Type VI Collagen in Extracellular, 100-nm Periodic Filaments and Fibrils: Identification by Immunoelectron Microscopy

Romaine R. Bruns,* William Press,* Eva Engvall,‡ Rupert Timpl,§ and Jerome Gross*

*Developmental Biology Laboratory, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114; ‡Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037; and §Max-Planck Institut für Biochemie, D-8033 Martinsried, Munich, Federal Republic of Germany

Abstract. Filaments and fibrils that exhibit a 100-nm axial periodicity and occur in the medium and in the deposited extracellular matrix of chicken embryo and human fibroblast cultures have been tentatively identified with type VI collagen on the basis of their similar structural characteristics (Bruns, R. R., 1984, *J. Ultrastruct. Res.*, 89:136–145). Using indirect immunoelectron microscopy and specific monoclonal and polyclonal antibodies, we now report their positive identification with collagen VI and their distribution in fibroblast cultures and in tendon. Primary human foreskin fibroblast cultures, labeled with anti-type VI antibody and studied by fluorescence microscopy, showed a progressive increase in labeling and changes in distribution with time up to 8 d in culture. With immunoelectron microscopy and monoclonal antibodies to human type VI collagen followed by goat anti-mouse IgG coupled to colloidal gold, they showed in thin sections specific 100-nm periodic labeling on extracellular filaments and fibrils: one monoclonal antibody (3C4) attached to the band region and another (4B10) to the interband region of the filaments and fibrils. Rabbit antiserum to type VI collagen also localized on the band region, but the staining was less well defined. Control experiments with antibodies to fibronectin and to procollagen types I and III labeled other filaments and fibrils, but not those with a 100-nm period. Heavy metal–stained fibrils with the same periodic and structural characteristics also have been found in both adult rat tail tendon and embryonic chicken tendon subjected to prolonged incubation in culture medium or treatment with adenosine 5′-triphosphate at pH 4.6. We conclude that the 100-nm periodic filaments and fibrils represent the native aggregate form of type VI collagen. It is likely that banded fibrils of the same periodicity and appearance, reported by many observers over the years in a wide range of normal and pathological tissues, are at least in part, type VI collagen.

With the discovery of the genetic heterogeneity of collagen (38), it became apparent that the composition and organization of the extracellular matrices were far more complex than were ever anticipated. To date there are at least 10 known, distinct collagen gene products, not including non-matrix collagen sequences such as those in Clq (47, 48), acetylcholine esterase (50), and surfactant (60). The molecular structure and tissue supramolecular aggregates of most of these are established. For several, however, the forms in which they exist in situ and their relations with other matrix components are not yet known. Type VI collagen is an example of the latter.

Extracellular filaments and fibrils that have a 100-nm periodic cross-banding have been observed in chicken embryo fibroblast cultures (7). The basic unit, a "beaded" filament, consists of a thread measuring 2–3 nm in diameter and pairs of "beads" distributed along its length at regular intervals of 90–110 nm. In the aggregated form (i.e., bundles of ordered "beaded" filaments), the laterally aligned "beads" appear as dark bands averaging 44 nm in width and the filamentous segments, as light bands ~67 nm wide. These structures are distributed widely in the extracellular compartment, on the surface of fibroblasts, and around or between collagen fibrils in native tissues. They are clearly distinguishable from the 67-nm periodic types I (9, 9a, 61) and III (61) collagen fibrils by their longer axial period and lateral organization. Detailed structural similarities between single, negatively stained "beaded" filaments and molecular models of type VI collagen, constructed according to the appearance of rotary-shadowed monomers and linear aggregates (23, 43), led to their tentative identification with type VI collagen (7).

In this paper we present definitive immunoelectron microscopic evidence that type VI collagen is a major component of such 100-nm periodic filaments and fibrils, and we show its distribution in fibroblast cultures and in both chicken embryo and adult rat tail tendon.

Materials and Methods

Cell Cultures

The epidermis of human foreskins was removed by trypsinization (55), and fibroblasts were isolated from the remaining dermis by digestion with bacterial collagenase and trypsin (15, 16). The cells, in Ham's F-12 medium con-

taining 10% bovine calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 25 μg/ml L-ascorbic acid (45), were plated onto 22-mm² glass (for fluorescence microscopy) or carbon-coated glass (for electron microscopy) coverslips at a density of 5×10^5 cells per 35-mm Falcon plastic culture dish. The medium and ascorbate were changed daily. Cells were examined from 2 h after isolation to 8 d in culture.

Antibodies

Preparation of monoclonal antibodies to type VI collagen has been described (18, 29). Antibodies 4B10 and 3C4 were used in the form of hybridoma culture medium or ascites fluid diluted 1:10–1:50 with PBS or with F-12 culture medium. In addition, antiserum against purified collagen VI was raised in rabbits (~200 μg/ml) (56, 59) and was used at a dilution of 1:10 with PBS. The secondary antibodies used were goat anti-mouse IgG bound to tetramethylrhodamine isothiocyanate (rhodamine) (Cappel Laboratories, Cochranville, PA; #2211-0231), goat anti-mouse IgG coupled to colloidal gold (10.7-nm diameter) (Janssen Pharmaceutica, Inc., Piscataway, NJ; #GAM G10), or goat anti-rabbit IgG with a ferritin marker (Cappel Laboratories; #4212-0081). Immediately before use, all antibody solutions were centrifuged for 2 min in a Beckman Microfuge to remove large aggregates.

Indirect Immunofluorescence Microscopy

In the usual procedure, the cell cultures were rinsed for 10 min in three changes of PBS to remove bovine serum. The coverglass, with cells attached, was supported on an "O" ring gasket, covered with 200 μl of a centrifuged antibody solution, and agitated gently on a rotating table for 30–60 min at room temperature (~21°C). Staining at 4°C was not noticeably different from that at 21°C. The unbound antibody was removed with three washes in PBS over 10 min and the cells were stained with the secondary antibody (goat anti-mouse IgG coupled with rhodamine) for 30–60 min, and washed as above. Coverslips were mounted on glass slides with Elvanol (49) and stored at −20°C. The cells were examined and photographed with a Zeiss Universal Microscope fitted with an epi-illuminating system.

Indirect Immunoelectron Microscopy

Primary fibroblasts in culture for 1–8 d were rinsed with PBS, pH 7.3, and incubated with 200 μl of the primary antibody solution overnight. After 8–10 washes with PBS for 8 h, the cells were incubated overnight with the secondary antibody labeled with colloidal gold or ferritin. They were again rinsed extensively in PBS for ~8 h, transferred into 0.1 M cacodylate buffer, pH 7.4, and fixed for 30 min in Karnovsky's fixative (36). In experiments with rabbit anti-serum, the fibroblast cultures were fixed before incubation with the primary antibody as described by Fleischmajer et al. (21). Since this method produced variable results in our experiments, most of the cultures were fixed after incubation with antibodies. All of the antibody reactions were carried out at ~4°C, with gentle agitation on a rotary shaker to facilitate penetration of reagents throughout the layers of fibroblasts. After fixation, the cells were transferred to room temperature, postfixed with buffered 2% OsO_4, stained with aqueous 1% uranyl acetate or tannic acid in cacodylate buffer (53), and embedded in epoxy resin. Thin sections of the cell layer, cut parallel or perpendicular to the substratum, were stained with uranyl acetate and lead citrate (35, 58). Thin sections were examined with a JEOL 100B electron microscope operated at 80 kV with a 20-μm objective aperture. Micrographs were taken at magnifications up to 50,000×. Magnifications were accurately determined from micrographs of a carbon-stabilized replica of a diffraction grating having 2,160 lines/mm. Measurements on periodic structures were made on glossy prints with a steel rule calibrated to 0.5 mm.

