proteins have resulted from these efforts.

It is an interesting aside in this history that one of the best characterized proteins, a "collagen-like glycoprotein," CLGP (Gibson and Cleary, 1982), which had been isolated from Ross and Bornstein type "reductive dissociative" extracts turned out to be a larger form of type VI collagen, which had been originally described as fragments, isolated from pepsin digests of tissue (Jander, Troyer and Rauterberg, 1984; Knight, et. al., 1984; Gibson and Cleary, 1985). When the structure of type VI collagen was first visualized by the rotary shadowing technique (Furthmayer, et. al., 1983), it was proposed that type VI collagen could be a microfibrillar component because it appeared to aggregate by end-to-end polymerization and because it was extracted from aortic intima, an elastic fiber-rich tissue containing abundant microfibrils. However, antiserum made against CLGP gave immunofluorescence patterns which were similar to those for an interstitial collagen, and not as would be expected for elastic microfibrils (Gibson and Cleary, 1983).

Yet, the idea that type VI collagen was a microfibrillar component persisted. For that reason, in the first manuscript presented in this thesis, we demonstrated the immunoelectron microscopic localization of type VI collagen to fine, small diameter filaments which appeared to interweave among the thick banded collagen fibers, and we showed that this filamentous network was distinct from fibrillin-containing microfibrils.

The main approach to identifying proteins in the reductive dissociative extracts as microfibrillar components has been immunological. The first reported antiserum made to a reductive dissociative extract (Kewley, Steven, and Williams, 1977a) showed immunolocalization to elastic fiber microfibrils as well as to collagen fibers (Kewley, Steven, and Williams, 1977b). Since reductive dissociative extracts contain heterogeneous materials, as analyzed by SDS-PAGE (Cleary and Gibson, 1983), antibodies elicited by this immunogen are likely to be directed toward different antigens. Still, it was possible that a microfibrillar protein was common to both the elastin and collagen fiber systems. It was used by Sear, et. al., 1978, to immunoprecipitate two radioactive fucose-labeled proteins, called MFP I ( $M_r$  150 k) and MFP II ( $M_r$  300 k), from the medium of fetal bovine nuchal ligament cells (nuchal ligament is rich in elastic fibers). Later, MFP I was shown to be type VI collagen (Knight, et. al., 1984), but this data was then subsequently questioned by the same group (Ayad, et. al., 1986). MFP II was not further studied, after its presence in the immunoprecipitate was noted. Anyway, whether MFP I or II are genuine microfibrillar components cannot be reevaluated since Kewley's antiserum has been lost (Ayad, et. al., 1986).

Two small proteins, isolated from reductive dissociative extracts of nuchal ligament, have been reported. The first, called SGP for "structural glycoprotein," had a molecular weight of 34 k as measured by SDS-PAGE and by equilibrium sedimentation, was digested by bacterial collagenase to a polypeptide of  $M_r$  12 k, and oxidized both free lysine and peptidyl-lysine (Serafini-Fracassini, et. al., 1981). Evidence that SGP might be a component of extracellular microfibrils was suggested by an in vitro aggregation experiment showing that, if exposed to copper ions, SGP formed cylindrical tactoids whose diameter was measured to be 11 nm, comparable to the diameter of tissue microfibrils. Immunological evidence localizing SGP to microfibrils has not been forthcoming, and no additional biochemical data has been reported. It is interesting to note that lysyl oxidase, an enzyme which might well interact with elastic fibers, also has a molecular weight of around 30 k.

The other small protein was recently described by Cleary's group (Gibson, et. al., 1986) as the "major antigen of elastin-associated microfibrils." It was a 31 k glycoprotein which had a distinctive amino acid composition (22% glutamic acid, was high (6.8%) in cysteine, and low (4.5%) in glycine) and was resistant to bacterial collagenase. Antiserum specific for this protein was localized to elastic fiber microfibrils. After the discovery and publication of fibrillin, Gibson and Cleary, 1987, found that the tissue distribution of their protein matched that of fibrillin, demonstrating again that these morphologically similar microfibrils have the same protein constituent(s) whether or not they are a part of the mature elastic fiber.

In thinking about this literature, it may be important to bear in mind that these microfibrillar protein extraction procedures are

lengthy, harsh, and extensive. It is possible that many of the resulting proteins are degradation products of larger proteins which did not fully survive these extraction procedures.

Another suggestion for a microfibrillar component has come from outside the field of elastin. Serum amyloid P component has been immunolocalized to elastic fiber microfibrils (Breathnach, et. al., 1981). Serum amyloid P is a normal plasma glycoprotein which was given the name P (for plasma) component of amyloid, deposits of which can be seen to contain two elements: fine fibrils, which are selectively stained by alkaline Congo Red, and small pentagonal structures, which are probably composed of the P component (Cathcart, Shirahama, and Cohen, 1967). Amyloid P can be easily isolated from plasma (Binette, Binette, and Calkins, 1974; Pepys, et. al., 1977). Electron microscopy of negatively stained purified amyloid P revealed pentagonal structures, 8.5nm in diameter with a lumen, which sometimes formed disc-like rods (Cathcart, Shirahama, and Cohen, 1967; Pepys, et. al., 1977; Inoue, et. al., 1986a). Amyloid P component is both a normal plasma component and a constituent of amyloid deposits; it is composed of noncovalently associated subunits ( $M_r$  24 k) arranged as pentagonal structures, and it has an affinity for certain polymers like agarose (and perhaps the anionic amyloid fibrils) and more specifically binds to the R isomer of the cyclic acetal, methyl 4,6-O-(1-carboxy-ethylidene)-B-D-galactopyranoside, a synthetic form of the galactoside component of agarose (Hind, et. al., 1984).

In a series of articles over the last few years, Inoue and LeBlond (Inoue and LeBlond, 1985a,b; Inoue, et. al., 1986a,b; Inoue and LeBlond, 1986) have developed a complicated ultrastructural model of microfibrils

and proposed that the central tubular portion of microfibrils are composed of amyloid P. Since amyloid P is known to associate into high molecular weight multimers by noncovalent interactions, and since at least its serum form is soluble in physiological buffers, something must stabilize amyloid P in the tissues if it is to perform its role as the central structural element of microfibrils. That stabilizing element was described as a "surface band" which wrapped itself irregularly, perhaps helically, around the microfibril. "Spikes" also appeared to be attached to the bands.

Rather than being considered as a structural component of microfibrils, it may be more appropriate to describe amyloid P as binding to the microfibrillar structure, since amyloid P has been shown to bind to certain carbohydrate structures and since microfibrils show affinity for cationic stains and for lectins (Fanning and Cleary, 1985). In addition, amyloid P can be immunolocalized to elastic fibers in the skin of children over 4 years of age, but not to dermal elastic fibers in children under 2, who have abundant microfibrils, suggesting that amyloid P becomes bound with age to elastic fibers (Khan and Walker, 1984). Moreover, as shown in the first manuscript in this thesis, microfibrils have a wider tissue distribution than the elastic fiber. Amyloid P has been reported to be present by immunofluorescence in glomerular basement membrane (on the endothelial cell side, where there are microfibrils), but not in tubular basement membrane or mesangium (where there are also microfibrils); and in lung, in alveolar capillary walls, but presumably not other surrounding structures which also contain plenty of microfibrils (Dyck, et. al., 1980). Finally, amyloid P was extracted from the basement membrane producing tumor (the mouse

Engelbreth-Holm-Swarm (EHS) tumor), in which it had been immunolocalized to microfibrils, by using a 0.01M Tris-0.05M citrate saline buffer, including collagenase (Inoue, et. al., 1986a). Based on the work by Ross and Bornstein, one would expect a true structural constituent of microfibrils to resist extraction by these methods.

## FIGURE LEGENDS

Figure 1. Indirect immunofluorescence, with monoclonal anti-collagen types VII and IV, of human tissues showing unique localization of type VII collagen to some basement membrane zones, but not to others. All other known basement membrane components are found, like type IV collagen, in all basement membranes. Magnification is 200x. This figure is taken from Sakai, et. al. 1986a.

ANTI TYPE VII  
COLLAGEN

ANTI TYPE IV  
COLLAGEN

SKIN

PLACENTA

CHORIOAMNIOTIC  
MEMBRANES

MUSCLE

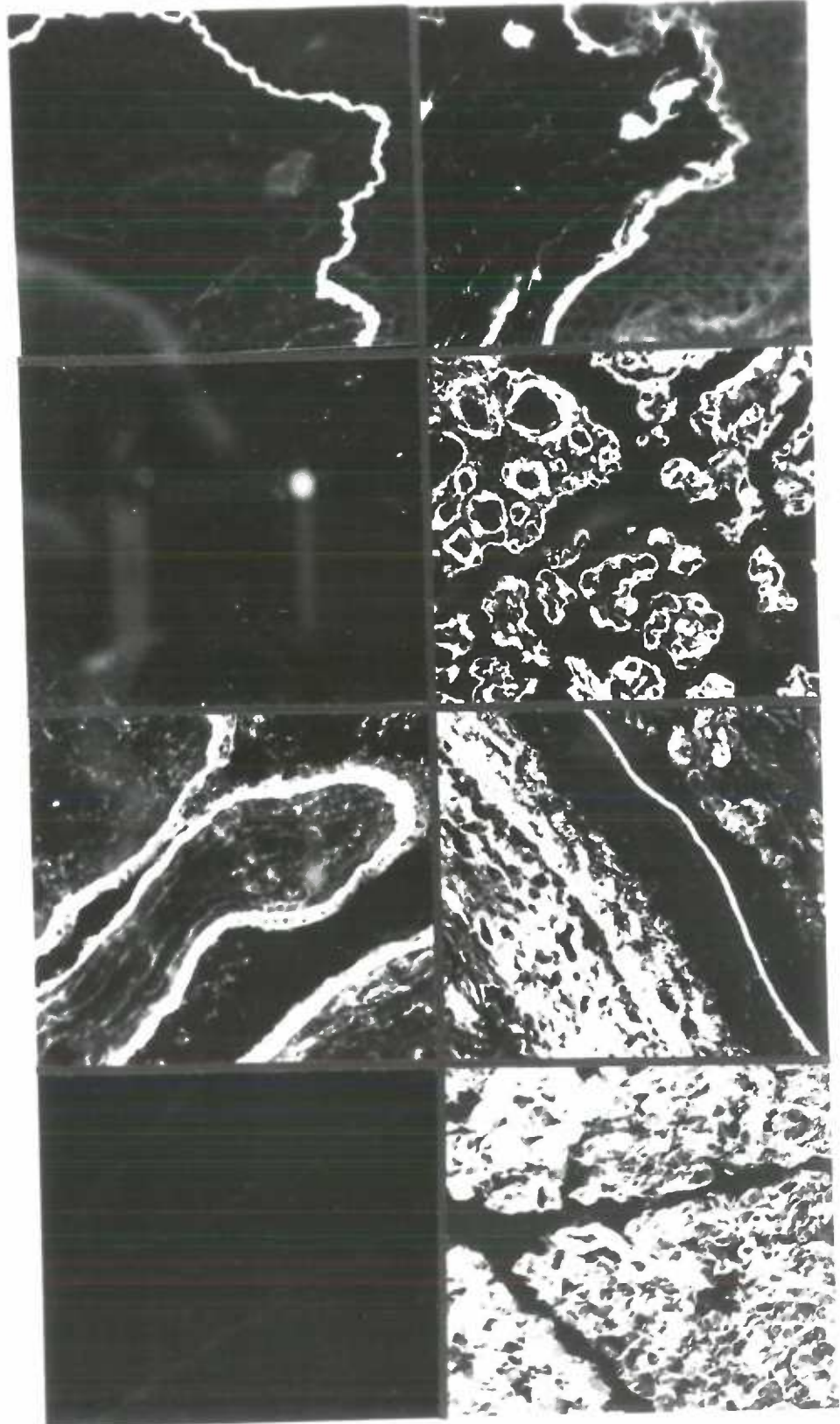


Figure 2. Indirect immunofluorescence, with monoclonal anti-type VIII collagen (panel on left) and with monoclonal anti-fibrillin (panel on right), of bovine tissues, showing type VIII distribution restricted to Descemet's membrane in the cornea (a) and to the surrounding sclera (c). Type VIII was not found in aorta (e) or ligament (g). Fibrillin distribution in cornea (b), sclera (d), aorta (f), and ligament (h) is shown for comparison. Magnification is 200x; bar=50 $\mu$ m. From Kapoor, et. al., submitted manuscript.

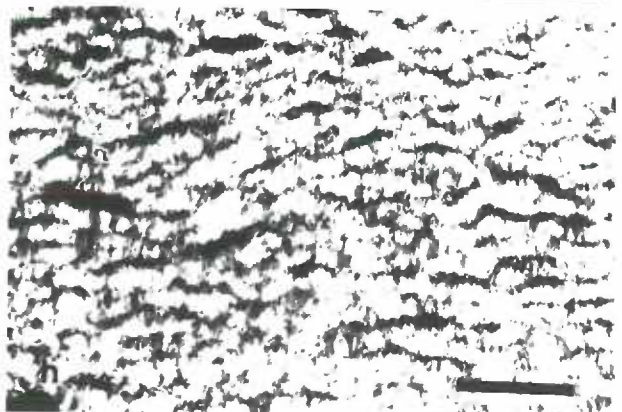
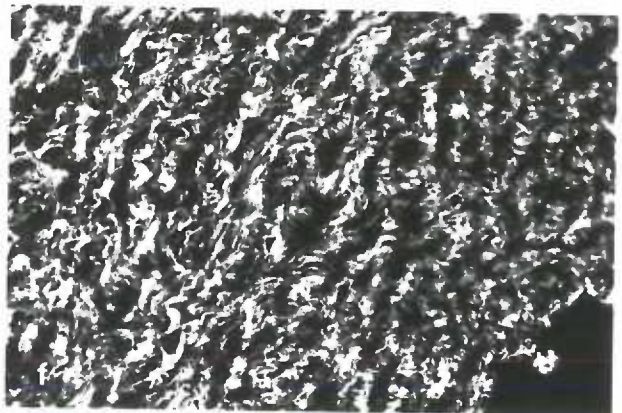
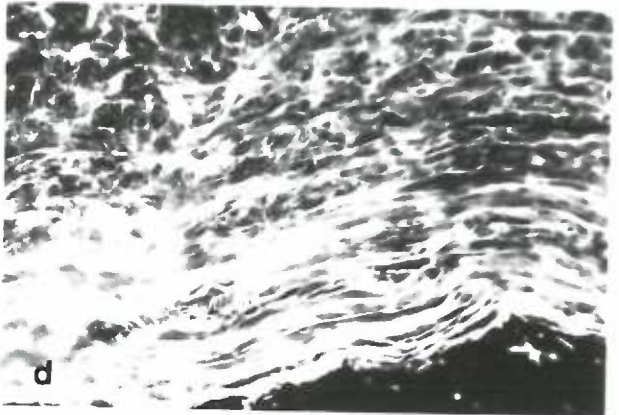
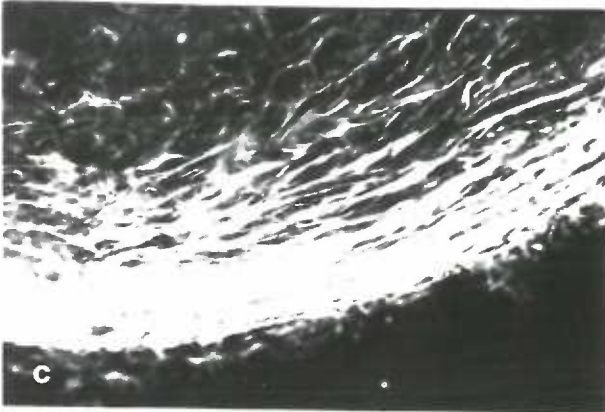
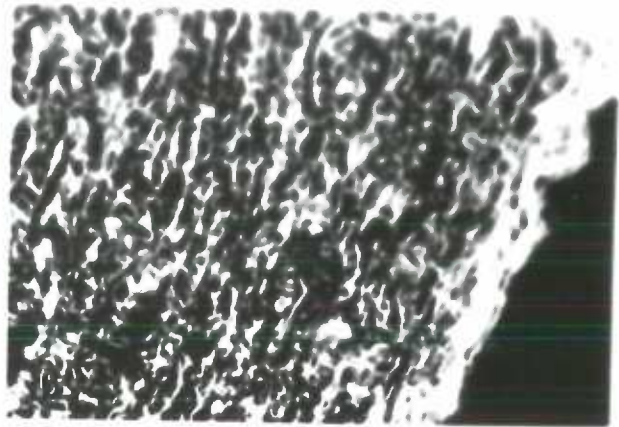
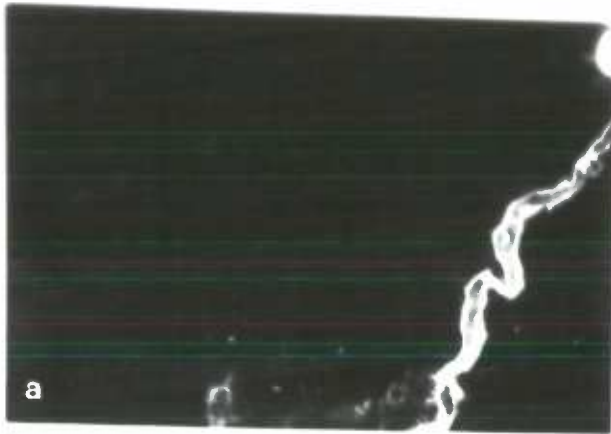


Figure 3. Indirect immunofluorescence, with monoclonal anti-type VIII collagen (panel on left) and with monoclonal anti-fibrillin (panel on right), of bovine elastic cartilage, digested with hyaluronidase (a,b), perichondrium (c,d), periosteum (e,f), and calvaria (g,h). Magnification is 200x. From Kapoor, et. al., submitted manuscript.

