Ultrastructure of Type VI Collagen in Human Skin and Cartilage Suggests an Anchoring Function for This Filamentous Network

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Abstract. An mAb was used in conjunction with immunoelectron microscopy to study the ultrastructure and distribution of the type VI collagen network. Type VI collagen in femoral head and costal cartilage was found distributed throughout the matrix but concentrated in areas surrounding chondrocytes. Three-dimensional information gained from high voltage stereo pair electron microscopy showed that the type VI collagen network in skin was organized into a highly branched, open, filamentous network that encircled interstitial collagen fibers, but did not appear to interact directly with them. Type VI collagen was also found concentrated near basement membranes of nerves, blood vessels, and fat cells although in a less organized state. Labeling was conspicuously reduced close to the epithelial basement membrane in the region of the anchoring fibrils. No labeling of basement membranes was seen. Based on these observations it is suggested that the type VI collagen forms a flexible network that anchors large interstitial structures such as nerves, blood vessels, and collagen fibers into surrounding connective tissues.

TYPE VI collagen forms a filamentous network in most extracellular matrices. The basic structural subunit of the filaments is a tetramer of type VI collagen molecules (for recent review see Timpl and Engel, 1987). The structure of the subunits has been described in some detail from electron microscope studies of rotary-shadowed molecules, initially on pepsin-solubilized and later intact type VI collagen tetramers (Furthmayr et al., 1983; von der Mark et al., 1984; Engvall et al., 1986). The type VI collagen molecule consists of a short triple helix, ∼105 nm in length, which has a very large globular domain at each end. From biosynthetic studies in fibroblasts, it is believed that dimers are first formed by two molecules aligning themselves in an antiparallel fashion so that their helices are staggered and overlap by 75 nm. Tetramers are formed by lateral association of two dimers with their ends in register (Engvall et al., 1986). These structures are stabilized by disulfide bonds. The end-on-end aggregation of tetramers gives rise to filaments that have been observed in cell culture as beaded filaments, the beads being a structure formed by the interaction of globular domains from two tetramers (Bruns, 1984).

In tissues, type VI collagen filaments are difficult to visualize without the aid of an immunolabel and also difficult to distinguish from microfibrillar structures such as those containing fibrillin (Sakai et al., 1986). Several mAbs have been used to immunolocalize type VI collagen and have shown that it has a very broad distribution in a wide variety of tissues (Table I). It has also not yet been detected in the calcified matrix of bone, and immunofluorescent staining of hyaline cartilage has produced conflicting results (Ayad et al., 1984; von der Mark et al., 1984).

Ultrastructural studies using ferritin or gold-labeled secondary antibodies have been much more restricted. In placenta, type VI collagen was localized to thin filaments 6–10 nm in diameter in amorphous stromal regions of placental villi (von der Mark et al., 1984), and surrounding type I/III collagen fibers (Amenta et al., 1986). In the aorta, heavy staining of filaments between the thick collagen fibers was observed with no labeling of thick collagen or elastin fibrils (von der Mark et al., 1984). Both studies gave the impression that type VI collagen formed a filamentous network that was independent of the type I/III collagen fibrils and frequently ran perpendicular to them. Similar observations were made in studies of a neurofibroma (Fleischmajer et al., 1985).

Banded aggregates of collagen with a periodicity of ∼100 nm have been frequently found in cell culture systems and many pathological tissues, particularly tumors (reviewed by Pillai, 1964; Sun and White, 1975; Buckwalter et al., 1979). This is distinctly different from the 64-nm periodicity of the interstitial collagens and corresponds well to a model for laterally aggregated type VI filaments. These structures, referred to as broad-banded material (Yardley and Brown, 1965), Luse bodies (Luse, 1960), or fibrous long spacing

Table I. Tissue Distribution of Type VI Collagen Using Immunofluorescence

Tissue	References
Skin	Gibson and Cleary, 1983; von der Mark et al., 1984; Hessle and Engvall, 1984; Sakai et al., 1986; McComb et al., 1987
Kidney	Jander et al., 1981; Marton and Arnason, 1982; Gibson and Cleary, 1983; von der Mark et al., 1984; Hessle and Engvall, 1984; McComb et al., 1987
Muscle	Marton and Arnason, 1982; Hessle and Engvall, 1984; Linsenmayer et al., 1986
Brain, liver	Marton and Arnason, 1982; McComb et al., 1987
Blood vessels	Jander et al., 1981; Gibson and Cleary, 1983; von der Mark et al., 1984
Amnion/chorion	Hessle and Engvall, 1984
Cornea, sclera, perichondrium	Linsenmayer et al., 1986
Alveolar bone, dentine	Becker et al., 1986
Spleen	Gibson and Cleary, 1983; McComb et al., 1987
Thyroid, pancreas, heart	McComb et al., 1987
Elastic cartilage	Sakai et al., 1986
Nuchal ligament, lung, tendon	Gibson and Cleary, 1983

(Silberberg et al., 1963; Ramsey, 1965; Cravioto and Lockwood, 1968) have also been found in normal nucleus pulposus (Buckwalter et al., 1979; Cornah et al., 1970).

The results presented here address four issues: the presence of type VI collagen in cartilage, the three-dimensional structural relationship between the type VI filaments and interstitial collagen, the relationship of type VI collagen filaments to broad-banded collagen aggregates, and the function of type VI collagen.

Materials and Methods

Antibody

The mouse mAb (5C6) which recognizes the helical domain of human type VI collagen used throughout this study has been characterized previously (Engvall et al., 1986).

Immunolabeling of Tissues

Enbloc immunolabeling of tissues was carried out using a previously described protocol (Sakai et al., 1986) with some modification. Freshly obtained human neonatal foreskins, skin obtained at autopsy, and adult skin biopsies were sliced perpendicular to the epithelium into strips 0.5-1 mm in thickness. Unfixed tissues were washed in PBS, pH 7.4, at 4°C for ∼2 h and then incubated in mouse ascites containing human type VI collagen antibody, diluted 1:5 in PBS overnight at 4°C. Following a 6-h wash in PBS at 4°C, the samples were incubated in goat anti-mouse (GAM) 5-nm gold conjugate (Janssen Life Science Products, 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, overnight at 4°C, followed by an extensive rinse in PBS as above. The samples were then rinsed briefly in 0.1 M sodium cacodylate buffer,

pH 7.4, fixed in cacodylate-buffered 3% formaldehyde/3% gluteraldehyde, rinsed again in cacodylate buffer, then postfixed in buffered 1.0% OsO_4. The samples were then rinsed in buffer, dehydrated in a graded series of ethanol dilutions to 100% EtOH, washed in propylene oxide, infiltrated in Spurr's epoxy (hard formula), and embedded so as to obtain cross sections of the epithelium. Some skin samples were removed from the above mentioned enbloc-treated samples after 5-nm gold incubation and exposed to freshly prepared 3% formaldehyde/3% gluteraldehyde containing 1,500 ppm ruthenium red, rinsed in 0.1 M cacodylate buffer, pH 7.4, containing 400 ppm ruthenium red, postfixed in 1% buffered OsO_4 containing 400 ppm ruthenium red, and then dehydrated without a buffer wash (modified from Myers et al., 1969).