Positive control cultures consisted of primary, human skin fibroblasts incubated with rabbit antisera directed to the amino-propeptides of bovine type I or type III collagen (56), or incubated with monoclonal antibody to human fibronectin (30). Negative controls were accomplished with normal mouse serum diluted 1:10 with PBS or PBS alone.

Enhancing the Appearance of Type VI Collagen in Cultures and Tendons

We used two methods to increase the size and visibility of the axial periods in heavy metal-stained type VI fibrils. Fibroblast cultures, 17–18 d chicken embryo metatarsal tendons, and adult rat (230 gm) tail tendons were incubated for 2 h at 37°C in 10–15 ml of Ham's F-12 culture medium containing 20 mM adenosine 5'-triphosphate, disodium, crystalline (ATP) (P-L Biochemicals, Milwaukee, WI; #27-1006-01), pH 4.6. The cultures were washed with PBS and processed for immunoelectron microscopy before fixation. Tendons were fixed immediately after ATP treatment. In other experiments, 1-cm pieces of fresh tendon were incubated in sterile F-12 medium (without added ATP) at 37°C for 3 d before fixation and embedding in Epon.

Results

Indirect Immunofluorescence Microscopy

Primary human foreskin fibroblasts, cultured for various time periods after plating, were prepared for immunofluorescence microscopy as described. After 4–6 h in culture, the attached and spread cells, incubated with monoclonal antibody to type VI collagen (3C4) followed by secondary goat anti-mouse IgG coupled to rhodamine, were not fluorescent (Fig. 1, *a* and *d*). The control culture, incubated with non-immunoreactive mouse serum, showed similar unstained spread cells (Fig. 1, *g* and *j*). After 1 d in culture, the fibroblasts revealed small patches of fluorescent material which appeared to arise on some individual cells, but more often within small clusters of cells (Fig. 1, *b* and *e*). At 4 d, the confluent cell layer was covered with a dense, intensely fluorescent, fibrillar network (Fig. 1, *c* and *f*). The corresponding control cultures, incubated with non-immune mouse serum, showed no significant fluorescence (Fig. 1, *h* and *k*, and *i* and *l*). The cells at 8 d (not shown) resembled those at 4 d, but they were more dense and tended to detach from the coverslips.

In positive control experiments, fibroblasts incubated with monoclonal antibody to human fibronectin (30), or with affinity-purified polyclonal rabbit antibodies to bovine procollagen I, or procollagen III, showed intense fluorescence of the extracellular matrix with all three antibodies (not shown; see references 20, 21, and 30).

Indirect Immunoelectron Microscopy

Human skin fibroblast cultures, incubated with monoclonal antibody to type VI collagen followed by secondary goat anti-mouse antibody conjugated with colloidal gold, showed a 100-nm periodic labeling pattern on numerous extracellular filaments and fibrils (Figs. 2, *a–c*, 3, *a–c*, 4, *a* and *b*). With monoclonal antibody 3C4, the gold particles were localized on the "bead" region of the filaments (Fig. 2 *a*); with antibody 4B10, the gold was located primarily on the filamentous interbands (Figs. 2 *b* and 3, *a–c*); and with the polyclonal antibody (rabbit antiserum), periodic clusters of ferritin appeared to be diffusely localized on the bead regions (Fig. 2 *c*).

The labeled filaments were widespread throughout the extracellular matrix among wider (~35 nm) cross-banded, apparently type I collagen fibrils and between and near to fibroblasts (Fig. 4, *a* and *b*). The preservation of cell structure was inadequate for us to observe regular structural relations between the filaments and the cell surface or intracellular structures. The filaments occasionally ran perpendicularly between parallel collagen fibrils or near and parallel to the fibril surface, often appearing to spiral loosely around the larger type I fibrils. Wide 100-nm periodic fibrils, consisting of parallel filaments in register, were encountered occasionally in sections cut through the cell layer exactly parallel to the substratum, presumably because of their preferred orientation in flattened intercellular compartments (24). Sections cut perpendicular to the substratum showed that the