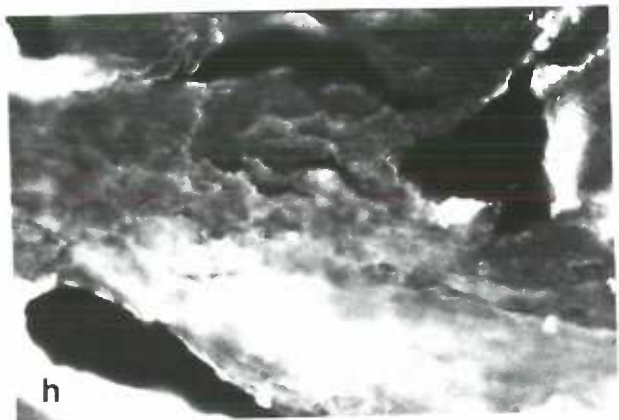
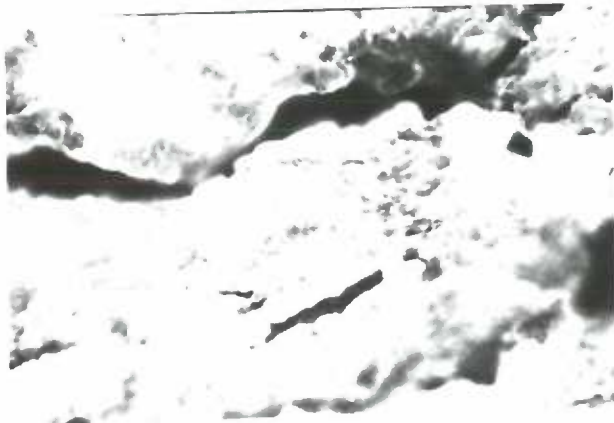
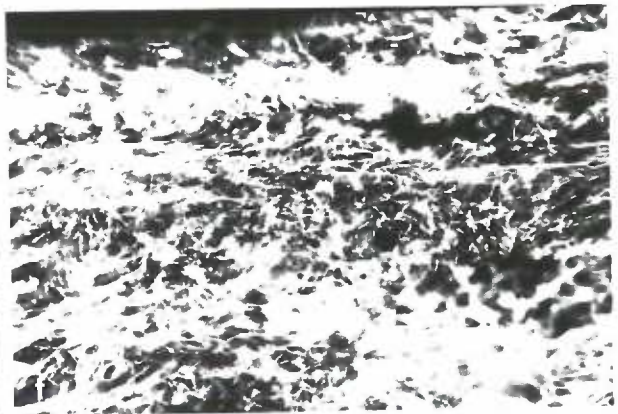
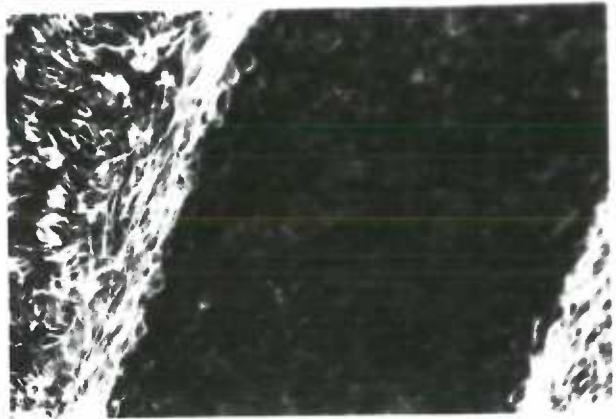
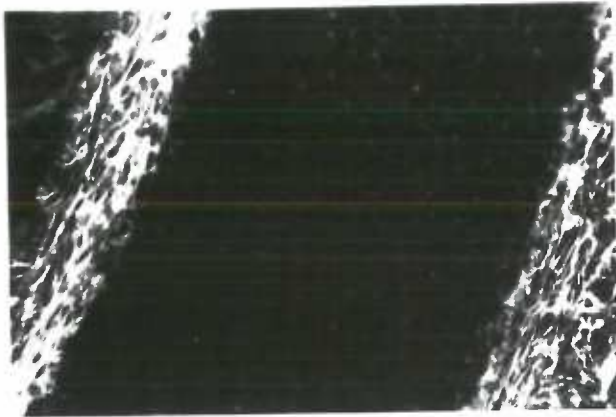
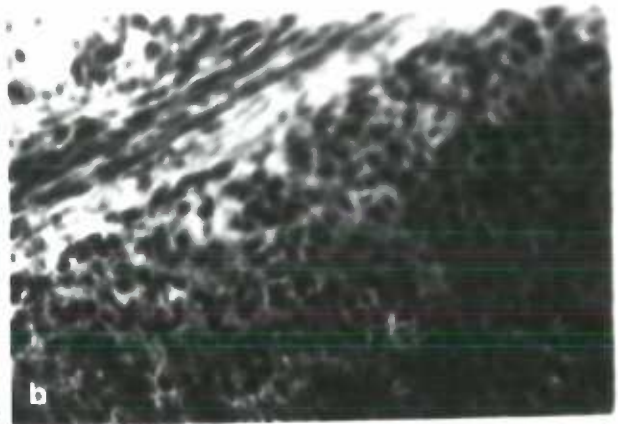
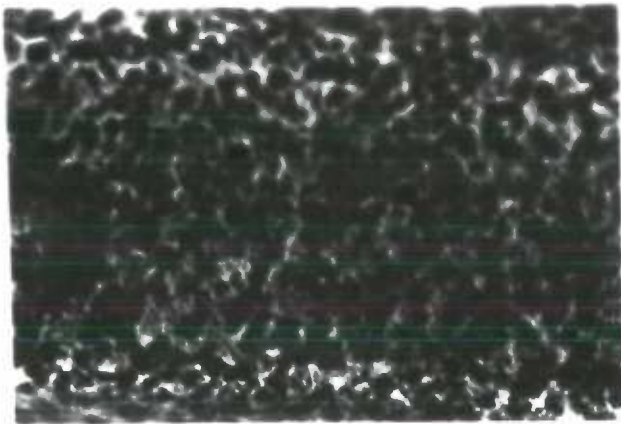
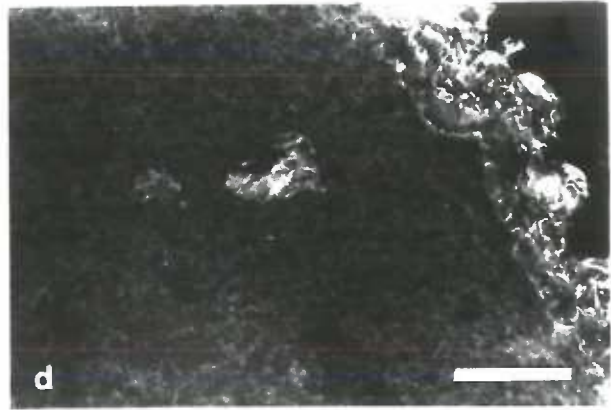
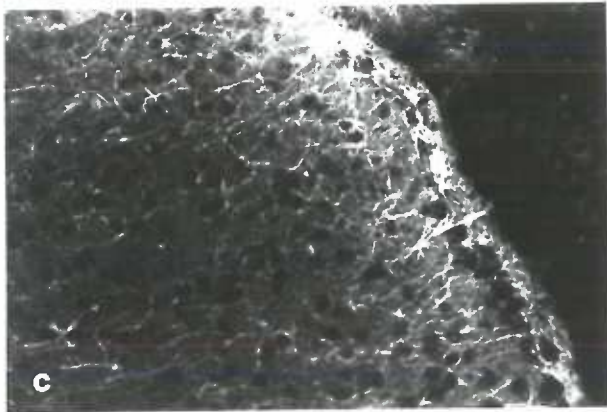
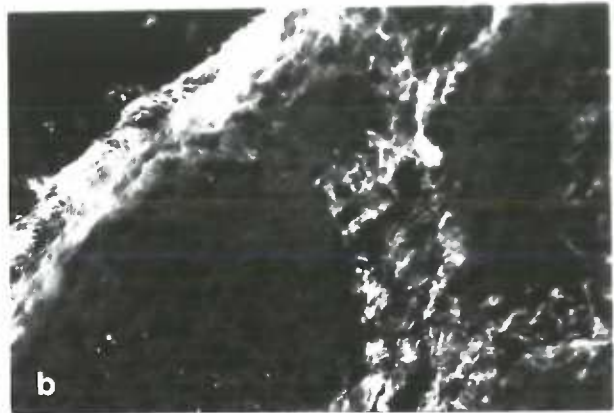
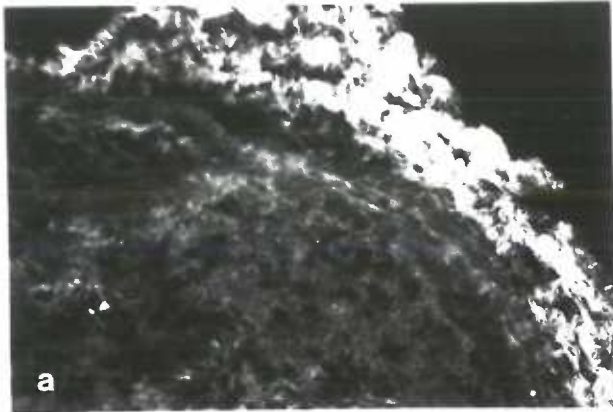


Figure 4. Indirect immunofluorescence, with monoclonal anti-type VIII collagen (panel on left) and with monoclonal anti-fibrillin (panel on right), of bovine optic nerve (a,b) and spinal cord (c,d). Type VIII collagen is present in the meninges and in fine fibrils of the spinal cord, but is not in perineurium or endoneurium or blood vessels. Fibrillin is present in the meninges as well as in the perineurium, endoneurium, and blood vessels, but does not stain the fine type VIII fibrils in the spinal cord. Magnification is 200x. From Kapoor, et. al., submitted manuscript.



FIBRILLIN, A NEW 350 KD GLYCOPROTEIN,  
IS A COMPONENT OF EXTRACELLULAR MICROFIBRILS

Condensed Title: a new component of microfibrils

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## ABSTRACT

A new connective tissue protein, which we call fibrillin, has been isolated from the medium of human fibroblast cell cultures. Electrophoresis of the disulfide bond reduced protein gave a single band with an estimated molecular weight of 350,000 daltons. This 350kd protein appeared to possess intrachain disulfide bonds. It could be stained with periodic acid-Schiff reagent, and after metabolic labeling, it contained [<sup>3</sup>H]glucosamine. It could not be labeled with [<sup>35</sup>S]sulfate. It was resistant to digestion by bacterial collagenase. Using monoclonal antibodies specific for fibrillin, we demonstrated its widespread distribution in the connective tissue matrices of skin, lung, kidney, vasculature, cartilage, tendon, muscle, cornea, and ciliary zonule. Electron microscopic immunolocalization with colloidal gold conjugates specified its location to a class of extracellular structural elements described as "microfibrils." These microfibrils possessed a characteristic appearance and averaged 10 nm in diameter. Microfibrils around the amorphous cores of the elastic fiber system as well as bundles of microfibrils without elastin cores were labeled equally well with antibody. Immunolocalization suggested that fibrillin is arrayed periodically along the individual microfibril and that individual microfibrils may be aligned within bundles. The periodicity of the epitope appeared to match the interstitial collagen band periodicity. In contrast, Type VI collagen which has been proposed as a possible microfibrillar component was immunolocalized, using a specific monoclonal antibody, to small diameter microfilaments which interweave among the large banded collagen fibers and was not

associated with the system of microfibrils identified by the presence of fibrillin.

Footnote of Abbreviations: phosphate-buffered saline (PBS),  
sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

## INTRODUCTION

The extracellular matrix appears to contain a variety of fibrillar structural elements. In addition to the large banded collagen fibers, there are various nonstriated fibrils with small cross-sectional diameters. These fibrils can be classed on the basis of their average diameter as 8-10 nm "microfibrils" (24,25) or as 3-5 nm "microfibrils" (16).

The larger 8-10 nm microfibril usually appears in cross-section as an outer electron-dense shell surrounding an inner hollow or lucid core and in longitudinal section as a beaded chain. These microfibrils are most abundantly associated with basement membranes and with the elastic fiber, and are found in most tissues. They are the subject of a recent review (5). The smaller 3-5 nm microfibril or "microfilament" is often connected to proteoglycan granules (16,10).

Many attempts have been made to specifically extract microfibrillar proteins. Guided by electron microscopic analysis of the residue from each extraction step, a protocol for the isolation of microfibrillar proteins has been described (30). After digestion of tissue with collagenase, the residue is extracted with guanidine. Microfibrillar proteins are thought to be finally solubilized with disulfide bond reducing agent in guanidine. Biochemical analysis of this microfibrillar extract from tissue (12) and immunological and biochemical studies of cell culture products (34,35) utilizing an antiserum prepared with this microfibrillar extract as the immunogen (20) have led to the identification of a major periodic acid-Schiff positive electrophoretic band with an apparent molecular weight of

140,000 daltons. This 140kd glycoprotein has been shown to be a component of the undegraded tissue form of Type VI collagen (18,21,14).

Electron microscopic studies of Type VI collagen molecules suggest a model for Type VI collagen filament formation involving an end-to-end rather than a lateral aggregation of a basic tetrameric unit (11,8). This electron microscopical model has been useful in clarifying the relationship between Type VI collagen and microfibrillar components. In fibroblast cultures of chick leg tendon and cornea and of human skin, 3 nm "beaded filaments," which appear to be different from the chains of globules formed in 10 nm microfibrils, compare favorably with the filamentous model for Type VI collagen (2,3). Using a previously characterized monoclonal antibody specific for Type VI collagen (17), we here demonstrate immunolocalization of Type VI collagen to small diameter microfilaments associated primarily with large banded interstitial collagen fibers and not to 10 nm microfibrils.

In this paper, we are presenting new information on a glycoprotein which we have isolated from the medium of human fibroblast cell cultures. Using a monoclonal antibody specific for this glycoprotein, we show that this protein is a component of extracellular structural elements described as 10 nm microfibrils, but not the 3 nm microfilaments, and we call this new protein, "fibrillin."

## MATERIALS AND METHODS

### Monoclonal Antibodies

Monoclonal antibodies specific for fibrillin were prepared according to previously described methods (31,32). A/J mice (Jackson Laboratories, Bar Harbor, Maine) were immunized with a crude extract of human amnion which had been solubilized by pepsin digestion and salt precipitated and which had Type VII collagen (1) as its principal species. Clones were screened by ELISA (7) and selected by immunofluorescence. Antibody was used either in the form of spent culture medium or as ascites produced in A/J X Balb/c hybrid mice. One of the hybridomas, clone 201, was used to produce the results shown here.

The monoclonal antibody specific for Type VI collagen was prepared as described (17).

### Indirect Immunofluorescence

Tissues were frozen in hexane in liquid nitrogen, and 8  $\mu$ m sections were cut using an American Optics histostat microtome at  $-28^{\circ}\text{C}$ . Sections were air-dried for 30 minutes and fixed in cold acetone for 10 minutes. Primary antibodies, usually hybridoma supernatants, were incubated on the sections for 3 hours at room temperature. Following washing in phosphate-buffered saline (PBS), fluorescein-conjugated anti-mouse IgG (Sigma, St. Louis, MO) was incubated on the sections for 30 minutes, and the unbound second antibody was removed by washing in PBS. The sections were covered with 90% glycerol in PBS and were

viewed using a Zeiss Photomicroscope III.

With some tissue sections, immunofluorescent tests were conducted after enzymatic digestion with hyaluronidase (Worthington Biochemicals, Malvern, PA), employed at a concentration of 8,000 units/ml of 0.1 M phosphate buffer, pH 5.3, for 20 minutes at room temperature, 0.01% elastase (Worthington Biochemicals) in 0.067 M Tris buffer, pH 8.8, for 20 minutes at room temperature, and bacterial collagenase (a gift from Dr. Nicholas P. Morris) in 0.05 M Tris buffer, pH 7.5, including 0.01 M  $\text{CaCl}_2$ , 50  $\mu\text{M}$  N-ethylmaleimide, and 50  $\mu\text{M}$  phenyl methyl sulfonyl fluoride, for 30 minutes at room temperature.

For immunofluorescence of cell cultures, cells were plated onto glass chamber slides and were fixed either in cold acetone for 10 minutes or in 2% para-formaldehyde in PBS for 20 minutes. The assay then proceeded as above.

#### Electron Microscopic Immunolocalization

Freshly obtained tissue samples were cut into blocks and incubated with primary antibody for 7-15 hours at 4°C. After washing away unbound antibody, the tissue blocks were incubated with colloidal gold conjugated to anti-mouse IgG (Janssen Life Sciences, Piscataway, NJ) in a 0.02 M Tris buffer, pH 8.0, containing 0.9% NaCl and 0.1% bovine serum albumin, for 5-15 hours at 4°C. The blocks were washed in PBS, rinsed in 0.1 M sodium cacodylate, pH 7.4, fixed in cacodylate-buffered Karnovsky's fix (19) for 60 minutes at 4°C, rinsed again and finally fixed in 1%  $\text{OsO}_4$  in cacodylate for 60 minutes at 4°C. Samples were dehydrated at room temperature through 30, 50, 70, 90, 95, and 100% ethanol and were washed in 100% propylene oxide. Then, they were

infiltrated with Spurr's epoxy (hard formula) and polymerized at 70°C overnight. Sixty to 90 nm sections were cut on a Reichert Ultracut E Ultramicrotome and mounted on formvar coated grids. Grids were stained in 2% uranyl acetate in 50% ethanol for 15 minutes and Reynold's lead citrate (29) for 60 seconds. Specimens were examined at 60-80 kilovolts, using a 30  $\mu$ m objective aperture with a Philips 410 L.S. transmission electron microscope.

The same protocol was used for preparing samples for immunolocalization by scanning electron microscopy, except that following dehydration through 100% ethanol, samples were critical point dried through liquid CO<sub>2</sub>, sputter coated in an argon atmosphere with gold using a Balzers MED 010 evaporator, and examined at 10 kilovolts in the upper stage of an ISI DS-130 scanning electron microscope.

### Cell Cultures

Primary cultures of fibroblasts were established from normal human skin explants. They were then dissociated with trypsin (Sigma, St. Louis, MO) in PBS and passaged. Primary cultures of human amniocytes were derived from amnion digested with 0.1% crude bacterial collagenase (Worthington Biochemicals, Malvern, PA) in PBS supplemented with 0.001 M CaCl<sub>2</sub> and 0.0005 M MgCl<sub>2</sub>. Amniocytes were mixed cultures of epithelial and fibroblastic cell types and were used only as primary cultures. Established cell lines, MG63, KB, FL, WISH, and HT1080 were obtained from the American Type Culture Collection, Rockville, Maryland. All cell cultures were maintained in Dulbecco's Modified Eagle Medium (GIBCO), supplemented with 10% fetal bovine serum (GIBCO), 50 units/ml of penicillin and 50  $\mu$ g/ml of streptomycin (GIBCO, Grand

Island, NY).

For metabolic labeling experiments, cell cultures were fed with serum-free medium containing 50  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]proline, [ $^3\text{H}$ ]glycine, [ $^3\text{H}$ ]glucosamine, [ $^{35}\text{S}$ ]sulfate, or [ $^{35}\text{S}$ ]cysteine (Amersham, Arlington Heights, IL). In experiments measuring  $^{35}\text{S}$  incorporation, sulfate or cysteine were not included in the medium. Sometimes cultures were supplemented with 100  $\mu\text{g/ml}$  of ascorbic acid and 64  $\mu\text{g/ml}$  of  $\beta$ -aminopropionitrile. When cell culture medium was harvested, it was placed immediately on ice, and the following inhibitors were included: 5mM EDTA, 50  $\mu\text{M}$  N-ethylmaleimide and 50  $\mu\text{M}$  phenylmethyl sulfonyl fluoride. Radiolabeled proteins were separated by polyacrylamide gel electrophoresis (23), and the bands were visualized by soaking the gel in Amplify (Amersham), drying it, and exposing it to Kodak XAR-5 X-ray film.

#### Immunoisolation

Ascites antibody was partially purified using Affigel Blue (BioRad, Richmond, Ca) and was coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ). Cell culture medium was incubated with antibody coupled to Sepharose for 3 hours at room temperature. The gel was then washed extensively with 0.05 M Tris, pH 7.5, containing 0.5 M NaCl and 0.05% Tween 20. Bound material was eluted from the antibody beads with 1 M acetic acid. Contaminating fibronectin was removed by absorption with gelatin-sepharose.

#### IMMUNOBLOTTING

Proteins were separated by polyacrylamide gel electrophoresis, and

the separated proteins were transferred from the gel to nitrocellulose paper using a BioRad transblot system. After transfer, the paper was washed in PBS, blocked with 4% bovine serum albumin in PBS, incubated with monoclonal antibody for 3 hours at room temperature, washed, and incubated with peroxidase-conjugated anti-mouse IgG (BioRad, Richmond, CA) for 2 hours. After the final washing, substrate (BioRad) was added, and the antibody-bound electrophoretic band was visualized.

#### Collagenase Digestion

Immunoisolated or partially purified sample was incubated with collagenase (a gift from Dr. Nicholas P. Morris and from Dr. Hans Peter Bachinger), 50  $\mu$ M N-ethylmaleimide, 50  $\mu$ M phenylmethyl sulfonyl fluoride, and 0.01 M  $\text{CaCl}_2$  in 0.05 M Tris, pH 7.5, for 30 minutes at 33°C. Proteins were separated by electrophoresis and visualized by fluorography or Coomassie Blue staining.

## RESULTS

### Isolation of Fibrillin From Cell Cultures

Hybridomas were selected on the basis of their immunofluorescent staining pattern. Supernatants from two different clones yielded identical patterns. Monoclonal antibodies from the two clones bind to the same protein antigen, which we call fibrillin, in immunoblotting and immunoprecipitation experiments, but their epitopes are different. Both antibodies recognize epitopes in human and bovine tissues; one also binds to chick tissues. The results shown here were obtained using monoclonal antibody 201, although similar data was produced using monoclonal antibody F2.

Cell cultures producing fibrillin were identified by indirect immunofluorescence. Using the medium from cell cultures of amniocytes, fibroblasts, or MG63, an osteosarcoma line, which had been incubated with radioactive amino acids, immunoisolation experiments yielded a protein which, upon electrophoresis, had a slower mobility than fibronectin (250 kd) under disulfide bond reducing conditions. The size of the polypeptide was estimated to be around 350,000 daltons (Figure 1a). When gels were run without reducing agent, the polypeptide migrated faster, suggesting the presence of intrachain disulfide bonds. This electrophoretic identification of fibrillin was confirmed by immunoblotting (Figure 1b).

Reduction of disulfide bonds destroyed antibody binding in both immunoblotting and in ELISA as well as in immunofluorescence experiments. Therefore, the epitope recognized by this monoclonal antibody (and also the different epitope recognized by monoclonal

antibody F2) appears to be stabilized by disulfide bonds.

When [ $^3\text{H}$ ]glucosamine was included in the medium, a radioactive protein with identical electrophoretic mobilities was immunoisolated. However, when the label was [ $^{35}\text{S}$ ]sulfate, no radioactive material was detected in the immunoisolation, although proteins in the medium did incorporate [ $^{35}\text{S}$ ]sulfate and the immunoisolated protein with its characteristic mobilities could be demonstrated after silver staining (data not shown). Presence of carbohydrate was also shown by the periodic acid-Schiff staining of partially purified culture medium separated by electrophoresis (Figure 1c).

Incubation of immunoisolated fibrillin with bacterial collagenase did not result in degradation (Figure 2). This data confirmed immunofluorescence experiments performed on tissue sections treated with collagenase.

#### Distribution of Fibrillin in Tissues

In human skin, the immunofluorescent staining pattern of fibrillin appeared to represent a network of discrete fibers extending from the region of the dermal-epidermal junction, where they often seemed to intersect perpendicularly, down into the deep dermis (Figure 3a). In contrast, Type VI collagen immunofluorescence was more evenly distributed throughout the connective tissue stroma (Figure 3b).

Immunofluorescent studies of various tissues revealed a wide distribution of fibrillin (Figure 4). Anti-fibrillin staining demonstrated the presence of discrete fibers in the bovine corneal stroma and emphasized a broad band of immunofluorescence in Descemet's membrane (4f). Bovine kidney glomeruli (4a) were brightly stained.

Bovine bronchioles, adjoining blood vessel walls, and alveolar connective tissue (4b), human muscle perimyseum and endomyseum (4c), and human placental vessels and trophoblastic connective tissue (4g) displayed immunofluorescence. Discrete fibers were stained in the human tendon (4d). In bovine ciliary zonule, the suspensory ligament of the lens, extraordinarily fibrillar structures were identified (4e).

Fibrillin immunofluorescence in aorta was typical of elastic fiber staining (figure 5a,c). However, when tissue sections of bovine aorta were incubated with elastase using conditions which abolished (5b) immunofluorescence generated by anti-elastin antiserum (a gift from Dr. Judith Foster), staining with anti-fibrillin monoclonal antibody was unaltered (5c). Fibrillin was also present in elastic cartilage of human ear (Figure 6a,b). However, hyaluronidase digestion was necessary to unmask antigenic sites; staining in the perichondrium and dermal connective tissue were not affected by treatment with hyaluronidase (6b). The immunofluorescence pattern was undisturbed by prior digestion of the tissue sections with bacterial collagenase, using conditions where the immunofluorescence obtained with monoclonal antibodies specific for Type IV collagen (31) and for Type III collagen were completely abolished (data not shown).

Cell matrices were examined by immunofluorescence (Figure 7). Fibrillin was identified in the matrices of cultures of human amniocytes (7a), fibroblasts (7b), and MG63, an osteosarcoma cell line (7c). In the primary cultures of amniocytes, some fibroblastic cells were present amidst the epithelial cells; immunofluorescence appeared only around the fibroblastic cells. In addition, the FL and WISH transformed epithelial cell lines from human amnion displayed no

immunofluorescence. KB, an epidermal oral carcinoma, and HT1080, a fibrosarcoma which synthesizes basement membrane constituents, were also unreactive with the monoclonal antibody.