Human cartilage was obtained at autopsy within 24 h after death, and was incubated in hyaluronidase (Sigma Chemical Co., St. Louis, MO) (8,000 U/ml 0.1 M $NaHPO_4$-0.15 M NaCl, pH 5.3) for 1 h at room temperature followed by a rinse in 0.1 M Tris acetate buffer, pH 7.6, then incubated in chondroitinase ABC (Miles Scientific, Naperville, IL; 0.25 U/ml Tris acetate buffer, pH 7.6) for 90 min at room temperature followed by a rinse in PBS before incubation in primary antibody. Subsequent enbloc immunolabeling was as described above. Control samples of all tissues were treated in the same manner except antibodies of irrelevant specificity were substituted for type VI collagen antibody.

Conventional and High Voltage Transmission EM

For conventional transmission EM, 60-90 nm sections were cut using an ultramicrotome (Ultracut E; Reichert Scientific Instruments, Buffalo, NY) and mounted on 2 × 1 mm formvar-coated slot grids. Grids were stained in 2% uranyl acetate in 50% EtOH for 15 min followed by Reynolds' lead citrate (Reynolds, 1963) for 60 s, and examined using a transmission electron microscope (model 410LS; Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 60 kV with a 30-μm objective aperture. The sections from which stereo micrographs were taken varied from 0.25-1.0 μm and staining times varied according to section thickness. Thick sections were observed using either a JEM 1000 (Japan Electronic Optics, Corp., Peabody, MA) operated at 1,000 kV at the High Voltage Electron Microscope Laboratory, University of Colorado (Boulder, CO) or a transmission electron microscope (model 410LS; Philips Electronic Instruments, Inc.) equipped with a LaB_6 emitter operated at 100 kV with a 50- or 30-μm objective aperture. Tilt angles for stereo pairs varied dependent on section thickness and magnification.

Scanning EM

Skin samples prepared for scanning EM were removed from the pool of samples following dehydration through 100% ethanol and critical-point dried in liquid CO_2 in a critical-point drying apparatus (model 814; Tousimis Scientific Instruments, Rockville, MD), mounted onto stubs, and sputter-coated with gold in an evaporator (MED 010; Balzers, Hudson, NH). Samples were observed using the upper stage of a scanning EM (model DS 130; International Scientific Instruments, Inc., Milpitas, CA) equipped with an LaB_6 emitter operated at 10 kV with a spot size of 3-10 nm.

