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Original Article

Extraction of Extendable Beaded Structures and Their Identification as Fibrillin-containing Extracellular Matrix Microfibrils[1]

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High molecular weight aggregates were extracted from human amnion using buffers containing 6 M guanidine hydrochloride. Rotary shadowed preparations and negatively stained samples examined by electron microscopy showed that each aggregate appeared to be a string of globular structures joined by fine filaments, giving the appearance of beads on a string. The periodicity of the beads was variable. A mouse monoclonal antibody directed against a previously characterized pepsin fragment of fibrillin was used with gold-conjugated secondary antibody and immunoelectron microscopy to show that the aggregates contained fibrillin. Similar structures were found in non-denaturing homogenates of skin, tongue, ligament, ciliary zonule, cartilage, and vitreous humor. When immunogold-labeled beaded structures were prepared for electron microscopy in the same manner as tissue, the beaded structures could no longer be seen. Instead, gold-labeled microfibrils were found which appeared to be the same as the fibrillin-containing matrix microfibrils observed in connective tissues and often associated with elastin. Thus, standard TEM protocols including fixation, dehydration, and embedding alter the ultrastructural appearance of microfibrils as compared with negative stain or rotary shadowing techniques. When skin was stretched and prepared for electron microscopy while still under tension, beaded filaments were seen in the tissue sections, but were not visible in non-stretched controls. In addition, when stretched ligament was immunolabeled with antibody directed against fibrillin while still under tension, the periodicity of antibodies along the microfibrils increased compared with non-stretched controls. We propose that microfibrils contain globular structures connected by fine filaments composed at lease in part of highly ordered, periodically distributed fibrillin molecules, whose periodicity is subject to change dependent on the tensional forces applied to the tissue in which they are contained. (*J Histochem Cytochem 39:441–449, 1991*)

KEY WORDS: Microfibril; Immunoelectron microscopy; Beaded strings; Fibrillin; Extracellular matrix; Elastin-associated fibril; Skin; Ligament.

Introduction

Fibrillin is an extracellular matrix glycoprotein that is a constituent of microfibrils that are found in many connective tissues, including skin, lung, kidney, vasculature, cartilage, tendon, muscle, cornea, and ciliary zonule. It was first isolated from the medium of human fibroblast cell cultures and was shown to be a non-collagenous, intramolecularly disulfide-bonded glycoprotein with an apparent molecular weight of 350 KD. Monoclonal antibodies (MAb) were used with immunofluorescence microscopy to show its widespread distribution in tissues, and with immunoelectron microscopy to localize it to structural elements called microfibrils (Sakai et al., 1986). Fibrillin-containing microfibrils are approximately 10 nm in diameter and in cross-section often appear to be hollow (Cleary and Gibson, 1983; Low, 1962). They are found associated with amorphous elastin (Greenlee et al., 1966), e.g., in skin and ligament, but also in tissues that lack amorphous elastin, such as hyaline cartilage and ciliary zonule. They appear to have a periodic structure, as several fibrillin MAb bind to the surface of microfibrils giving rise to an induced 52-nm periodic banding pattern, equivalent to that of banded collagen fibrils in the same preparations after tissue dehydration and embedding (Maddox et al., 1989; Sakai et al., 1986).

Studies on the biosynthesis of fibrillin and its extractability in cultured chick aorta have revealed that the monomer quickly assembles, initially into a disulfide-bonded aggregate and then into a non-reducible structure (Sakai, 1990). Three pepsin fragments

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of the tissue form of fibrillin have recently been characterized and fragment specific antibodies prepared (Maddox et al., 1989). Rotary shadowed images of one of the fragments (PF3) revealed a unique structure composed of a dense central globular domain with several protruding arms, indicating a complex structure for the tissue form of this protein. Here we describe a polymeric beaded structure that can be extracted from several microfibril-containing tissues. We identify these beaded fibrils as microfibrils in three ways: (a) beaded fibrils can be processed in a manner resulting in the morphological appearance of microfibrils; (b) beaded fibrils can be immunolabeled with antibodies specific for the PF3 fragment of fibrillin and by negative staining their periodicities can be shown to elongate; and (c) in tissues that have been stretched and this tension maintained during routine processing for TEM, microfibrils are shown to increase in periodicity and have a beaded appearance.

Materials and Methods

Preparation of Fibrillin Aggregates. Fibrillin aggregates were isolated from 6 M guanidine hydrochloride extracts of human amnion using a procedure recently described for the isolation of Type VI collagen (Kuo et al., 1989). Briefly, the extract was dialyzed against 1% acetic acid, the precipitated material collected, taken up in a neutral buffer containing 6 M urea, and passed through DEAE-cellulose. Proteins that did not bind were size-fractionated in the presence of 6 M urea using Sephacryl S-500 and the first fraction, eluting at the void volume of the column, contained the fibrillin aggregates, whereas type VI collagen was in another fraction (Figure 2 in Kuo et al., 1989). The fibrillin-containing fraction, which was slightly opalescent, was centrifuged at 18,000 × g for 60 min. A small gelatinous-residue was collected and re-suspended in 0.2 M ammonium bicarbonate. This suspension, which contained fibrillin aggregates, was contrasted either by rotary shadowing or negative staining, or was immunolabeled as described below before viewing in the electron microscope.