#### Electron Microscopic Immunolocalization

Electron microscopic immunolocalization studies demonstrated the presence of fibrillin in fibrous structural elements described as "microfibrils" (Figures 8,9). In human skin, anti-fibrillin monoclonal antibody directed the deposition of gold specifically to microfibrils whose average diameter was 10 nm. Gold-decorated microfibrils were seen around amorphous cores of the elastic fiber system (Figure 8). In addition, bundles of microfibrils without amorphous cores were labeled equally well. At the dermal-epidermal junction, these bundles of microfibrils appeared to intersect the basal lamina (Figure 9). There was no labeling of any other connective tissue components.

Scanning electron microscopic immunolocalization displayed a three-dimensional image of anti-fibrillin labeled fibers (Figure 10). In a region of human dermis which has been shown by transmission electron microscopy to contain mature elastic fibers, gold identified the presence of fibrillin on fibers, of varying diameters, which branch and which appear to form a discrete fiber system.

In contrast to the localization of fibrillin, Type VI collagen was immunolocalized in human skin to the fine filamentous structures that lie among the large banded interstitial collagen fibers (Figure 11). Type VI collagen-directed gold deposition did not occur on bundles of microfibrils or on microfibrils around elastin cores.

The epitope on fibrillin which is recognized by monoclonal

antibody 201 seemed to occur periodically along the microfibrils (Figure 12). Interestingly, the fibrillin periodicity, as detected by gold deposition, matched the interstitial collagen band periodicity of 67 nm. This periodicity appeared along the length of individual microfibrils as well as across bundles of individual microfibrils, suggesting an ordered aggregation of this component into microfibrils and microfibrillar bundles. However, it is also possible that the seemingly ordered aggregation of individual microfibrils into bundles may be due in part to aggregation and alignment by the antibodies.

## DISCUSSION

We have demonstrated that a new 350 kd glycoprotein, which we call fibrillin, is a major structural component of a special class of microfibrils. Fibrillin is present in both elastin-associated microfibrils and in bundles of microfibrils without a visible amorphous element. Because of their visual similarities, it has been thought that elastin-associated microfibrils might be related to the oxytalan fibers of skin (6,22,38), to microfibrils described in the basement membrane region of the alveolar wall (24), to the bundles of discrete tubular fibrils in the subendothelial spaces of kidney glomeruli (9), and to the microfibrils of the ciliary zonule (28,36). Our data supports this idea. Using a monoclonal antibody and electron microscopic immunolocalization, we have shown that fibrillin is common to microfibrils at the basement membrane of the dermal-epidermal junction and to microfibrils at the periphery of true elastic fibers in skin, to microfibrils in elastic tissues such as aorta and ear cartilage (data not shown), and to microfibrils in elastin-poor tissues such as cornea, tendon, and ciliary zonule (data not shown). In addition, immunofluorescence localized this glycoprotein to alveolar connective tissue, kidney glomeruli and muscle sheaths. Thus, our immunological data has specified the presence of fibrillin to all morphologically similar 10 nm microfibrils which we have so far examined and suggests that a unique system of microfibrils exists in the connective tissue space.

In addition, our data, utilizing a monoclonal antibody and immunogold electron microscopy, localized Type VI collagen specifically

to small diameter microfilaments. Immunofluorescence microscopy has shown Type VI collagen to be distributed in tissues in a pattern similar to the interstitial collagens (17,13). Electron microscopic immunolocalization using monospecific antisera directed against the pepsinized form of Type VI collagen has shown ferritin labeling "very close" to 5-10 nm microfilaments present in the amorphous ground substance of aortic media and placental villi (39). In that study, some ferritin can be seen along elastic fibers, but the labeling is not prominent. In our study, the microfibrillar component of the elastic fiber as well as the bundles of 10 nm microfibrils were never labeled by monoclonal anti-Type VI collagen and immunogold.

It has become increasingly clear that, in addition to the large banded collagen fibers, different collagen types can form different extracellular structures. In basement membranes, Type IV collagen may aggregate in an end-to-end manner to form a network (37) and may associate laterally as well (40). Type VII collagen appears to be a major structural component of the anchoring fibril in the basement membranes of certain epithelia like skin and cornea (32). In accord with Bruns' analysis (2,3) of cell culture microfilaments, immunolocalization of Type VI collagen to extracellular microfilaments supports the suggestion that Type VI collagen may be the major structural element of a special class of microfilaments. Whether these Type VI collagen microfilaments are identical to the small microfilaments observed in early chick development and what function they serve in the extracellular matrix, particularly with respect to the large banded collagen fibers among which they appear to interweave, are not known.

We have characterized fibrillin as a microfibrillar component and as a fibroblast cell culture product. Fibrillin is a collagenase resistant nonsulfated glycoprotein with an estimated molecular weight of around 350,000 daltons. A comparison with Sear's MFPII (34,35) cannot be made adequately, except to say that MFPII possessed a similar apparent molecular weight (300 kd) by electrophoresis under disulfide bond reducing conditions and that it appeared to be resistant to collagenase digestion.

The tissue form of fibrillin is currently under investigation. Ross and Bornstein (30) have demonstrated the resistance of microfibrillar structural components to extraction by collagenase digestion and dissociating agents. Their data also suggests that disulfide bridges form the principal crosslinks which stabilize the fibril structure. Our preliminary studies (not shown) indicate that, in chick aorta organ cultures, in cell culture matrices, and in extracts of aorta, immunodetection of fibrillin results in an electrophoretic band similar in size to the fibroblast product in the culture medium and that, in organ cultures, fibrillin forms intermolecular disulfide-bonded aggregates which become insoluble.

It has been speculated that microfibrils consist of a precursor elastin core surrounded by glycoprotein (4). Fibronectin may be associated with microfibrils (26,33,15). The association of Types III and IV collagens and laminin with microfibrils in the cornea has also been reported (27). The "67 nm" periodicity of fibrillin which we have described may be suggestive of a relationship between fibrillin and an interstitial collagen. The identification of fibrillin as a principal structural component of microfibrils will allow potential interactions

of microfibrils with other extracellular matrix constituents to be explored.

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## FIGURE LEGENDS

Figure 1 (a) Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 3-5% acrylamide gel of fibrillin isolated from [ $^{35}\text{S}$ ]cysteine-labeled fibroblast culture medium. Samples were run without (-2ME) or with (+2ME) 2-mercaptoethanol. Traces of contaminating fibronectin (Fn) in this preparation are used here for molecular weight estimation.

(b) and (c) SDS-PAGE using a 3-5% acrylamide gel of 50% saturated  $(\text{NH}_4)_2\text{SO}_4$  precipitated fibroblast culture medium proteins. Lanes 1 are stained with Coomassie Blue. Lane 2 in (b) shows medium proteins transferred to nitrocellulose and immunoblotted with anti-fibrillin monoclonal antibody. Lane 2 in (c) is stained with the periodic acid-Schiff reagent. Samples were prepared without reducing agent. Positions of molecular weight standards are marked.

Figure 2 . SDS-PAGE using a 3-5% acrylamide gel of fibroblast culture medium proteins labeled with [ $^3\text{H}$ ]proline and [ $^3\text{H}$ ] glycine (lanes 1,2,7,8) and of immunoisolated fibrillin (lanes 3,4,5,6). Lanes 2,4,6, and 8 show samples which were incubated with bacterial collagenase. Lanes 1,2,3, and 4 were run without reducing agent, and lanes 5,6,7, and 8 were run with reducing agent.

Figure 3 . Indirect immunofluorescent staining of human skin using anti-fibrillin monoclonal antibody (a) and monoclonal anti-Type VI collagen (b). Magnification=200x; bar=50 $\mu$ m.

Figure 4 Fibrillin immunofluorescence of sections of (a) bovine kidney, (b) bovine lung, (c) human muscle, (d) human tendon, (e) bovine ciliary zonule, (f) bovine cornea, and (g) human placenta. Magnification=200x; bar=50 $\mu$ m.

Figure 5 Resistance of fibrillin immunofluorescence to elastase digestion. Bovine aorta tissue sections were stained with anti-elastin antiserum before (a) and after (b) elastase treatment of the sections. Panel (c) shows anti-fibrillin staining of an elastase-digested tissue section. Magnification=200x; bar=50 $\mu$ m.

Figure 6    Hyaluronidase digestion of human elastic (ear) cartilage. Panels (a) and (b) show immunofluorescence using monoclonal anti-fibrillin, and (c) and (d) show staining using monoclonal anti-Type VI collagen. Immunofluorescence of hyaluronidase digested tissue sections is shown in (b) and (d). Magnification=300x; bar=50 $\mu$ m.

Figure 7     Immunofluorescence demonstrating fibrillin in the matrices of cultured cells: human amniocytes (a), fibroblasts (b), and MG63 (c). Magnification=200x; bar=50 $\mu$ m.

Figure 8      Electron microscopic immunolocalization of fibrillin to microfibrils around amorphous cores of elastic fibers in human skin. Longitudinal sections (a-d, g) and cross-sections (e, f) of 5 nm gold-decorated microfibrils are shown. Undecorated collagen fibers (a-d) as well as undecorated filamentous structures (d) can be seen. Magnifications: (a-c)=30,000x (bar=0.5 $\mu$ m); (d, g)=50,000x (bar=0.25 $\mu$ m); (e, f)=60,000x (bar=0.25 $\mu$ m).

Figure 9 Anti-fibrillin labeled microfibrils in the region of the dermal-epidermal junction of human skin. Panel (a) (magnification=25,000x; bar=0.5 $\mu$ m) shows a microfibrillar bundle, amidst unlabeled collagen fibers, in its approach toward the basal lamina; anchoring fibrils, the lamina densa, and basal cell structures are also unlabeled. Panel (b) (magnification=50,000x; bar=0.25  $\mu$ m) depicts 5 nm gold-decorated microfibrils appearing to insert into the lamina densa.

Figure 10 Immunolocalization of fibrillin by scanning electron microscopy of 40 nm gold-decorated fibers, among unlabeled collagen fibers, in human dermis. Magnification=13,200x; bar=1 $\mu$ m.

Figure 11 Electron microscopic localization of Type VI collagen to fine filaments associated with banded collagen fibers in human skin. Unlabeled microfibrils can be seen. Magnification=50,000x; bar=0.25 $\mu$ m.

Figure 12 Longitudinal periodicity of fibrillin demonstrated by 5 nm gold deposition. Similarity to the interstitial collagen banding period can be seen by comparison with the adjoining collagen fiber. Magnification=75,000x; bar=0.2 $\mu$ m.

# Fibrillin, A New 350-kD Glycoprotein, Is a Component of Extracellular Microfibrils

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**Abstract.** A new connective tissue protein, which we call fibrillin, has been isolated from the medium of human fibroblast cell cultures. Electrophoresis of the disulfide bond-reduced protein gave a single band with an estimated molecular mass of 350,000 D. This 350-kD protein appeared to possess intrachain disulfide bonds. It could be stained with periodic acid-Schiff reagent, and after metabolic labeling, it contained [<sup>3</sup>H]glucosamine. It could not be labeled with [<sup>35</sup>S]sulfate. It was resistant to digestion by bacterial collagenase. Using mAbs specific for fibrillin, we demonstrated its widespread distribution in the connective tissue matrices of skin, lung, kidney, vasculature, cartilage, tendon, muscle, cornea, and ciliary zonule. Electron microscopic immunolocalization with colloidal gold conjugates specified its location to a class of extracellular structural elements described as

microfibrils. These microfibrils possessed a characteristic appearance and averaged 10 nm in diameter. Microfibrils around the amorphous cores of the elastic fiber system as well as bundles of microfibrils without elastin cores were labeled equally well with antibody. Immunolocalization suggested that fibrillin is arrayed periodically along the individual microfibril and that individual microfibrils may be aligned within bundles. The periodicity of the epitope appeared to match the interstitial collagen band periodicity. In contrast, type VI collagen, which has been proposed as a possible microfibrillar component, was immunolocalized with a specific mAb to small diameter microfilaments that interweave among the large, banded collagen fibers; it was not associated with the system of microfibrils identified by the presence of fibrillin.

THE extracellular matrix appears to contain a variety of fibrillar structural elements. In addition to the large banded collagen fibers, there are various nonstriated fibrils with small cross-sectional diameters. These fibrils can be classed on the basis of their average diameter as 8–10-nm microfibrils (24, 25) or as 3–5-nm microfibrils (16).

The larger 8–10-nm microfibril usually appears in cross-section as an outer electron-dense shell surrounding an inner hollow or lucid core, and in longitudinal section as a beaded chain. These microfibrils are most abundantly associated with basement membranes and with the elastic fiber, and are found in most tissues. They are the subject of a recent review (5). The smaller 3–5-nm microfibril or microfilament is often connected to proteoglycan granules (16, 10).

Many attempts have been made to specifically extract microfibrillar proteins. Ross and Bornstein, guided by electron microscopic analysis of the residue from each extraction step, have described a protocol for the isolation of microfibrillar proteins (30). After digestion of tissue with collagenase, the residue is extracted with guanidine. Microfibrillar proteins are thought to be finally solubilized with disulfide bond reducing agent in guanidine. Biochemical analysis of this microfibrillar extract from tissue (12) and immunological and biochemical studies of cell culture products

(34, 35) using an antiserum prepared with this microfibrillar extract as the immunogen (20) have led to the identification of a major periodic acid-Schiff-positive electrophoretic band with an apparent molecular mass of 140,000 D. This 140-kD glycoprotein has been shown to be a component of the undegraded tissue form of type VI collagen (18, 21, 14).

Electron microscopic studies of type VI collagen molecules suggest a model for type VI collagen filament formation involving an end-to-end rather than a lateral aggregation of a basic tetrameric unit (11, 8). This electron microscopical model has been useful in clarifying the relationship between type VI collagen and microfibrillar components. In fibroblast cultures of chick leg tendon and cornea and of human skin, 3-nm beaded filaments, which appear to be different from the chains of globules formed in 10-nm microfibrils, compare favorably with the filamentous model for type VI collagen (2, 3). Using a previously characterized mAb specific for type VI collagen (17), we demonstrate here immunolocalization of type VI collagen to small diameter microfilaments associated primarily with large banded interstitial collagen fibers and not with 10-nm microfibrils.

In this paper, we are presenting new information on a glycoprotein that we have isolated from the medium of human fibroblast cell cultures. Using a mAb specific for this glyco-

anti-mouse IgG (Bio-Rad Laboratories) for 2 h. After the final washing, substrate (Bio-Rad Laboratories) was added, and the antibody-bound electrophoretic band was visualized.

## Collagenase Digestion

Immunoisolated or partially purified sample was incubated with collagenase (a gift from Dr. Nicholas P. Morris and Dr. Hans Peter Bachinger, Shriners Hospital for Crippled Children), 50  $\mu$ M *N*-ethylmaleimide, 50  $\mu$ M phenylmethylsulfonyl fluoride, and 0.01 M  $\text{CaCl}_2$  in 0.05 M Tris, pH 7.5, for 30 min at 33°C. Proteins were separated by electrophoresis and visualized by fluorography or Coomassie Blue staining.

## Results

### Isolation of Fibrillin from Cell Cultures

Hybridomas were selected on the basis of their immunofluorescent staining pattern. Supernatants from two different clones yielded identical patterns. mAbs from the two clones bind to the same protein antigen, which we call fibrillin, in immunoblotting and immunoprecipitation experiments, but their epitopes are different. Both antibodies recognize epitopes in human and bovine tissues; one also binds to chick tissues. The results shown here were obtained using mAb 201, although similar data was produced using mAb F2.

Cell cultures producing fibrillin were identified by indirect immunofluorescence. Using the medium from cell cultures of amniocytes, fibroblasts, or MG63, an osteosarcoma line, which had been incubated with radioactive amino acids, immunoisolation experiments yielded a protein, which, upon electrophoresis, had a slower mobility than fibronectin (250 kD) under disulfide bond-reducing conditions. The size of the polypeptide was estimated to be  $\sim 350,000$  D (Fig. 1 a). When gels were run without reducing agent, the polypeptide

migrated faster, suggesting the presence of intrachain disulfide bonds. This electrophoretic identification of fibrillin was confirmed by immunoblotting (Fig. 1 b).

Reduction of disulfide bonds destroyed antibody binding in both immunoblotting and in ELISA as well as in immunofluorescence experiments. Therefore, the epitope recognized by this mAb (and also the different epitope recognized by mAb F2) appears to be stabilized by disulfide bonds.

When [ $^3\text{H}$ ]glucosamine was included in the medium, a radioactive protein with identical electrophoretic mobilities was immunoisolated. However, when the label was [ $^{35}\text{S}$ ]sulfate, no radioactive material was detected in the immunoisolation, although proteins in the medium did incorporate [ $^{35}\text{S}$ ]sulfate and the immunoisolated protein with its characteristic mobilities could be demonstrated after silver staining (data not shown). Presence of carbohydrate was also shown by the periodic acid-Schiff staining of partially purified culture medium separated by electrophoresis (Fig. 1 c).

Incubation of immunoisolated fibrillin with bacterial collagenase did not result in degradation (Fig. 2). This data confirmed immunofluorescence experiments performed on tissue sections treated with collagenase.

### Distribution of Fibrillin in Tissues

In human skin, the immunofluorescent staining pattern of fibrillin appeared to represent a network of discrete fibers extending from the region of the dermal-epidermal junction, where they often seemed to intersect perpendicularly, down into the deep dermis (Fig. 3 a). In contrast, type VI collagen immunofluorescence was more evenly distributed throughout the connective tissue stroma (Fig. 3 b).

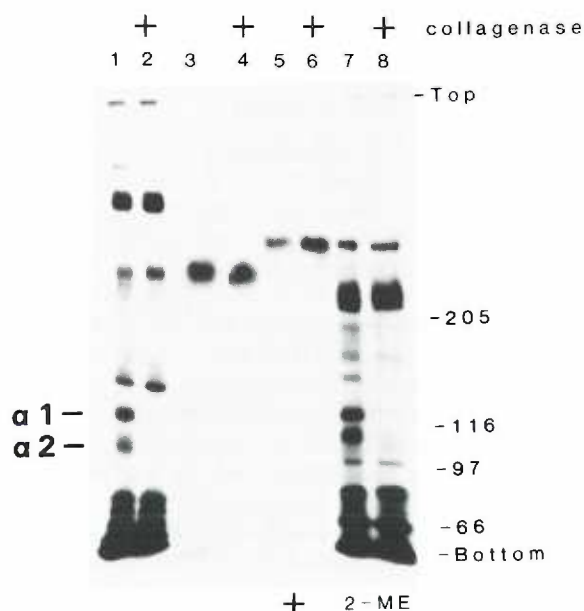


**Figure 2.** SDS PAGE using a 3–5% acrylamide gel of fibroblast culture medium proteins labeled with [ $^3\text{H}$ ]proline and [ $^3\text{H}$ ] glycine (lanes 1, 2, 7, and 8) and of immunoisolated fibrillin (lanes 3, 4, 5, and 6). Lanes 2, 4, 6, and 8 show samples that were incubated with bacterial collagenase. Lanes 1, 2, 3, and 4 were run without reducing agent, and lanes 5, 6, 7, and 8 were run with reducing agent.

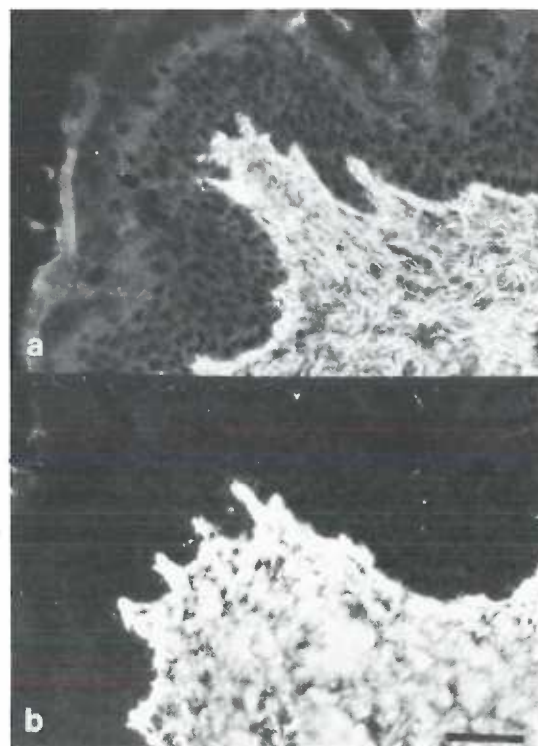


**Figure 3.** Indirect immunofluorescent staining of human skin using antifibrillin mAb (a) and monoclonal anti-type VI collagen (b). Bar, 50  $\mu$ m.

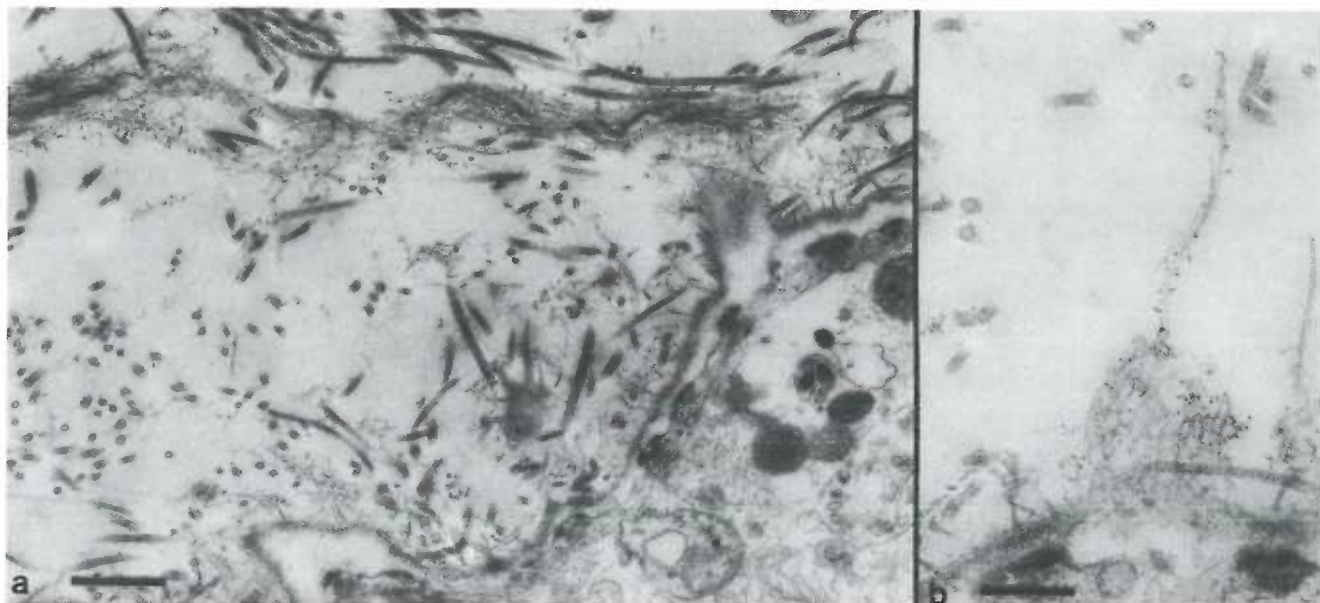


**Figure 9.** Antifibrillin-labeled microfibrils in the region of the dermal-epidermal junction of human skin. *a* shows a microfibrillar bundle, amidst unlabeled collagen fibers, in its approach toward the basal lamina; anchoring fibrils, the lamina densa, and basal cell structures are also unlabeled. *b* depicts 5-nm gold-decorated microfibrils appearing to insert into the lamina densa. Bars: (*a*) 0.5  $\mu\text{m}$ ; (*b*) 0.25  $\mu\text{m}$ .

