Type III Collagen Can Be Present on Banded Collagen Fibrils Regardless of Fibril Diameter

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Abstract. Monoclonal antibodies that recognize an epitope within the triple helix of type III collagen have been used to examine the distribution of that collagen type in human skin, cornea, amnion, aorta, and tendon. Ultrastructural examination of those tissues indicates antibody binding to collagen fibrils in skin, amnion, aorta, and tendon regardless of the diameter of the fibril. The antibody distribution is unchanged with donor age, site of biopsy, or region of tissue examined. In contrast, antibody applied to adult human cornea localizes to isolated fibrils, which appear randomly throughout the matrix. These studies indicate that type III collagen remains associated with collagen fibrils after removal of the amino and carboxyl propeptides, and suggests that fibrils of skin, tendon, and amnion (and presumably many other tissues that contain both types I and III collagens) are copolymers of at least types I and III collagens.

The morphology of connective tissues is largely determined by the size and orientation of collagen fibrils. Fibril diameters vary with the tissue studied and the developmental stage of that tissue. These observations suggest that the fibril-forming process is a well controlled series of events. A number of regulatory mechanisms have been suggested. These include direct participation by cells in fibrillogenesis (Birk and Trelstad, 1986) as well as the involvement of posttranslational proteolytic processing of type I and III procollagens (Miyahara et al., 1984; Fleischmajer et al., 1985) and possibly type V collagen (Fitch et al., 1984) in the control of this process. The latter postulates are supported by the observations that types I, III, and V collagen share common structural features (Miller, 1985), and that all can form fibrils in vitro with the same periodic D-banding seen in vivo (Adachi and Hayashi, 1985).

Types I, III, and V collagen all are present in human skin at all ages but the interrelationships of these molecules are unknown. In vitro studies of fibrillogenesis have indicated the importance of helix–helix interactions in the regulation of this process (Birk and Silver, 1984). Initial studies of in vitro fibrillogenesis of mixtures of the triple-helical domains from types I and III collagens indicated that the resultant fibril diameter was inversely proportional to the I/III molar ratio (Lapiere et al., 1977). There is growing evidence that the pN- and pC-forms of both types I and III collagens are involved in fibrillogenesis. pN-type I and pN-type III collagens are present on fibrils of small diameter, but absent from fibrils of larger diameter (Fleischmajer et al., 1981, 1983, 1985; Sato et al., 1986). The reported persistence of the amino propeptide of pN-type I collagen in the thin and hieroglyphic fibrils of dermatosparactic animals ( Lenaers et al., 1971; Becker et al., 1976) suggests that these globular regions further deter fiber growth or stabilization. The further observation that the amino propeptide of pN-type III collagen is excised far more slowly than the equivalent peptide of pN-type I collagen under physiological growth conditions (Fessler and Fessler, 1979; Fessler et al., 1981) suggests that the limitation to fiber growth may be at least partially related to the proteolytic excision of the type III amino propeptide. In vitro fiber formation observed during pN-type I to type I collagen conversion demonstrated a dependence of the rate of fibril formation upon the rate of removal of the aminoterminal peptide, but the final fibril diameter was unaffected. In similar experiments using pC-type I collagen, the fibril formed concurrent with removal of the carboxyl-terminal peptide were of very large diameter (Miyahara et al., 1984). These observations suggest that the amino and carboxyl propeptides of both types I and III collagens may be directly involved in regulating fibril growth. The subsequent fate and function of both the excised propeptides and the type III collagen molecule are unclear. Existing evidence suggests that the type III collagen triple helix is absent from the surface of large diameter fibrils (Nowack et al., 1976; Fleischmajer et al., 1980). The reported absence of type III collagen from the surface of mature fibers suggests that another mechanism may exist that limits the ultimate size of the fibrils, since type III collagen, which was observed on fibrils of small diameter, must have either been removed or buried within the interior of the fibril.

