The $\alpha 1$ Chain of Type VIII Collagen is Associated with Many but Not All Microfibrils of Elastic Fiber System

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Key words: Type VIII collagen/Elastic fiber/Microfibril/Immunoelectron microscopy

ABSTRACT. The antigen of monoclonal antibodies which had labeled the hexagonal lattice of Descemet's membrane in a specific manner was shown to be the $\alpha 1$ chain of type VIII collagen by immunoblotting followed by amino acid sequence analysis. With this antibody, the localization of $\alpha 1$ (VIII) in various tissues was studied by several immunocytochemical methods. Under light microscopy, the $\alpha 1$ (VIII) was found in a fine fibrillar form in various capsular tissues such as capsules of the liver, kidneys, adrenals, lungs and so on. It was also present in dense connective tissues such as the Achilles tendon, and periodontal and perivertebral ligaments. When some dense connective tissues which had been negative to the label including the intima of aorta, perimysium and Glisson's sheath of the liver, were subjected to pepsin digestion, epitopes were revealed which showed a specific immunofluorescence pattern. In many locations the pattern of localization coincided with that of elastic fiber components, and full or partial colocalization with tropoelastin or costaining with resorcin-fuchsin staining was observed. In immunoelectron microscopy, the antigen ($\alpha 1$ (VIII)) was localized on the surface of, but not inside, elastic fibers. However, some tissues which are rich in elastic fibers or microfibrils remained unlabeled. These included elastic fibers of the aortic media and ligamentum nuchae as well as ciliary zonules. Therefore it is suggested that $\alpha 1$ (VIII) is a collagen associated with microfibrils of some elastic fiber systems, but is not an intrinsic component of either elastic fibers or of microfibrils.

Extracellular matrix constitutes a major portion of the body, and plays important roles in many biological activities. The number of known extracellular matrix constituents is growing rapidly. Collagens are among them, being the most widely distributed and having many subspecies. Up to now, fourteen types have been accepted as genetically distinct types (5). We have been studying a collagen type which constitutes hexagonal lattices in Descemet's membrane (DM) of the cornea (37, 38, 39, 40). By immunochemical analysis with monoclonal antibodies against the lattice structure (named 6A2 and 9H3), it was shown that the lattice contains as its major component a 64 kD peptide containing a 50 kD pepsin-resistant collagenous domain (50K-B) (40).

This 50 kD collagenous domain (50K-B) was postulated to be derived from type VIII collagen (17, 18, 20, 23). There were, however, discrepancies in the molecular mass reported for type VIII collagen chains; 125 kD for EC2 (18, 32, 34, 35) and 60 kD for VIII-3 (1), and tissue form from DM (14). Yamaguchi *et al.* (43) cloned and sequenced cDNA clones which encode a collagen-

ous chain in VIII-3. This chain was 60 kD and was named $\alpha 1$ (VIII). It contained an amino acid sequence highly homologous to the N-terminal region of 50K-B. The lattice collagen is very similar in molecular mass and other characteristics to the $\alpha 1$ (VIII) chain, but the direct relation is still ambiguous. On the other hand the lattice collagen does not have immunological crossreactivity to the 125 kD species, EC2 (18, 40). Recently, the amino acid sequence of the triple helical domain of the other 50 kD collagenous component, 50K-A, from DMs was determined and was shown to belong to the same family as $\alpha 1$ (VIII). This chain was named $\alpha 2$ (VIII) (24) and its gene was partially sequenced (27). Thus there is more than one distinct collagenous peptide in what has been called type VIII collagen.

Therefore, although immunolocalization studies of type VIII collagen were performed either with antibodies against EC2 (18) or with antibodies against whole type VIII collagen (a mixture of 50K-A and 50K-B) (21, 33), and its relation to neural crest derived cells or possible relations to elastic fibers and microfibrils were proposed, there is no guarantee that these peptides colocalize. Thus, localization study of each chain is

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needed. There have been no electron microscopic localization studies done on type VIII collagen except ours on DMs.

In the present work we confirmed that the antigen of 6A2 mAb is $\alpha 1$ (VIII), and immunolocalization with the antibody showed the collagen is demonstrated in various connective tissues, including some capsular tissues and some dense connective tissues. It was shown that $\alpha 1$ (VIII) is associated with many, but not all, elastic fibers and by immunoelectron microscopy this association is seen to be with the microfibrillar components of the reactive elastic fiber systems.