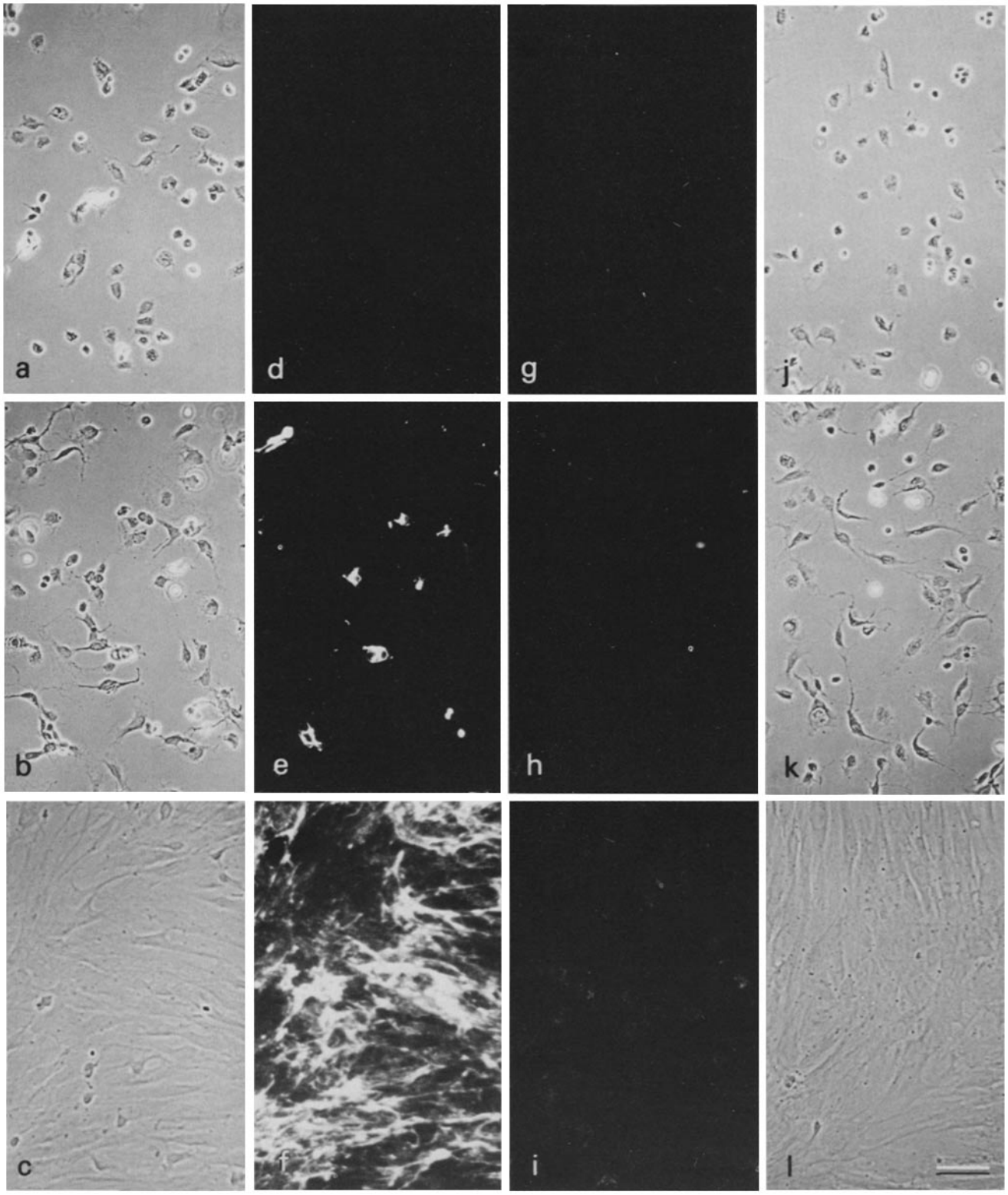

Figure 1. 6-, 24-, and 96-h cultures of human foreskin fibroblasts (rows *1*, *2*, and *3*, respectively) incubated with monoclonal antibody to type VI collagen (columns *1* and *2*) or with mouse serum (control) (columns *3* and *4*) and with secondary goat anti-mouse IgG coupled to rhodamine. Columns *2* and *3* represent cultures viewed with fluorescence optics, and columns *1* and *4* represent corresponding panels viewed with phase-contrast optics. Note that *d*, *e*, and *f* show a gradually increasing amount of fluorescent material (type VI collagen) with time in culture. Corresponding control cultures (*g*, *h*, and *i*), given an identical treatment except that antibody to type VI collagen was replaced with mouse serum, showed no significant fluorescent staining. Bar, 100 μm.

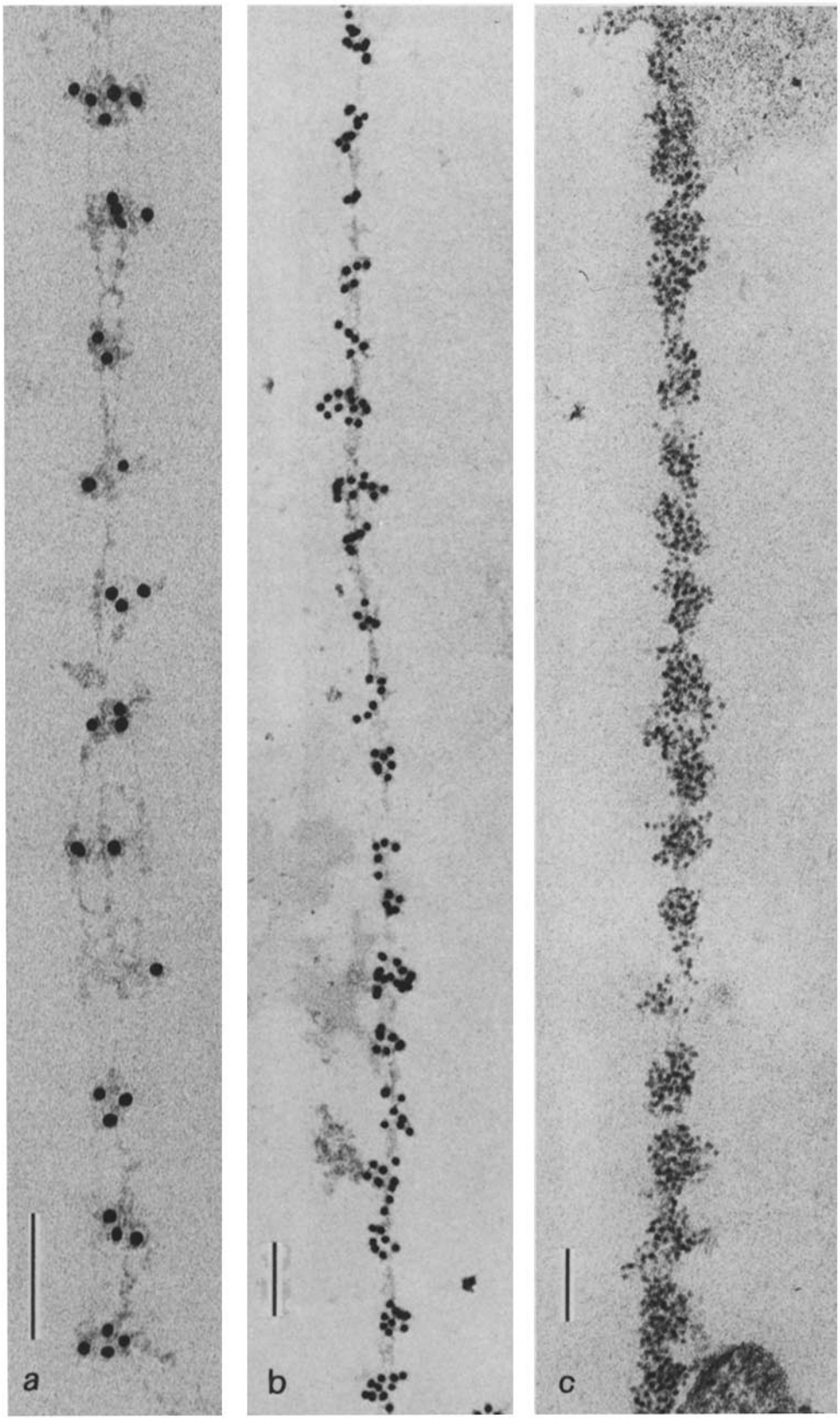

Figure 2. Type VI collagen, in human foreskin fibroblast cultures, identified by indirect immunoelectron microscopy. (*a*) 7-d fibroblast culture incubated with monoclonal antibody (3C4) to human type VI collagen and with secondary goat anti-mouse IgG labeled with colloidal gold particles (10.7-nm diameter). (*b*) 5-d fibroblast culture incubated with monoclonal antibody (4B10) as in *a*. (*c*) 48-h culture fixed with glutaraldehyde and subsequently incubated with rabbit immune serum containing antibody to type VI collagen and with secondary goat anti-rabbit IgG coupled to ferritin. *a–c* show thin sections of cultures that were incubated with antibodies and then embedded in Epon. Bars, 100 nm.

marker particles extended throughout the cell layer down to the substratum.