The present study uses a monoclonal antibody to a defined epitope within the triple-helical domain of human type III collagen to investigate the location of type III collagen in human dermis, tendon, amnion, aorta, and cornea. The results indicate that in skin, tendon, aorta, and amnion, type III collagen is present on all banded collagen fibrils regardless of...
Materials and Methods

Monoclonal Antibody Production

B10.A(4R) mice (Jackson Laboratories, Bar Harbor, ME) were immunized with 100 μg of human type III collagen emulsified in complete Freund's adjuvant. 2 wk later mice were boosted on three consecutive days with 200 μg of human type III collagen (prepared from human amnion by limited pepsin solubilization as described [Burgeson et al., 1976]), and spleen cells were fused with the myeloma cell line P3-NS1/Ag4-1 (Kohler et al., 1976) the fourth day, according to previously described methods (Hollister et al., 1982; Sakai et al., 1982). Hybridomas were screened for the production of antibody by ELISA and by indirect immunofluorescence, and selected colonies were cloned by limiting dilution.

Antibody was collected from selected hybridoma clones either in the form of spent tissue culture medium or as ascites from B10.A(4R) × BALB/c hybrid mice, pristane-primed, and injected with at least 10⁶ hybridoma cells.

Electron Microscopy

En bloc immunolabeling of tissues by secondary gold conjugates (Sakai et al., 1986a), and by the visualization of "naked" IgM primary antibodies (Keene et al., 1987) has been previously described. Briefly, human neonatal foreskin, autopsy samples including aorta, skin, and cornea obtained within 24 h after death, and term amnion obtained within 30 min after birth were sliced into 0.5-1-mm thick strips. Unfixed tissues were washed in PBS, incubated overnight in murine monoclonal anti-human type III collagen IgM (mAb-III) diluted 1:5 in PBS, then extensively washed in PBS. Samples were then rinsed in sodium cacodylate buffer, fixed in buffered Karnovsky's fixative, pH 7.4 (Karnovsky, 1965), and postfixed in buffered OsO₄. Samples were dehydrated in a graded series of ethanol dilutions, washed in propylene oxide, and embedded in Spurr's epoxy. Grids supporting 70-80-nm thick sections were stained in 2% uranyl acetate in 50% ethanol for 15 min followed by Reynold's lead citrate (Reynolds, 1963) for 60 s. Control samples were treated in the same manner except that the first antibody was omitted, or human IgM and other antibodies of irrelevant specificity were substituted for mAb-III. "Naked" IgM visualization by transmission electron microscopy (TEM) immediately followed. Cornea sections were incubated in mAb-III and washed in PBS, and additionally incubated in 5-nm colloidal gold conjugated goat anti-mouse IgM (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:3 in BSA buffer, pH 8.2 overnight at 4°C, rinsed in PBS, cacodylate buffer, and fixed, dehydrated, and embedded as above. Tendon samples were prepared for TEM by swelling the fibers in 0.2 M acetic acid overnight at 4°C, after which they were rinsed in PBS, pH 7.4 for 15 min, then incubated in mAb-III overnight at 4°C as above. After an extensive PBS wash, they were either fixed as above and examined without secondary conjugate, or incubated in 5-nm colloidal gold conjugated anti-IgM.

Skin samples prepared for scanning electron microscopy (SEM) were removed from the above pool of samples after dehydration through 100% ethanol, critical point dried, then sputter coated with gold. Tendon samples prepared for SEM were first dehydrated in a graded series of ethanol, critical point dried, sliced longitudinally with respect to the collagen fibers to allow direct antibody access to the tendon fibrils, then rehydrated directly in antibody. Subsequent steps were as above.

Rotary shadowed replicas of mAb-III-type III complexes were prepared as described previously (Morris et al., 1986).

TEM samples were observed using a Philips 410 L.S. electron microscope operated at 60 or 80 kV using a 30-μm objective aperture. SEM samples were observed using the upper stage of a LaB₆ emitter equipped ISI DS 130 operated at 9 or 10 kV with the smallest available spot size.