The pepsin fragment of fibrillin (PF3) was prepared as described previously (Maddox et al., 1989).

Preparation of Antibodies. Monoclonal antibody MAb69 has been previously characterized and shown to be specific for the pepsin-resistant "PF3" domain in human and bovine fibrillin; polyclonal antibody 7075 has been previously characterized and shown to be specific for the pepsin-resistant "PF2" domain in human and bovine fibrillin (Maddox et al., 1989). The control MAb used recognized Type III collagen (Keene et al., 1987a) and Type VI collagen (Keene et al., 1988), and a polyclonal antibody to Type VII collagen (Keene et al., 1987b) was also used.

Preparation of Tissue Homogenates. Bovine nuchal ligament, aorta, femoral head cartilage, tongue, skin, and suspensory ligaments of the eye were obtained from 10-week-old fetal calves within 28 hr after death. Fetal calf vitreous humor was obtained by cutting the eye globe at the equator and allowing a portion of the vitreous body to fall free. This was combined with an equal volume of 0.2 M ammonium bicarbonate buffer, pH 7.4. The mixture was gently shaken but not homogenized. Small pieces of bovine nuchal ligament, femoral head cartilage, isolated suspensory ligaments, tongue, skin, and aorta were homogenized in 0.2 M ammonium bicarbonate buffer on ice using a polytron tissue homogenizer (Brinkman Instruments) until no visible tissue fragments remained. The resulting homogenates were centrifuged briefly at 500 × g to remove large tissue debris. Each sample was deposited on a carbon-coated grid and negatively stained with 2% phosphotungstic acid, pH 7.0, and also sprayed, using an airbrush, from 70% glycerol onto freshly cleaved mica and rotary shadowed as previously described (Morris et al., 1986).

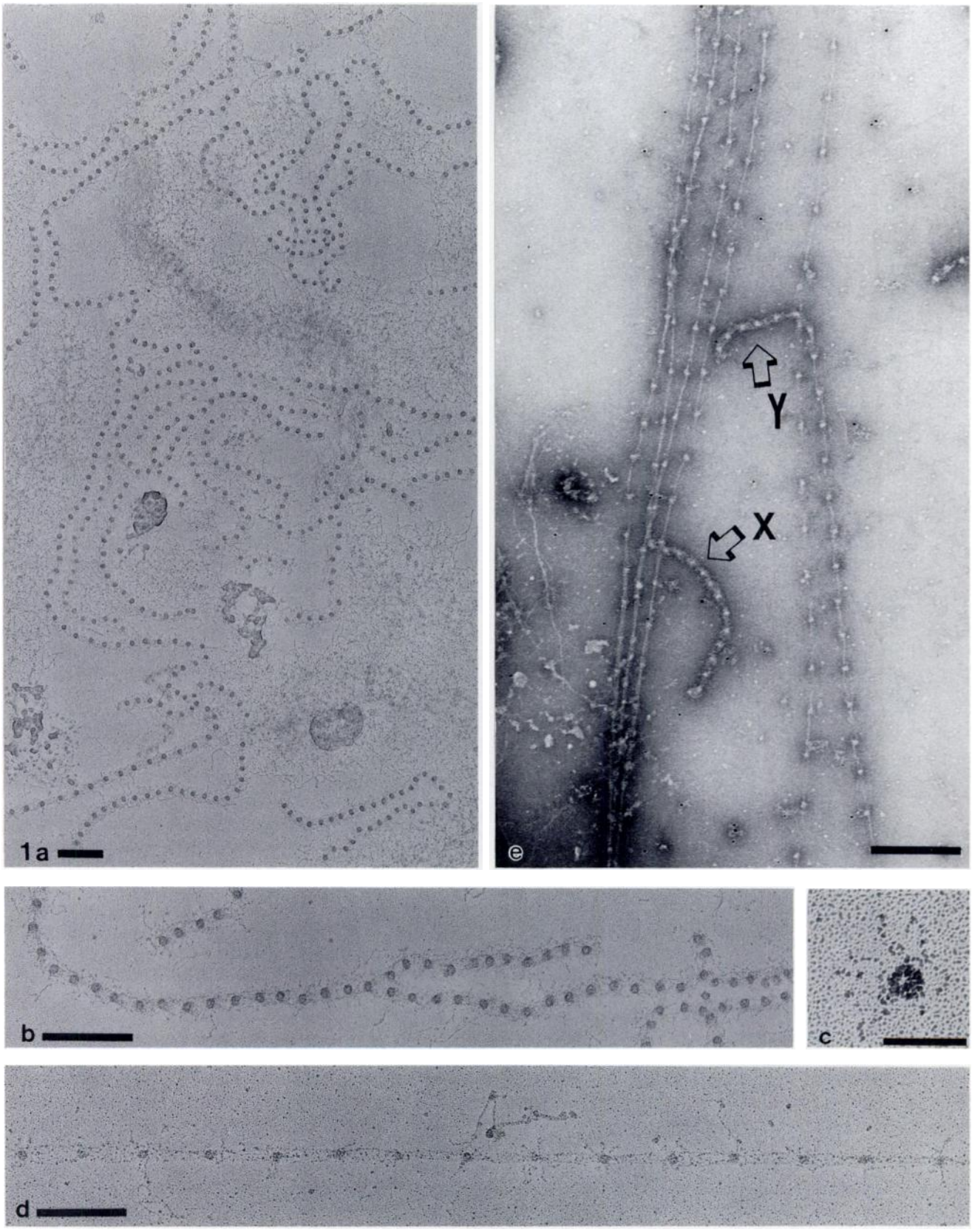
Immunolabeling of Tissue Homogenates. Each tissue homogenate (150 µl) was incubated overnight at 4°C with 50 µl of undiluted antibody. Excess primary antibody was rinsed away by washing the sample pellet three times in 500 µl of 0.2 M ammonium bicarbonate buffer and centrifuging at 11,600 × g for 15 min. The washed pellet was re-suspended in the appropriate goat anti-mouse or anti-rabbit–5-nm gold conjugate (Janssen Life Sciences; Piscataway, NJ) diluted 1:3 in BSA buffer (20 mM Tris-HCl, 0.9% NaCl, 1 mg/ml BSA, 20 mM NaN_3), pH 8.0, and incubated overnight at 4°C. Unbound secondary conjugate was washed away as above. After incubation in primary and secondary antibodies, the samples were deposited on carbon-coated 600-mesh grids and contrasted with 2% PTA, pH 7.0. One sample of immunolabeled ligament homogenate was pelleted, fixed, dehydrated in acetone, and embedded following the same protocol described below for intact tissues. All samples were examined using a Philips 410 L.S. operated at 60 kV with a 30-µm objective aperture.