Measurements were made on various labeled and unlabeled periodic fibrils to further confirm their identity (Table I). Fibroblast cultures incubated with monoclonal or polyclonal anti-type VI collagen antibodies showed clusters of the markers distributed on the filaments and fibrils at mean intervals of ~93 nm. In control cultures, where labeling was

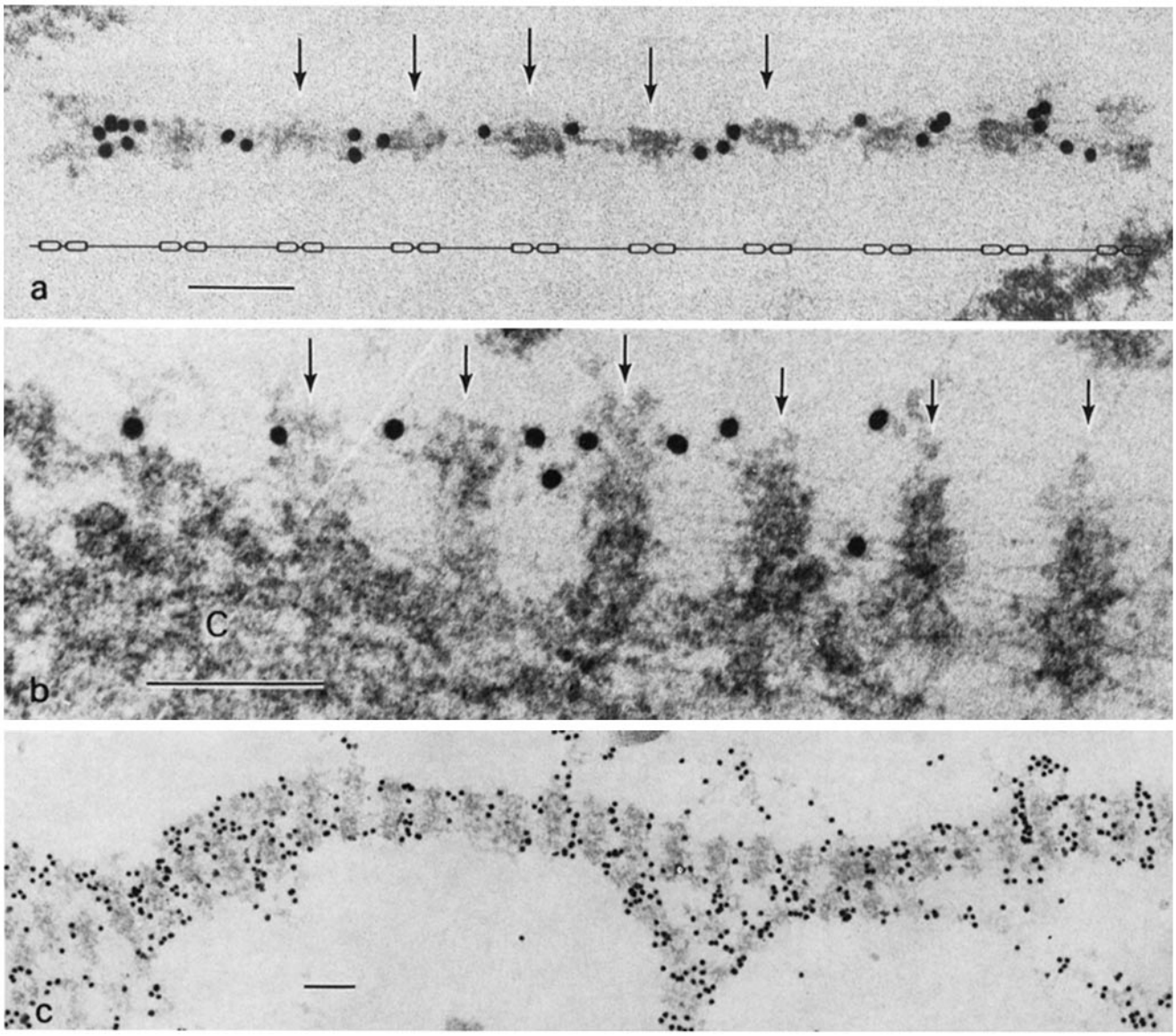

Figure 3. Human skin fibroblast cultures incubated with anti-type VI monoclonal antibody (4B10) and with secondary goat anti-mouse IgG conjugated with colloidal gold. (*a*) A slender type VI fibril showing gold particles localized on the filamentous interbands between the dark bands (*arrows*). The schematic diagram of a single "beaded" filament (taken from reference 7 and drawn to the same linear magnification as the gold-labeled type VI fibril) shows the axial correspondence of the "beads" with the dark bands of the type VI fibril (*arrows*). (*b*) A 100-nm periodic fibril occurring on the surface of a fibroblast (*C*) showing gold particles labeling the longitudinal, filamentous interbands that extend between the transverse dark bands (*arrows*). (*c*) Lower power view of a 100-nm periodic fibril heavily labeled with gold particles. Note that most of the particles are localized on the interbands (filaments). The cultures in *b* and *c* were treated with sodium adenosine triphosphate before incubation with antibodies (see Materials and Methods). Bars, 100 nm.

absent but the periodic structure of the type VI fibrils was clearly visible from the heavy metal staining, the mean axial period was ~95 nm. Control experiments with affinity-purified polyclonal antibodies directed against procollagen types I and III (21) showed clusters of ferritin label distributed along fibrils at intervals averaging 61 nm, as has been described in human dermis (20). In control cultures not exposed to primary antibodies, cross-banded fibrils (presumably type I collagen) had a mean repeating period of ~54 nm. In fields containing both type I cross-banded fibrils and type VI fibrils, the axial periods averaged 54 nm and 85 nm, respectively (not shown). Such reduced values for the axial period as compared with those of hydrated and stretched specimens (3, 6, 17, 19) probably result from shrinkage occurring during dehydration of the specimens.

In control experiments with antibody against human fibronectin, the gold particles occurred in a non-periodic distribution on small patches of filamentous material lying on the surface of filopodia and on many of the larger collagen fibrils as was reported by Hynes and Yamada (30) and by Fleischmajer and Timpl (22). It did not bind to 100-nm periodic filaments and fibrils (Fig. 5). Control cultures prepared for immunoelectron microscopy with the types I and III procollagen appeared essentially as shown by Fleischmajer et al. (20, 21). Such fibrils are not shown, but measurements made on them appear in Table I (see below).