Results

Cartilage

When hyaluronidase- and chondroitinase-treated femoral head cartilage from a 30-mo-old human was incubated in type VI collagen primary antibody followed by 5-nm gold-conjugated secondary antibody, the gold localized to randomly arranged 100-nm periodic branching filaments present throughout the cartilage matrix (Fig. 1 *a*). No direct association or alignment to the type II collagen fibrils was seen. However, a higher concentration of type VI-containing fibrils was found immediately adjacent to chondrocytes (Fig. 1, *b* and *c*). Without pretreatment of the cartilage with hyaluronidase and chondroitinase, no gold label was found. These filaments were not labeled in cartilage treated with mAbs directed against irrelevant antigens, for example type III collagen, fibrillin, and elastin.
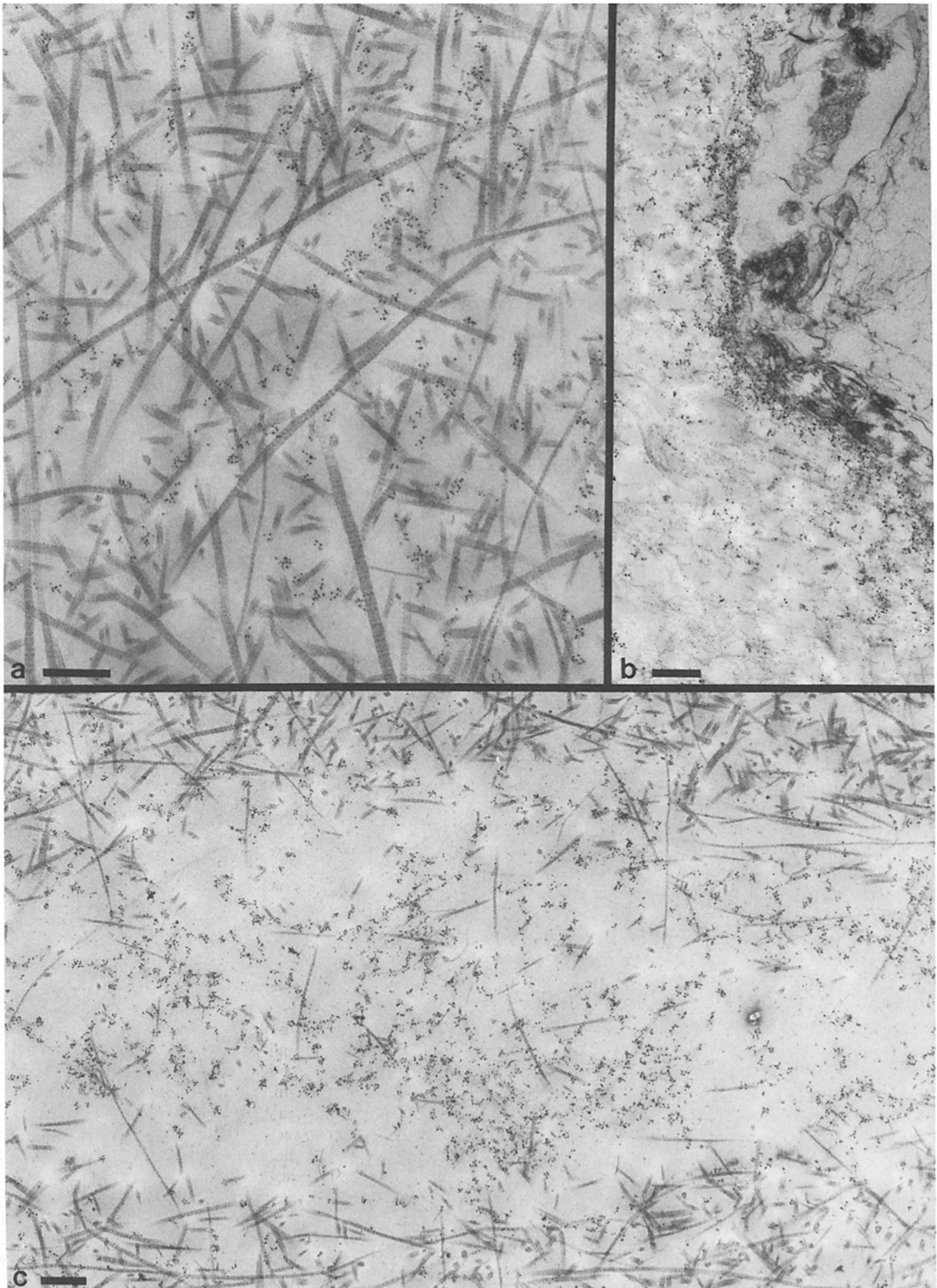
Figure 1. Electron microscopic immunolocalization of type VI collagen in human cartilage. Type VI collagen is distributed as randomly arranged periodic fibrils in cartilage matrix (*a*, 36-mo costal cartilage). It is present in higher concentration immediately adjacent to chondrocytes, for example in a 24-wk fetus femoral head chondrocyte cut in cross section (*b*), and in a grazing section through the pericellular matrix of a 36-mo costal cartilage chondrocyte (*c*). Bars, 250 nm.
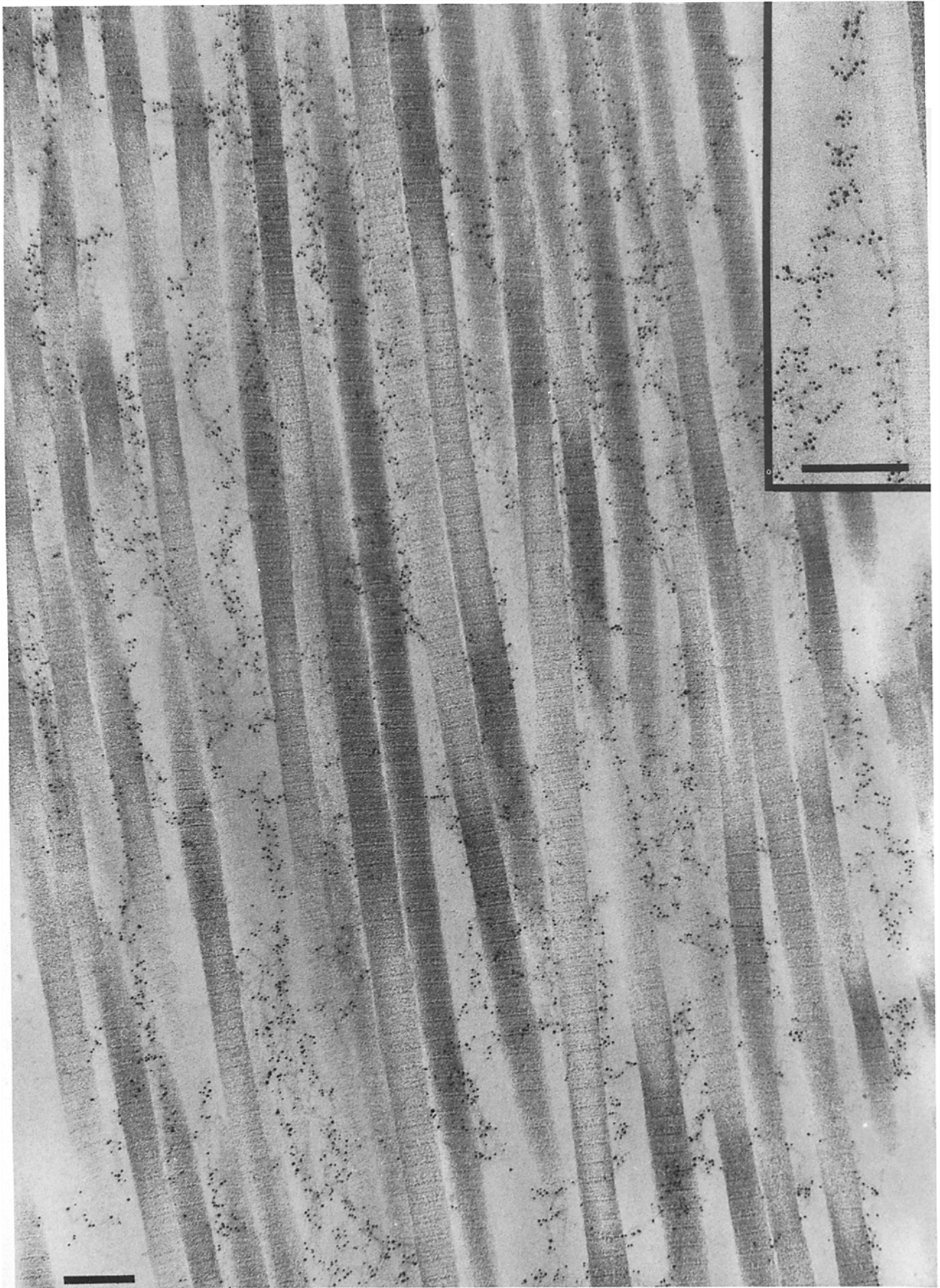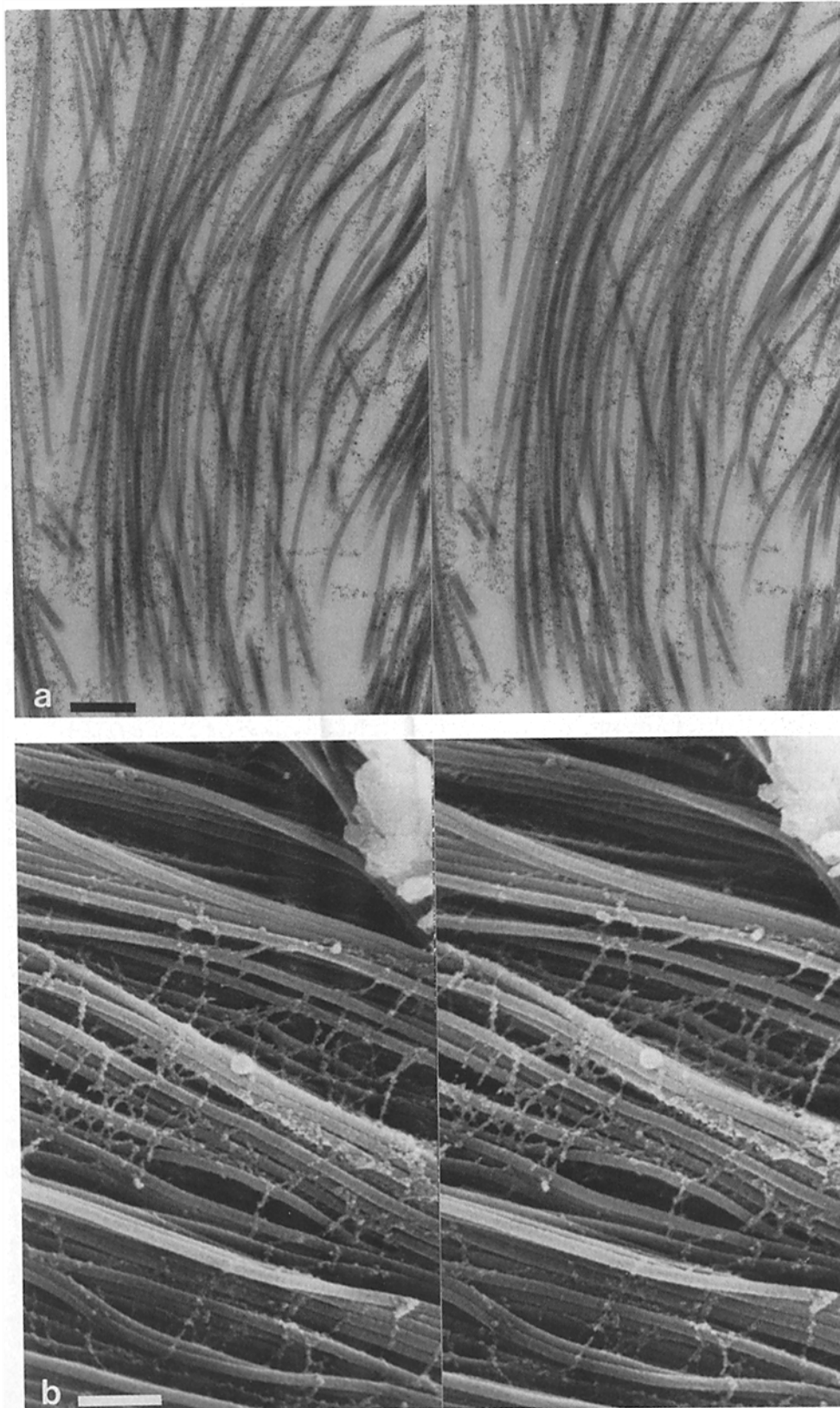
Figure 3. Stereo pairs of type VI–antibody immunolabeled skin. (*a*) High voltage EM stereo pair of a 0.5-μm-thick section of 45-yr-old human skin. A branching network of type VI collagen, interdigitating amongst banded fibrils is clearly visible. (*b*) Scanning EM stereo pair of similarly treated 9-yr-old skin. The type VI micofibrillar network is clearly visible, extended across many loosened interstitial collagen fibrils. Bars, 500 nm.