1. Abbreviations used in this paper: mAb-III, murine monoclonal anti-human type III collagen IgM; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

In vitro Preparation of Collagen Fibrils

Human type III collagen was prepared from amnion as previously described (Burgeson et al., 1976). Human type I collagen was solubilized from bone after decalcification of neonatal femurs by extraction with five changes of 0.5 M EDTA, Tris-HCl pH 7.5, 1 wk each at 4°C. Bones were homogenized using a polytron homogenizer (Brinkmann Instruments Co., Westbury, NJ) and digested with 0.5 mg/ml pepsin (Boehringer-Mannheim Diagnostics, Inc., Houston, TX) in 0.5 M acetic acid at 4°C for 48 h with constant agitation. Solubilized protein was clarified by centrifugation and precipitated by the addition of solid NaCl to 1.2 M. The harvested proteins were redissolved in 1 M NaCl, 50 mM Tris-HCl, pH 7.5, clarified by centrifugation and dialyzed versus 2.0 M NaCl, reclarified, and type I collagen was precipitated by dialysis versus 2.8 M NaCl. The precipitate was dissolved in 0.5 M acetic acid and reprecipitated by the addition of solid NaCl to 1.0 M. The precipitate was redissolved in 0.5 M acetic acid, extensively dialyzed versus 0.5 M acetic acid, and lyophilized. The purity of the collagen preparations was tested by polyacrylamide gel electrophoresis and determined to be >95% pure. The type III preparation contained 1-5% type I collagen; the type I preparation was free of type III collagen as determined by ELISA (not shown).

Collagen types I and III were dissolved in 10 mM HCl at 4°C to ~2 mg/ml. The exact concentration was determined by amino acid analysis, and adjusted to 0.5 mg/ml. Fibrils were formed for 24 h at 10°C as previously described (McPherson et al., 1985).

Miscellaneous Procedures

Type III collagen renaturation, trypsin digestion, and characterization of tryptic fragments have been previously described (Bächinger et al., 1980). Triple-helical fragments for ELISA were prepared by inactivating trypsin with TLCK at a final concentration of 1.0 mg/ml. ELISA was performed as previously described (Engvall and Perlmann, 1977).

Results

Antibody Specificity

IgM monoclonal anti-human type III collagen triple-helical domain (mAb-III) has been previously reported (Keene et al., 1987). The antibody shows no crossreactivity with any
Antigen–antibody complexes were analyzed by rotary shadowing. Antibody was most commonly localized to a region ~90 nm from one end (Fig. 2 F). While end binding was relatively rare, further studies were undertaken to determine the site of the epitope. As previously reported, exposure of partially renatured type III collagen to trypsin results in the generation of fragments with lengths reflecting the extent of triple-helix re-formation (Bächinger et al., 1980). Refolding starts at the carboxyl-terminal disulfide bonds and with increasing renaturation time larger and larger fragments appear upon tryptic digestion. Each of these timed digestions was tested for their ability (a) to react with mAb-III (Fig. 3 A), (b) to inhibit mAb-III binding to native type III collagen (Fig. 3 B), and the sizes of the trypsin-resistant peptides were evaluated by gel electrophoresis (not shown). mAb-III reactivity is detected by ELISA first with 10-min fragments, and increases linearly until a maximum is established with 20-min fragments (Fig. 3 A). Likewise, inhibition was negligible with 10-min fragments and maximal with 20-min fragments (Fig. 3 B) coinciding with the appearance of electrophoretic bands g and h (Bächinger et al., 1980), which approximately correspond to peptides including residues 350–1,018 and 250–1,018 respectively. Therefore, these results indicated that the epitope is contained within 250–350 residues from the amino terminus or ~75–105 nm from the amino terminus and confirmed the majority of the rotary shadowing evidence.