MATERIALS AND METHODS

Monoclonal antibody. Balb/c mice were immunized with homogenate of DMs, and monoclonal antibodies (mAbs) against type VIII collagen were obtained as described previously (40).

Immunoblotting and Amino Acid Sequence. Type VIII collagen and related peptides (50K-DM, 50K-A ($\alpha 2$ (VIII)), and 50K-B ($\alpha 1$ (VIII))) were prepared as previously described (17). HPLC purified 50K-B collagen was separated by 5% SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) (25). Immunoblotting was performed as described previously (38). After staining with Coomassie brilliant blue, the lower half of the 50K-B protein band was cut out and applied to a protein sequencer ABI 477A/120A (Applied Biosystems, Foster City, CA).

Tissues. Various bovine tissues were obtained from a local slaughterhouse. Many of them are three years of age. They were fixed by immersion in periodate-lysine-paraformaldehyde (PLP) fixative for two hours at room temperature. After fixation, the tissues were immersed overnight in Holt's sucrose-gum arabic solution (0.88 M sucrose, 1% gum arabic, 0.1 g/l thymol) and frozen in liquid nitrogen. Some tissues were immersed in 10% dimethylsulfoxide in phosphate-buffered saline (PBS) for 1 h and frozen in liquid nitrogen without fixation.

Light Microscopy. Six- to 10- μ m sections were obtained from either fixed or unfixed tissues in a Ripshaw model 1900 cryostat and transferred onto glass slides pretreated with 0.1% poly-L-lysine. Then the sections were treated with PBS containing 0.02 M glycine (glycine-PBS), then with 50% calf serum in PBS (CS-PBS) and labeled with the mAbs. The mAbs were diluted to 1 : 100 in CS-PBS from stock solutions. Labeling was performed at room temperature for 2 h. After three rinses in PBS, the sections were labeled with FITC-labeled goat anti-mouse IgGs (MBL Co, Ltd., Nagoya, Japan) (1 : 100 diluted in CS-PBS) for 2 h at room temperature. Then the specimens were rinsed three times with PBS and mounted in 90% glycerol in PBS containing 0.2% *p*-phenylene diamine, and observed with an Olympus BH-2 epifluorescence microscope (Olympus Optical Company, Ltd., Tokyo, Japan).

Photographs were taken with Kodak Ektachrome 400 film.

In some tissues with strong autofluorescence from elastic fibers, the specimens were labeled with the mAb, washed with PBS, and labeled with rabbit anti-mouse IgGs (MBL Co., Ltd.) diluted to 1 : 1,000 in CS-PBS for 2 h at room temperature, and then with goat anti-rabbit IgG gold (Auro Probe GARG5, Janssen Life Science Products, Beerse, Belgium) diluted to 1 : 20 in CS-PBS overnight at room temperature. After rinsing in PBS the gold labels on sections were enhanced with silver lactate solution as described by Birrell *et al.* (2) for 20 min in the dark. The tissues were observed with an Olympus light microscope.

For double-label immunofluorescence, rabbit anti-bovine tropoelastin antiserum (Elastin Products Company, Owensville, MO) was added to the 1st antibody solution at a ratio of 1 : 50 and rhodamine-labeled anti-rabbit IgG antibody (Cooper Biomedical Inc. Malvern, PA) to the 2nd antibody solution.

Digestion with pepsin. Some sections from unfixed frozen tissues were digested with 1% pepsin in 0.1 N acetic acid for 1 h at room temperature according to the procedure of Kittelberger *et al.* (20) to expose possible masked epitopes.

Elastin histochemistry. After immunofluorescence microscopy, the sections were fixed with 4% neutral formalin, rinsed with distilled water, and stained with 0.6% resorcin-fuchsin (Tokyo Kasei, Tokyo Japan) in 1% HCl in 90% ethanol for 10 min, and observed with an Olympus light microscope.

Electron microscopy. Fixed tissues were frozen as specimens for light microscopy. 20 μ m frozen sections were cut, and treated with 0.02% glycine in PBS, then with CS-PBS, and labeled with primary antibodies diluted to 1 : 100 in CS-PBS overnight at room temperature on a rotator. They were rinsed five times with PBS for 15 min each time, treated with rabbit anti-mouse IgGs (1 : 1,000 in CS-PBS), rinsed with PBS, and labeled with Auro Probe GARG5 overnight (1 : 20 in CS-PBS). After labeling, they were rinsed with PBS, fixed with 1% glutaraldehyde in PBS and postfixed with 1% osmium tetroxide, stained en block with uranyl acetate, dehydrated, and embedded in Epon 812. Thin sections were cut, stained with uranyl acetate and with lead citrate, and observed in a JEOL 100C transmission electron microscope operated at 80 kV.