Figure 2. Electron microscopic immunolocalization of type VI collagen in 20-yr-old human skin. The type VI network in adult skin matrix associated with longitudinally section banded collagen fibrils appears to align roughly parallel with interstitial collagen fibrils. The label displays ∽100-nm periodicity (*inset*). Bars, 200 nm.

Skin

In the reticular layers of skin, 5-nm gold deposition directed by mAbs to type VI collagen revealed a branching network of type VI closely associated with banded collagen fibrils (Fig. 2). Type VI antibody-directed gold label often displayed a periodicity of $\sim$ 100 nm (Fig. 2, *inset*) as previously reported (Bruns et al., 1986). When this filamentous network was examined in longitudinal section through the associated banded collagen fibrils, the general orientation of the type VI network appeared to be parallel to that of the banded fibrils. However, the type VI filamentous network branches repeatedly, and in so doing traverses many individual banded fibrils. This is most readily seen by viewing stereo pictures (Fig. 3), or in the scanning EM (Fig. 4). In areas where banded collagen fibers were cut in cross section, type VI collagen filaments were seen to traverse between individual banded fibrils but were often not uniformly distributed (Fig. 5 *a*). Local concentrations of type VI sometimes appeared to subdivide banded fibrils into groups (Fig. 5 *b*, *arrows*). Generally, the type VI network formed a weblike network of branching filaments which intertwined with banded collagen fibrils.

In the papillary dermis closely apposed to the epithelial basement membrane, type VI collagen associated with banded collagen fibrils was seen to lack the weblike arrangement seen in the deeper reticular layers of skin and appeared to be more uniformly distributed. In this region, the banded fibrils were similar to type II collagen in cartilage as they exist as individual fibrils (Fig. 6 *a*). Type VI–directed gold particles were not detected within the basement membranes of skin nor within anchoring plaques, which are connected to the epithelial basement membrane by way of the anchoring fibril network (Keene et al., 1987*b*).

Adjacent to basement membranes associated with fat cells (Fig. 6 *b*), nerve fibers (Fig. 6 *c*), and capillaries (not shown), the concentration of type VI–containing filaments was markedly increased relative to the concentration noted in the matrix contained within the reticular and papillary layers. As seen by viewing stereo pairs (Fig. 7), type VI was associated with disorganized interstitial collagen fibrils surrounding the nerve and accumulated along the basement membrane. Fibrillin, a noncollagenous microfibrillar structure often associated with collagens and easily mistaken for type VI collagen, is also visible (also see Fig. 4 *a*).

When immunolabeled tissues were stained enbloc with ruthenium red, the type VI–containing filaments stained intensely and were easily visualized between sites labeled with gold particles (Fig. 8, *arrowheads*), whereas in untreated preparations filaments were only faintly visible (Fig. 2, *inset*).

When the amount of type VI–directed colloidal gold distributed in 24-wk-old fetal skin was compared to that amount distributed in neonatal or older skin, it was our impression that there is significantly more type VI collagen present in the fetal skin. Additionally, in fetal skin which had undergone enbloc treatment with type VI antibody, discreet broad-banded aggregates displaying $\sim$100-nm periodicity were commonly distributed in areas beyond the limits of antibody penetration (Fig. 9). This is in contrast to similarly treated older skin in which their occurrence was much rarer and almost entirely limited to areas immediately adjacent to nonepithelial basement membranes.

Discussion

Immunofluorescent studies previously undertaken to localize type VI collagen in cartilage have produced contradictory results, none of which agree completely with the results reported here. In one study (von der Mark et al., 1984) no type VI was detected in cartilage, while in another (Ayad et al., 1984) only pericellular staining was observed. These discrepancies may be caused by differences in tissue age or pretreatment conditions before antibody labeling procedures. The results reported here show it to be distributed throughout the matrix of the cartilage, and somewhat more concentrated directly around chondrocytes. It is not yet possible to say whether the increased labeling around chondrocytes corresponds to the pericellular matrix described by Poole et al. (1984) and also by Weiss et al. (1968), or to the pericellular capsule which has been described as a basket-weave network of fine fibrillar material which surrounds the pericellular network (Poole et al., 1984). Farther away from the chondrocyte, the labeled VI filaments are found in decreasing numbers, forming an interlacing network between type II collagen fibrils. Type VI collagen has been described as a beaded filament (Bruns et al., 1984), and the distribution described for the beaded filaments in cartilage based purely on ultrastructural appearance (Weiss et al., 1968) agrees well with the distribution of type VI described here.

The pericellular matrix of chondrocytes also stains with ruthenium red (Luft, 1971; Poole et al., 1984) as do filaments observed in several other tissue structures including synovium (Myers et al., 1969), skin, capillaries (Kajikawa et al., 1970), nerve (Luft, 1971), and aorta (Myers et al., 1973). It was shown that mucopolysaccharides are a component of these filaments (Myers et al., 1969) and it was suggested that the mechanical properties of tissues would be influenced by their composition (Myers et al., 1973). As shown here, type VI collagen is a component of a ruthenium red–stainable network.