Banded collagen fibrils were formed in vitro from pure type I collagen obtained from human bone by pepsin solubilization and from pure type III collagen isolated and purified from pepsin digests of human amnion as described (Materials and Methods). Molecules of IgM were directly visualized upon the surface of the type III collagen fibril by either TEM or SEM, but no binding to type I collagen fibrils was detected (Keene et al., 1987). Fibrils formed from mixtures of types I and III collagens were similarly tested for maximal antibody binding by TEM. Fibrils formed from a mixture of 5% type III and 95% type I were labeled uniformly with antibody, indicating that as little as 5% type III collagen in fibrils is easily detectable (Fig. 4). In a separate experiment (not shown), uniform labeling was also observed upon fibrils made from solutions containing 2.5% type III, non-uniform labeling was observed upon fibrils containing 1% type III collagen, and no detectable antibody bound fibrils of 0.2% or 0.1%.

Figure 2. mAb-III binds the type III collagen within the triple-helical domain. mAb-III, type III collagen antigen–antibody complexes, were visualized after rotary shadowing. The predominant site of binding is 90 nm from one end (A–E), but binding is occasionally seen at the end as well (F). Bar, 100 nm.

Figure 3. The epitope recognized by mAb-III is about one-third the distance from the amino terminus of the type III triple helix. Heat-denatured type III collagen was renatured at 25°C. At various renaturation times (x-axis), aliquots were tested for their ability (A) to demonstrate reactivity to mAb-III or (B) to inhibit mAb-III binding to native type III collagen by ELISA (y-axis). Peptides first appearing at 10 min, and abundant at 15 and 20 min were analyzed by gel electrophoresis (not shown), which indicated these peptides included about two-thirds of the triple helix from the COOH-terminus (the COOH-terminus is the nucleation point of renaturation due to the presence of interchain disulfide bonds). Therefore, the recognized epitope lies approximately one-third the distance from the amino terminus, consistent with the data in Fig. 2, A–E.
67-nm Periodically Banded Fibrils in Human Dermis Uniformly Label with mAb-III Regardless of Fibril Diameter, Tissue Age, or Location within the Dermis

Type III collagen triple helix was immunolocalized within human dermis using mAb-III. As shown by SEM in Fig. 5, A and C, all fibrils in human neonatal foreskin observed within the field were uniformly labeled with antibody. A corresponding region incubated with human IgM did not cause visible IgM deposition (Keene et al., 1987), nor did antibodies of irrelevant specificity (Fig. 5, B and D). TEM localization of type III at the dermal epidermal junction of neonatal foreskin (Fig. 6 A), the reticular dermis of 14-yr-old toe skin (Fig. 6 B), the reticular collodion 11-yr-old back skin (Fig. 6 C), and the deep reticular dermis from 14-yr-old back skin, adjacent to the subcutaneous fat cells (Fig. 6 D), indicated that all fibrils were strongly labeled. As expected for a monoclonal antibody that recognizes a single epitope, the observed antibody binding occurred at intervals ~67 nm. The very fine microfibrils seen at the dermal–epidermal junction (Fig. 6 A) more closely resembled fibrillin microfibrils (Sakai et al., 1986b) and anchoring fibrils (Sakai et al., 1986a) than uniformly banded collagen fibrils, but it is possible that very fine banded fibrils may not label with mAb-III.

Banded Fibrils in Human Tendon of any Age, Amnion, and Aorta Label Uniformly with mAb-III

Human Achilles tendon (ages 14 to 80 yr), term amnion, and aorta (age 14 yr) were also examined after exposure to mAb-III. The vast majority of collagen fibrils in tendon were seen to be uniformly labeled when examined by either TEM without (Fig. 7 A) or with colloidal gold–conjugated second antibody (Fig. 7 B), or SEM (Fig. 7, C and E). Tendon fibrils incubated by the same procedures with antibodies of irrelevant specificity appeared relatively smooth, although a greater amount of debris is found along the control tendon surface (Fig. 7 D) than was seen with skin (Fig. 5). As demonstrated for skin, this observation held for tendon regardless of donor age (tissue samples shown in Fig. 7 range from 77- to 80-yr-old; results from 14-yr-old samples were identical [not shown]) and for fibrils with obvious differences in diameters (Fig. 7 B). Occasional fibrils (not obvious in these micrographs) were unlabeled. These tended to be small in diameter, and were unlabeled along their entire observable length. Adjacent fibrils were uniformly labeled. TEM examination of human amnion (Fig. 8 A) and aorta (14-yr-old, Fig. 8 B) showed uniform deposition of mAb-III.