Preparation of Tissues for Electron Microscopy. Human amnion was obtained shortly after term delivery and fixed in buffered 2% glutaraldehyde/3% paraformaldehyde followed by 1% OsO_4, then dehydrated and embedded in Spurr's epoxy for TEM. In addition, fetal calf nuchal ligament and neonate human foreskin were cut into strips measuring approximately 1 mm × 10 mm, then placed under tension in the following way. One end of a tissue strip was tied to a wooden dowel and the other end was pulled, using forceps, so that the tissue was effectively stretched. Maintaining tension, this end was also secured to the dowel. Tension was maintained throughout the subsequent protocol until embedding. En bloc immunolabeling of relaxed and stretched tissue was carried out using modifications of a protocol described previously (Sakai et al., 1986). Briefly, tissue pieces were submerged overnight at 4°C in ascites fluid containing the appropriate antibody, diluted 1:5 in PBS. After washing, tissues were incubated overnight in goat anti-mouse 5-nm gold conjugate (Janssen) diluted 1:3 in BSA buffer, pH 8.0. Tissues were then washed, fixed in buffered 3% gluteraldehyde/3% paraformaldehyde followed by buffered 1% OsO_4, then dehydrated and embedded in Spurr's epoxy. Stretched tissues were sectioned in a plane parallel with the direction of tension. All sectioned material was contrasted with uranyl acetate (2% in 50% ETOH for 15 min) followed by Reynolds lead citrate (Reynolds, 1963) for 60 sec.

Results

Beaded aggregates of fibrillin isolated from 6 M guanidine hydrochloride extracts of amnion were not in solution even after passage through two chromatography columns. They could easily be sedi-