Some sections were stained with tannic acid-uranyl acetate followed by lead citrate according to the method of Kajikawa *et al.* (16) to specifically stain elastic fibers.

For ultrathin cryosections, tissues were immersed in 1.6 M sucrose, 10% polyvinylpyrrolidone, 0.1 M phosphate buffer, pH 7.4, overnight at 4°C, and frozen in liquid nitrogen. Ultrathin cryosections were cut with a Sorvall LTC-2 cryosection device (RMC Cryosystems Inc., Tuscon, AZ) (42). Grids with cryosections were treated with glycine-PBS and CS-PBS, labeled with mAbs, rinsed with PBS, and then labeled with 10 nm goat anti-mouse IgG gold (GAMG10, Janssen Life Science Products) diluted to 1 : 20 in CS-PBS, for 6 h at room temperature. They were rinsed in PBS, and fixed with 1%

glutaraldehyde in PBS, then with 1% osmium tetroxide, stained en block with 0.5% uranyl acetate, dehydrated, and embedded in LR white resin according to the method of Keller *et al.* (19).

RESULTS

Immunoblotting and Amino Acid Sequence Analysis. The material used for amino acid sequence is shown in Fig. 1. Since our previous study showed that the two mAbs (6A2 and 9H3) might recognize different areas of the 50K-B band (40), only the lower half of the 50K-B band, where 6A2 showed strong reaction and

Fig. 1. Immunoblotting of the 6A2-reactive area in 50K-B. Lane A shows Coomassie brilliant blue stained SDS-PAGE of 50K-B band and lane B shows immunoblotting reaction of the material in lane A with 6A2 mAb. Lane C shows 50K-B band after transfer to polyvinylidene difluoride membrane. The lower half of the 50K-B band was excised (the rectangle indicated by the arrow in lane C and processed for amino acid sequence analysis). Lane D shows crude 50K-DM collagen (upper band of the doublet corresponds to the migration of 50K-A, and 50K-B contains polypeptides of both upper and lower bands). The black arrow indicates the position of migration of tracking dye. The white arrow indicates the top of the gel.

9H3 did not, was used for the amino acid sequence. The amino-terminal 12 amino acid sequence of the band was identical to part of the amino-terminal sequence of the collagenous portion of the $\alpha 1$ (VIII) chain predicted by the $\alpha 1$ (VIII) chain cDNA reported by Yamaguchi *et al.* (43) (Fig. 2) and to that of the amino acid sequence of the 50K-B band reported by Kapoor *et al.* (17).

Localization in ocular tissues. As reported previously immunofluorescence microscopy with 6A2 antibody showed wide distribution of the antigen in ocular tissues. It was distributed in Descemet's membrane of the cornea, in the sclera, the optic nerve sheath, and in the choroid (40).

Localization in extraocular tissues. The antigen was not detected in the parenchyma of the liver, in cartilage, cerebrum, spinal cord and other organs. In the kidney most of the parenchyma was devoid of label except around arteries and around a few Bowman's capsules. However, the $\alpha 1$ (VIII) chain was found in the capsular area of these organs. The liver capsule (Fig. 3A) and kidney capsule (Fig. 3B) were labeled in a fine fibrillar pattern parallel to the surface of these organs. Similar reactions were also seen in the adrenal capsule (Fig. 3C) and the perineurium (Fig. 3D). They also existed in the perichondrium and in the dura mater of the spinal cord as reported previously (40). In the lungs, the pleura was always positive to the label (Fig. 3E). Often, with a high concentration of antibodies, some elastic fibers in the lung parenchyma were also labeled. The peripheral area of the bronchi and large blood vessels were also labeled. The diameter of positive structures in the pleura was somewhat thicker than in other organs. The connective tissues in skeletal muscle were unreactive with the label (Fig. 3F).