Most Periodically Banded Fibrils in Human Cornea Are Unlabeled by mAb-III

Human cornea (age 15 yr) was similarly examined by TEM using mAb-III. As shown in Fig. 8, C and D, only occasional fibrils were labeled. In this case, to be certain that the deposits observed were IgM, a 5-nm colloidal gold–conjugated anti–mouse IgM antibody was additionally used. The antibodies had adequate access to the tissue, as the labeled fibrils were uniformly decorated along their entire exposed length, while fibrils immediately adjacent were negative.

Discussion

The present studies indicate that 67-nm periodically banded fibrils in human skin, amnion, aorta, and Achilles tendon have type III collagen uniformly upon their surfaces. This observation contradicts previous studies that reported that only fibrils of relatively small diameter were immunologically reactive to type III collagen specific antibodies. This discrepancy may arise from the antibodies used in previous studies. The antisera used in previous studies (Nowack et al., 1976; Fleischmajer et al., 1980) were made to neutral salt extracted type III collagen. Both antisera were absorbed against other collagen types, and both reacted with only a portion of the total collagen fibers in skin. It is most likely that these antisera reacted with portions of either the amino or carboxyl propeptides of type III collagen. Several lines of evidence support this conclusion. First, since type III collagen is only slowly processed in vivo (Fessler et al., 1979) it is almost certain that the immunogen contained type III molecules with at least partially intact propeptides. Absorption with other collagens would remove crossreacting anti–helical antibodies, but not those specific either to the type III helix or to the propeptides. In addition, ultrastructural immunolocalization...
using one of these antisera (and presumably the other would behave similarly since they were made according to identical procedures) showed binding at regular intervals of 65 nm, indicating recognition of highly clustered epitopes, and little or no reactivity of the remainder of the molecule. And also, the immunolocalization of type III collagen using this antisera was indistinguishable from that obtained with antibodies to type III procollagen (Nowack et al., 1976) or from that seen using antibodies specific to the amino propeptide of type III procollagen (Fleischmajer et al., 1981, 1983).

Because of the above ambiguities, the specificity of the monoclonal antibody used in the present study has been

Figure 5. Scanning electron microscopic immunolocalization of type III collagen within human dermis. The dermis of neonatal human foreskin was examined by SEM after incubation with mAb-III (IgM). Naked bound IgM is visualized along all fibrils and exhibits an ~67-nm periodicity (A and C). Foreskin incubated with an antibody of irrelevant specificity demonstrates no binding (B and D). Bars: (A) 250 nm; (B) 150 nm.