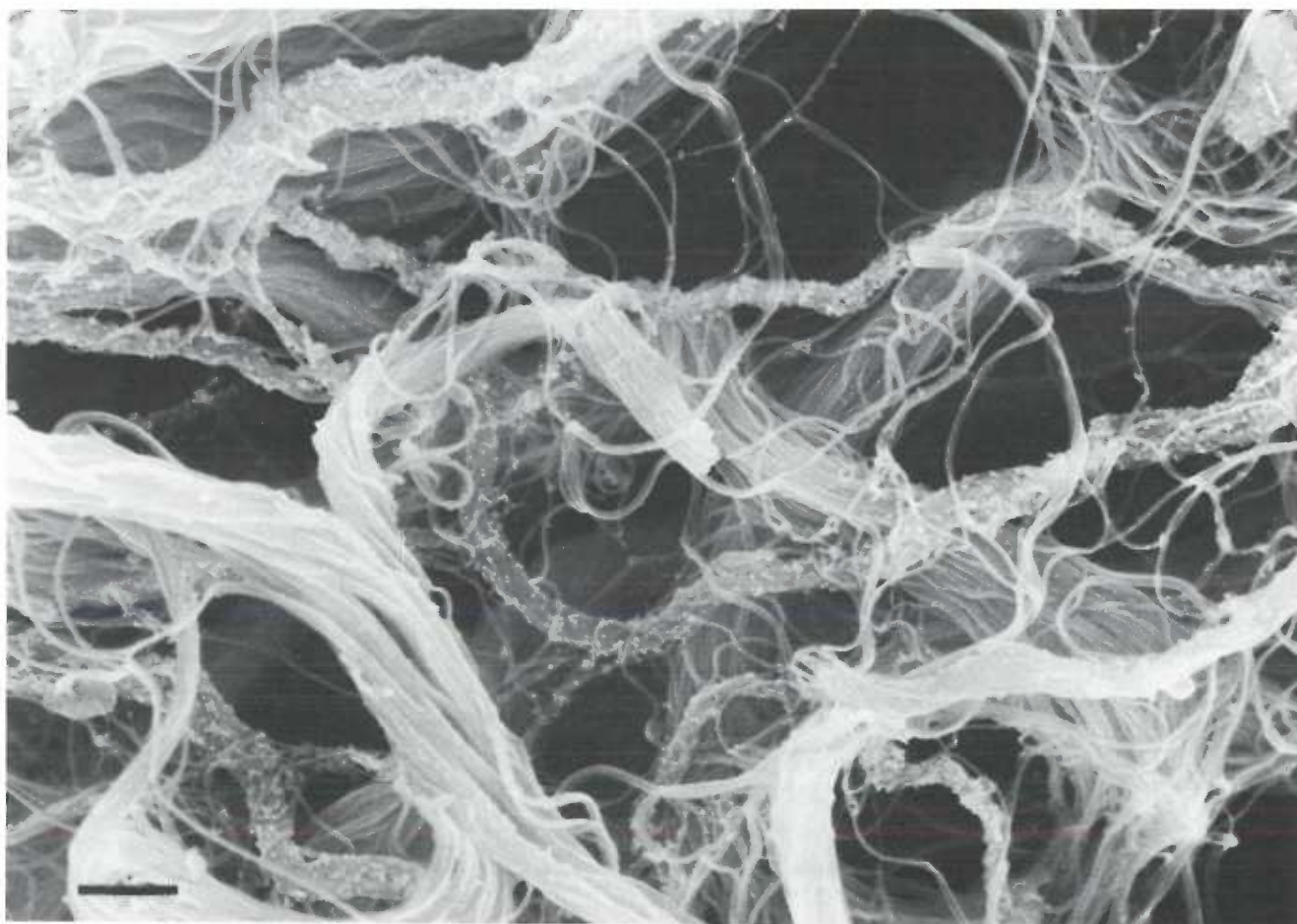


**Figure 10.** Immunolocalization of fibrillin by scanning electron microscopy of 40-nm gold-decorated fibers, among unlabeled collagen fibers, in human dermis. Bar, 1  $\mu\text{m}$ .

FIBRILLIN, A COMPONENT OF EXTRACELLULAR MICROFIBRILS, FORMS  
INTERMOLECULAR DISULFIDE CROSSLINKS IN THE CONNECTIVE TISSUE MATRIX

Condensed Title: Fibrillin in Connective Tissue

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## SUMMARY

Fibrillin is a large noncollagenous glycoprotein which has been immunolocalized electron microscopically to a special class of connective tissue microfibrils. Fibrillin-containing microfibrils are ubiquitous in the extracellular space. However, these microfibrils are extremely difficult to solubilize in order to isolate their protein components. We report here new conditions for the extraction of fibrillin from embryonic chick aortae, and we compare this tissue fibrillin with fibrillin obtained from the medium of human fibroblasts in cell culture. Based upon SDS-PAGE and immunoblotting, fibrillin from these two sources appears not to be significantly different in size, indicating that there is little, if any, processing entailed in its extracellular assembly. Radioactive labeling with cysteine, followed by sequential extraction, reveals selective solubilization by dithioerythritol of one cysteine-rich protein, fibrillin. In addition, pulse-chase experiments demonstrate that fibrillin becomes increasingly insoluble through the formation of intermolecular disulfide crosslinks. Disulfide-crosslinked fibrillin multimers are analyzed on agarose gels where they appear to be greater than 8 million kDa. We hypothesize that fibrillin is a constitutive structural element of connective tissue microfibrils which utilizes intermolecular disulfide crosslinks in its assembly process.

Ultrastructural examinations of the different connective tissues have revealed a variety of complex fibrous networks. The extracellular matrix is trussed by long thick fibers which are enmeshed in a delicate web of fine filaments; it is also bridged with extensive trunklines of anastomosing fibrils. In addition, there are special fibrous structures which occur in only certain connective tissues: the anchoring fibril, for example, lies subjacent to the lamina densa of skin, but is absent in most other tissues. Recently, progress has been made in identifying protein constituents of these different fibrous elements of the connective tissue.

Fibrillin has been shown to be a component of a specific kind of connective tissue fibril (Sakai, Keene, and Engvall, 1986). Extracellular microfibrils, which have been described as strings of hollow beads with an approximate diameter of 10 nm, can be found in most connective tissue spaces: they are part of the mature elastic fiber system where they appear as a peripheral microfibrillar mantle surrounding amorphous electron dense centers; they are also present in fiber bundles of microfibrils, apparently independent of the amorphous elastic material. Although the ultrastructure of these microfibrils has been similarly described, their identity or difference has been a matter of controversy (for a review, see Cleary and Gibson, 1983). The demonstration that these ubiquitous 10 nm microfibrils, whether they are associated with the elastic fiber system or apparently independent of it, contain fibrillin suggests that fibrillin may be a major constitutive structural element of these microfibrils.

Attempts have been made to isolate and characterize the protein constituents of these connective tissue microfibrils. However, the task

of extracting microfibrillar protein candidates from various tissues, making antibodies specifically reactive with the candidate protein, and immunolocalizing it to microfibrils has proven extremely difficult. A different approach led to the isolation and characterization of fibrillin as a component of extracellular microfibrils: monoclonal antibodies were produced using pepsin-digested tissue extracts; antibodies that bound to microfibrils were selected and used to isolate the antigen, fibrillin, from the cell culture medium of fibroblasts.

Fibrillin has been characterized as a large nonsulfated, noncollagenous glycoprotein with an approximate molecular weight of 350 kD. Since fibrillin was first isolated from fibroblast culture medium, its properties as a component of the connective tissue matrix are unknown. Microfibrils in the connective tissue have been shown to be extremely resistant to extraction, but apparently sensitive to disulfide-bond reducing agents (Ross and Bornstein, 1969). If fibrillin is a constitutive structural element of these microfibrils, as the immunolocalization suggests, it should not be easily extracted from the tissue. The object of this work is to investigate the following questions: what conditions will successfully extract fibrillin from tissues? In the tissue, is fibrillin the same size as in cell culture, or is it processed in the connective tissue space? Does fibrillin form intermolecular disulfide bonds? Do these disulfide crosslinks help to cement fibrillin into the connective tissue? In this paper, we show that fibrillin can be extracted in small quantities from embryonic chick aortae, that tissue fibrillin appears not to be significantly different in size from fibrillin secreted into the medium of cultured fibroblasts, and that fibrillin is stabilized in the connective tissue matrix by

intermolecular disulfide bonds.

## EXPERIMENTAL PROCEDURES

### Extraction of Chick Embryonic Tissue

The embryos of White Leghorn chickens were subjected to 5mg of B-aminopropionitrile (BAPN) in phosphate buffered saline, pH 7.4 (PBS) injected into the air sack on day 14. Embryos were sacrificed on day 17, and aortae were dissected and placed either in liquid N<sub>2</sub> or in organ culture medium.

Aortae were extracted sequentially with the following reagents: PBS, 1M NaCl in 50mM Tris-HCl, pH 7.5, 8M urea in 50mM Tris-HCl, pH 7.5 (two times), and 50mM dithioerythritol (DTE) in 8M urea/50mM Tris-HCl, pH 7.5. All extractions were performed at 4<sup>0</sup> C with the following protease inhibitors present: 0.001M N-ethylmaleimide, 0.004M ethylenediaminetetraacetic acid (EDTA), and 0.001M phenylmethylsulfonyl fluoride. The second 8M urea extraction went overnight at 4<sup>0</sup> C. Tissue was dissociated in extraction buffers with a Brinkmann polytron. Extracts were centrifuged at 4<sup>0</sup> C for 1 hour at 14,000 rpms in a Sorvall RC-5B centrifuge, and supernatants were stored at -90<sup>0</sup> C. Pellets were then extracted with the next buffer, using the polytron, and centrifuged. All chemicals were from Sigma, unless otherwise indicated.

### Organ Culture Experiments

Aortae from batches of 50-60 embryos at a time were dissected and incubated in Dulbecco's Modified Eagle Medium, minus cysteine (sometimes minus methionine as well), in a shaking water bath at 37° C. In preliminary experiments, they were radioactively labeled with medium containing 50µCi/ml [<sup>35</sup>S]cysteine (Amersham) and 50µCi/ml [<sup>35</sup>S]methionine (Amersham). In subsequent experiments, [<sup>14</sup>C]cystine (New England Nuclear) was used at a concentration of 1-5µCi/ml of medium. The medium was also supplemented with 0.02M HEPES and penicillin-streptomycin (50U/ml and 50µg/ml, respectively) (GIBCO). The aortae were then chased with medium containing cysteine (and methionine) and extracted as above. The medium usually contained 64µg/ml BAPN, unless otherwise specified. For each experiment, equal numbers of aortae were removed at specified time points and immediately frozen in liquid N<sub>2</sub>.

#### Immunochemical Analyses

Monoclonal anti-fibrillin used in this study was prepared as described by Sakai, Keene, and Engvall, 1986. It recognizes an epitope stabilized by disulfide bonds.

A polyclonal antiserum was prepared using a pepsin-resistant fibrillin peptide (M<sub>r</sub>=75,000) isolated from human amnion and selected by its reactivity with the monoclonal anti-fibrillin (Maddox, Sakai, and Glanville, manuscript in preparation). This peptide was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using disulfide bond reducing conditions. Bands were cut out of the gel, homogenized with PBS by passage through needles, emulsified in

complete Freund's adjuvant, and injected into New Zealand white rabbits. Subsequent boosters were given with material similarly prepared, but emulsified in incomplete Freund's adjuvant. The antiserum was tested for reactivity with the pepsin digest fragment and with intact fibrillin obtained from cell culture medium. Immunoblots demonstrated specific binding to both the pepsin generated fragment and cell culture fibrillin.

Immunoblots and immunoprecipitation were performed as described (Sakai, Keene, and Engvall, 1986). For immunoblots, samples of the various extracts were directly applied to SDS-PAGE. Monoclonal antibody (purified from ascites) was used at a dilution of 1:250 (5µg/ml antibody), and polyclonal antiserum was diluted 1:500. Second antibodies conjugated to peroxidase (Biorad) were used at a dilution of 1:2000 in PBS with 2% bovine serum albumin and 0.05% Tween 20. For preliminary immunoprecipitation experiments, the extracts were dialyzed against 50mM Tris-HCl, 0.5M NaCl, pH 7.5. After centrifugation, the supernatants were run over a monoclonal anti-fibrillin Sepharose column. Pellets, especially from the 8M urea extracts, also appeared on gels to contain fibrillin, so in later immunoprecipitation experiments, the extracts were dialyzed against 50mM Tris-HCl, 0.5M NaCl, and 2M urea pH 7.5, in which almost all the material remained in solution. Before application to monoclonal anti-fibrillin Sepharose, the samples were diluted with cold PBS to a urea concentration of 0.5M.

#### Molecular Sieve Chromatography

Extracts dialyzed against 50mM Tris-HCl, 0.5M NaCl, and 2M urea, pH

7.5 were centrifuged and filtered through a 0.45 $\mu$ m filter (Schleicher and Schuell). Aliquots were applied to two Superose 6 HR 16/50 columns in tandem (Pharmacia) with a flow rate of 0.5ml/min. Absorbance was monitored at 220nm, and 1.5 ml fractions were collected.

### Electrophoresis

SDS-PAGE was performed as described (Burgeson, et. al., 1976). The gels shown here are 3-5% gradients of polyacrylamide. Agarose gels were 1.5% agarose in 0.19M Tris-HCl, 0.25% SDS, pH 8.8. Gels were prepared for fluorography by soaking in Amplify (Amersham).

## RESULTS

One of the monoclonal antibodies can be used to detect fibrillin in chick as well as human and other tissues (Sakai, Keene, and Engvall, 1986). Because the chick embryo is a rapidly growing tissue which has proven useful in studies of extracellular procollagen conversion to collagen and of the soluble form of elastin, tropoelastin, the chick embryo was chosen for study.

### Extraction from Tissue

Figure 1 shows the electrophoretic pattern of [ $^{14}\text{C}$ ]cys-labeled bands obtained after sequential extraction of chick aortae with PBS, 1M NaCl in 50mM Tris, pH 7.5, 8M urea in 50mM Tris, pH 7.5, and 50mM DTE in 8M urea, 50mM Tris, pH 7.5. A band with a mobility similar to fibrillin, which had been isolated from human fibroblast medium, (lane 1) was present in all extracts. The tissue was extracted twice with 8M urea, the second time over 16 hours (lane 5). Few radioactive counts were obtained after the second extraction with 8M urea. Then, the disulfide bond reducing agent, DTE, further removed one prominent [ $^{14}\text{C}$ ]cys-labeled protein which migrated in the gel to a position matching that of fibrillin (lane 6). Extraction with higher concentrations of DTE did not increase the yield of this band (data not shown).

### Identification of the Tissue Form of Fibrillin

In order to ask whether the [ $^{14}\text{C}$ ]cys-labeled protein which appeared to comigrate in gels was fibrillin, immunoblots were performed using a rabbit antiserum which binds to reduced fibrillin peptides. In Western blots, this antiserum specifically reacted with the reduced fibrillin chain in samples of human fibroblast culture medium containing many other proteins (Figure 2a, lanes 6, 7). In addition, this polyclonal anti-fibrillin identified a similar band in all extracts of chick aortae (lanes 1-5). A Coomassie-stained gel of these extracts is shown for comparison (Figure 2b), demonstrating the large heterogeneity of these samples.

Monoclonal anti-fibrillin was also used to identify the tissue form of fibrillin. After dialysis against a buffer (50mM Tris, 0.5M NaCl, pH 7.5) suitable for immunoprecipitation, each extract from radioactively-labeled aortae was centrifuged to remove the insoluble material, and the supernatant was applied to monoclonal anti-fibrillin coupled to Sepharose. When viewed after electrophoresis and fluorography, the PBS, 1M NaCl, 8M urea, and 50mM DTE extracts contained immunoprecipitated proteins which comigrated in the gel with fibrillin (data not shown). Since the monoclonal anti-fibrillin reacts with an epitope stabilized by intrachain disulfide bonds, the material immunoprecipitated from the DTE extract presumably was able to reform the epitope when the reducing agent was dialyzed out of the sample.

### Pulse-Chase Experiments

After exhaustive prior extraction, chick aortae contained one major [ $^{14}\text{C}$ ]cys-labeled protein which could be removed with DTE. This protein was fibrillin. However, small amounts of fibrillin were detected in all of the extracts of chick aortae. Pulse-chase experiments were conducted in order to determine whether the formation of intermolecular disulfide crosslinks enables fibrillin to become increasingly insoluble with time.

Figure 3 shows the results of one representative pulse-chase experiment. Chick aortae were labeled with [ $^{14}\text{C}$ ]cystine for 30 minutes, chased for 0, 1, 3 and 19 hours, and extracted with PBS, 1M NaCl, 8M urea (two times), and 50mM DTE. After 30 minutes of labeling, newly synthesized fibrillin was present in the PBS and 8M urea extracts (lanes 1 and 5), but was not present in the DTE extract (lane 14). After only 1 hour of chase, fibrillin was found in all extracts (lanes 2, 6, 11), including the DTE extract (lane 15). After 3 hours of chase, fibrillin was decreased in the PBS (lane 3) and 8M urea (lane 7 and 12) extracts and increased in the DTE extract (lane 16). After 19 hours, PBS extract no longer contained fibrillin (lane 4); 8M urea extracts contained smaller amounts of fibrillin (lanes 8 and 13) than at 3 hours of chase. Most of the radioactively labeled fibrillin present at this time had been resistant to prior extractions and was solubilized by DTE (lane 17). Since the 1M NaCl extracts contained very little fibrillin, they are not shown here.

Based on these results, we hypothesized that PBS-extracted fibrillin was newly synthesized and not yet disulfide-crosslinked.

Pulse-chase extracts were separated by molecular sieve chromatography. PBS extracts gave the chromatographic profile shown in Figure 4a, and 8M urea extracts yielded the profile in Figure 4b. Gels were run to analyze the profiles. The positions of monomeric and multimeric fibrillin are marked. For Figure 4c, samples were taken from the sieve column fractions which represented the elution positions of fibrillin aggregate and monomer. Figure 4c summarizes the information gained from gels: fibrillin monomer, which runs faster on gels presumably due to the presence of intrachain disulfide bonds, was extracted by PBS after 0 (lanes 3 and 11) and 4 (lanes 5 and 13) hours of chase; disulfide-crosslinked fibrillin multimers were not present in the PBS extracts (lanes 2 and 10; 4 and 12; 6 and 14), but fibrillin multimers were extracted by 8M urea after 4 hours of chase (lanes 8 and 16). Small amounts of fibrillin monomers also appeared to be present after 4 hours of chase in the 8M urea extracts (see Figures 5 and 6). PBS extracts of aortae chased for 21 hours did not contain fibrillin monomer (lanes 7 and 15) or aggregate (lanes 6 and 14).

#### Intermolecular Disulfide Bonds Crosslink Fibrillin in the Tissue

In order to examine the intermolecular disulfide bonds in fibrillin, chick aortae were labeled with 250 $\mu$ Ci/ml of [ $^{35}$ S]cysteine for 1 hour, chased for 4 hours, and sequentially extracted with PBS, 1M NaCl and 8M urea. Material immunoprecipitated from the 8M urea extract by monoclonal anti-fibrillin coupled to Sepharose was applied to a gel without reducing agent. Similar to the result shown in Figure 4c (lane 8), most of the counts failed to enter the stacking gel (Figure 5a,

lane 1). Fibrillin monomer (arrow) was also immunoprecipitated, demonstrating the existence of a pool of monomer at this time point. With reducing agent, the immunoprecipitated material was primarily fibrillin (Figure 5a, lane 3). Some additional material, which could be either contaminants of the immunoprecipitation or non-reducibly crosslinked species of fibrillin, appeared to be present. On a 1.5% agarose gel, the unreduced fibrillin still remained at the top of the gel (Figure 5b, lane 1), except for a small amount of fibrillin monomer (arrow). When compared with mouse type IV procollagen markers (Figure 5b, lane 2), the minimum molecular weight calculated for the fibrillin aggregate was greater than 8 million daltons.

In order to confirm this information, 8M urea extracts of radioactively-labeled chick aortae were applied directly to a 1.5% agarose gel. After fluorography, only two high molecular weight cys-labeled bands were seen (Figure 6a, lane 1). When these two bands were cut out of the agarose gel, solubilized in hot gel sample buffer including 2-mercaptoethanol, and were applied to a 3-5% polyacrylamide gel, the material at the top of the agarose gel was reduced to a band which comigrated with fibrillin (Figure 6b, lane 2). The conditions required for immunoprecipitation of the 8M urea sample thus did not create the multimeric aggregation of fibrillin; these disulfide-bonded multimers were extracted directly from the tissue. Residual counts that were at the top of the stacking gel probably represented incomplete solubilization of the dried agarose gel pieces. However, the possibility exists that this material may contain species, of either fibrillin or unrelated proteins, which were either extremely large or joined by nonreducible crosslinks.