We also examined various dense connective tissues. In the Achilles tendon (Fig. 3G), immunofluorescence of two different orientations was observed: in the tendon proper fine fibrils ran parallel to the axis of the tendon, while in the inner and outer peritendineum, they

Fig. 2. Amino acid sequence of the lower half of the 50K-B band (Fig. 1 lane C) is compared to the amino acid sequence of collagenous portion of $\alpha 1$ (VIII) chain deduced from cDNA sequence (43). The sequence in the rectangle (RGEQGPRGEPGP) is the amino acid sequence of the N-terminal portion of the lower half of 50K-B band determined in the study.

The sequence is identical to the amino-terminal sequence of collagenous portion of $\alpha 1$ (VIII) chain predicted by Yamaguchi *et al.*

Fig. 3.

Fig. 4. Localization of $\alpha 1$ (VIII) shown after pepsin digestion. A) Immunofluorescence localization of $\alpha 1$ (VIII) in the intima of the aorta. Conspicuous fibrillar fluorescence appeared just beneath endothelium (arrow). $\times 100$. Bar = $100\ \mu\text{m}$. B) Immunogold localization in aortic media after pepsin digestion. Fibrillar labeling has appeared. Hollow tubular structures, possibly corresponding to vasa vasorum (arrow), are surrounded by immunoreactive structures. Areas occupied with elastic fibers (E) are negative to the labels. $\times 200$. Bar = $200\ \mu\text{m}$. C) In skeletal muscle, perimysium and endomysium became labeled in a fine fibrillar manner after pepsin digestion. Compare with Fig. 3F. $\times 200$. Bar = $100\ \mu\text{m}$. D) The ligamentum nuchae remains negative even after pepsin treatment. $\times 200$. Bar = $100\ \mu\text{m}$.

were aligned perpendicular to the axis, around the circumference of the tendon fiber. Throughout ligaments around vertebrae, groups of parallel fibrillar immunofluorescence were observed among collagenous fibrils. In the skin, the dermis and the area around hair follicles

showed fibrillar immunofluorescence with high concentration of antibodies (data not shown).

We also checked tissues abundant in elastin and elastin-associated microfibrils. Periodontal ligaments showed fibrillar fluorescence in some areas (see Fig.

Fig. 3. Immunofluorescence localization (A–G) and immunogold localization (H) of $\alpha 1$ (VIII) chain in various tissues. A) Capsule of the liver. Fluorescent label is localized in the capsule but not in the parenchyma (P). $\times 180$. Bar = $100\ \mu\text{m}$. B) Surface area of the kidney. In the capsule fine fibrillar fluorescence is observed. The parenchyma (P) is negative to the label. $\times 190$. Bar = $100\ \mu\text{m}$. C) Capsule of the adrenal cortex. Fine fibrillar fluorescence is observed. The inner elastic lamina of an artery in the capsule looks bright, but only because of autofluorescence. $\times 160$. Bar = $100\ \mu\text{m}$. D) A nerve in the tail. The perineurium is heavily labeled. Fibrillar fluorescence is also seen in the nerve bundle. $\times 160$. Bar = $100\ \mu\text{m}$. E) In the pulmonary pleura, thick fibrillar fluorescence is observed. $\times 260$. Bar = $100\ \mu\text{m}$. F) Muscle fibers in the tail. No fluorescence is observed without pepsin digestion. $\times 190$. Bar = $100\ \mu\text{m}$. G) Cross section of Achilles tendon. Fine fibrillar fluorescence is observed. It is oriented longitudinally in the tendon fiber, whereas it is oriented perpendicular to the fiber axis in the inner and outer peritendineum. $\times 190$. Bar = $100\ \mu\text{m}$. H) Localization of $\alpha 1$ (VIII) in the spleen shown by immunogold labeling followed by silver enhancement. Fibers in cross section of a trabecula are heavily labeled. Other area (red pulp) is devoid of labels. $\times 160$. Bar = $100\ \mu\text{m}$.

6C). In the ligamentum nuchae there was no reaction (data not shown, see Fig. 4D). The ciliary zonule was also negative to the labels (data not shown, see Fig. 8B). Elastic fibers in the aortic media were negative to the labels (data not shown, see Fig. 4B). Control experiments in which normal mouse serum was substituted for monoclonal antibodies showed virtually no fluorescence in the tissues listed above.