Figure 1. Beaded aggregates in guanidine extracts of human amnion. (**a**) Rotary shadowed preparation containing long segments of beaded strings interdispersed among partially denatured banded collagen fibers. (**b**) Higher magnification of relaxed rotary shadowed beaded strings, showing the network of fine filaments surrounding the beads. The periodicity in these beaded strings averages about 33 nm. (**c**) Rotary shadowed fibrillin pepsin fragment PF3. Note the similarity between this fragment and a single globular domain of the beaded strings. (**d**) Occasionally, some beaded strings become tangled among debris present in the sample and are stretched, causing the periodicity to increase. The periodicity of this beaded string averages 94 nm. (**e**) A negatively stained preparation in which the periodicity of individual beaded strings varies. In this field, each of two beaded strings (labeled X and Y) become tangled among additional strands which are held under tension at either end by debris. At the point of entanglement, the average periodicity of strand X elongates from approximately 42 nm to 82 nm, whereas the periodicity of strand Y elongates from 38 nm to 110 nm. Bars: **a,b,d,e** = 200 nm; **c** = 50 nm.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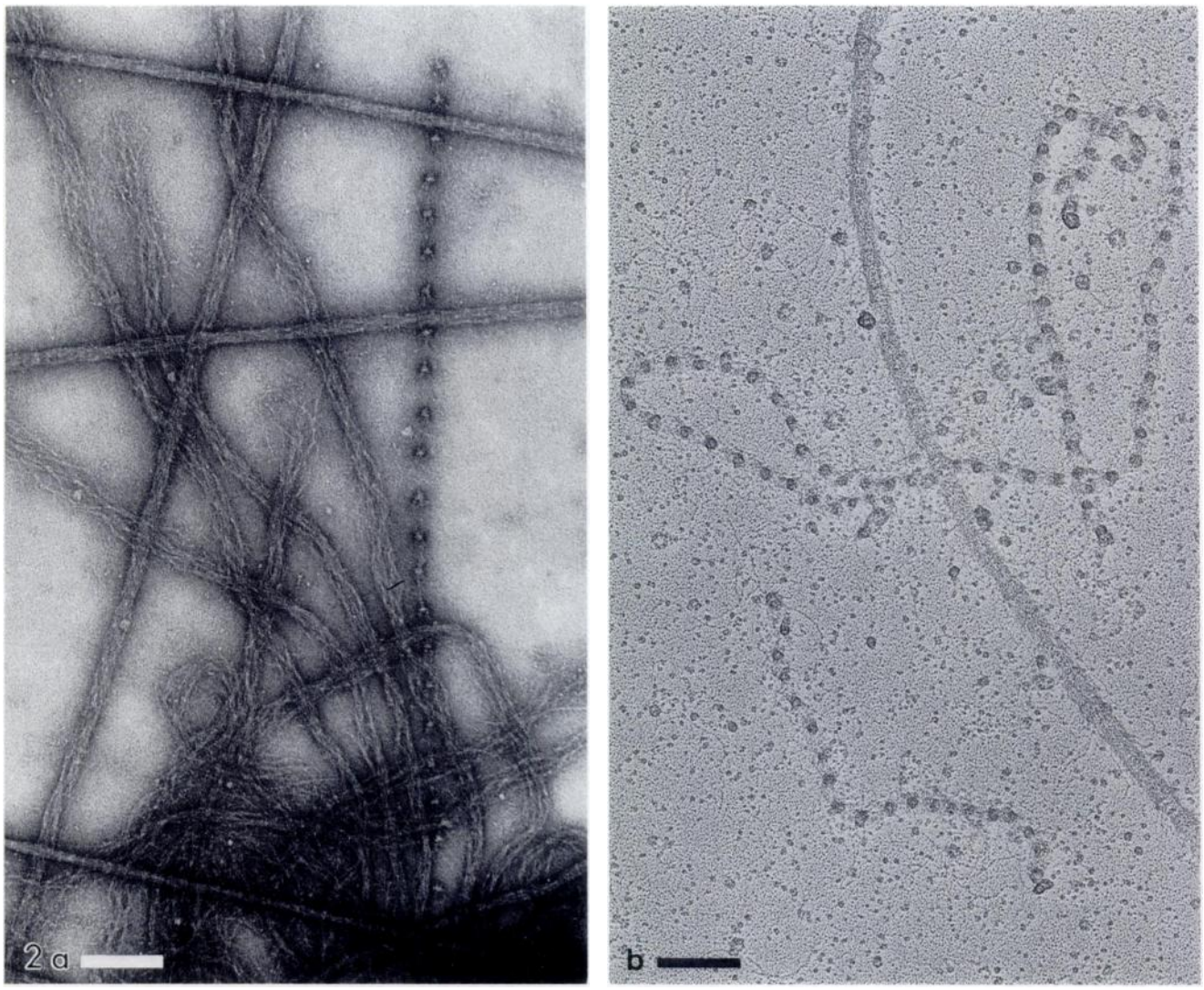
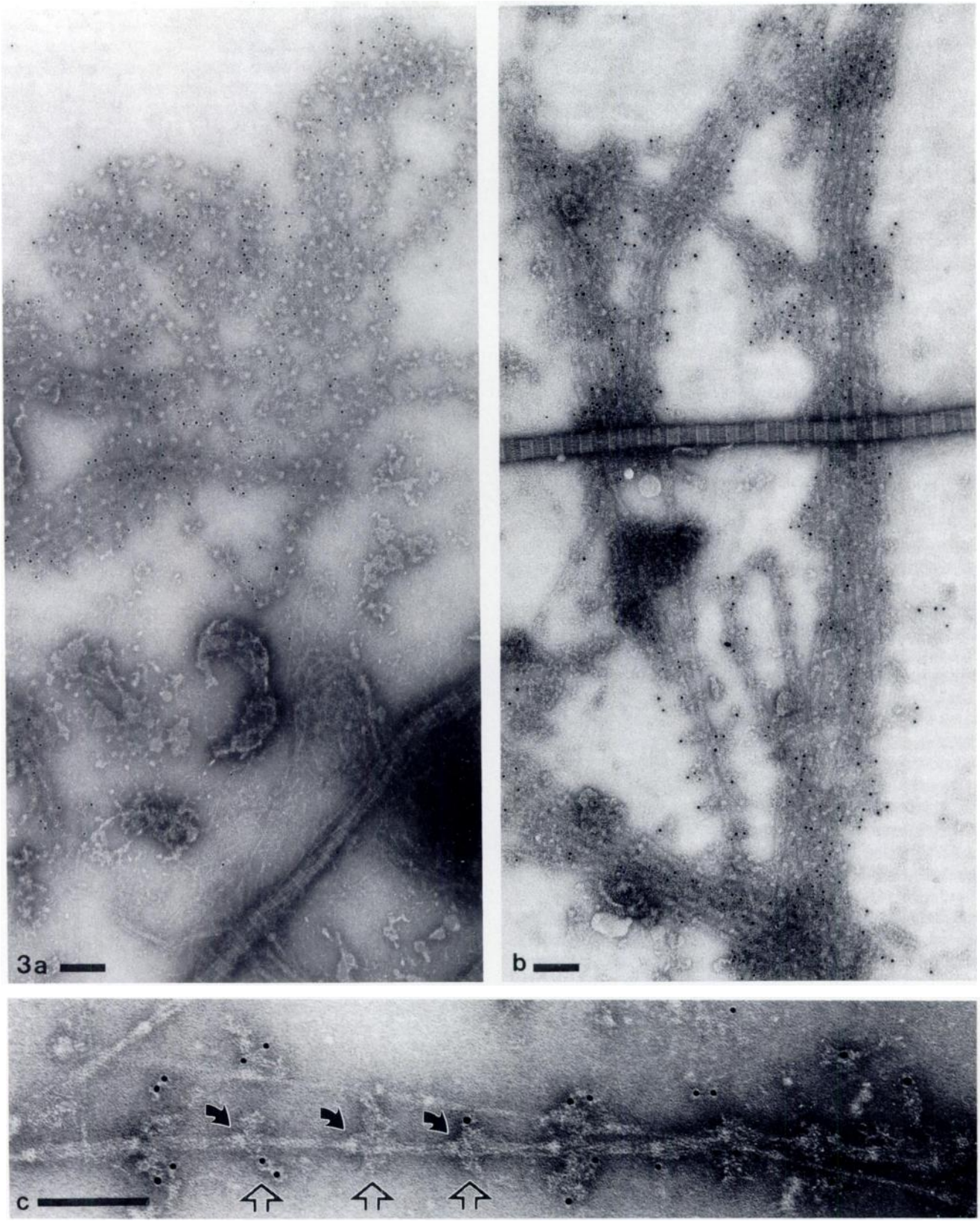