Local accumulation of type VI filaments is not only observed around chondrocytes in cartilage but also around basement membranes associated with fat cells, nerves, and capillaries of the skin. In contrast, no accumulation of type VI is found at the epithelial basement membrane. The epithelial basement membrane is a sheetlike structure that is attached to the underlying connective tissue by a system of anchoring plaques and fibrils which contain type VII collagen (Keene et al., 1987*b*). Other basement membranes lack an anchoring fibril network, so the accumulation of a type VI filamentous network encircling these structures and its continuation into the surrounding matrix may serve to anchor them in the surrounding connective tissue. Type VI collagen around endothelial basement membranes may in fact be synthesized by endothelial cells (Sawada et al., 1987). Thus type VI could contribute significantly to the mechanical properties of connective tissues, binding various components such as collagen fibers, nerves, blood vessels, cells, and ground substance together into a functional tissue while still allowing flexibility. Although there are no covalent bonds between the tetrameric subunits of type VI collagen (Wu et al., 1987), the strength of the network may be provided by the numbers of filaments involved. The absence of covalent linkages may in fact facilitate growth and remodeling of tissues and per-
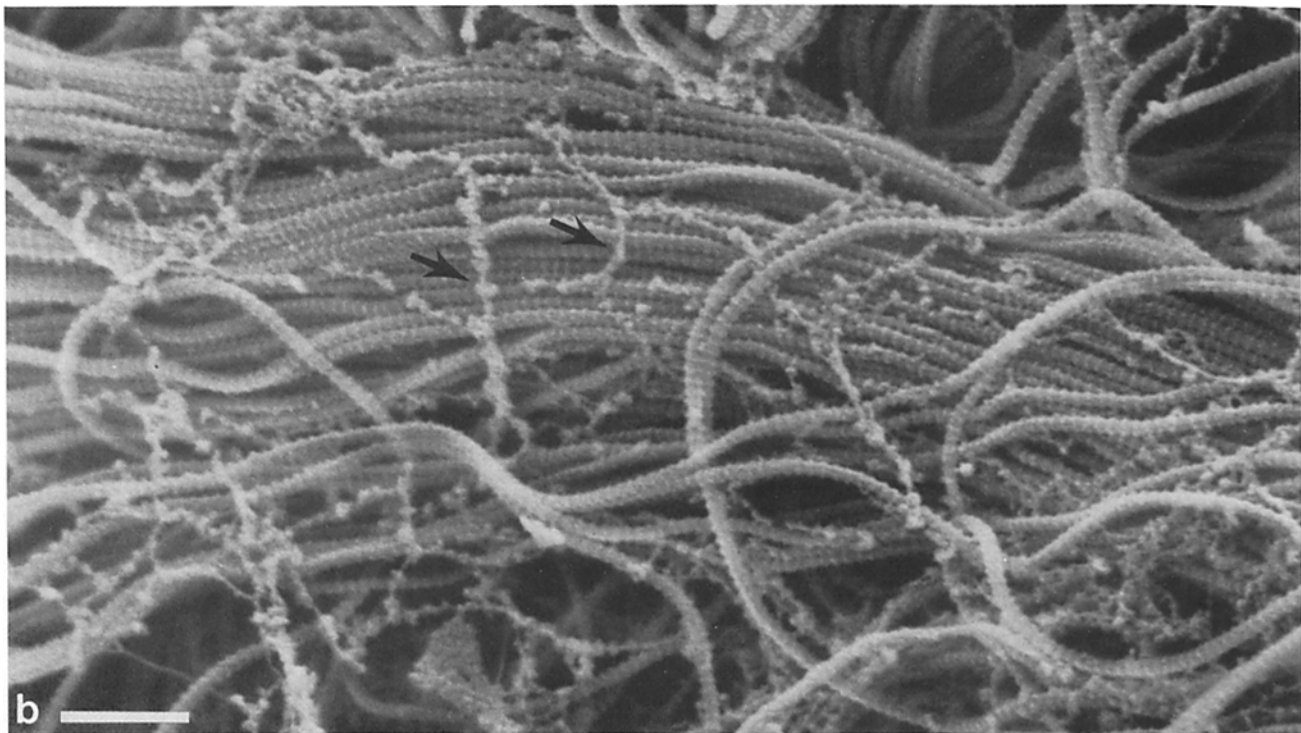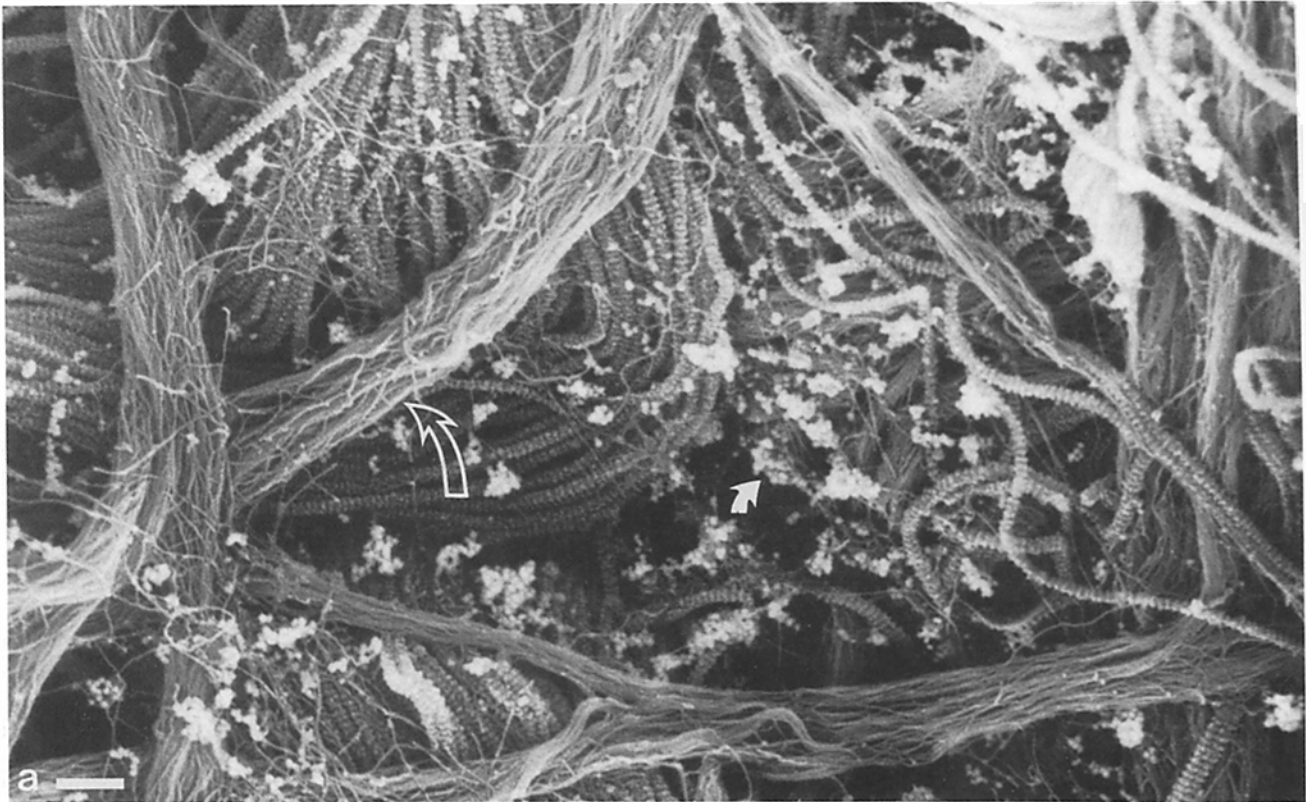
Figure 4. Scanning EM of human neonatal foreskin. (*a*) Scanning EM reveals the type VI network, decorated with antibody-directed 40-nm gold clusters (*solid arrow*), which can be easily distinguished from thick fiber bundles containing fibrillin (*open arrow*). (*b*) The branching type VI network labeled with 5-nm gold forms an open basketweave-like network which girds bundles of interstitial collagen fibrils. Type III collagen is also immunolabeled in this micrograph by the "naked" IgM technique in which no gold conjugates were used (Keene et al., 1987*a*). Bars, 500 nm.
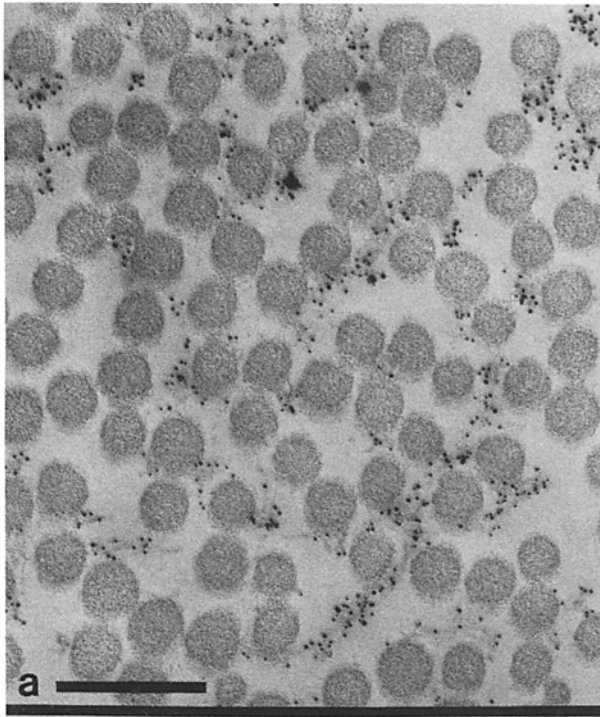
Figure 5. A cross section of interstitial collagen fibrils showing the distribution of immunolabeled type VI collagen in 18-yr-old human skin. Type VI collagen is not uniformly distributed (*a*). Occasionally, local concentrations of type VI collagen seem to divide banded collagen fibers into groups of fibrils (*arrowheads, b*). Bars, 250 nm.