Light microscopic immunocytochemistry with gold. In some tissues with elastic fiber systems, the orange autofluorescence from elastic fibers obscured the specific yellow-green fluorescence by FITC. Therefore, we were obliged to use an alternative method to confirm immunofluorescence observations. Immunogold labeling followed by silver enhancement gave satisfactory results, and it was confirmed that the splenic capsule and trabeculae contained antibody-positive fibrillar structures (Fig. 3H), whereas the elastic fibers in the aorta were negative to the label (see Fig. 4B).

Pepsin digestion followed by immunocytochemistry. Since it was reported that pepsin treatment revealed epitopes of anti-50K-DM (a mixture of 50K-B ($\alpha 1$ (VIII)) and another 50 kD collagenous peptide 50K-A ($\alpha 2$ (VIII)) antibodies which had been masked (20), we also tried pepsin digestion on unfixed tissue sections followed by immunofluorescence labeling or by immunogold labeling with silver enhancement (2). This digestion revealed immunoreactivity which was not evident without digestion in many tissues.

In the aorta, a fine fibrillar label appeared in the intima just beneath the endothelial cells (Fig. 4A). Labeling also appeared in other tunics. It was scattered in the media and in the adventitia, and very often surrounded vascular structures. However, most of the elastic fibers seemed to remain unlabeled (Fig. 4B). The endomysium and the perimysium also became labeled with the mAb

(Fig. 4C). In the liver labeling appeared in areas possibly corresponding to Glisson's sheath (see Fig. 6A). The ligamentum nuchae remained unlabeled even after digestion (Fig. 4D).

Immunofluorescence combined with elastic fiber histochemistry and double-label immunofluorescence. When a section from the spleen was observed with immunofluorescence with anti- $\alpha 1$ (VIII) antibody (Fig. 5A) and then by resorcin-fuchsin staining for elastic fiber (Fig. 5B), colocalization of $\alpha 1$ (VIII) with elastic fiber in the capsule and trabecula is shown. Fibrillar immunofluorescence with anti- $\alpha 1$ (VIII) antibody was also observed in the white pulp.

In the liver treated with pepsin, circumferential fluorescence was shown with both anti- $\alpha 1$ (VIII) (Fig. 6A) and anti-tropoelastin (Fig. 6B) antibodies possibly corresponding to vessels in Glisson's sheath, though exact identity was not certain because the strong pepsinization significantly destroyed the tissue architecture as claimed by Kittelberger *et al.* (21).

In the periodontal ligament, however, the parallel distribution of $\alpha 1$ (VIII) (Fig. 6C) and tropoelastin (Fig. 6D) was incomplete. This is probably because $\alpha 1$ (VIII) localizes not only in some elastic fibers but also in oxytalan fibers in which no elastin is distributed (see also Fig. 8A).

Immunoelectron microscopy. To gain insight into the detailed localization of the antigen, several tissues fixed with PLP fixative were processed for electron microscopy.

In the perichondrium of the tracheal cartilage, two major extracellular structures were identified: collagenous fibrils of various diameters and elastic fibers surrounded by microfibrils. Preembedding immunolabeling with gold probes showed heavy labeling exclusively around elastic fibers (Fig. 7A). In the pleura also, elastic

Fig. 5. A) Immunofluorescence with anti- $\alpha 1$ (VIII) mAb followed by B) resorcin-fuchsin histochemistry for elastin on the same section of the spleen. The colocalization of $\alpha 1$ (VIII) and elastic fiber in a portion of capsule (C) and a trabecula (T) is shown. $\times 100$. Bar = 100 μm .

Fig. 6. Double-label immunofluorescence with anti- $\alpha 1$ (VIII) mAb followed by FITC-labeled anti-mouse IgGs (A and C) and anti-tropoelastin antibody followed by rhodamine-labeled anti-rabbit IgGs (B and D) on the same section of pepsin-treated liver (A and B) and untreated periodontal ligament (C and D). In the liver, the two proteins show the same localization around a hollow structure, possibly corresponding to a vessel in Glisson's sheath. On the other hand, in the periodontal ligament, partial coexistence of these two proteins was shown. Arrows indicate a blood vessel around which there is moderately positive reaction to anti- $\alpha 1$ (VIII) and positive reaction to anti-tropoelastin. A and B: $\times 100$. Bar = $100 \mu\text{m}$. C and D: $\times 200$. Bar = $100 \mu\text{m}$.

fibers were the only structures labeled (Fig. 7B). On close inspection, although the surfaces of elastic fibers were labeled, microfibrils were not always labeled and bare areas were very often observed (Fig. 7B). In the splenic capsule, the surface of some elastic fibers was labeled with gold, at relatively low density, possibly due to the inaccessibility of gold to densely packed tissues (photograph not shown). The gold-labeled structures in the perichondrium were densely stained by tannic acid-uranyl acetate stain in a fashion typical of amorphous

elastic fibers (Fig. 7C). This confirmed that the $\alpha 1$ (VIII) antibody labeled a component located on the surface of some elastic fibers.