Figure 2. Material in non-denaturing homogenates of tissues contains readily identifiable beaded aggregates and banded collagen fibrils. (a) Fetal calf vitreous humor visualized by negative staining. (b) Fetal calf femoral head cartilage visualized by rotary shadowing. Bars = 150 nm.

mented out of a buffer containing 6 M urea, along with fragments of collagen fibers and other unidentified material. The beaded structures were clearly visible in rotary shadowed samples, as shown in Figure 1a. Strings with more than 60 beads were frequently seen. The strings of beads were clearly very flexible, although individual beads within a string never touched. In rotary shadowed preparations, the diameter of the globular portion of the beaded strings was approximately 22 nm, whereas in negative stain preparations the beaded portion was approximately 15 nm. As seen by rotary shadowing at higher magnification, each bead was surrounded by a network of fine filaments (Figure 1b). They appeared to be polymers of PF3, previously characterized and shown to contain a fragment of fibrillin (Figure 1c). The periodicity of the globular portion along individual beaded strings varied. In figures 1b and 1d, the average periodicities were 33 nm and 94 nm, respectively. Periodicities as high as 165 nm have been measured. The periodicity

Figure 3. Beaded aggregates labeled with fibrillin-specific monoclonal antibody mAb69 and 5-nm gold-conjugated secondary antibody and then negatively stained with 2% phosphotungstic acid, pH 7.0. (a) Beaded aggregates in a guanidine extract of human amnion. The beaded structures can clearly be seen labeled with gold, indicating that they are fibrillin-containing structures. At the top of the picture, other fibrous material and a collagen fiber are not labeled. (b) Low-magnification field of fetal calf ligament homogenate demonstrates that the antibody also labels the beaded string structures directly extracted from tissue under non-denaturing conditions. (c) At higher magnification, antibody labeling is seen to be located consistently to one side of the "beaded" area of the string, indicating that the structure is not symmetrical. Solid arrows point to beaded areas; open arrows point to antibody binding sites. Bars = 100 nm.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within an individual string was usually constant, although if the string becomes entangled, as described in the legend to Figure 1e, the periodicity may change as much as threefold.