Are There Additional Crosslinks in Tissue Fibrillin?

Chick aortae from embryos which were not injected with BAPN were extracted sequentially as above and compared with the results shown here. Additionally, pulse-chase experiments were similarly conducted, but omitting BAPN, and using aortae from uninjected embryos. No difference in results from the data shown here was obtained.

Additional experiments were performed to ask whether our extraction procedures removed all or only part of the fibrillin present in the tissue. Since fragments of fibrillin can be obtained from pepsin digests of amnion (Maddox, Sakai, and Glanville, manuscript in preparation), residues of extracted aortae were further treated with pepsin. With both pulse-chased and nonradioactive samples, no evidence of fibrillin could be obtained by fluorography or immunoblotting. Since the extraction procedures (8M urea and DTE) may have rendered whatever fibrillin was remaining in the tissues sensitive to pepsin, equal numbers of aortae were extracted first with 1M NaCl and then with either DTE or pepsin. Coomassie-stained gels and immunoblotting of these materials demonstrated less fibrillin in the samples digested with pepsin than in those extracted with DTE (data not shown). Since there is no way as yet to quantitate amounts of fibrillin in tissues, the question of additional crosslinks cannot be answered.

## DISCUSSION

In this paper, we have identified the tissue form of fibrillin, which appears on gels to be not very different in size from cell culture fibrillin. In some gels, chick tissue fibrillin appeared to have a slightly slower mobility than human cell culture fibrillin, but this finding may be due to a species difference or to slightly different glycosylation. Fibrillin obtained from tissue, however, is significantly different from cell culture fibrillin: tissue fibrillin is extremely difficult to solubilize, and it is extensively crosslinked by disulfide bonds to form large aggregates.

Pulse-chase experiments demonstrated that disulfide bonds formed between fibrillin molecules within 2 hours from the time of secretion. Pools of newly synthesized monomers (extracted by PBS) decreased with time and were depleted within 20 hours; disulfide-crosslinked aggregates which could be released from the tissue with 8M urea increased within the first 4 hours and then decreased. Crosslinked aggregates which could be released from the tissue only with disulfide-bond reducing agent increased steadily with chase times. The formation of intermolecular disulfide crosslinks proceeded concomitantly with increasing resistance to solubilization by neutral salt buffers and by denaturing reagents.

In order to examine the process of disulfide crosslinking, we chose a time point, based upon our pulse-chase experiments, which would contain both fibrillin monomers and aggregates in the 8M urea extraction. Both immunoprecipitation and two-dimensional electrophoresis revealed that disulfide-crosslinked fibrillin aggregates

are extremely large. No "ladder" of crosslinked species was observed, even though fibrillin monomer was also present, confirming that we were looking at an appropriate time point. Comparison on agarose gels with disulfide-crosslinked type IV procollagen molecules suggested that fibrillin aggregates could be at least 8 million molecular weight and possibly much larger. Assuming that the electrophoretic mobility of these aggregates is determined primarily by molecular weight, these aggregates could be composed of more than 23 fibrillin molecules. It is possible, however, that these aggregates are composed of fewer molecules which together have a conformation (for example, end-to-end alignment) which restricts migration through gels. It is also possible that intermediate species do exist, but in pools too small for us to detect.

The formation of intermolecular disulfide crosslinks probably plays an important role in the assembly of fibrillin into microfibrils. Although disulfide bonds are formed in vitro with relative ease, care was taken here to perform extractions at neutral pH, 4° C, and with EDTA, conditions which do not encourage the air oxidation of free thiols to disulfides. The conditions used in these experiments with both cell culture fibrillin and tissue fibrillin were adequate to preserve fibrillin monomer, and so it is assumed that the formation of disulfide-crosslinked fibrillin aggregates observed in these experiments occurred in the connective tissue space and reflects the in vivo assembly of fibrillin in the connective tissue.

Disulfide bonds are present in many extracellular matrix proteins, including fibronectin and laminin. In the biosynthesis of collagens, disulfide bonds form both within and between the terminal domains of individual collagen chains and are important to the process of collagen

folding (Fessler, et. al., 1985). In addition, at least collagens type IV (Glanville, 1987), VI (Timpl and Engel, 1987) and VII (Burgeson, 1987) utilize disulfide bonds to covalently stabilize the assembly of these molecules into networks, filaments, and fibrils. Most of the information about these latter molecules has been gained through biochemical studies of their structure and through electron microscopical observations. However, the cellular and developmental processes involved in the attainment of crosslinked structures in the connective tissue space are not known.

Fibrillin is found in cell culture medium as an intra-chain disulfide-bonded monomer (Sakai, Keene, and Engvall, 1986), unlike type VI collagen which forms disulfide-bonded tetramers within the cell before secretion (Engvall, Hessle, and Klier, 1986). If both of these proteins utilize disulfide crosslinks to assemble in the extracellular space into long fibrils with a prescribed diameter, it may be that the cell must play an important role in controlling assembly of these individual units, perhaps by providing specific environments ("compartments") in which fibril assembly can occur (Birk and Trelstad, 1986).

In mature tissues, both collagen and elastin contain lysyl-oxidase derived crosslinks. Our experiments showed no influence of lysyl oxidase on the extractability of fibrillin. However, our data was obtained from embryonic tissue. It may be that fibrillin develops additional covalent crosslinks as the tissue matures.

Almost twenty years ago, Ross and Bornstein (1969) tried to solubilize elastic fiber microfibrils with a variety of reagents, examining the tissue residue through the electron microscope after each

extraction. They found that microfibrils were still visible after digestion with collagenase and after extraction with 5M guanidine hydrochloride. Reducing agent in guanidine appeared to remove the microfibrils from the elastic fiber core. Our data that fibrillin becomes insoluble through intermolecular disulfide crosslinking is consistent with this early finding.

Many attempts have been made to isolate microfibrillar protein components (Muir, Bornstein and Ross, 1976; Serafini-Fracassini, et. al., 1981; Gibson, et. al., 1986); most have followed the Ross and Bornstein procedure of removing collagen and other matrix proteins by subjecting tissues to collagenase and guanidine hydrochloride and then examining the proteins released by a final reductive guanidine extraction. We have modified this procedure for isolating fibrillin from tissues. Since the electrophoretic pattern for fibrillin was known and antibody reagents specific for fibrillin were available, we were able to examine a variety of extraction methods and found that fibrillin is easily degraded. Therefore, the extraction method used here is designed to avoid lengthy extractions, digestions, or dialysis. For our experiments, urea was used in place of guanidine hydrochloride.

Although fibrillin is easily degraded, it is a very large molecule, and it may possess a number of interesting and stable domains. The original immunogen used to prepare the fibrillin monoclonal antibodies was a sample of pepsin-digested human amnion. Monoclonal anti-fibrillin was then used to isolate and characterize a relatively stable pepsin-digested fragment of fibrillin (Maddox, B. K., Sakai, L. Y., and Glanville, R. W., manuscript in preparation). We have created additional fibrillin monoclonal antibodies which will be used to look

for other stable domains of fibrillin.

Whether or not there is any relationship between fibrillin and two small (34kD and 31kD) proteins characterized as elastic microfibrillar components (Serafini-Fracassini, et. al., 1981; Gibson, et. al., 1986) is not known. However, since fibrillin is larger and is easily degraded, the possibility exists that these proteins may be degradation products of fibrillin.

Together with previous studies of tissue distribution and periodic immunolocalization to specific microfibrils, the data contributed in this paper strongly support the suggestion that fibrillin is a constitutive structural element of connective tissue microfibrils.

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## FIGURE LEGENDS

Figure 1. SDS-PAGE (3-5% polyacrylamide gradient) of [ $^{14}\text{C}$ ]cystine-labeled proteins extracted from chick aortae which had been continuously pulsed for 17 hours. Lane 1: fibrillin marker immunoisolated from the medium of [ $^{35}\text{S}$ ]cysteine-labeled human fibroblasts; lane 2: PBS extract; lane 3: 1M NaCl extract; lane 4: 8M urea extract; lane 5: second 8M urea extract; lane 6: DTE extract. Single arrow marks the top of the stacking gel; double arrow indicates the beginning of the running gel.

1 2 3 4 5 6

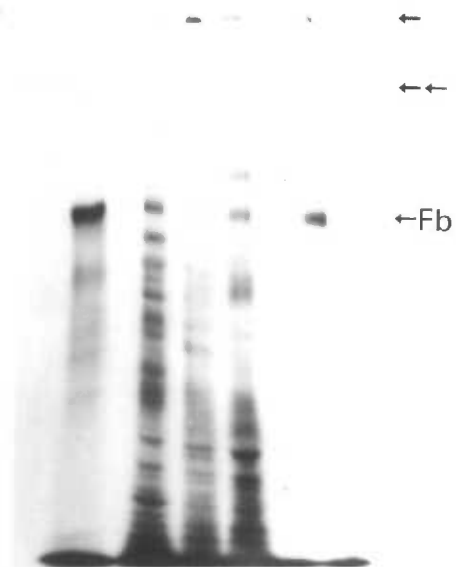


Figure 2. A. Immunoblot of PBS extracts (lanes 1, 2), 1M NaCl extract (lane 3), 8M urea extract (lane 4), DTE extract (lane 5), and human fibroblast medium (lanes 6, 7) with polyclonal anti-fibrillin.

B. Coomassie-stained gel of PBS extract (lane 1), 1M NaCl extract (lane 2), 8M urea extract (lane 3), DTE extract (lane 4), and human fibroblast medium proteins (lane 5). Fibrillin is pointed out with the arrow.

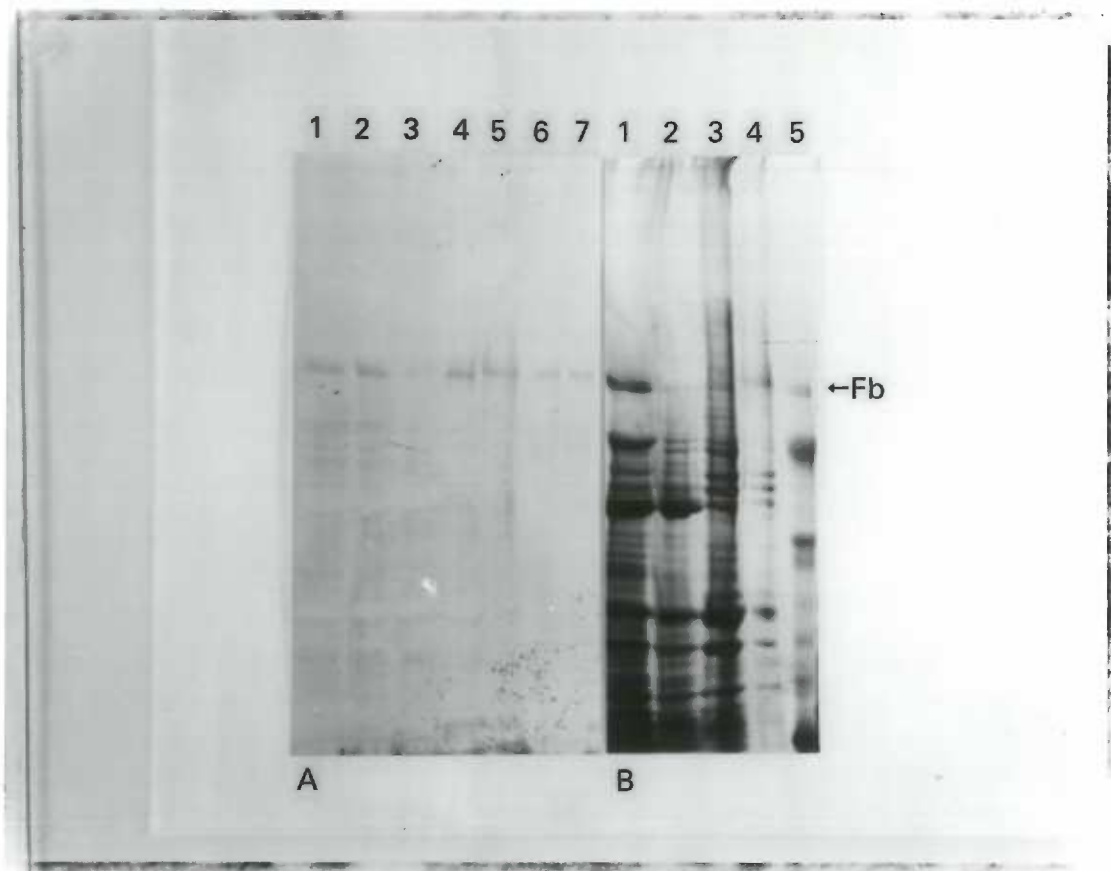
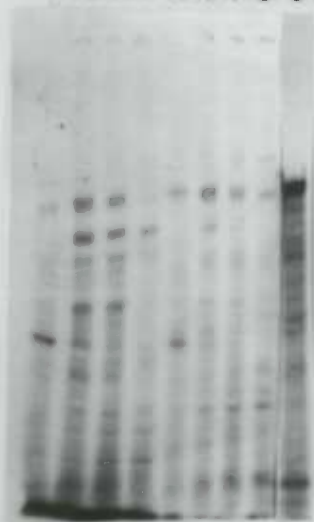


Figure 3. SDS-PAGE of [ $^{14}\text{C}$ ]cystine-labeled (pulsed for 30 minutes) proteins extracted from equal numbers of chick aortae after different periods of chase. Equal volumes of extract were applied to each channel. Lanes 1-4: PBS extracts after 0 minutes chase (1), 1 hour chase (2), 3 hours chase (3), and 19 hours chase (4). Lanes 5-8: 8M urea extracts after 0 minutes chase (5), 1 hour chase (6), 3 hours chase (7), and 19 hours chase (8). Lane 9: fibrillin marker from human fibroblast medium. Lanes 10-13: second 8M urea extracts after 0 minutes chase (10), 1 hour chase (11), 3 hours chase (12), and 19 hours chase (13). Lanes 14-17: DTE extracts after 0 minutes chase (14), 1 hour chase (15), 3 hours chase (16), and 19 hours chase (17).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



←Fb

Figure 4. A. Chromatographic profile of a PBS extract of chick aortae fractionated by molecular sieving on Superose 6. Single arrow indicates the elution position of monomeric fibrillin. Double arrows point to the elution position of multimeric fibrillin.

B. Chromatographic profile of an 8M urea extract of chick aortae fractionated by molecular sieving on Superose 6. Single arrow indicates the elution position of monomeric fibrillin; double arrows, multimeric fibrillin.

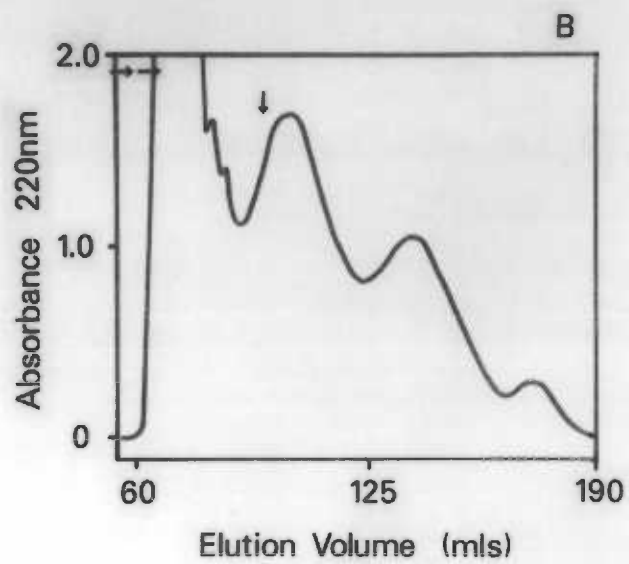
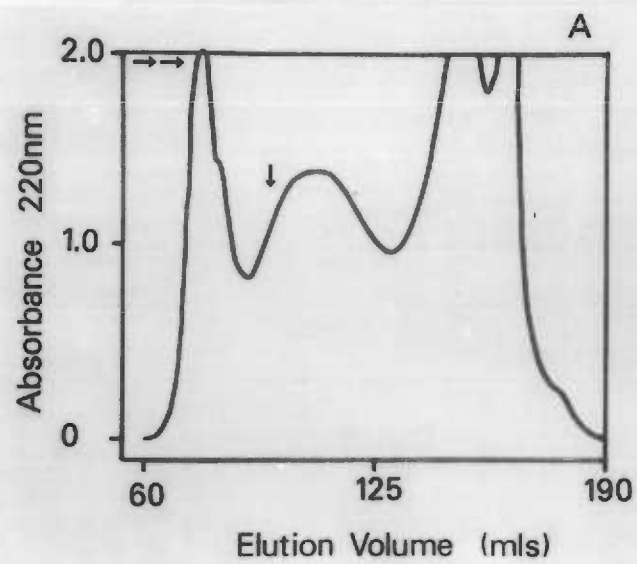


Figure 4. C. SDS-PAGE of sieve fractions selected from multimeric and monomeric fibrillin elution positions of pulse-chase extracts chromatographed on molecular sieve. Samples in lanes 1-8 do not include 2-mercaptoethanol (-), while samples in lanes 9-16 do have 2-mercaptoethanol (+). Lanes 1 and 9: nonreduced and reduced [ $^{35}\text{S}$ ]cysteine-labeled fibrillin marker from human fibroblasts. Lanes 2 and 10: "multimer" fraction of PBS extract chased for 0 minutes. Lanes 3 and 11: "monomer" fraction of PBS extract chased for 0 minutes. Lanes 4 and 12: "multimer" fraction of PBS extract chased for 4 hours. Lanes 5 and 13: "monomer" fraction of PBS extract chased for 4 hours. Lanes 6 and 14: "multimer" fraction of PBS extract chased for 21 hours. Lanes 7 and 15: "monomer" fraction of PBS extract chased for 21 hours. Lanes 8 and 16: "multimer" fraction of 8M urea extract chased for 4 hours.

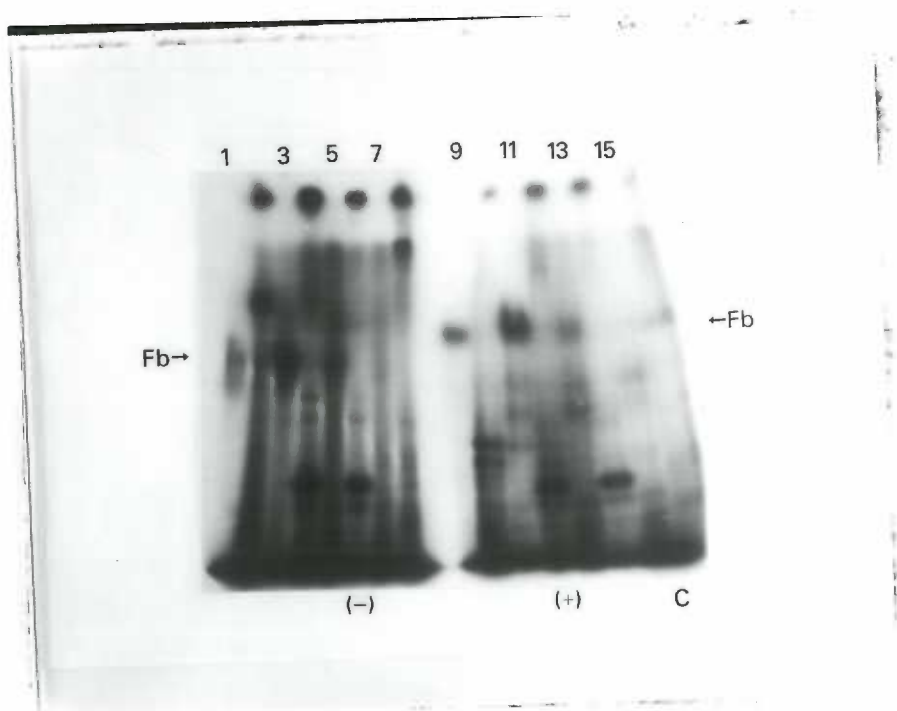


Figure 5. A. SDS-PAGE of immunoprecipitated fibrillin from 8M urea extracts of chick aortae pulsed for 1 hour with 250 $\mu$ Ci/ml [ $^{35}$ S]cysteine and chased for 5 hours. Lane 1: nonreduced (-2 mercaptoethanol) fibrillin immunoprecipitate; arrow indicates the position of monomeric fibrillin. Lane 2: reduced (+2 mercaptoethanol) fibrillin and fibronectin markers from human fibroblasts. Lane 3: reduced fibrillin immunoprecipitate.

B. 1.5% agarose gel of immunoprecipitated nonreduced fibrillin (lane 1) shown in 5A. The arrow indicates the position of fibrillin monomer. Lanes 2 and 3: nonreduced type IV collagen markers, with estimated molecular weights of 0.54 million kDa (monomer), 1.08 million kDa (dimer), 1.62 million kDa (trimer), and 2.16 million kDa (tetramer).