To check if all the elastin-associated microfibrils were labeled or not, the periodontal ligament (Fig. 8A) and ciliary zonules (Fig. 8B) were also processed for electron microscopy. Although oxytalan fibers in the periodontal ligament, which are composed of elastin-associated microfibrils, were heavily labeled, the fibers of ciliary zonules were negative to labeling.

Fig. 7. Immunoelectron microscopic localization of $\alpha 1$ (VIII). A) In the perichondrium of the tracheal cartilage, the surface of an elastic fiber (E) is heavily labeled with gold. Collagen fibrils (C) are negative to the label. $\times 42,250$. Bar = $1 \mu\text{m}$. B) An elastic fiber (E) in the pleura. The surface of the fiber is labeled with gold (circles), though other structures remained unlabeled. Although labels are localized around elastic fibers, some structures similar to elastin-associated microfibrils remain unlabeled (arrows). $\times 46,500$. Bar = $0.5 \mu\text{m}$. C) Elastic fibers in the tracheal cartilage labeled with anti- $\alpha 1$ (VIII) and gold followed by tannic acid-uranyl acetate staining of the elastic fibers. Periphery of the densely stained elastic structures (E) is labeled with gold. $\times 65,000$. Bar = $0.5 \mu\text{m}$.

Fig. 7.

Fig. 8. Two structures known to contain microfibrils of elastic fiber systems without amorphous elastin cores are labeled with 6A2 and the gold probes. A) Although oxytalan fibers (o) in the periodontal ligament are heavily and specifically labeled, B) the ciliary zonule microfibrils remain unlabeled. A: $\times 63,000$. B: $\times 54,000$. Bar = $0.5 \mu\text{m}$.

To investigate whether the interior of elastic fibers bound these antibodies or not, ultrathin cryosections were made of the spleen, which contains dense bundles of elastic fibers (Fig. 9). Consistently, only the surface of the elastic fibers was labeled, and the antibody was shown to be localized only on the surface of elastic fibers.

We also immunolabeled unfixed tracheal perichondrium. In these specimens, the gold-labeled structures occasionally showed faint periodicity, which was similar to the D-period of collagen fibrils (Fig. 10).

DISCUSSION

The 6A2 antibody reacted preferentially with the lower half of the 50K-B band in our previous study (40). The partial amino acid sequence of that band was identical to the amino acid sequence of an area in the collagenous domain of the $\alpha 1$ (VIII) chain deduced from rabbit cDNA sequences reported by Yamaguchi *et al.* (43). Thus it was confirmed that the antigen of 6A2 mAb is $\alpha 1$ (VIII). The sequence is also identical to that of the N-terminus of the whole 50K-B band as reported by Kapoor *et al.* (17).

The investigation of various organs with the antibody showed wide distribution of the antigen. Capsules of

many tissues, i.e., the lungs, kidneys, adrenals, liver, spleen, nerves, sclera, dura mater of the spinal cord, and tendons, showed specific fibrillar immunofluorescence. Although endomysium did not show fluorescence, pepsin treatment revealed masked epitopes in that tissue. The antigen was also localized in some dense connective tissues; the tendon, ligament, and dermis. These localizations of antigen by immunofluorescent techniques are generally consistent with the observations by Kittelberger *et al.* (21), who used polyclonal antibody against mixture of $\alpha 1$ (VIII) and $\alpha 2$ (VIII) in the same tissues which we investigated so far. This indicated either that $\alpha 1$ (VIII) coexists with $\alpha 2$ (VIII) in most tissues, or that $\alpha 1$ (VIII) has much higher antigenicity than $\alpha 2$ (VIII).

In some tissues, antibody bound in a distribution corresponding to that of elastin or of tropoelastin reported by immunocytochemistry (3, 6).