To demonstrate that the beaded string was a physiological structure and not induced by the harsh procedure used to extract amnion, various tissues were homogenized in non-denaturing buffers and examined in the electron microscope after rotary shadowing or negative staining. A negatively stained sample from fetal calf vitreous humor (Figure 2a) and a rotary shadowed preparation from fetal calf femoral head cartilage (Figure 2b) are shown. In both pictures the beaded structure is clearly identifiable. Similar results (not shown) were obtained from ligament, aorta, tongue, skin and suspensory ligament.

Although beaded aggregates could not be completely purified from guanidine extracts, it was possible to show that they contained fibrillin by using immunogold labeling techniques in combination with negative staining. Figure 3a shows a preparation of beaded aggregates in which gold particulate label is directed by MAb69, a monoclonal antibody specific for pepsin fragment "PF3" of fibrillin. Only the beaded structures were labeled with gold, indicating that they are fibrillin-containing structures. Controls using primary antibodies specific for Types III, VI, and VII collagen did not result in gold labeling of the beaded strings.

To confirm that the beaded strings observed in tissue homogenates are the same as those found in guanidine extracts of human amnion, some of the homogenates were immunolabeled as described above. Ligament, which contains large numbers of microfibrils, contained masses of beaded strings which could be specifically labeled using the fibrillin antibody MAb69 (Figure 3b). Furthermore, close examination of individual immunolabeled strings (Figure 3c) showed that the binding site for the antibody is located consistently to one side of a bead, indicating that the globular domain is asymmetric. In controls without antibody or with irrelevant antibody, including those specific for collagen Types III, VI, and VII, no attached material was seen (see Figure 1e, control antibody specific for type VI collagen).

After conventional fixation, dehydration, and embedding, matrix microfibrils present in tissues measured approximately 10–12 nm in diameter. Typically, in longitudinal section, a scalloped appearance to the microfibrils could be resolved, but a definitive substructure or periodicity was difficult to distinguish (Figure 4b). Because a beaded structure similar to that seen in negative stain preparations is not visible in tissue sections, it is probably altered by the processing, particularly dehydration, necessary to prepare tissues for conventional electron microscopy. To demonstrate this, a pelleted immunolabeled ligament homogenate, similar to that shown in Figure 3b, was subjected to fixation, dehydration, and embedding. Before pelleting, it was confirmed by negative staining that the only labeled structures in the homogenate were beaded strings. TEM revealed that the beaded strings were no longer apparent in the embedded preparation (Figure 4a). Instead, the only immunolabeled structures present were those that are indistinguishable from gold-labeled matrix microfibrils visualized following en bloc immunolocalization techniques using antibodies specific for fibrillin (compare Figure 4a with Figure 4d, upper left, or with Figure 12 in Sakai et al., 1986).

Tissues were manually stretched, then fixed, dehydrated, and embedded while still under tension to determine if the appearance of tissue microfibrils as seen in conventional TEM preparations could be altered. Although difficult to adequately contrast, Figure 4c shows a beaded microfibril in stretched skin. These structures were never observed in unstretched tissues.

When antibodies recognizing fibrillin are used by en bloc methods to label microfibrils in tissues, a banding pattern, induced by primary antibody, is visible regardless of the antibody used (Maddox et al., 1989; Sakai et al., 1986). After fixation, dehydration, and embedding the periodicity measured about 52 nm, the same as banded collagen fibrils present in similarly processed tissues. When stretched tissue was immunolabeled while still under tension, the distance between antibody-directed gold particulates increased as compared with unstretched controls. In some areas of stretched skin the periodicity was measured to be over 75 nm, demonstrating that even though a definitive beaded appearance was not evident in this preparation, the periodicity of the microfibril was nevertheless extended (Figure 4d).

Discussion

The ultrastructure of connective tissue microfibrils was described in early literature as being an approximately 10-nm diameter fibril with a vesicular cross-section and a 3–4-nm lucent core (Cleary et al., 1981; Fahrenbach et al., 1966; Greenlee et al., 1966; Low, 1962). The longitudinal section was variously described as a "light and dark staining pattern" (Cleary et al., 1981), "beaded appearance" (Fahrenbach et al., 1966), and "chain-like aggregates of tiny vesicles" (Haust, 1965). Their periodicity was not uniformly reported. In one report (Fahrenbach et al., 1966), structures resembling beaded strings were extracted from fetal nuchal ligament and observed after negative staining. They were described as 13 nm long × 13 nm wide cylindrical segments separated by an electron-opaque region 10 nm long × 15 nm wide, and were thought to originate from "pre-elastin filaments." Similar beaded fibrils have been described as "beaded protofibrils" after staining (Hayes and Allen, 1967). Because these structures appeared to display a periodicity similar to collagen fibers, it was speculated that they were immature collagen fibrils. More recently, similar beaded structures were found in rotary shadowed samples of chick vitreous humor, where the beads were clearly visible and had a diameter of 22 nm with a spacing of 50 nm (Wright and Mayne, 1988). These authors speculated that the shadowed beaded fibrils they observed in preparations of vitreous humor may have been zonular microfibrils. However, no direct data were obtained to support this hypothesis. The source, appearance, and measured diameters of the beaded structures visualized here, either by rotary shadowing or negative staining, indicate that they are the same as those described in these earlier studies.