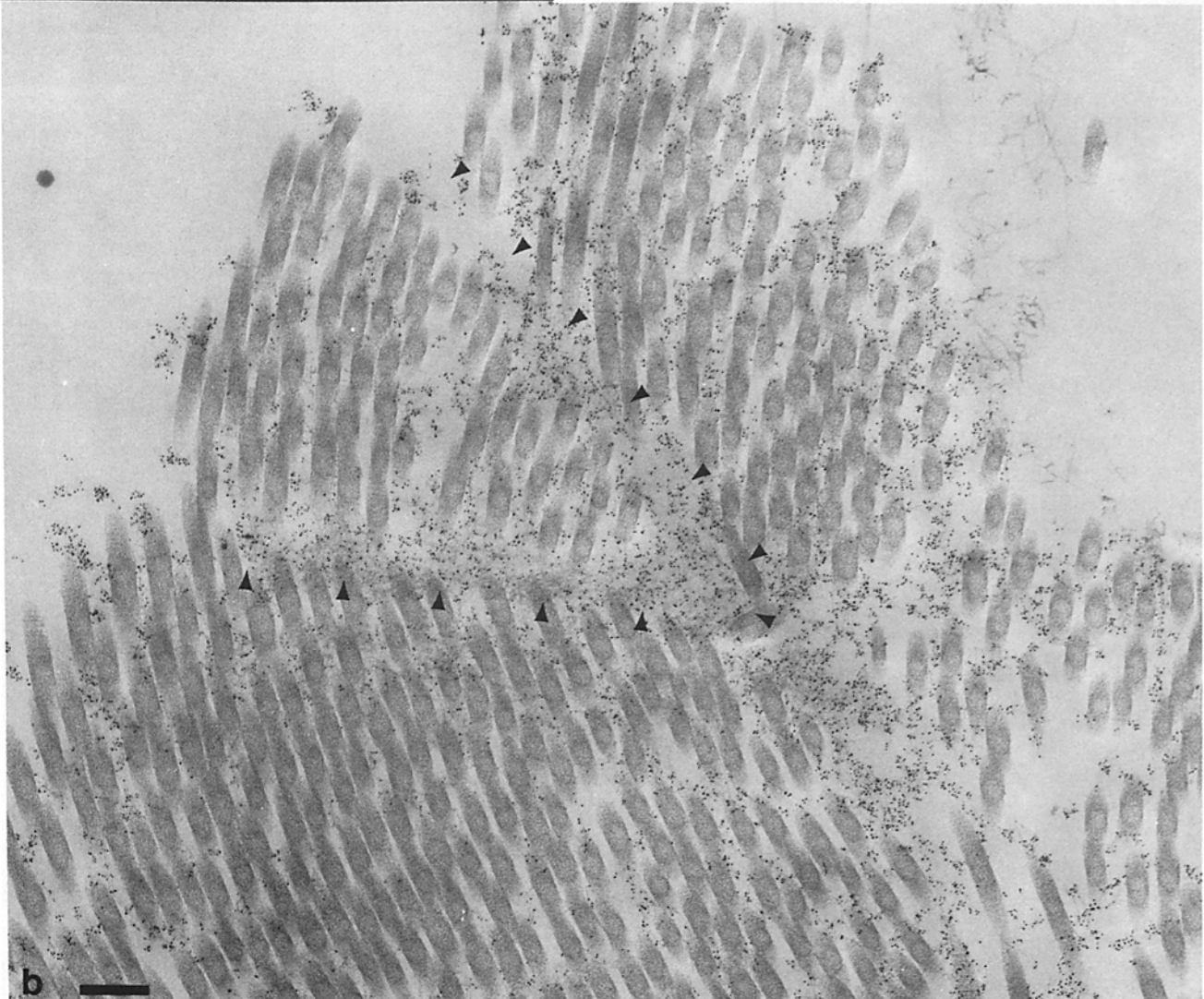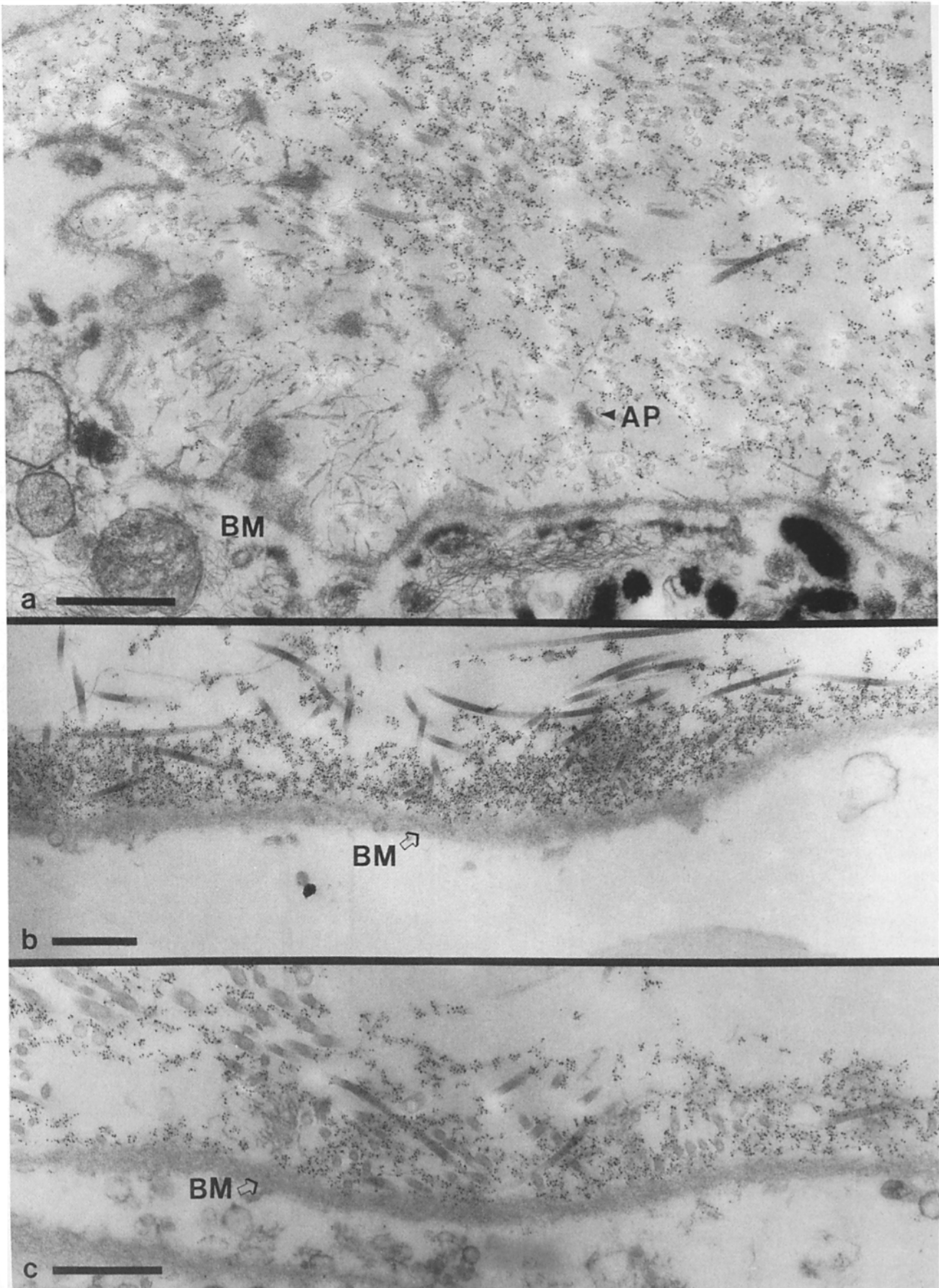
Figure 6. Electron microscopic immunolocalization of type VI collagen adjacent to basement membranes in human skin. The distribution of type VI collagen along basement membranes in skin differs from its distribution within the matrix. Adjacent to the epithelial basement membranes (*a*), type VI collagen is closely associated with banded collagen fibrils but lacks the orderly arrangement seen in deeper layers. It is not localized to the basement membrane (*BM*) or to anchoring plaques (*AP*). Areas immediately surrounding basement membranes of fat cells (*b*), nerves (*c*), and capillaries (not shown) show a marked increase in the concentration of type VI relative to the surrounding matrix. Bars, 500 nm.
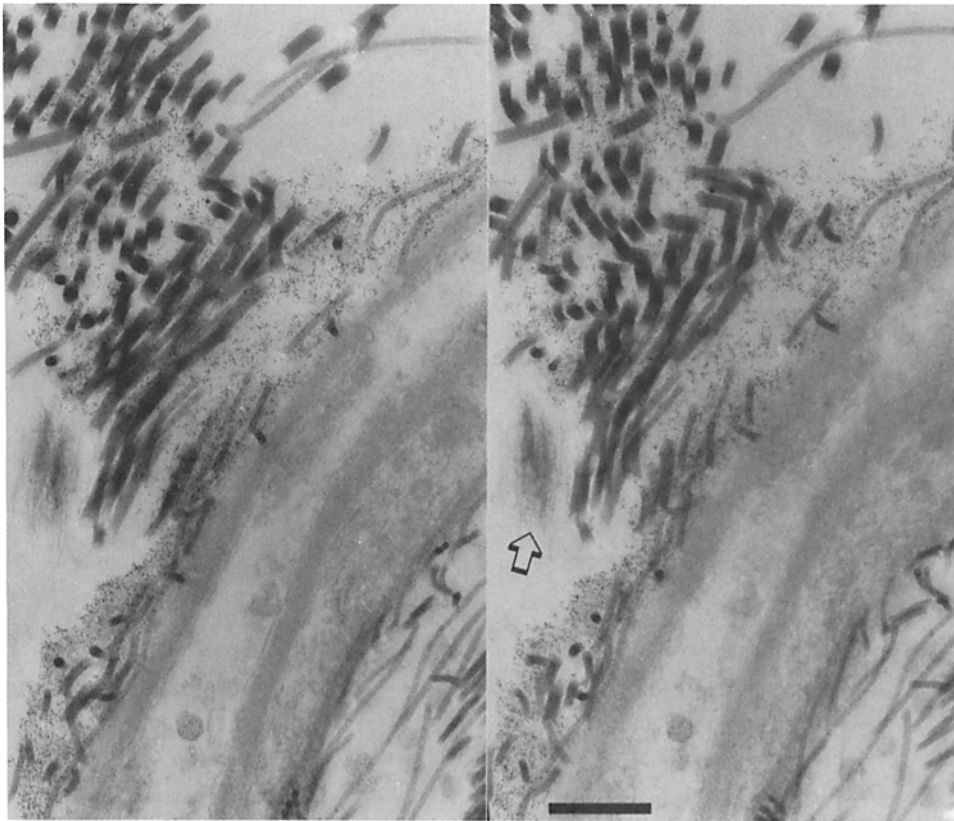
Figure 7. Stereo pair produced using high voltage EM of a 0.5-μm-thick section of nerve basement membrane in the skin of a 45 yr old reveals a high concentration of disorganized type VI fibrils closely associated with the basement membrane. The type VI antibody does not direct gold deposition to elastin-associated microfibrils containing fibrillin (*open arrow*). Bar, 500 nm.