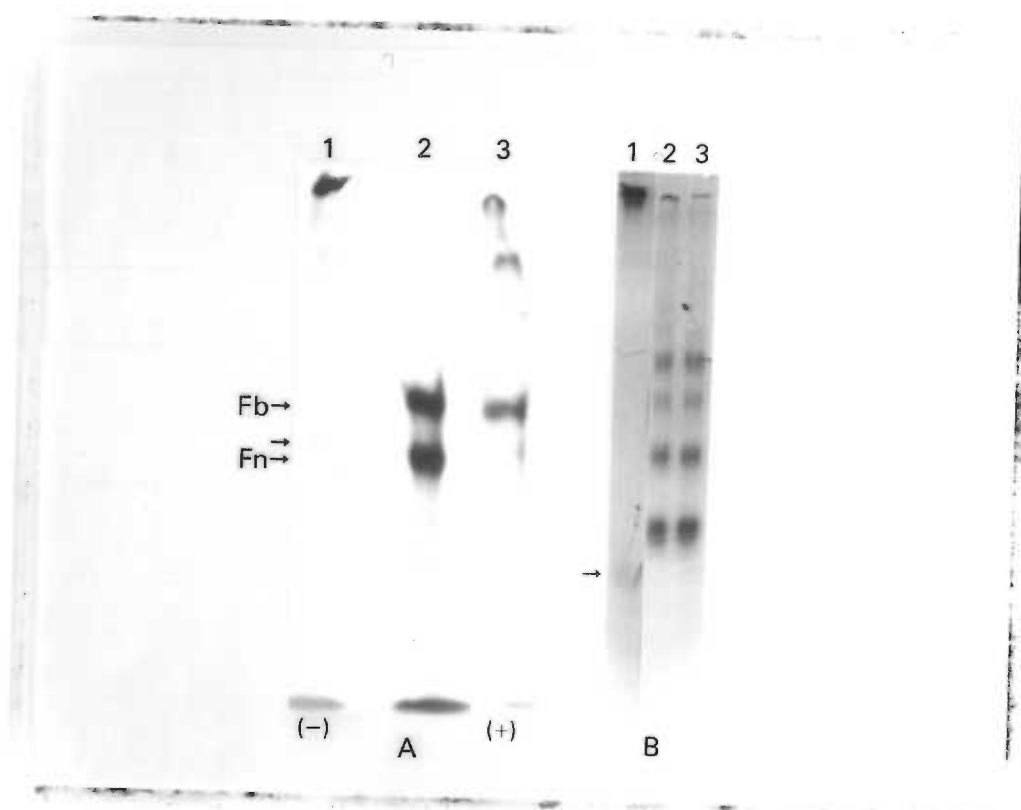
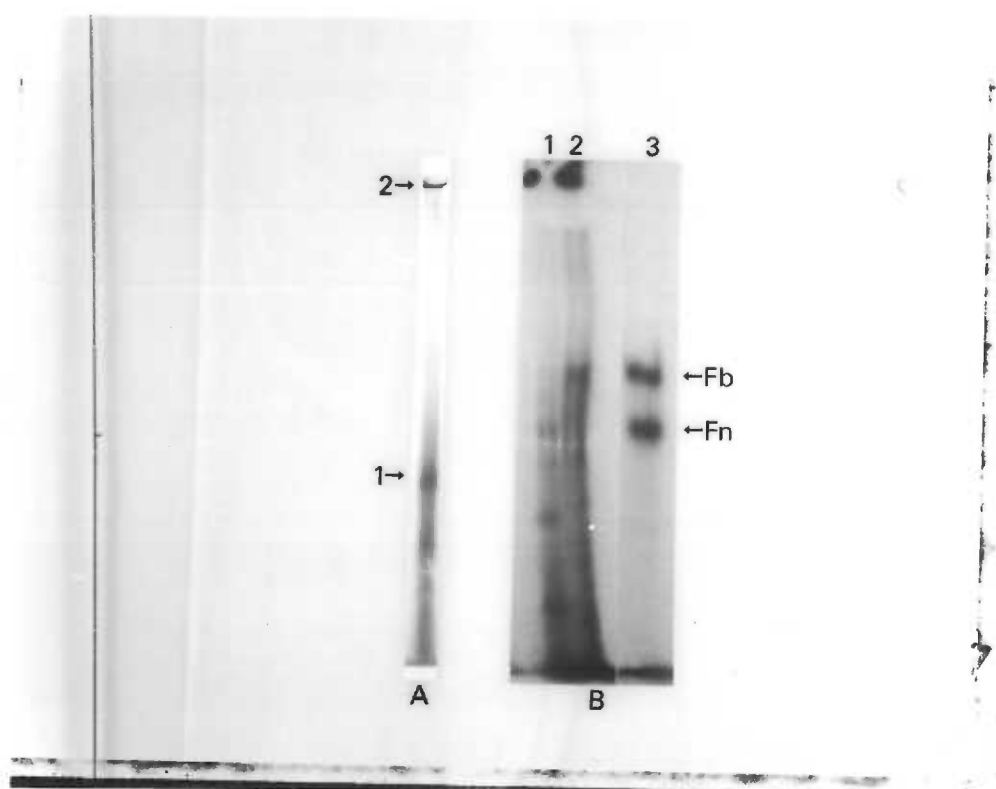


Figure 6. A. 1.5% agarose gel of 8M urea extract of chick aortae pulsed for 1 hour with 250 $\mu$ Ci/ml [<sup>35</sup>S]cysteine and chased for 5 hours. This sample was applied directly to the gel. Numbers 1 and 2 indicate the bands which were excised, solubilized in boiling sample buffer with 2 mercaptoethanol, and run on SDS-PAGE.

B. SDS-PAGE of excised agarose gel bands. Lane 1: band 1. Lane 2: band 2. Lane 3: fibrillin and fibronectin markers from human fibroblasts.



VITRONECTIN IS LOCATED WITH FIBRILLIN  
IN SOME CONNECTIVE TISSUES, BUT NOT IN OTHERS

Condensed Title: immunolocalization of vitronectin and fibrillin

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## ABSTRACT

Vitronectin, a multifunctional blood protein which is also a component of connective tissue, is here specifically localized by immunoelectron microscopy, with a monoclonal antibody, to certain connective tissue microfibrils, often referred to as elastic fiber microfibrils or as "oxytalan" fibrils. The immunolocalization demonstrates a periodic distribution of vitronectin along the individual microfibril. We have previously shown (Sakai, Keene, and Engvall, J. Cell Biol. 103:2499-2509) that a new connective tissue protein, fibrillin, is a constituent of these microfibrils. Immunofluorescence studies of the distribution of these two proteins in various tissues demonstrates that vitronectin and fibrillin often colocalize, as would be expected based on the immunoelectron microscopic data. However, vitronectin and fibrillin do not always appear to be present together in the same tissues. This data suggests that vitronectin is probably not a structural constituent of fibrillin-containing microfibrils. Nevertheless, vitronectin appears to interact very specifically with these microfibrils.

Vitronectin is a serum and plasma protein which was first isolated on glass beads and which was shown to promote the in vitro attachment and spreading of cells (Holmes, 1967). It was originally referred to as "serum spreading factor" (Barnes, et. al., 1980). Vitronectin is composed of two polypeptides with molecular weights of 75 and 65 kDa (Hayman, et. al., 1983), although the molecular weight calculated from the amino acid residues deduced from cDNA is about 52 kDa (Suzuki, et. al., 1985). Based on sequence data, vitronectin is identical to S-protein (Jenne and Stanley, 1985), which forms complexes with terminal complement components to inhibit membrane insertion of C5b-7 (Podack and Muller-Eberhard, 1978) and participates in the coagulation pathway by forming complexes with thrombin and antithrombin III (Podack, Dahlback and Griffin, 1986). Vitronectin is a multidomain protein with an amino-terminal domain which is identical to a 44 residue serum peptide called somatomedin B, an RGD cell attachment domain, and a heparin-binding domain (Suzuki, et. al., 1984).

This multifunctional blood protein has been shown to be a product of human fibroblasts in cell culture and was immunolocalized by light microscopy to some examples of loose connective tissue (Hayman, et. al., 1983). Vitronectin has been additionally immunolocalized to the elastic fibers of human dermis as well as to the thin fibers of the papillary dermis (Dahlback, Lofberg, and Dahlback, 1986) and to blood vessel walls and amyloid deposits in kidneys (Dahlback, Lofberg, and Dahlback, 1987).

Fibrillin is a component of connective tissue microfibrils, which include the elastic fiber microfibrils as well as other ubiquitous microfibrils like the oxytalan fibrils of the papillary dermis and the microfibrils of the ciliary zonule and glomerular basement membrane

(Sakai, Keene, and Engvall, 1986). It has been characterized as a very large (350 kDa) noncollagenous glycoprotein which is secreted into the medium of human fibroblasts in cell culture. Electron microscopic immunolocalization of fibrillin reveals periodic labeling along individual microfibrils. Based upon its wide tissue distribution, its periodic immunolabeling, and its assembly into disulfide-crosslinked multimers which stabilizes it in the connective tissue (Sakai, Morris, and Bachinger, manuscript submitted for publication), fibrillin is thought to be a constitutive structural element of connective tissue microfibrils.

We have compared the distribution of vitronectin and fibrillin in a number of different connective tissues, and we have immunolocalized vitronectin through the electron microscope.

## MATERIALS AND METHODS

### Proteins and Antibodies

Vitronectin was purified by affinity chromatography on glass beads and fractionation by FPLC on a Mono Q column (Pharmacia), an anion exchanger, as described (Ruoslahti, et. al., 1987). Monoclonal and polyclonal antibodies specific for vitronectin were prepared and characterized as described (Hayman, et. al., 1983).

Fibrillin was obtained from 50% saturated ammonium sulfate precipitates of medium from human fibroblasts, which was made 0.001M N-ethylmaleimide, 0.004M ethylenediaminetetraacetic acid, and 0.001M phenylmethylsulfonyl fluoride at the time of collection. Medium was collected and precipitated on ice. Pellets were resuspended in 0.05M Tris-HCl, 2M urea, pH 8.3, treated with 0.40% diisopropyl fluorophosphate, and dialyzed overnight against the same buffer. Fibrillin was then partially purified by fractionation on HPLC DEAE 5PW (Waters), chromatography on gelatin-Sepharose (Pharmacia) to remove most of the fibronectin, and FPLC Superose 6 (Pharmacia) molecular sieve chromatography. Monoclonal anti-fibrillin was prepared and characterized as previously described (Sakai, Keene, and Engvall, 1986).

### Immunofluorescence

Tissues were obtained from two human fetuses (24 and 30 weeks gestation) soon after death, frozen in hexane, and stored at  $-90^{\circ}\text{C}$  until use. Tissue from a 17 year old was obtained at surgery and was

similarly frozen. Blocks were mounted in O.C.T. Compound (Miles) and frozen in liquid N<sub>2</sub>. Approximately 8  $\mu$ m sections were cut at -28<sup>o</sup> C on an A0 Histostat microtome and were picked up on glass slides. Sections were air dried under a fan for 30 minutes and were fixed in cold acetone for 10 minutes. After washing in phosphate buffered saline (PBS), primary antibodies (vitronectin and fibrillin ascites antibodies diluted 1:100 in PBS) were applied to the sections for 3 hours at room temperature in a humidified chamber. After washing away unbound antibodies, fluorescein conjugated anti-mouse IgG (Sigma), diluted 1:50, was incubated on the sections for 30 minutes. Sections were washed again in PBS, covered with 90% glycerol in PBS, and viewed with a Zeiss Photomicroscope III. Cartilage sections were also digested with hyaluronidase (Worthington Biochemicals), 8,000 U/ml of 0.1M phosphate buffer, pH 5.3, for 20 minutes at room temperature.

#### Electron Microscopy

Tissues were obtained from human neonatal foreskin obtained at the time of circumcision and were prepared for immunoelectron microscopy as described (Sakai, Keene, and Engvall, 1986). Antibody incubations were performed on the freshly obtained tissue blocks prior to fixation and embedding. Samples were viewed with a Philips 410LS transmission electron microscope.

#### Other Immunochemical Tests

Enzyme-linked immunosorbent assays (ELISA) were performed as

described (Sakai, et. al., 1986). Immunoblotting procedures were also as described (Sakai, Keene, and Engvall, 1986).

## RESULTS

The specificities of monoclonal and polyclonal antibodies to vitronectin have been previously characterized (Hayman, et. al., 1983). Monoclonal anti-fibrillin has also been described (Sakai, Keene, and Engvall, 1986). Since the cDNA sequence of vitronectin includes a 61 bp untranslated region at the 5' end and 109 bases containing the polyadenylation signal at the 3' end (Suzuki, et. al., 1985; Jenne and Stanley, 1985), vitronectin is probably not structurally related to fibrillin. However, since the antibodies used in these tissue studies could have contained contaminating antibodies, they were tested for crossreactivity. The vitronectin antibodies showed no crossreactivity with fibrillin when tested either in ELISA or in immunoblotting experiments, and anti-fibrillin showed no crossreactivity with vitronectin in ELISA (data not shown).

### Colocalization in Skin

The immunofluorescence patterns of monoclonal anti-vitronectin and monoclonal anti-fibrillin in human neonatal foreskin were almost identical (Figure 1a,b).

Immunoelectron microscopy demonstrated periodic binding of monoclonal anti-vitronectin along the lengths of both elastic fiber microfibrils and papillary microfibrils, which are not associated with the amorphous (elastin) component (Figure 2).

### Distribution in Other Tissues

Vitronectin and fibrillin appeared to codistribute in some tissues but not in others. In the annulus fibrosus (fibrocartilage) of the intervertebral disc, staining for the two proteins revealed similar fibrillar arrays, which were somewhat brighter if the tissue was digested with hyaluronidase before incubation with antibody (Figure 2b,d). However, in the elastic cartilage of the epiglottis, fine fibrils were seen within the hyaluronidase-digested cartilage matrix in sections incubated with anti-fibrillin (Figure 2a), but these were not visible in sections stained with anti-vitronectin (Figure 2c). Perichondrium was stained by both antibodies and showed no effect from the hyaluronidase digestion (Figure 2a,c).

A difference in immunofluorescence localization was also obtained in muscle sections. Skeletal muscle excised from the fetuses were well stained by anti-fibrillin both in the perimysium, including the walls of blood vessels, and in the endomysium (Figure 3b). However, vitronectin appeared to be present primarily in the walls of blood vessels of the perimysium; vitronectin immunofluorescence in the endomysium was only faint (Figure 3a). The same results were obtained when skeletal muscle from a 17 year old individual was examined (Figure 3c,d). When polyclonal anti-vitronectin was used, the staining pattern was the same (data not shown). Fetal skin, stained at the same time with the two antibodies showed no difference in immunofluorescence (Figure 3g,h), demonstrating that poor reactivity of anti-vitronectin was not due to antibody titer. In addition, cardiac muscle contained both fibrillin and vitronectin in the intercellular spaces (Figure 3e,f).

Similar data was demonstrated in nerves of the brachial plexus. The connective tissue investment of the nerve fascicles, the perineurium, was brightly stained with both anti-fibrillin and anti-vitronectin (Figure 4a,b). However, while fibrillin was evident in the delicate connective tissue surrounding each individual nerve fiber, the endoneurium (Figure 4a), anti-vitronectin immunofluorescence was weak in that region (Figure 4b).

We also examined the spinal cord and found vitronectin staining in the meninges and in the blood vessels of the spinal cord (Figure 4c,d). Fibrillin immunofluorescence of this tissue was identical (data not shown). Identical patterns in the cornea were also found with the two antibodies (data not shown).

## DISCUSSION

Vitronectin, a multifunctional protein found in large amounts in the blood, is also present in the connective tissue. Since vitronectin was first described in muscle, lung, chorion, skin, and kidney (Hayman, et. al., 1983), knowledge of its distribution in the connective tissue has not been substantially extended. We have documented here the specific immunoelectron microscopic localization of vitronectin to special connective tissue microfibrils, which have been shown to contain fibrillin (Sakai, Keene, and Engvall, 1986).

In addition, we have extended the distribution of vitronectin and fibrillin to connective tissues not previously examined. Vitronectin and fibrillin were shown to codistribute in fibrocartilage, in perichondrium, in blood vessel walls, in the meninges, and in the perimysium and perineurium. However, vitronectin does not appear to be present in all the tissues where we can find fibrillin. It was not evident in the matrix of elastic cartilage nor in the delicate connective tissues between individual muscle and nerve fibers, even though fibrillin was clearly demonstrated in these regions.

It is possible that vitronectin is equally present in these regions, but masked to our procedures, since only one epitope was available for binding by the monoclonal antibody. To check this possibility, we also tested vitronectin polyclonal antisera, which yielded the same results as monoclonal anti-vitronectin. Still, it may be that vitronectin is masked in these regions in a way that does not mask the binding of our antibody to fibrillin.

Alternatively, vitronectin may not be a structural constituent of these fibrillin-containing microfibrils. While vitronectin is secreted by connective tissue cells in culture, fibroblasts, and it has been extracted from tissue (Hayman, et. al, 1983), detailed biochemical study of its form in the tissue has not been performed. On the other hand, the intermolecular disulfide crosslinking of fibrillin into insoluble multimers in the tissue has been demonstrated in pulse-chase experiments, suggesting that fibrillin is a constitutive structural element of microfibrils (Sakai, Morris, and Bächinger, manuscript submitted for publication).

If vitronectin is not a structural component of microfibrils, the immunoelectron microscopical localization suggests that there is a very specific interaction between vitronectin and microfibrils. Since vitronectin has so many known biological roles (it promotes cell adhesion, and it participates in the complement and coagulation pathways), it seems likely that vitronectin may serve an important, although yet unknown, function in its interaction with microfibrils. It may be interesting to speculate that, since the RGD cell attachment site in vitronectin immediately follows the somatomedin B sequence in the molecule (Suzuki, et. al., 1985), vitronectin may bind to a vitronectin cell surface receptor (Pytela, Pierschbacher, and Ruoslahti, 1985) and communicate some growth-factor-like message to the cell via the adjacent somatomedin B sequence. The function of somatomedin B, which is cleaved from vitronectin in the blood, interestingly splitting the RGD sequence, is unknown. Laminin has recently been shown to have an epidermal growth factor like sequence contained within its chains. Perhaps, the extracellular matrix carries within itself special messages to regulate

cell behavior.

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## FIGURE LEGENDS

Figure 1. Indirect immunofluorescence of human neonatal foreskin with (a) monoclonal anti-vitronectin and (b) monoclonal anti-fibrillin. PBS control section (c) shows no autofluorescence or background binding of the second antibody to the dermal connective tissue. Magnification is 250x.

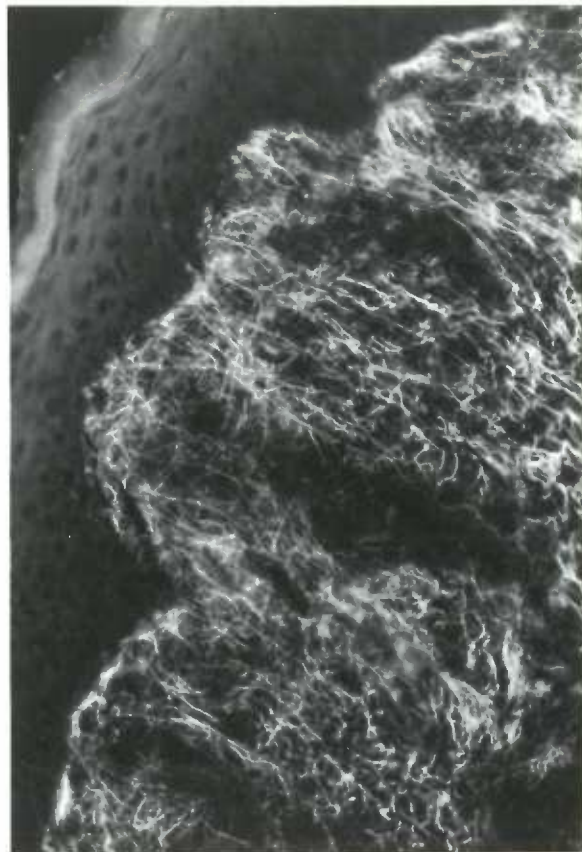
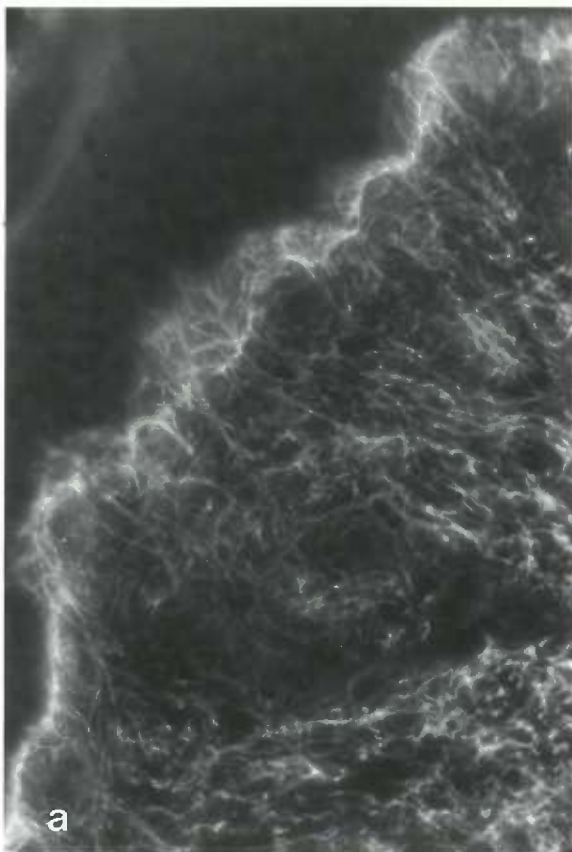


Figure 2. Immunolectron microscopical localization of monoclonal anti-vitronectin in human neonatal foreskin demonstrating periodic colloidal gold deposition along the microfibrils.