Several proteins including fibronectin, vitronectin, and amyloid P have been found to associate with microfibrils but are not considered true structural components of microfibrils (Dahlbäck et al., 1990; Kobayashi et al., 1989; Schwartz et al., 1985; Breathnach et al., 1981). MAGP, a 31 KD glycoprotein isolated from reductive extracts of bovine nuchal ligament, has been partially characterized (Gibson et al., 1986). Immunofluorescent studies using polyclonal antibodies prepared against MAGP labeled both elastin-associated

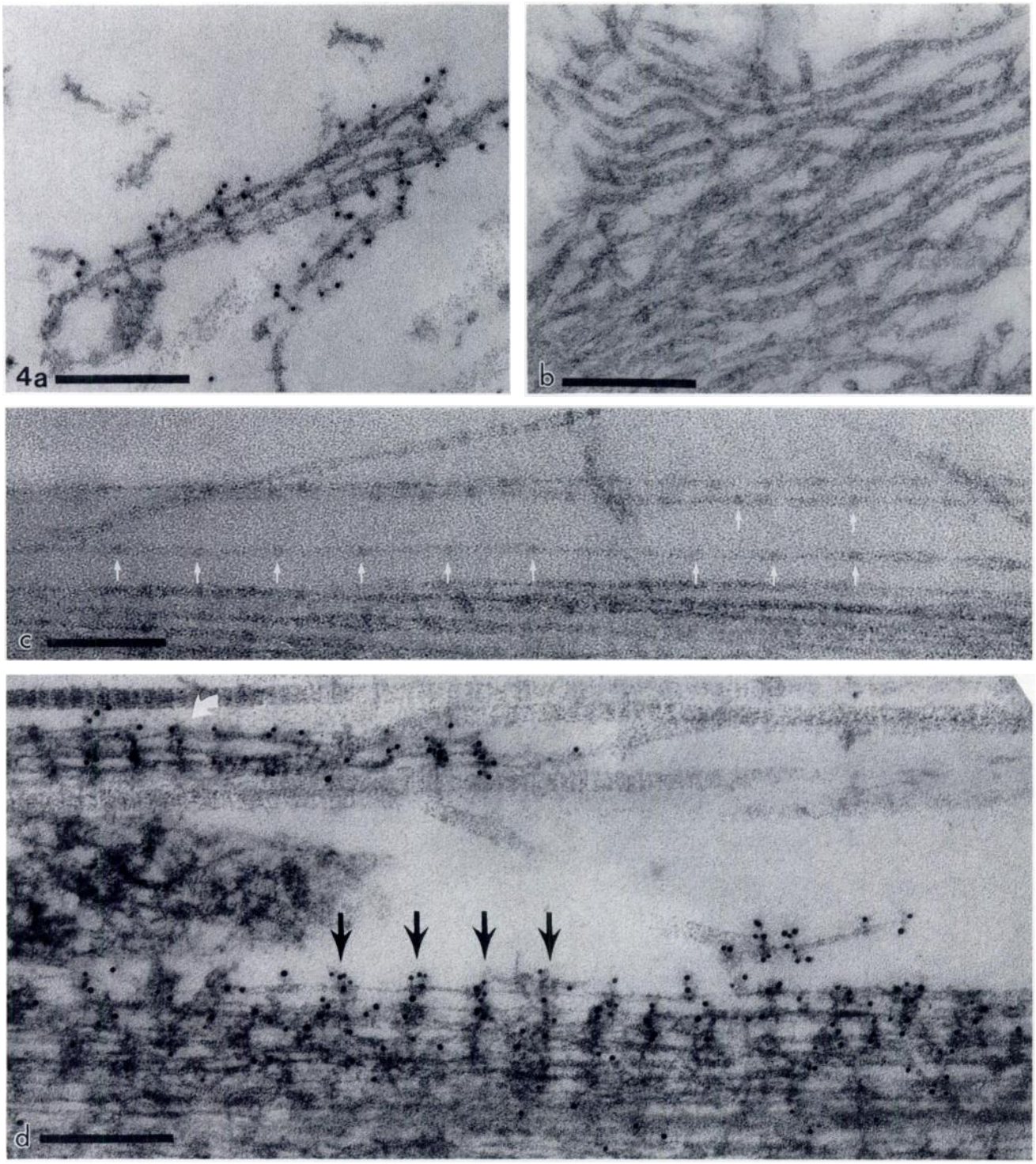

Figure 4. (**a**) Ultra-thin sections of immunolabeled fetal calf ligament homogenate labeled with MAb69 and 5-nm gold secondary antibody conjugate (as shown in Figure 3b) observed after fixation, dehydration, embedding, and sectioning do not contain the beaded string structures, but instead contain immunogold labeled microfibril-like structures. (**b**) Normal tissue matrix microfibrils in well-fixed human amnion. (**c**) Human skin placed under tension before and during conventional processing for TEM. Some of the microfibrils are sufficiently stretched to take on the appearance of beaded aggregates. The periodicity in these areas averages 76 nm. (**d**) Immunolabeling of fetal calf ligament with MAb69 and 5-nm gold-conjugated secondary antibody while under tension results in microfibrils with varying periodicity. Some microfibrils (upper left, solid white arrow) demonstrate a periodicity of approximately 50 nm and are apparently not under tension. The periodicity of other microfibrils (solid black arrows) is substantially increased (in this example, to 80 nm), suggesting that these microfibrils are under tension. Bars = 150 nm.