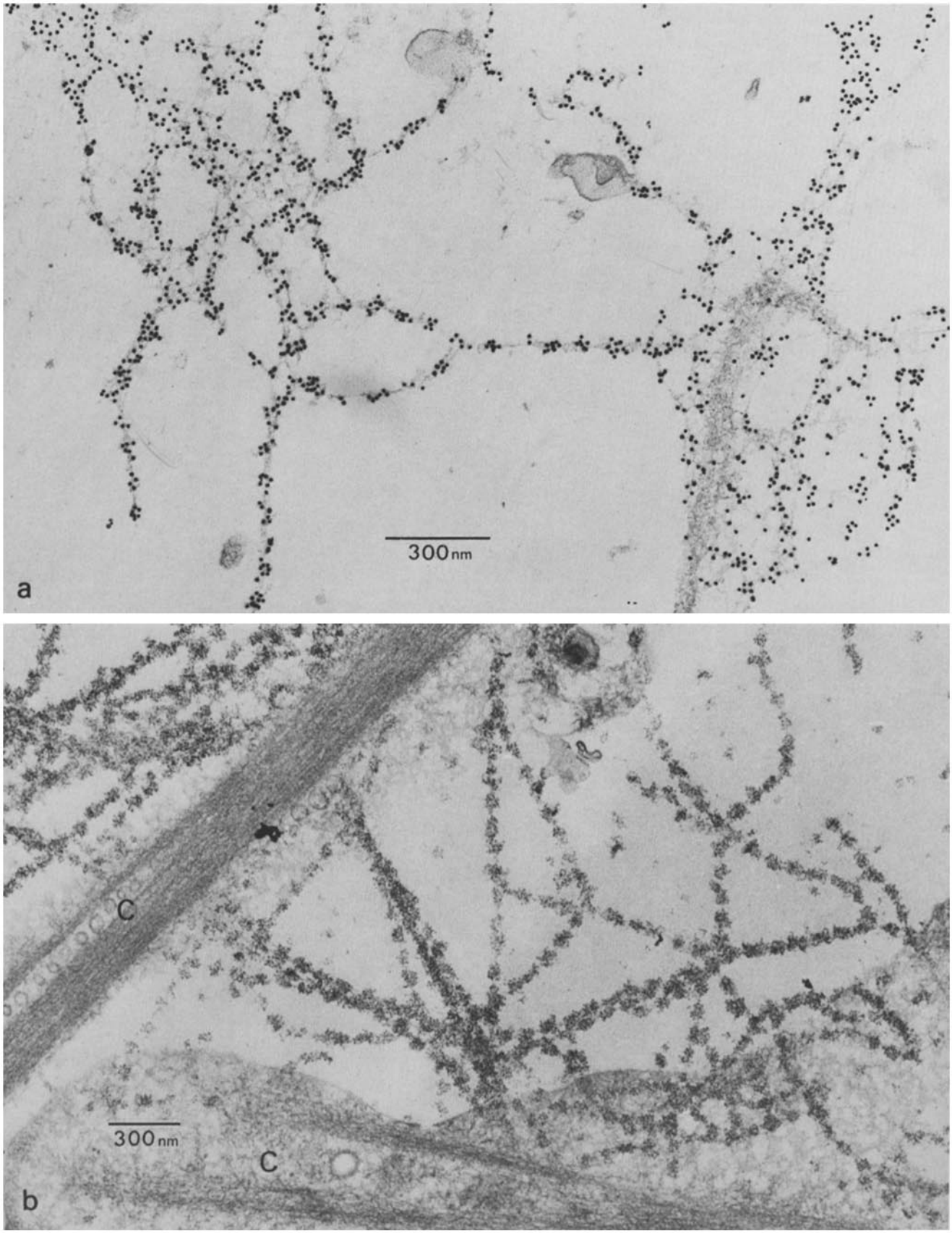

Figure 4. (*a*) 5-d culture of unfixed, human foreskin fibroblasts incubated with monoclonal antibody to human type VI collagen and with secondary goat anti-mouse IgG labeled with colloidal gold. (*b*) A 48-h culture fixed with glutaraldehyde and incubated with rabbit immune serum to human type VI collagen and with goat anti-rabbit IgG coupled to ferritin. Sections of a fibroblast are marked *C*. In both *a* and *b*, clusters of electron-dense label are distributed along filaments at intervals of 100 nm.

*Table I. Measurements on the Axial Period of Various Extracellular Fibrils**

Method	Antibody against‡	Electron dense marker§	Axial period¶ mean ± SD	*n*	Range
100-nm Filaments (Type VI)					
Ur-Pb Stain Only	–	–	95.4 ± 6.3	110 (30)	76–108
Immuno-EM	Collagen-VI (monoclonal)	Gold	90.1 ± 8.0	132 (22)	79–111
	(Antiserum)	Ferritin	95.0 ± 7.0	144 (12)	77–105
"67-nm" Fibrils (Types I & III)					
Ur-Pb Stain Only	–	–	53.6 ± 1.7	118 (12)	50–56
Immuno-EM	Procollagen I	Ferritin	61.8 ± 2.1	76 (14)	58–64
	Procollagen III	Ferritin	60.5 ± 0.3	149 (5)	54–65
Aperiodic Fibrils					
Immuno-EM	Fibronectin	Gold	–	–	–

* Human foreskin fibroblast cultures.
‡ Antigen the primary antibody was directed against.
§ Type of electron-dense marker coupled to secondary antibody.
¶ Mean is given in nm; *n*, the total number of periods measured. The number of different electron micrographs used for the measurements is in parentheses.

Fibroblast Cultures Treated with ATP

During the examination of normal, 5–8 d cultures, we occasionally encountered large, 100-nm periodic-type fibrils that appeared to be ordered aggregates of the filamentous type VI collagen. In light of our other observations, it was of interest to examine such structures with specific antibody labeling to identify them and possibly to refine the localization of the antibodies. Since they were encountered only rarely in cultures, we attempted to induce aggregation of the numerous thin filaments by the use of ATP, as in the preparation of segment-long spacing forms from native, soluble type I collagen (8, 25). In fibroblast cultures incubated for 2 h at 37°C in F-12 medium containing 20 mM ATP, pH 4.6, we found an increased number of large, wide 100-nm periodic struc-