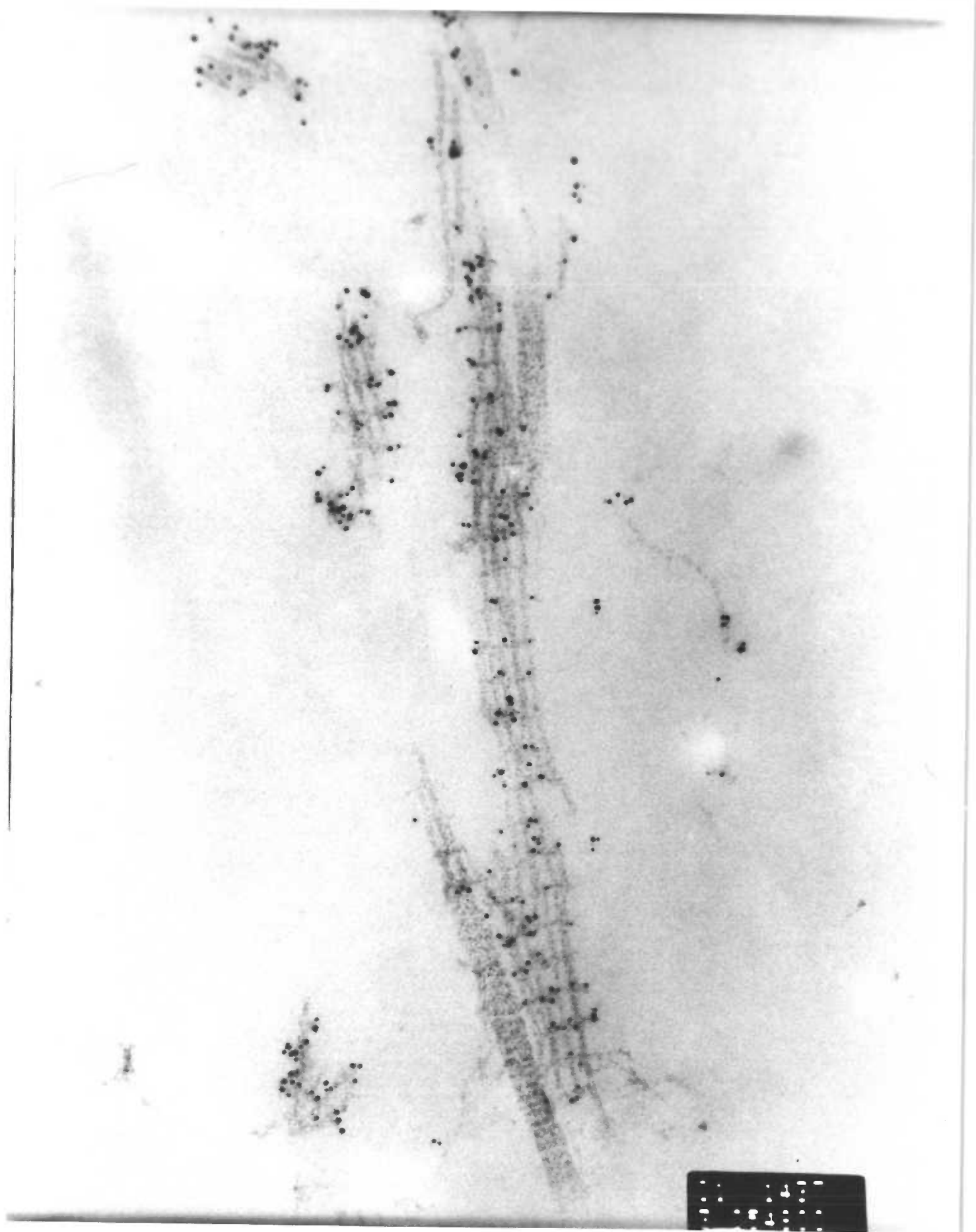


Figure 3. Indirect immunofluorescence of human elastic cartilage and perichondrium (digested with hyaluronidase) (a,c,e) and of anulus fibrosis (digested with hyaluronidase) (b,d,f). Vitronectin staining is shown in c and d; fibrillin staining in a and b. PBS control sections (e,f) show very little background. Magnification is 200x.

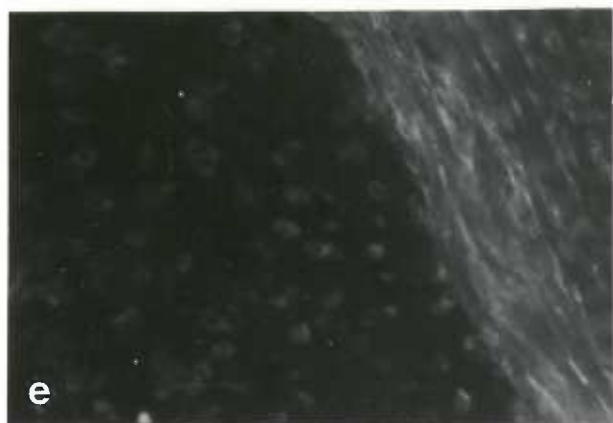
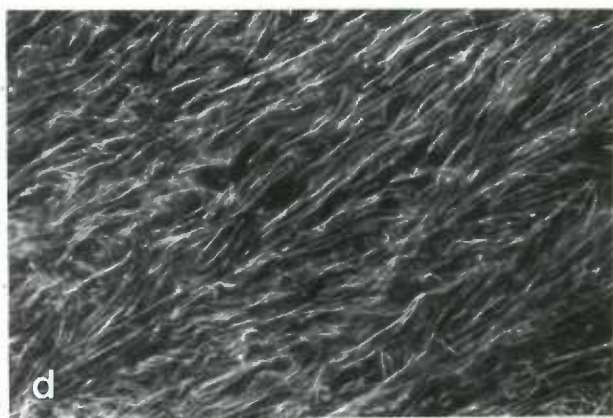
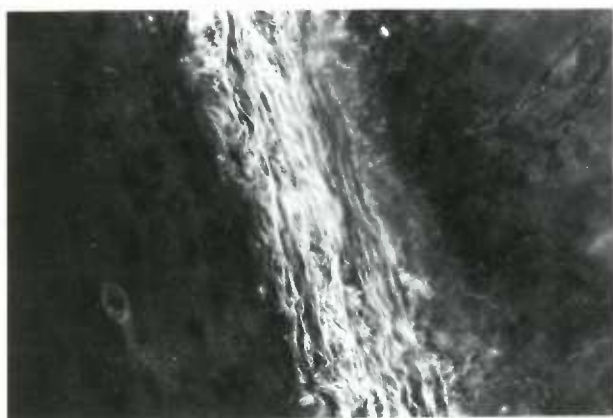
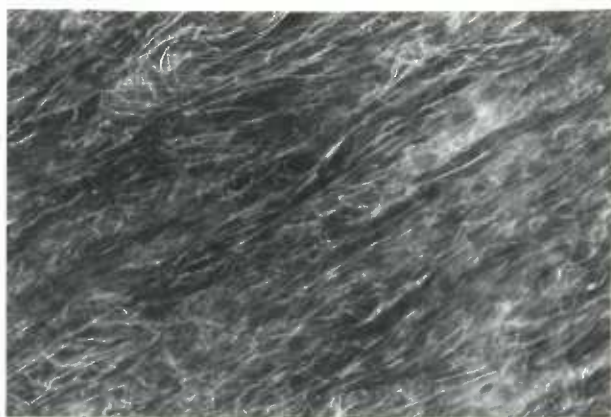
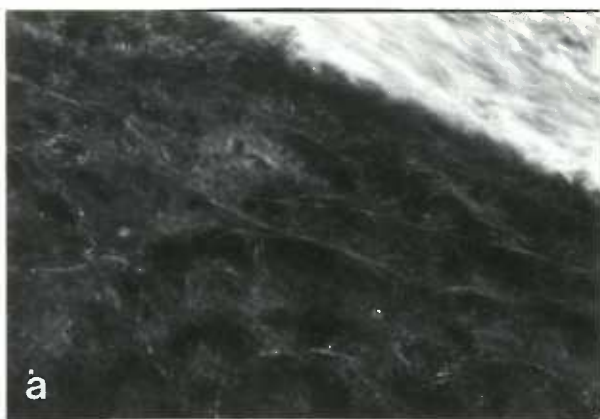


Figure 4. Indirect immunofluorescence of human fetal skeletal muscle (a,b), of human 17 year old skeletal muscle (c,d), of fetal cardiac muscle (e,f) and of fetal skin (g,h). Monoclonal anti-vitronectin was used in a, c, e, and g. Monoclonal anti-fibrillin was used in b, d, f, and h. Magnification is 200x, except in d, which is 128x.

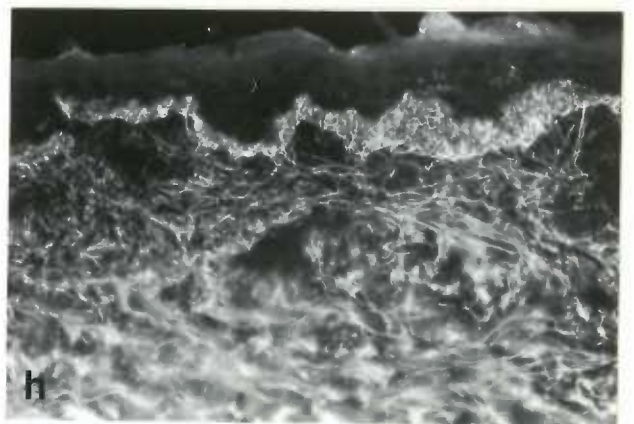
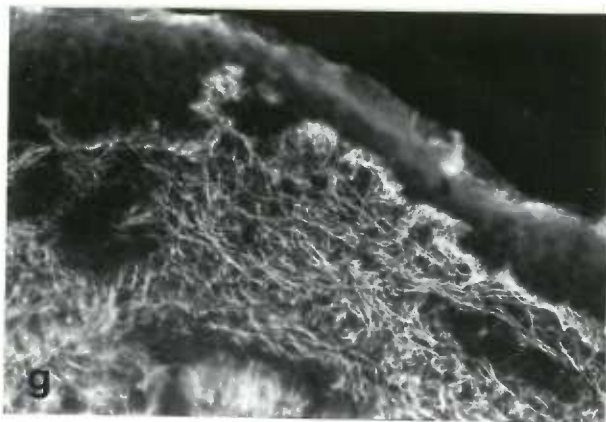
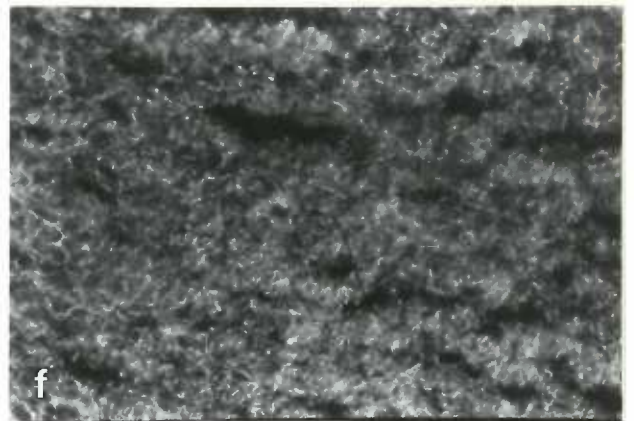
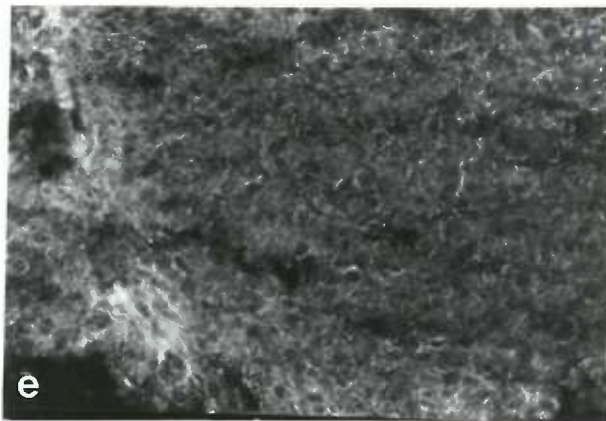
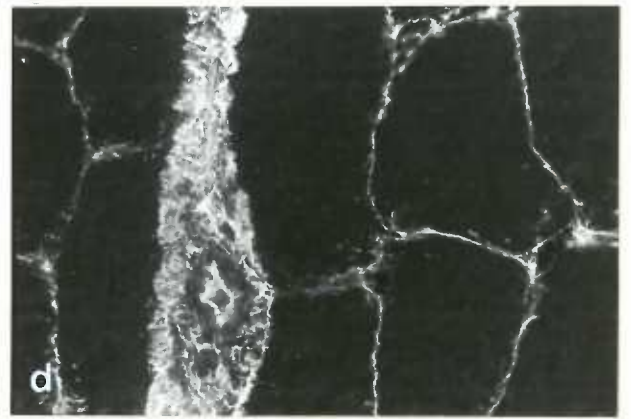
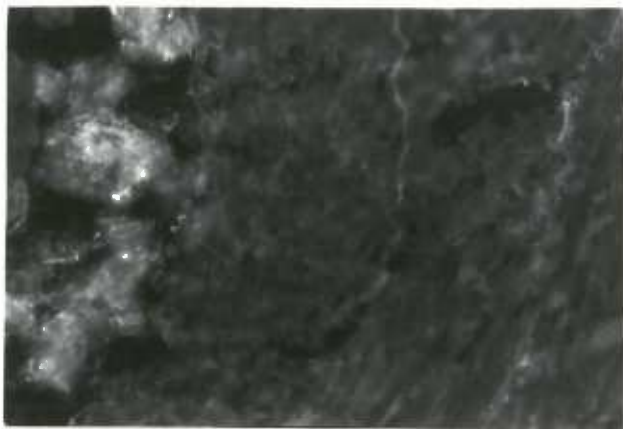
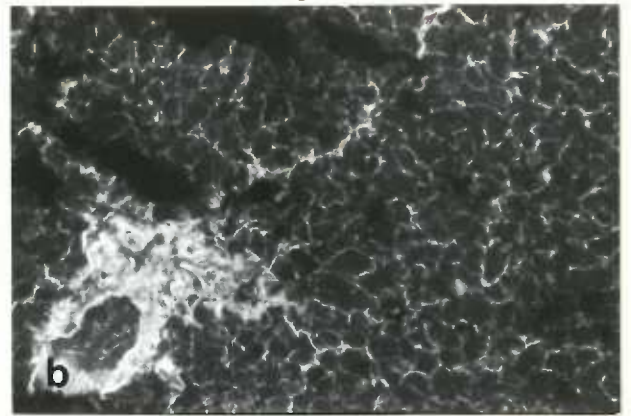
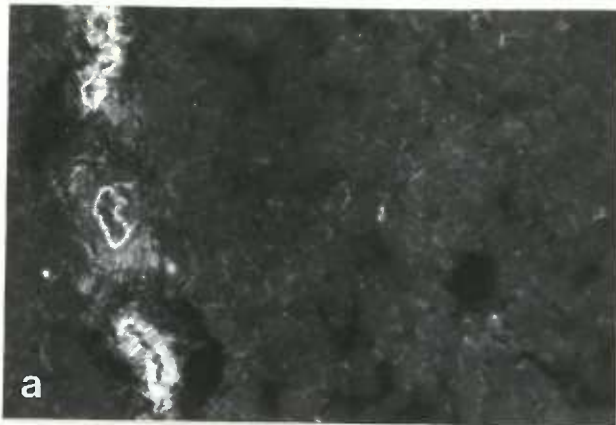
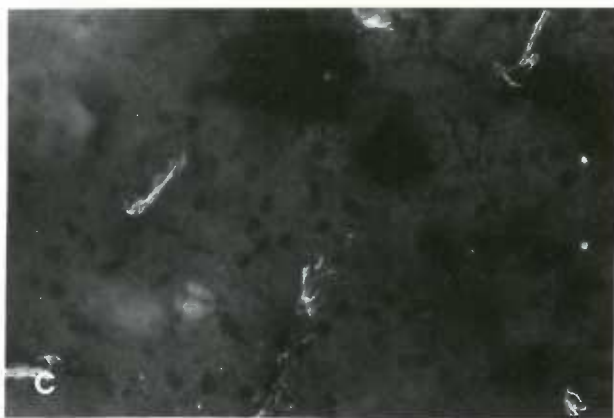
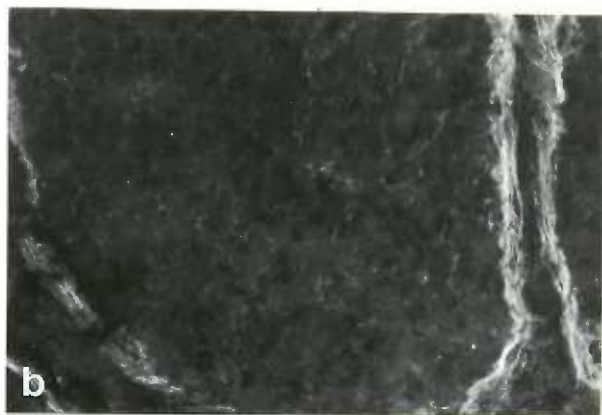
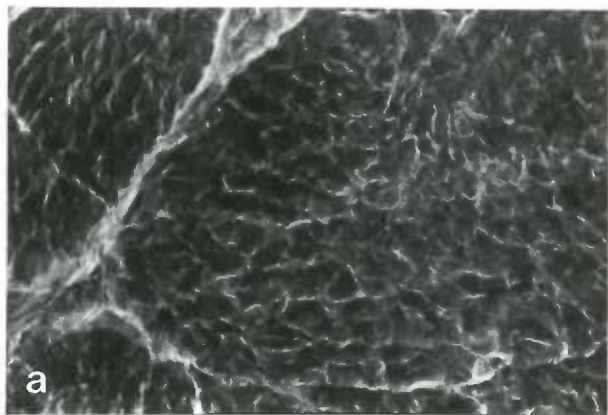


Figure 5. Indirect immunofluorescence of human fetal nerve (a,b), spinal cord matrix (c) and meninges surrounding the spinal cord (d). Monoclonal anti-vitronectin was used in b, c, and d. Monoclonal anti-fibrillin was used in a. Magnification is 200x.



## DISCUSSION

### CONCLUSIONS

The data in the manuscripts presented here support the hypothesis that fibrillin is a constitutive structural element of certain morphologically identifiable connective tissue microfibrils. Monoclonal antibodies, which bind specifically to these microfibrils, have been produced and characterized. They have been used to isolate a new protein, fibrillin, from the medium of cultured human fibroblasts. Using immunoaffinity and immunoblotting techniques to guide the biochemical procedures, fibrillin was extracted from embryonic chick aortae. It was found that, in this tissue, fibrillin is first secreted as a monomer (identifiable by immunoprecipitation and by a unique electrophoretic pattern) which then becomes disulfide bonded into multimers within 1-2 hours. With time, this process of intermolecular disulfide crosslinking rendered fibrillin resistant to solubilization except by reducing agent. Fibrillin appeared to be the only large, abundant disulfide crosslinked protein which could be finally released from the tissues with reducing agent. This data confirms an early suggestion (Ross and Bornstein, 1969) that microfibrillar components are resistant to extraction, except by disulfide bond reducing agent.

Additional microscopic evidence was presented which also supports the hypothesis that fibrillin is a constitutive structural element of microfibrils. Fibrillin was immunolocalized to the individual microfibril and to microfibrillar bundles, and the labeling was periodic, implying that the arrangement of fibrillin along the

microfibril is ordered. The tissue distribution of fibrillin was found to be broader than that which would be expected for elastic fiber microfibrils. Specifically, there was localization of fibrillin to the microfibrils of the ciliary zonule and of the papillary dermis, which are the classic examples of microfibrils without amorphous elastin cores. This was the first clear demonstration that these microfibrils and elastic fiber microfibrils, in which fibrillin was also specifically identified, are identical, with respect to at least one protein constituent. Fibrillin has been found in every tissue for which there is ultrastructural evidence of microfibrils, as would be expected for a constitutive structural element. On the other hand, amyloid P component, which has been proposed to be a component of microfibrils (Inoue, et. al., 1986b), has not been.

There may be additional structural constituents of fibrillin microfibrils. Based upon a report that vitronectin could be immunolocalized to microfibrils in the skin (Dahlback, Lofberg, and Dahlback, 1986), I obtained, from Drs. Erkki Ruoslahti and Eva Engvall, a monoclonal antibody specific for vitronectin, and I conducted a tissue survey. While vitronectin was localized by immunoelectron microscopy to microfibrils, and even though this labeling was periodic, vitronectin was not present in the tissue everywhere there are fibrillin microfibrils. It is possible that vitronectin, for some, as yet unknown, reason, binds to these microfibrils, but is not a structural constituent of the microfibril itself. It is interesting to bear in mind that both vitronectin and amyloid P component are blood proteins. Perhaps, fibrillin microfibrils serve as a scaffold for the deposition of these other proteins which serve important yet currently unknown

biological functions in the connective tissue, or in mediating the interaction between the connective tissue and the cell or cellular processes.

The relationship between fibrillin and two small (around 30 k) proteins isolated from "microfibrillar protein" extracts (Serafini-Fracassini, et. al., 1981; Gibson, et. al., 1986) is not known. They may be degradation products of fibrillin. On the other hand, they may be unrelated to fibrillin. Whether or not they are structural constituents of microfibrils is also not known.

Fibrillin is the first noncollagenous glycoprotein which (probably) assembles into a connective tissue fibril system. Other glycoproteins, like fibronectin and laminin, have been identified within the extracellular matrix, but they do not form obvious and distinctive fibrillar structures in the connective tissue space. Moreover, fibrillin microfibrils are major structural elements: it was surprising how ubiquitous they are, and yet how specially oriented they seem to be in the various tissues. Fibrillin microfibrils compose the major structural element of the ciliary zonule, the suspensory ligament of the lens; at the same time, they encircle the elastic fiber, and they form the fine structures bridging basement membranes and subjacent stroma (for example, in kidney glomerulus and lung). Thus, while fibrillin microfibrils appear to be structurally similar, they must serve multiple different structural functions. Since fibrillin is a very large molecule, it may possess the different domains necessary to provide the versatility required by the different tissues. Whether fibrillin self-assembles, utilizing disulfide crosslinks, or whether it requires the presence of other components for assembly will be an interesting

question for future investigations. In addition to its significance to connective tissue structural biochemistry, the discovery of fibrillin may impact, over the years, our knowledge of developmental processes (microfibrils appear very early in avian embryology, coinciding with the first mesenchymal cell migrations (Mayer, Hay, and Hynes, 1981)), of cell-matrix interactions and of processes like wound healing, and of the abnormal processes involved in heritable disorders of connective tissue.

#### **CURRENT PROBLEMS**

Two years ago I intended to perform experiments which would contribute to an understanding of how fibrillin might assemble into microfibrils and how it may interact with other proteins. Fibrillin is a very large protein, and if the suggestions that fibrillin microfibrils are diverse in structural function, which are implied from tissue morphology, are correct, there must be domains within fibrillin which not only interact with other fibrillin domains to form disulfide bonds and otherwise self-assemble, but also domains which interact with elastin, perhaps with basement membrane proteins, and with proteins like vitronectin. Perhaps fibrillin may have a specific cell binding domain. In order to begin to investigate these possibilities, fibrillin must be purified, and structural domains of fibrillin must be identified. I have discovered that this is a very difficult task. I have foundered upon the rock of protein purification. And, I have come to appreciate my friend, Nick Morris' encouragement that "protein purification is its own reward." Still, the interesting questions of assembly and protein-protein interaction can only be answered if proteins can be

purified.