Although no direct correlation was made, Kittelberger *et al.* suggested that type VIII collagen is associated with elastic fiber or elastin-associated microfibrils (21). Our immunofluorescence combined with elastic fiber histochemistry and double-label immunofluorescence studies gave support to the idea. The electron microscopic observation that the surface of elastic fibers was labeled with the antibody also confirmed that $\alpha 1$ (VIII)

Fig. 9. (left) Ultrathin cryosection of the splenic capsule labeled with 6A2 mAb and 10 nm anti-mouse IgG gold. The label is located exclusively on the periphery of an elastic fiber (E). Collagenous fibrils (C) and cytoplasm of a cell (P) are not labeled. $\times 46,000$. Bar = $0.5 \mu\text{m}$.

Fig. 10. (right) Perichondrium of a tracheal cartilage labeled with the mAb and gold before fixation, and processed for electron microscopy. Occasionally the labeled structure shows a faint banding pattern (arrowheads) the periodicity of which is similar to that of collagenous fibrils (C). $\times 82,000$. Bar = $0.1 \mu\text{m}$.

is associated with the elastic fiber system in some tissues.

Elastic fibers are composed of amorphous elastin in the center and elastin-associated microfibrils 10–12 nm in diameter at the periphery (7). They differ in chemical characteristics (31). The latter are also seen independent of elastin in structures such as ciliary zonules and oxytalan fibers of periodontal ligaments (7).

The association of $\alpha 1$ (VIII) along the periphery of elastic fibers and along the periodontal ligament suggests that $\alpha 1$ (VIII) is a component of microfibrils. The fact that only the periphery of elastic fiber was labeled on ultrathin cryosections in which even the central region of elastic fibers is freely accessible to antibodies strengthens the idea. However, ciliary zonules (30), and the mesangial matrix of the kidney (26), which were shown to contain elastic fiber-related microfibrils, were not labeled with the antibody. Therefore, not all the microfibrils of the elastic fiber system were labeled. Furthermore, elastic fibers in the aortic media and ligamentum nuchae were also devoid of significant labels. Thus, not all elastic fibers were labeled either.

There are two explanations for this lack of coincidence. One is that microfibrils are chemically heterogeneous among elastic fiber systems, and $\alpha 1$ (VIII) is a in-

trinsic component of one of them. The other is that $\alpha 1$ (VIII) is not an intrinsic component of microfibrils and is very often, but not always, associated with the system. There are many candidate molecules for microfibrils of the elastic fiber system: fibrillin (36), MAGP (9, 22), amyloid P component (12), fibronectin (11) and others (4, 10, 15). Since some of them more consistently colocalize with microfibrils (8, 36), we prefer the latter idea.

In some tissues fibrillin shares a coincidence with $\alpha 1$ (VIII) in that anti-fibrillin antibody showed the same periodicity as collagenous fibrils, along microfibrils. The significance of the observation remains to be further elucidated.

$\alpha 1$ (VIII) was shown to be responsible for the regular hexagonal nature of Descemet's membrane lattice (40). None of the tissues in which it was demonstrated in the present study contain hexagonal lattices or long-spacing structures as their normal major components. Although it is also possible that our monoclonal antibody recognized two unrelated proteins, one against hexagonal lattices and the other against elastic fiber systems, this is not likely since the polyclonal antibodies reported by Kittelberger *et al.* showed a similar distribution pattern to ours in Descemet's membrane and in other tissues.

$\alpha 1$ (VIII) may take the lattice form only in a special environment.

$\alpha 1$ (VIII) has a strong similarity in structure to type X collagen (43). Type X collagen is known to localize in hypertrophic cartilage and has some role in calcification in enchondral ossification (41). However, $\alpha 1$ (VIII) does not seem to have any relationship with ossification so far, and the functional analogy between these two collagen types was not successful. It is also known that type X collagen is developmentally regulated (28). Recently, contribution of type VIII collagen in angiogenesis and cardiac development was suggested (13, 29, 33). At present we have few data about the developmental change in the expression of $\alpha 1$ (VIII) chain.

Acknowledgements. The authors would like to thank Dr. Yoriko Tamura for her assistance with the biochemical experiments, Ms. Michiyo Esaki for her technical assistance with the immunofluorescence techniques, and Mr. Tatsuo Maejima for photographic help. We are also indebted to Dr. Richard C. Goris for critical review of the manuscript.

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(Received for publication, July 9, 1991

and in revised form, September 13, 1991)