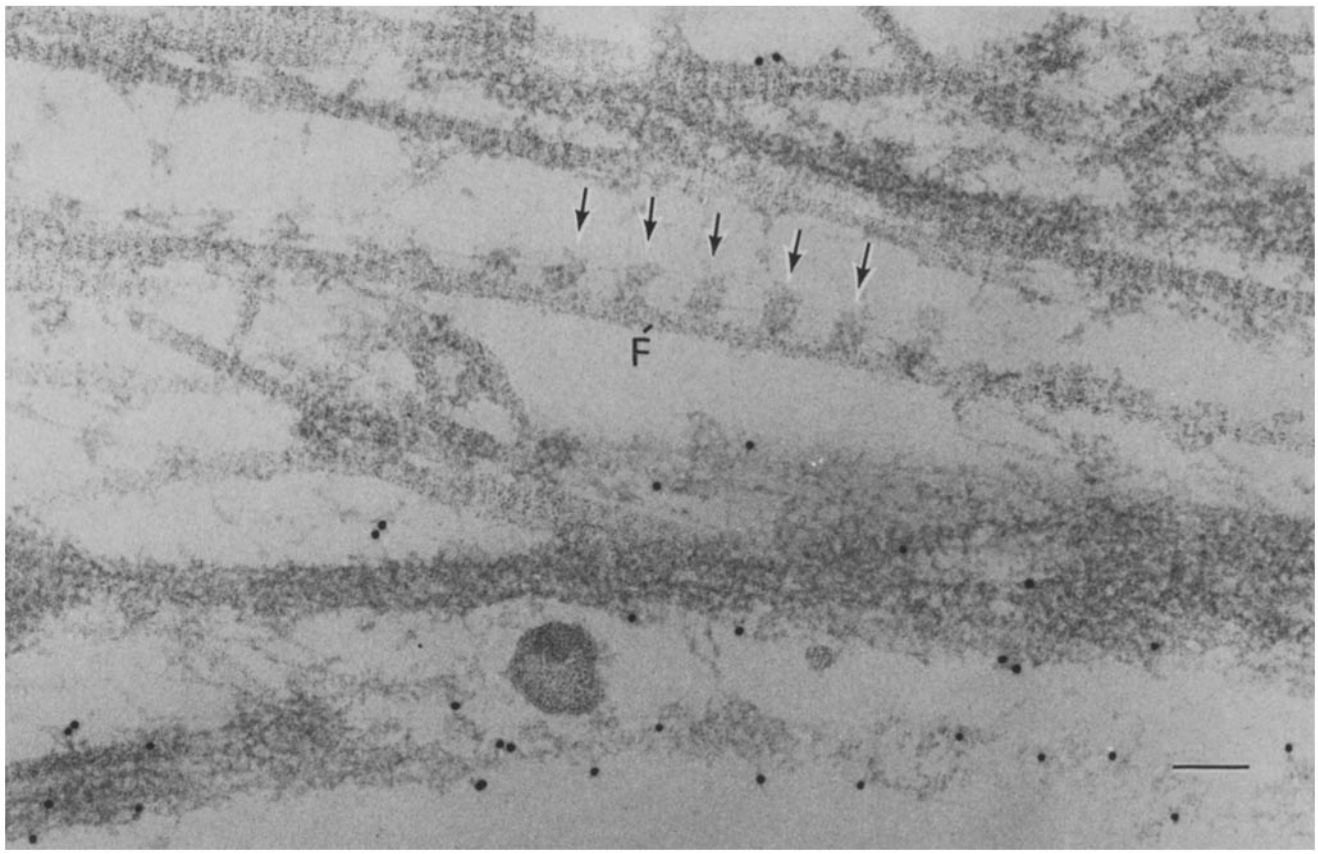

Figure 5. Thin section of 5-d, positive control, human skin fibroblast culture treated with polyclonal rabbit antibody to fibronectin and secondary goat anti-rabbit IgG coupled to colloidal gold particles of 10-nm diameter. Note that the gold particles distributed in the lower part of the field on what appears to be a grazing section of a fibroblast filopodium. A 100-nm periodic, type VI fibril (*arrows*), attached to a 20-nm wide, cross-banded fibril (*F*), is not labeled with gold particles. Bar, 100 nm.

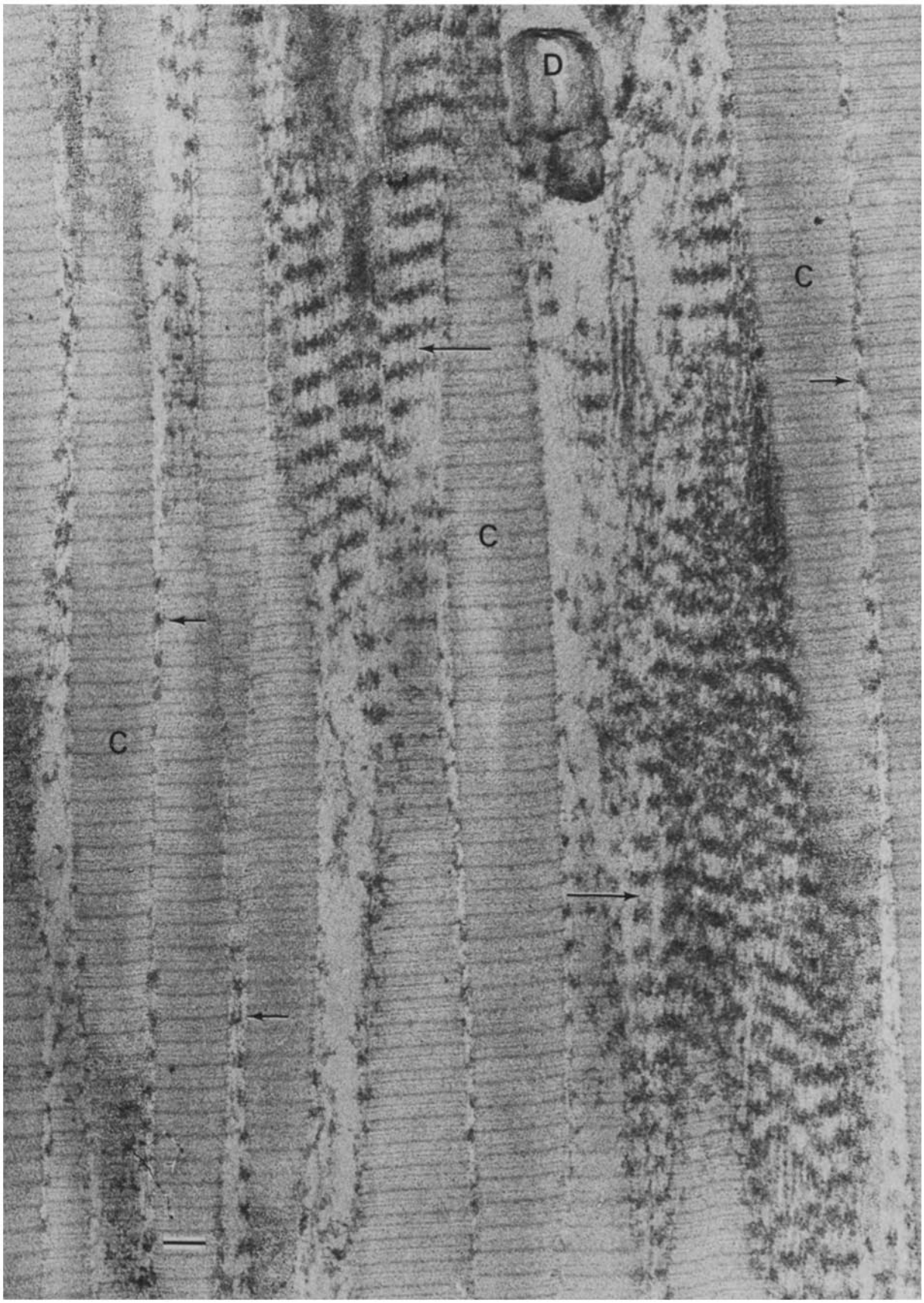

Figure 6. Longitudinal thin section of adult rat tail tendon showing 100-nm periodic fibrils (*long arrows*) distributed among type I collagen fibrils (*C*) and near to what appears to be a cell fragment (*D*). The narrow (*short arrows*) and wide (*long arrows*) type VI fibrils give the impression that they "coat" or partially cover the type I fibrils. Note that the regular period of the type VI fibril is larger than the period of the adjacent type I fibril (*small arrows*). This tendon was incubated at 37°C for 3 d in Ham's F-12 culture medium before fixation. Bar, 100 nm.

tures. Immunogold staining with the 4B10 antibody showed gold particles localized over the filamentous segments of the large fibrils (Figs. 3, *b* and *c*). In most of such large fibrils, the gold marker particles were found near to the surface, as if the gold antibody complex had difficulty penetrating the fibrils.