I chose to obtain starting materials from the serum-free cell culture medium of human fibroblasts. Of all the high molecular weight proteins in this medium, fibronectin is the most abundant, and relative to fibronectin, fibrillin staining on gels appears to be about 1%. By substantially scaling up cell culture efforts, I think the amount of fibrillin in the pooled starting materials is enough to do biochemical studies.

The medium is collected into containers on ice and protease inhibitors (0.001M N-ethylmaleimide, 0.004M EDTA, and 0.001M phenylmethylsulfonyl fluoride) are added. Proteins are precipitated by the addition of 50% saturated ammonium sulfate. After centrifugation, pellets are resuspended in 0.05M Tris-HCl, 0.5M NaCl, pH 7.5, including protease inhibitors, and stored frozen until pooled. Samples are thawed and pooled into a container on ice, and 0.40% diisopropyl fluorophosphate is added. Samples are dialyzed in the cold against 0.05M Tris-HCl, 2M urea, pH 8.3, containing EDTA.

These medium samples are extremely heterogeneous. Visualized on SDS-PAGE, the bands are numerous and not well separated. This material is first fractionated on HPLC DEAE (Waters 5PW, which is a 21.5mm x 15cm preparative column) using a flow rate of 5 mls per minute and a linear gradient of 0 to 0.3M NaCl in 0.05M Tris-HCl, 2M urea, pH 8.3. Fractions are collected, and gels of these fractions are run with and without reducing agent. Immunoblotting of the nonreduced fractions determines the elution position of fibrillin, which, with these conditions, begins at approximately 0.2M NaCl. At first, this column was tried using 0.05M Tris-HCl, 2M urea, pH 8.0. In fractions

containing fibrillin (by immunoblotting), there were two bands which ran more slowly than fibronectin, after reduction (Appendix, figure 1). The identity of the top band as fibrillin was confirmed by passing fibrillin DEAE fractions over two molecular sieve FPLC Superose 6 HR 16/50 (Pharmacia) columns in tandem and checking the elution fractions by immunoblotting. The faster migrating band in figure 1 eluted slightly before fibrillin in the molecular sieve run. This band was the only major contaminant of fibrillin after sieving (Appendix, figure 2). By raising the pH to 8.3, fibrillin stuck a little tighter to the DEAE and eluted with the fibronectin peak (Appendix, figure 3), thus separating fibrillin from the contaminating band which was difficult to separate from fibrillin by molecular sieve chromatography.

However, I traded one problem for another. I thought that I could remove fibronectin on gelatin Sepharose (Pharmacia), but the DEAE fractions had 2M urea in them, which would prevent fibronectin from binding to gelatin. I tried dialyzing into a buffer without urea, and I lost a lot of the fibrillin, probably due to degradation. I tried diluting the sample so that fibronectin would absorb to the column. Then I had a relatively clean, but dilute sample. I tried salt precipitating the sample and sieving it; I succeeded only in plugging the column filter. I tried removing the fibronectin on gelatin Sepharose before running the DEAE column and got mostly degradation products of fibrillin. I have tried running samples through the DEAE column, through the Superose column, diluting the sample to pass over gelatin Sepharose, and concentrating fibrillin on FPLC Mono Q, another anion exchanger. Everything looked good until the final step: as the fibrillin peak was just coming off, the back pressure went way up.

Either the salt concentration, or the eluting protein concentration, had possibly caused the protein to precipitate on the column. I have tried a different column, a chromatofocusing column, and my sample stuck to it as well.

The best results I have had were from a year ago when there were fractions from the Superose column which were relatively clean, as judged by silver staining. This sample was prepared by diluting DEAE fractions which contained fibrillin and fibronectin (Appendix, figure 3) in order to bind fibronectin to gelatin Sepharose. The dilute unbound material was concentrated on concanavalin A Sepharose (Pharmacia) and eluted with 0.5M alpha-D-methylglucoside and then with 0.5M alpha-D-methylmannoside (Appendix, figure 4). This material was checked by immunoblotting with monoclonal anti-fibrillin. The eluted material, which appeared to contain little, if any, fibronectin, or other high molecular weight contaminants, was then applied to the molecular sieve column. Fibrillin fractions from the sieve column were, however, extremely dilute. Efforts to concentrate them by vacuum dialysis failed totally. I did, however, have enough material for rotary shadowing. This material looked a lot like fibronectin. However, in ELISA, there was a very good reaction with anti-fibrillin, but no reaction with polyclonal anti-fibronectin. Still, it is possible that the molecules visualized by the shadowing were fibronectin and not fibrillin. I have tried to couple monoclonal antibody to these molecules and have found some preliminary binding. But, these events might still be random binding on fibronectin, not fibrillin, molecules. Gaining good visual images of fibrillin will depend on purifying larger amounts of fibrillin.

Assuming that good visual images will be obtained some day, I would like to know if the appearance of sprayed and shadowed fibrillin corresponds to its physical shape in solution. To this end, I have performed a few sedimentation experiments of radiolabeled fibrillin (Appendix, figure 5). Sedimentation was through a 5-20% sucrose gradient for 16 hours, at 45,000 rpms, 4<sup>0</sup> C, in a Beckman L8-80 ultracentrifuge, using an SW60 rotor. Sucrose gradients were dripped, using a specially calibrated dripper, and counted, and fractions were analyzed by SDS-PAGE. Sedimentation coefficients were calculated according to measurements originally reported by Nick Morris (Phd thesis, 1977). The estimated sedimentation coefficient for fibrillin is around 6.1. If fibrillin is a prolate or rod-shaped molecule, as the rotary shadowing suggests, it should have a length of around 130nm, assuming values for molecular weight (350,000), partial specific volume (0.704 ml/g), degree of hydration (0.1-0.2 ml/g), and a sedimentation coefficient of 6.1.

I have also tried to purify fibrillin from samples of chick material, and I have had similar problems. The largest obstacle to purification is that fibrillin is extremely sensitive to proteolysis. Protease inhibitors must always be present. In this regard, time is also an issue, since the longer crude fractions sit around, the more opportunity there is for degradation. The second most difficult problem has been to successfully concentrate fibrillin. Part of this problem may also be due to the first obstacle. However, it is also possible that fibrillin may be precipitating or polymerizing during the purification process, leading to excessive losses.

Different approaches to purifying fibrillin need to be tried,

bearing in mind these two problems.

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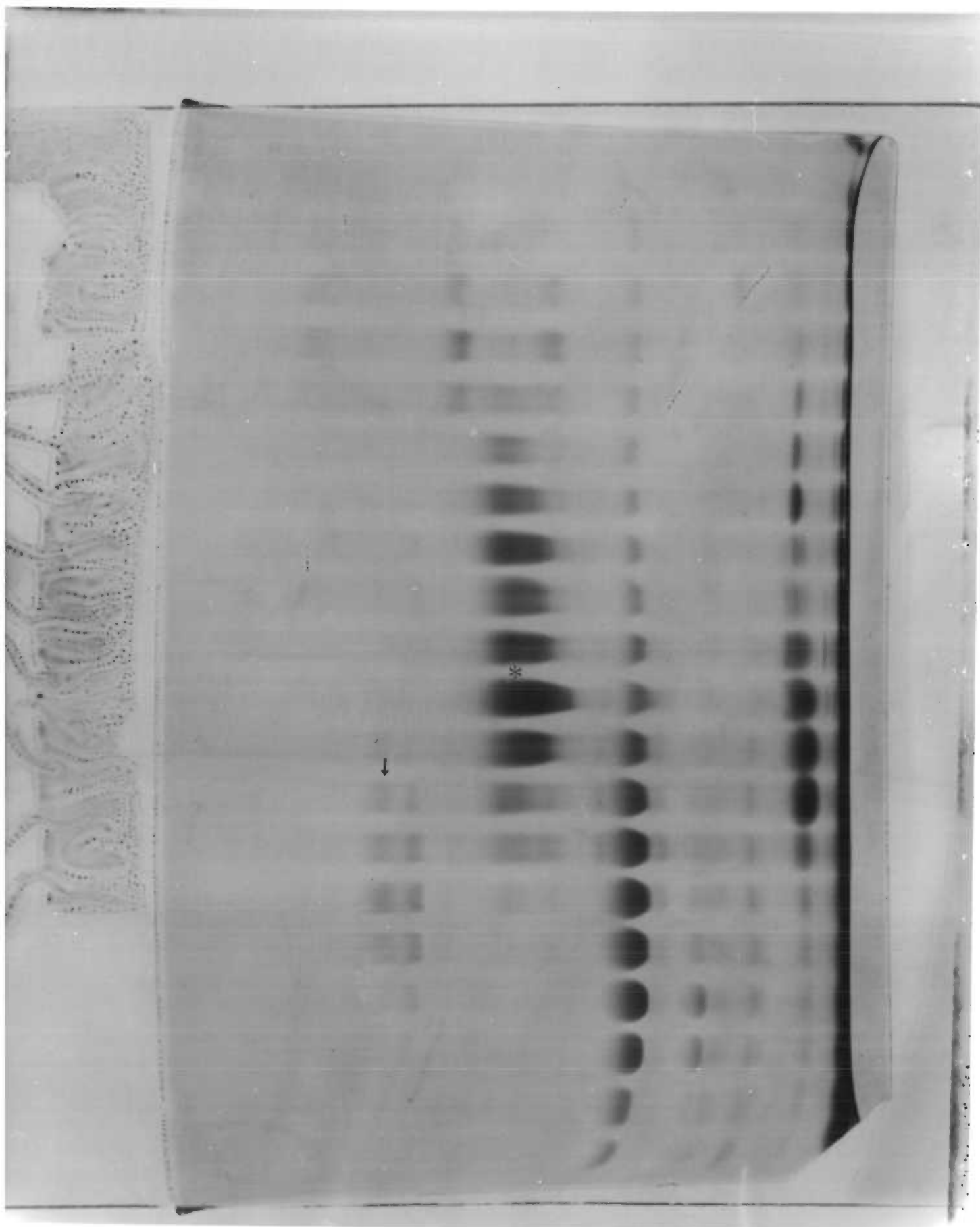
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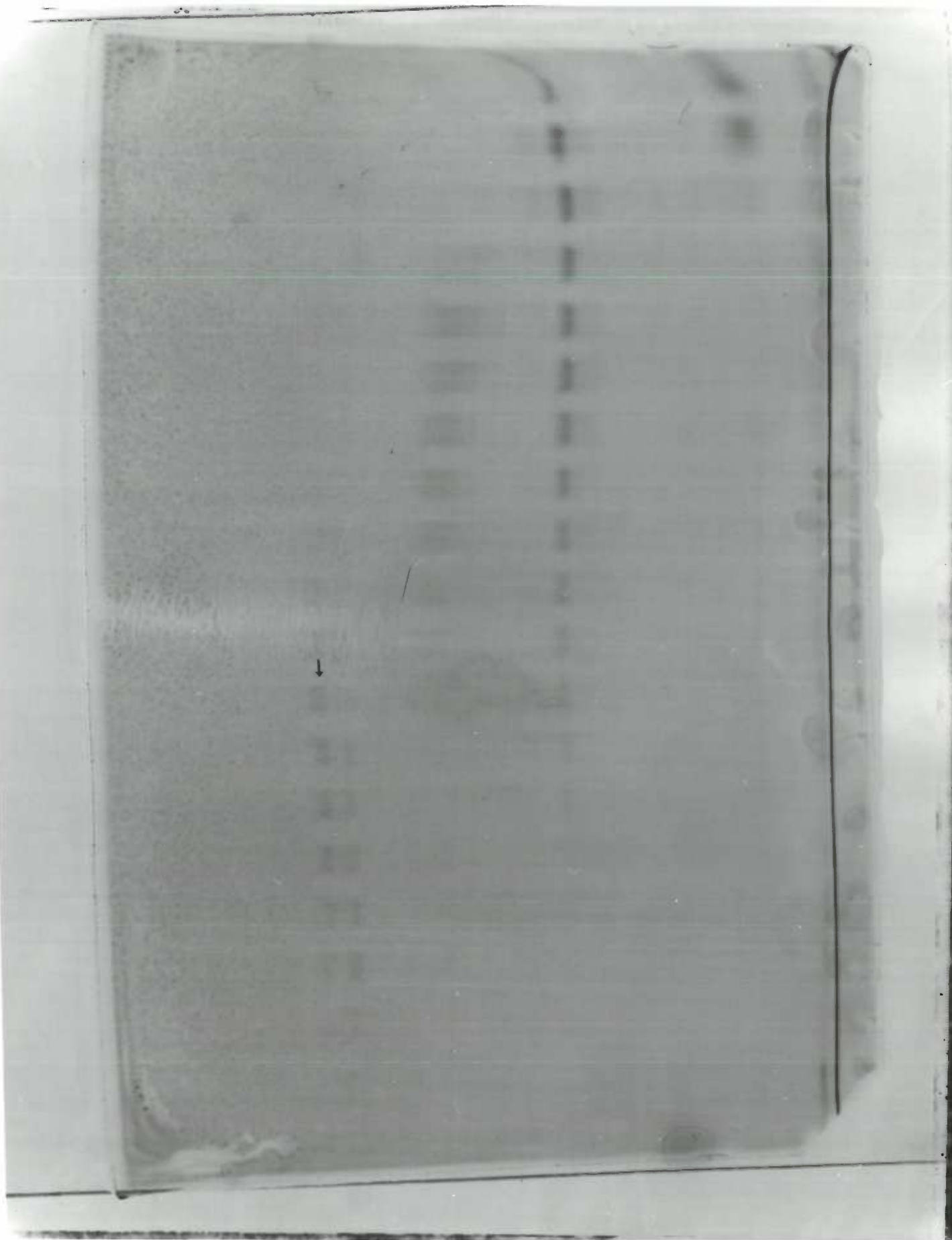
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## APPENDIX

Figure 1. Coomassie-stained 3-5% gradient SDS-PAGE of DEAE fractions. This separation was achieved using 0.05M Tris-HCl, 2M urea, pH 8.0 and a linear NaCl gradient of 0-0.3M. Fibrillin is marked with an arrow. There is a problematic contaminating band just below fibrillin. Fibronectin is marked with a \*.

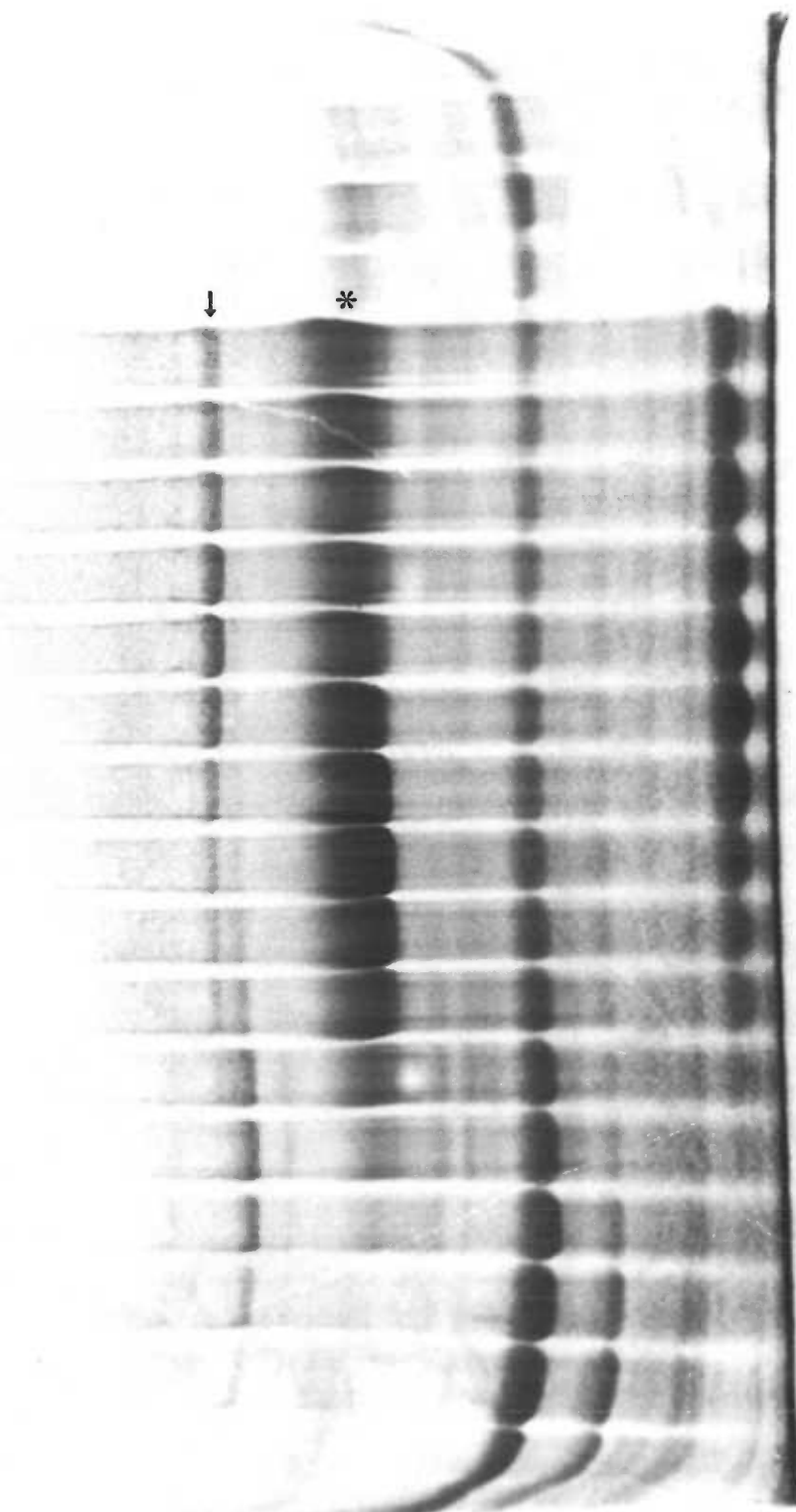


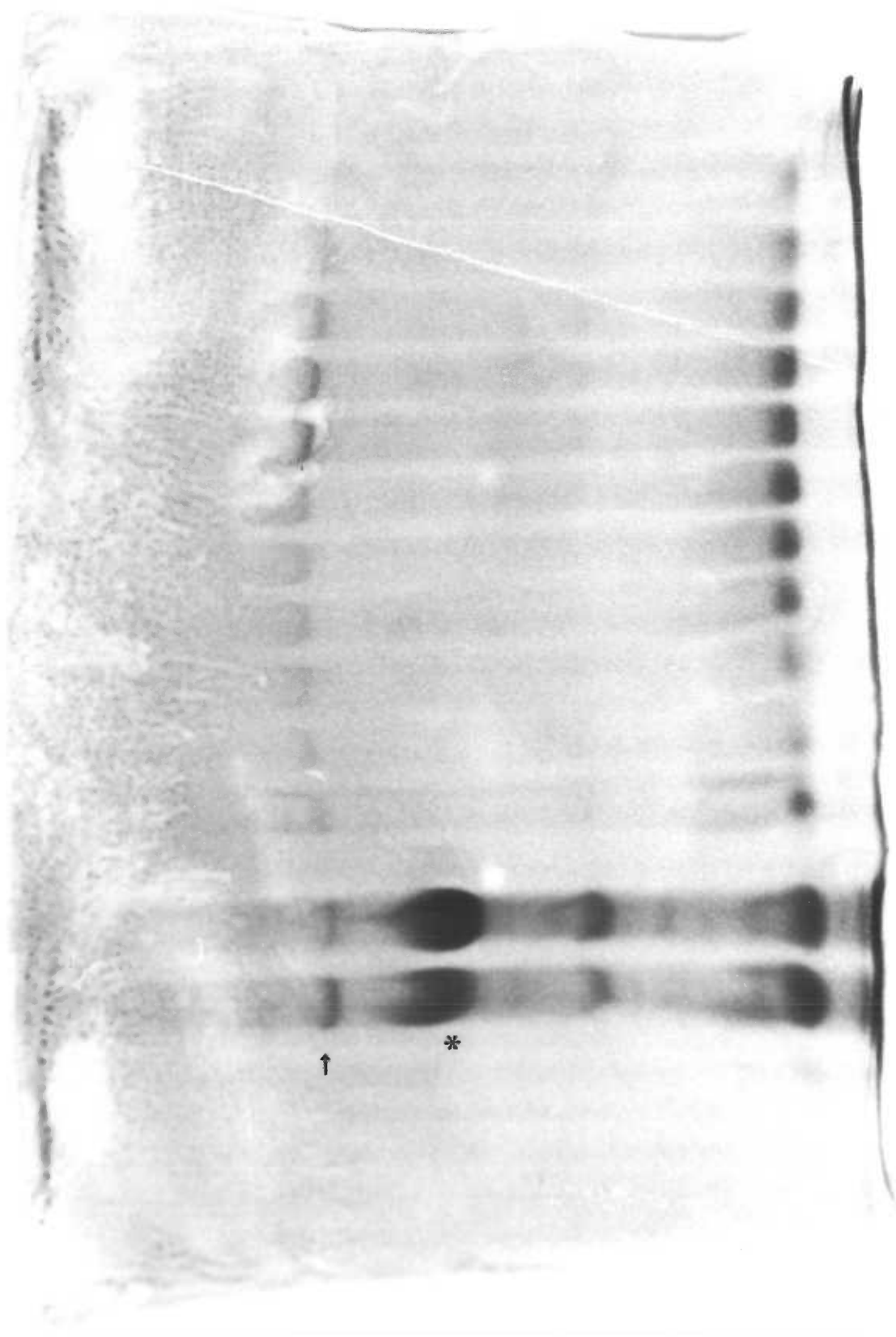


255 256 257

22 72 52 42 32 22 12 02 11 18 14 14 12 10 8

6





11 10 9 8 7 6 5 4 3 2 1  
mannose glucose

Figure 5. 3-5% gradient SDS-PAGE of [ $^{35}\text{S}$ ]cysteine-labeled fibrillin (peak is in lanes 13 and 14) from human fibroblast culture medium after sedimentation. Sedimentation was in the direction of the arrow.