haps the assembly and disassembly of aggregates. Skin fibroblasts from a patient with Cutis-Laxa, a disease which is characterized by loss of skin elasticity, were shown to deposit less type VI collagen into an insoluble extracellular matrix in vitro than control fibroblasts (Crawford et al., 1985), suggesting that abnormal synthesis and deposition of type VI collagen may result in changed mechanical properties of affected tissues.

Immunolocalization studies using mAbs directed against either fibrillin (Sakai et al., 1986) or type VI collagen (reported here), show fibrillin and type VI collagen to be components of separate filamentous systems. Fibrillin is a noncollagenous microfibrillar component often associated with elastin, whereas type VI collagen is predominantly associated with interstitial collagen fibers of types I and III collagen. Using a combination of scanning EM micrographs and thick-section microscopy, a more detailed description of the three-dimensional structure of the type IV collagen network was developed. The network appears to consist of an open irregular network of branching filaments that intertwine, in an apparently random manner, between collagen fibers and individual fibrils. They appear to entrap fibers and fibrils so that extreme lateral movement would be restricted, while longitudinal movement may be possible, when stress is applied.

Structures similar to those described previously as broad-banded collagen or fibrous long spacing collagen, seen frequently in pathological tissues or cell culture (see Introduction), are readily observed in normal fetal skin after incubation in buffer. In this study, the formation of aggregates was inhibited in areas of skin perfused by an antibody recog-

nizing type VI collagen, presumably because bound antibody sterically hinders the formation of aggregates. This supports the conclusion of Bruns (Bruns et al., 1986), who showed that broad-banded aggregates formed in cultures of skin fibroblasts could be immunolabeled with a mAb directed against the helical domain of type VI. As broad-banded aggregates are never seen in fresh, immediately fixed normal skin, they are probably an artifact produced by prolonged incubation of tissues in buffers. There is evidence that neutral proteases are involved in the formation of these aggregates. In tissue-cultured rat skin, the formation of aggregates was inhibited by EDTA and this effect was reversible by the addition of exogenous bacterial collagenase (Kajikawa et al., 1980). Pathological conditions displaying broad-banded collagen aggregates may therefore involve alterations in the molecular structure of type VI collagen or in the surrounding matrix, allowing type VI filaments to aggregate.
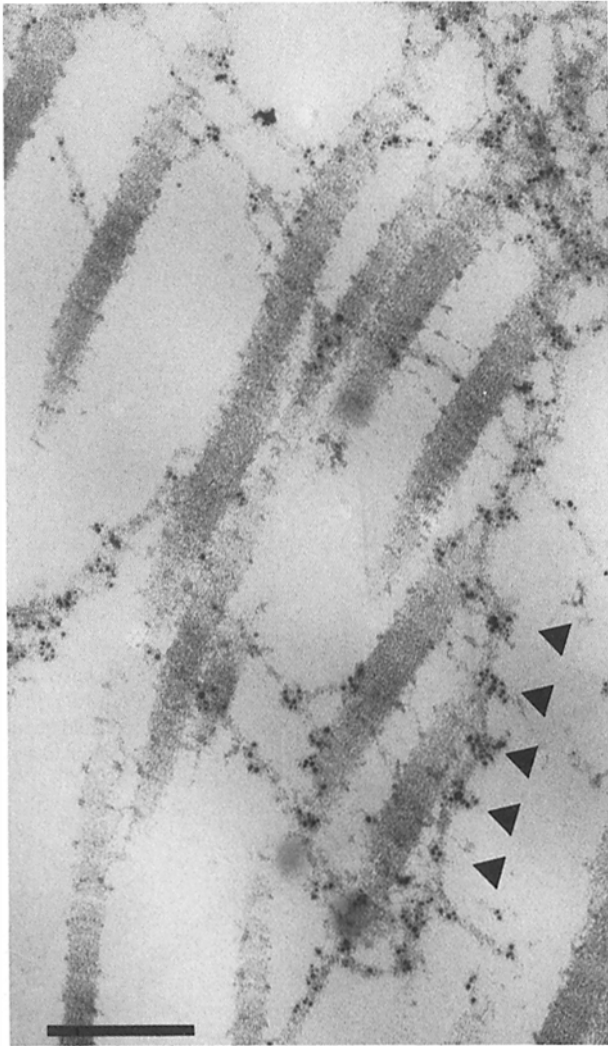
Figure 8. En bloc treatment with ruthenium red stains immunolabeled type VI collagen fibrils in neonatal foreskin. In contrast to staining only in uranyl acetate and lead citrate (Fig. 2, *inset*), type VI fibers are clearly visible between areas labeled with antibody-directed gold (*arrowheads*). Bar, 200 nm.
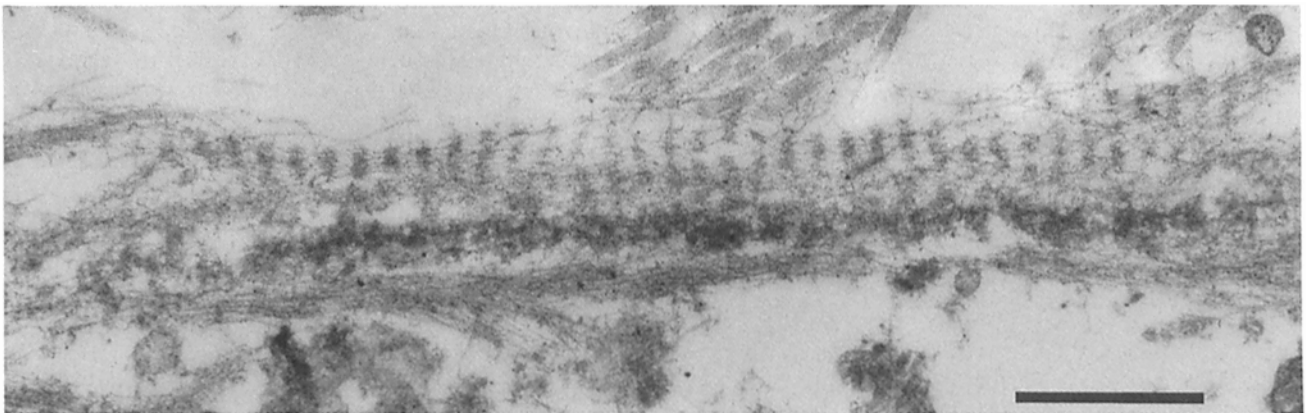
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Figure 9. Broad-banded collagen aggregate in human fetal skin. After enbloc treatment with type VI antibody, aggregates can be found in areas not penetrated by antibody. Bar, 500 nm.

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