100-nm Periodic Fibrils in Tendons

Adult rat tail tendon, incubated in vitro for 3 d at 37°C before fixation, contained numerous 100-nm periodic fibrils distributed adjacent to the plasma membrane of fibroblasts and among the heavy bundles of type I collagen fibrils (Fig. 6). They appeared as wide, cross-banded sheets, where they were cut in grazing section, and as a narrow line of 100-nm periodic, dense nodes, where the type I fibrils were close together and cut in their longitudinal axis (Fig. 6). The 100-nm period of the type VI fibrils showed no obvious regular relation to the 67-nm period of the type I fibrils (Fig. 6).

In occasional sections grazing a bundle of type I collagen fibrils, type VI-like fibrils appeared to be arranged in a hexagonal net (Fig. 7), which resembled the network described by Carlson et al. (Figs. 13 and 14 in reference 11) in rabbit renal tubular basement membranes and the hexagonal array in bovine Descemet's membrane (31, 51).

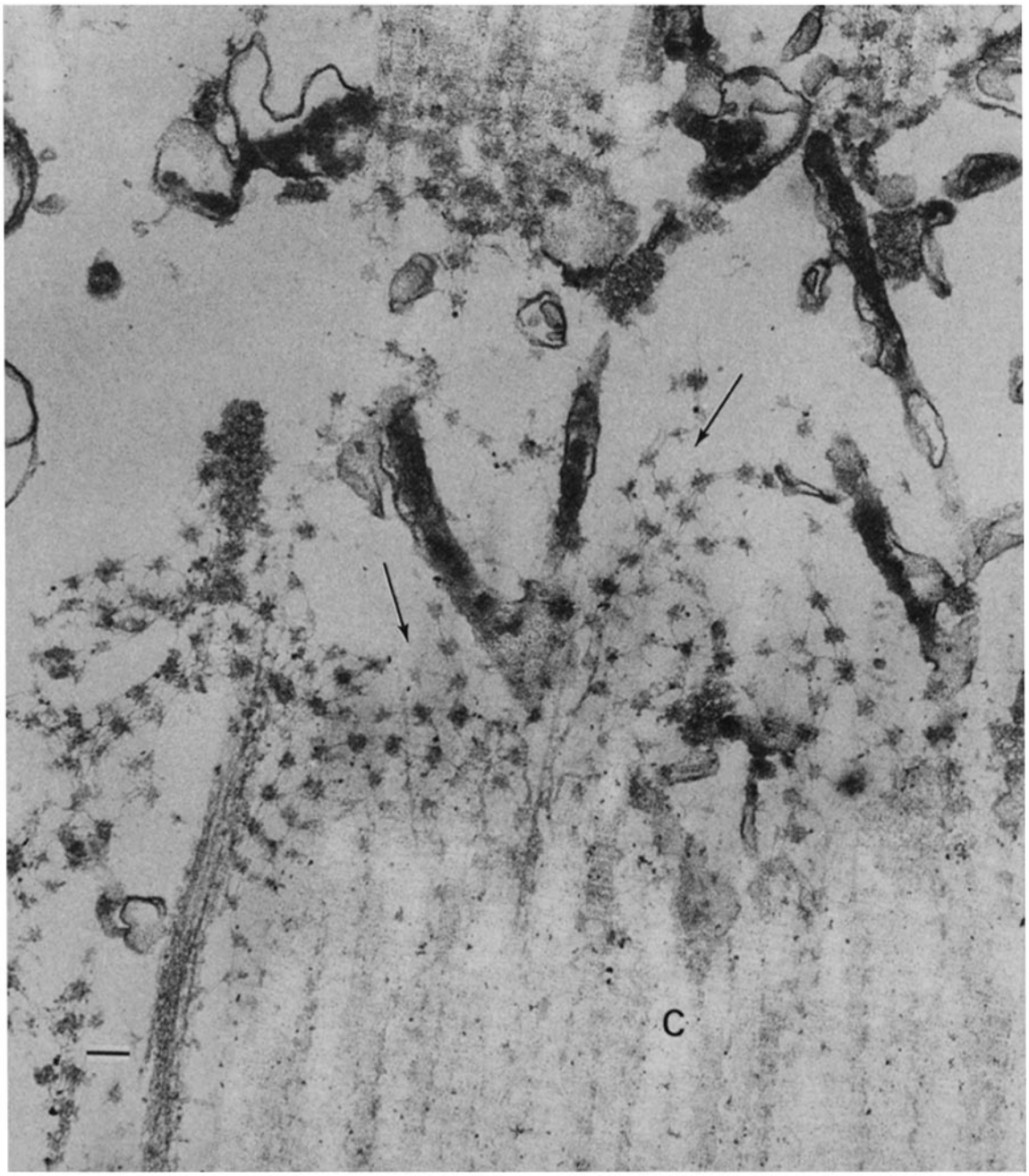

Figure 7. Thin section of chicken embryo metatarsal tendon grazing a bundle of type I collagen fibrils (*C*) and showing type VI fibrils (arrows) *en face*. Note that the dense nodes are connected by thin filaments and appear to be arranged in a hexagonal-type of pattern. Tendon was treated with ATP (see Materials and Methods). Bar, 100 nm.

The type VI fibrils were not distributed uniformly throughout the tendon. They seemed to be concentrated near fibroblasts, which were always disrupted by the prolonged incubation and exposure to ATP at low pH. Most fields within the tendon did not show any type VI fibrils. The 100-nm periodic fibrils were not visible in rat tail tendons fixed immediately after removal from the animal (4, 5, 26, 44).

Discussion

Type VI collagen is a "short chain" molecule consisting of a triple-helical segment (105 nm in length) with globular domains at each end (18, 23, 33, 43). In its natural form, it exists as an extensively disulfide cross-linked aggregate containing polypeptide chains of 140 kD and 260 kD (18, 57). The helical region is thought to contain the 140-kD polypeptide. The 260-kD polypeptide appears to be non-collagenous and its conformation in the aggregate is not known (18). Rotary-shadowed specimens suggest that the monomeric form of type VI associates in an antiparallel, staggered arrangement to form tetrameric, dumbbell shaped units which in turn assemble end-to-end to form oligomers (18) or "microfibrillar" structures (59). It has been proposed that "beaded" filaments and 100-nm periodic fibrils, which occur in the extracellular matrix of fibroblast cultures and tissues, contain type VI collagen (7).

Our results with immunoelectron microscopy on fibroblast cultures (the binding of specific antibody and the 100-nm periodic distribution of the antibody on filaments and fibrils), in addition to the structural similarities between 100-nm periodic filaments and the reported type VI collagen oligomers (7, 18, 23), all support the conclusion that the 100-nm periodic filaments and fibrils, found in the extracellular matrix of fibroblast cultures, contain type VI collagen. The occurrence of similar periodic fibrils in both adult rat tail and chicken embryo metatarsal (not shown) tendons suggest that they perform some significant function extending from embryonic to adult life.