microfibrils and microfibrils in non-elastin-containing tissue in a similar manner as do MAb to fibrillin (Gibson and Cleary, 1987). A second 340 KD fragment called MP340 was subsequently characterized and shown to be related to MAGP (Gibson et al., 1989). Because of the similar molecular weights of MF340 and fibrillin and the similarity of the antibody binding patterns of MAGP and fibrillin, it is likely that MP340 is fibrillin, and to date this is the only structural component of microfibrils identified.

The first indication that the beaded strings might contain fibrillin came from a study of three large pepsin fragments of fibrillin (Maddox et al., 1989). By rotary shadowing, the structure of one of the fragments (PF3) appeared to be the same as a single globular area along a beaded string (Figure 1c). Furthermore, other fractions contained two to five PF3 fragments joined into short strings, indicating that PF3 probably originated from a larger polymeric structure. In this report, we used an MAb specific for fibrillin and directed against PF3 to show that the beaded fibrils present in homogenates of various tissues contain fibrillin. The beaded filaments present in 6 M guanidine hydrochloride extracts of human amnion which had been partially purified in the presence of 6M urea were also recognized by the antibody. This indicates that fibrillin must be a covalently bound component of the beaded strings and not just an associated protein. Certainly, the presence of fibrillin in both microfibrils and in the beaded strings suggests a common entity.

Studies of the biosynthesis and assembly of fibrillin in embryonic chick aorta organ cultures have shown that fibrillin monomers quickly polymerize, within hours after initial secretion, into large multimers stabilized by intermolecular disulfide bonds (Sakai, 1990). With the exception of pools of newly synthesized fibrillin monomers, the tissue form of fibrillin has been shown to be multimeric. Estimates of the size of initially formed aggregates might accommodate more than 23 fibrillin molecules. The fibrillin-containing beaded structure described in this report is consistent with these studies. However, the details of the initial assembly process and of the stabilization of the final linear polymer are still under investigation. In addition, whether other molecules participate in the formation of this structure is presently unknown.

All monoclonal fibrillin antibodies bind to microfibrils in tissues with approximately a 52-nm periodicity, indicating that there is a regular arrangement of fibrillin molecules within this structure (Maddox et al., 1989; Sakai et al., 1986). The material deposited at each binding location along a beaded string represents a cluster of primary and secondary antibodies, and is not seen in unlabeled controls. The actual epitope on the fibrillin molecule is probably positioned along the arms very close to each bead. Although binding at regular intervals along the beaded string, MAb69 bound consistently to only one side of the bead structure. For this asymmetric binding to occur the beads must contain either the amino terminal end of one molecule and the carboxy terminal end of an adjacent molecule (head to tail alignment), or the central region of the fibrillin molecule so that the amino terminal region of the molecule is on one side and the carboxy terminal region on the other side of the bead.

The ultrastructural appearance of antibody-labeled beaded strings can be effectively converted to antibody-labeled microfibrils by processing beaded strings through a standard TEM fixation, dehydration, and embedding protocol as though they were tissue, indicating that the beaded strings and tissue microfibrils represent the same structural entity. However, the periodicity of the beaded strings present in guanidine extracts and tissue homogenates is variable, and significantly increases when subjected to tension. By stretching ligament before antibody labeling, the periodicity of microfibrils could be increased significantly beyond that of the surrounding collagen fibrils, indicating a unique extendability of the microfibril structure. It is likely that the 52-nm period demonstrated in standard tissue biopsy specimens represents the period present in relaxed tissue following standard processing for TEM.

The true structural appearance and the extendability characteristics of microfibrils are probably close to those of the beaded strings, since this structure can be demonstrated with minimal tissue processing. It is likely that the ultrastructural appearance of microfibrils in tissue sections represents an artifact of tissue processing, particularly of dehydration. Because the dimensions of the microfibrils in vivo are unknown, the results of the tissue stretching experiments are difficult to interpret, since the microfibrils may have been stretched or, alternatively, have been prevented from shrinking during dehydration. However, the beaded string is clearly present in stretched tissue and is therefore not an artifact of extraction. Because the beaded fibrils appear in our preparations to have widely different periodicities, and because the periodicity along microfibrils can be changed by stretching the tissue, we suggest that the physiological periodicity of fibrillin-containing microfibrils may reflect the organization of the surrounding matrix.

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