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Figure 6. TEM immunolocalization of type III collagen within human dermis. Skin samples from several sites of individuals of varying ages were incubated with mAb-III and visualized by TEM. In all cases, IgM is visualized along each fibril regardless of specimen age, site of biopsy, location within the biopsy, or fibril diameter. (A) Neonatal foreskin, dermal epidermal junction; (B) plantar surface toe skin, 14-yr-old, reticular dermis; (C) back skin, 11-yr-old, reticular dermis; (D) thigh skin, deep reticular dermis near subcutaneous fat layer, 14-yr-old. Bars: (A) 500 nm; (B) 150 nm.
Figure 7. Electron microscopical immunolocalization of type III collagen in human tendon. Achilles tendon specimens from 77- to 80-yr-old individuals were incubated with mAb-III and examined by TEM (A and B) and SEM (C and E). B was incubated with 5-nm colloidal gold-conjugated second antibody and shows periodic labeling of fibrils ranging in diameter from 32 to 130 nm. Almost all fibrils demonstrate IgM deposition. Unincubated control tendon (D) is not decorated with IgM when examined. Bars: (A) 500 nm; (D) 250 nm.
tested collagens by ELISA. It reacts with in vitro formed fibrils rigorously tested. It shows no crossreactivity with other collagen types in suspension culture, but does synthesize type III collagen. Chick tendon fibroblasts do not synthesize detectable amounts of type III collagen in suspension culture, but do synthesize type III when grown in monolayer culture. Previous indirect immunofluorescence studies indicate that type III contributes only a negligible proportion of the total collagen content. We were surprised to observe uniform labeling of tendon fibrils with anti-type III collagen antibody. Published chemical studies of tendon collagens indicate that type III contributes only a negligible proportion of the total collagen content. Chick tendon fibroblasts do not synthesize detectable amounts of type III collagen in suspension culture, but do synthesize type III when grown in monolayer culture. Previous indirect immunofluorescence studies suggest that type III collagen is restricted to the endotendineum of adult chick and human tendon, but its distribution may be somewhat broader in 13-d chick embryo. The former studies used antisera that reacted only with non-helical epitopes; the reactivity of the antibodies used in the latter studies to helical versus nonhelical epitopes was determined. Similarly, indirect immunofluorescence of human tendon using the IgM antibodies described in this present report is not readily distinguishable from background unless the tissue is first processed by the procedures described here for immunoelectron microscopy. The failure to detect type III collagen within the fiber bundles of tendon without swelling in acid or exposing epitopes by slicing critical point dried specimens, could be explained by poor penetration of antibody. The small amount of type III detected by chemical means is more difficult to understand, since the IgM antibody uniformly labels only in vitro-formed fibrils made from solutions containing at least 1–2.5% type III collagen. It is possible that this amount of type III might be below the limits of detection by the chemical methods used. It is unlikely that the periodic binding of IgM to tendon fibrils is an artifact. IgM of irrelevant specificity shows no labeling of tendon; the IgM antibody does not label in vitro fibrils. One possibility might be that the type III collagen epitope recognized by the IgM monoclonal antibody is blocked in human cornea, perhaps by proteoglycan, which localizes to specific sites along the corneal collagen fibrils. The apparent lack of labeling is not likely to result from problems of incomplete antibody penetration because when present, the antibody labels a single fibril along its entire observable length. Fibrils immediately adjacent are negative. We do not interpret the lack of antibody binding seen in these present experiments to demonstrate a sparsity of type III collagen in human cornea. They do suggest that corneal fibrils are in some way different from those in other tissues studied.

The finding that fibrils in skin, tendon, aorta, and amnion contain type III collagen upon their surfaces suggests that type III is either restricted to the fibril surface, perhaps at the end stages of fibril growth, or that type III is incorporated throughout all growing fibrils in proportion to the percent type III represents of total newly synthesized fibrillar collagen. The present study supports the latter conclusion, but the former cannot be excluded. The observation that type III collagen (or procollagen) is uniformly present on all fibrils in rapidly growing skin (neonatal foreskin) where small fibrils are likely to represent growth intermediates, strongly suggests that type III procollagen is involved in fibril growth regulation as previously suggested and as a consequence type III collagen becomes incorporated throughout the resulting fibrils.

The molecular events involved in the regulation of fibril length or girth remain unresolved, yet these studies do indicate that the triple-helical domains of pN- or pC-type III collagens seen on the surface of small diameter fibrils do become integral components of those fibrils. The presence of type III collagen or procollagen may accelerate or retard the fibril growth process, may modulate a structural/functional property of the fibrils themselves, or some combination of these possibilities.

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Figure 8. TEM immunolocalization of type III collagen in human amnion, aorta, and cornea. Neonatal amnion (A), 14-yr-old aorta (B), and 15-yr-old cornea (C and D) were incubated with mAb-III. The cornea samples were then incubated with goat anti-mouse IgM conjugated with 5-nm colloidal gold. All samples were then examined by TEM. All show periodic binding of IgM, but only occasional fibrils are labeled in cornea. Bars: (A) 250 nm; (D) 150 nm.

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