The binding of antibody 3C4 to the band region of type VI collagen fibrils (Fig. 2) and recent evidence that the 3C4 monoclonal antibody binds to the reduced and denatured 260-kD polypeptide component of VI (18), suggest that the antibody binding site for the 260 kD polypeptide is located in the band (non-helical) region of the type VI fibril. This location would correspond to the globular ends of tetrameric, type VI collagen models shown by Furthmayr et al. (23) and Engvall et al. (18). The conformation of the remaining part of the 260-kD chain in the native aggregate is unknown. The 4B10 antibody binds at the interband region of the fibril (Figs. 2 *b* and 3, *a–c*), but it is not known which polypeptide chain(s) contains its binding site.

The type VI filaments are widely distributed in the extracellular matrix of fibroblast cultures (Fig. 1). Immunofluorescent staining of the cultures as a function of time

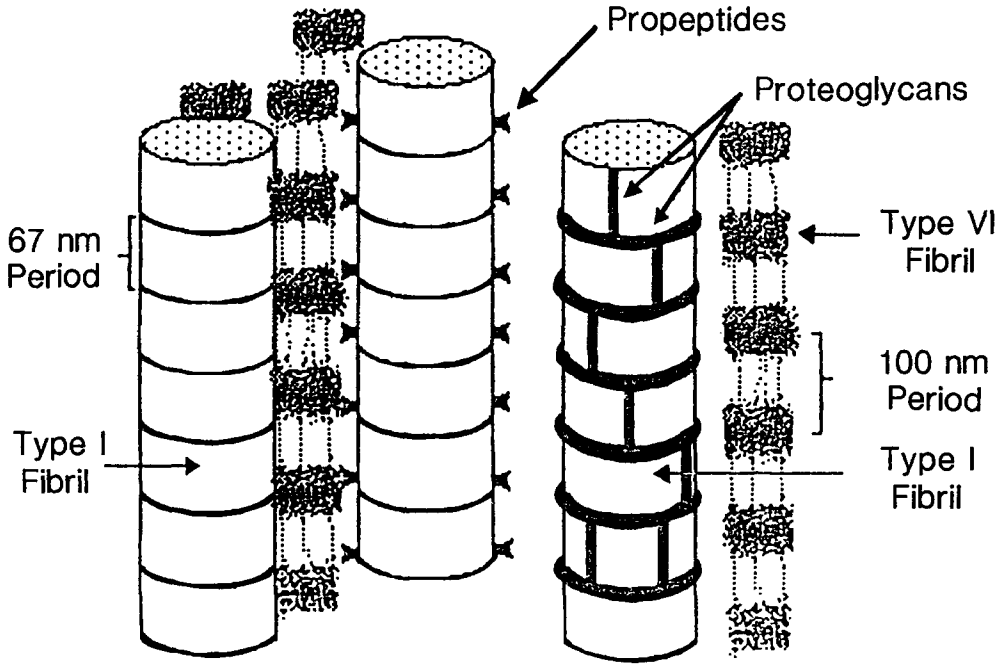

Figure 8. Summarizing diagram showing our current concept of the major fibrous components in the extracellular compartment of adult rat tail tendon. Dense collagenous areas in the tendon contain fascicles of type I collagen fibrils, which consist of "quarter-staggered" molecules and show a periodic cross-banding of 67 nm. The fibrils are covered with an orthogonal array of proteoglycan (52) and may have laterally projecting propeptides of assembled, but incompletely processed procollagen (21). The fibrils of type VI collagen, which occur among the type I fibrils, consist of laterally aggregated "beaded" filaments having a period of ~100 nm. No regular matching is evident between the 67-nm and 100-nm periods. In fascicles of type I fibrils, the type VI filaments and the type I fibrils generally lie in parallel, but other orientations also may occur (59). The natural aggregation state (fibrous, monomeric, complexed with other tissue components) and the function of the type VI is unknown.

shows that fluorescence first appears within or on clusters of cells and gradually increases to form a dense network throughout the entire culture. We find no indications that the extracellular type VI collagen is a transient component that disappears with time. Immunoelectron microscopy shows that the type VI filaments occur throughout the extracellular matrix, frequently near and around type I fibrils and sometimes close to the plasma membrane of filopodia. In cultures treated with ATP, large 100-nm periodic fibrils are occasionally encountered on plasma membranes (Fig. 3, *b* and *c*). They resemble the cross-banded fibrils formed in vitro by the addition of ATP to an acidic solution of type VI collagen (32).

The occurrence of 100-nm fibrils in tendons treated with ATP or subjected to prolonged incubation in culture medium (Fig. 6) and their absence (or our inability to observe them) in untreated tissues raises questions about their origin and native state. A careful look at thin sections of interfibrillar and pericellular areas of untreated tendons reveals small amounts of wispy, unorganized filaments (26, 39). If this material is type VI and exists naturally as unorganized filaments, then ATP or prolonged incubation must induce an ordered aggregation, while routine fixation would preserve the native, non-ordered state of the filaments. On the other hand, if the type VI filaments exist in situ in an ordered state, the ATP or incubation treatment would preserve the ordered state of the filaments against the disaggregating action of the fixative. Intuitively, we would expect as most likely the first possibilities, for there appears to be an insufficient number of thin filaments in routinely fixed tendon to account for the masses of 100-nm periodic filaments found in the treated tendons and, conversely, there appear to be too few thin filaments if masses of the naturally occurring 100-nm periodic fibrils became disorganized. How ATP and simple lengthy incubations control these various aggregation states is not clear at this time.

Several reports describe carbohydrate-containing material associated with the dark bands of what now appear to be type VI fibrils. Yardley and Brown (62) found in situ, colloidal iron staining material ("acid mucopolysaccharides") associated with the dense bands of "broad band material" in fibroblast cultures, and others have found ruthenium red–staining material on the bands of similar unidentified fibrils in nucleus pulposus and in skin (10, 27, 34). The thin filaments that extend perpendicularly between large collagen fibrils in aorta and synovium, and which stain with ruthenium red (39, 40) also could contain type VI structures. In rat tail tendon, Scott and Orford (52) have described three forms of proteoglycan-containing filaments that stain with the dye Cupromeronic Blue. One type occurs in orthogonal arrays on specific bands of type I collagen fibrils in rat tail tendon. The two other types of stained filaments appear either as long, densely staining, broad rods or as fine filaments that are not regularly related to the type I fibrils. These occur in some areas where the type VI fibrils occur, but any significant relations or interactions between the two remain to be demonstrated. Fig. 8 shows our concept of these structures in diagrammatic form.

The literature contains numerous examples of unusual, extracellular, cross-banded structures that have not been adequately characterized. Some of them show a 100-nm axial period consisting of only one major dark and one light band (1, 12–14, 24, 27, 28, 34, 37, 41, 42, 46, 54, 62). Interestingly, similar fibrils with the same periodicity and appearance occur in the byssus threads of the mollusk, *Mytilus galloprovincialis* (2). Such 100-nm periodic fibrils may be related to those identified here as type VI collagen. How type VI collagen relates to other components of the extracellular matrix and to cell function during development and in adult life is yet to be explored.

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