Localization of Type V Collagen and Type IV Collagen in Human Cornea, Lung, and Skin

Immunohistochemical Evidence by Anti-Collagen Antibodies Characterized by Immunoelectroblotting

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Tissue distribution of Type V collagen in comparison with Type IV collagen was investigated by indirect immunofluorescence microscopy. Affinity-purified rat antibodies to Type IV and Type V collagens obtained from human placenta reacted specifically only with the corresponding type of collagen in both native and denatured conformations. In indirect immunofluorescent stainings of human skin, lung, and cornea tissues, Type IV and Type V collagens showed distinct distributions. Type IV collagen was distributed exclusively in basement membrane. However, Type V collagen had two characteristic features; a diffuse distribution in interstitium and the locations on or adjacent to basement membrane. On the basis of these findings and of biochemical characterization of the collagens, it is postulated that Type V collagen is an intermediate collagen, possibly having a function of binding or connecting of interstitial collagen fibrils with membranous collagen networks. (Am J Pathol 1984, 116:417–426)

THE MOLECULAR heterogeneity of collagen involves at least five genetically distinct types (Types I–V).[1] Each molecule of the five types of collagen is composed of three polypeptide chains. There are at least nine genetically distinct collagenous polypeptides, called α-chains. The assembly of four chains, α1(I), α2(I), α1(II), and α1(III) to triple-helical molecules constructs collagens Type I, Type II, and Type III.[1] Type I collagen is located in extracellular matrices of skin, tendon, bone, uterus, cornea, and many other tissues; Type II collagen, in hyaline and elastic cartilage; and Type III collagen, in extracellular matrices of many tissues often together with Type I collagen.[1–7] Type IV collagen, which is composed of α1(IV) and α2(IV) with characteristic amino acid compositions, is the major skeletal macromolecule of basement membrane in various tissues.[1,3–7]

Type V collagen was first isolated from human placenta by Burgeson et al in 1976.[8] Thereafter, Type V collagen was identified in many tissues, such as blood vessel wall,[9] bone,[10] cartilage,[10] skeletal muscle,[11] corneal stroma[12,13] skin,[14,15] synovium,[16] lung,[17,18] and tendon.[19] The Type V collagen was initially considered to consist of α1(V) and α2(V) chains.[8] Some tissues contain a small amount of the third chain, α3(V).[14,20,21] The molecular organization of Type V collagen, in terms of α1(V), α2(V), and α3(V) chain compositions, still remains to be elucidated.[8–22] Evidence has accumulated that Type V collagen consists of at least two different molecules. Since the initial isolation of Type V collagen was from tissues rich in basement membrane, such as placenta, and the amino acid composition resembled that of Type IV collagen, it was suggested that Type V colla-

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gen was a constituent of basement membrane.[8,9] The distribution and function of Type V collagen are still controversial: 1) the codistribution of Type IV collagen and Type V collagen led to a hypothesis that Type V collagen, in addition to Type IV collagen, is a constituent macromolecule of basement membrane[17,23,24]; 2) the distribution of Type V collagen close to mesenchymal cells was taken to construct exocytoskeleton[25,26]; 3) diffuse staining in interstitium and stroma raised the possibility of the association of Type V collagen with interstitial collagen fibrils[27-30]; 4) the production by epithelial cells and localization on the surface of epithelial cells gave rise to the theory that there was an intimate relation of this type of collagen to the migration and the cytoskeletal components of epithelial cells[18,31]; 5) overdeposition of Type V collagen in some diseased tissues was discussed as a possible involvement of myofibroblasts or cells derived from smooth muscle cells which may produce Type V collagen.[32-34] In any case, apparently inconsistent data cannot be unified by a hypothesis that Type V collagen is a single form of the molecule or of the aggregate. The polymorphic structure of Type V collagen in terms of both molecular organization and aggregate structure seems to be a current hypothesis.[21,22,27] The aim of the present study is to examine the distribution of Type V collagen, particularly in comparison with Type IV collagen, by immunohistochemical methods using antibodies reactive with the separated chains derived from Type IV and Type V collagens. The polymorphic distribution of Type V collagen is discussed in relation to the biochemical characteristics of this type of collagen.

Materials and Methods

Preparation of Type IV and Type V Collagens

Two human placentas stored in a freezer were cut with a knife and minced with a Polytron homogenizer. The minced tissue were washed with 4.5 M NaCl, 1 mM Tris-HCl, pH 7.5, several times and then finally with 0.5 M acetic acid. Pellets of the minced tissues were resuspended in 4 liters of 0.5 M acetic acid and treated with 0.8 g of pepsin at 4 C for 3 days. The supernate by centrifugation at 10,000 *g* for 30 minutes was neutralized by addition of solid Tris and 20% NaOH and left standing at pH 8.2 overnight. Collagen Types I, III, IV, and V were fractionated essentially according to the method described by Rhodes and Miller.[10] Briefly, to the supernate at 0.7 M NaCl in 0.5 M acetic acid, solid NaCl was added to a final concentration of 1.2 M. The collected precipitates were dissolved in 0.5 M acetic acid and then dialyzed against 0.01 M Tris-HCl at pH 8.5, 0.02 M NaCl, and 2 M urea at 4 C. The precipitate at this point contained mainly Type V collagen, while the supernate contained Type IV-like collagen. The above procedure of salt fractionation was repeated several times. The purity of the collagen at each step was monitored by SDS-polyacrylamide gel electrophoresis and circular dichroism.

As a final step of purification, the Type V collagen dissolved in 0.02 M Tris-HCl, pH 8.5, 0.05 M NaCl, and 2 M urea was passed through DEAE-cellulose (DE52).

Phosphocellulose Chromatography of the Type IV Collagen in the Native State

Type IV collagen in 0.5 M acetic acid was dialyzed against 3 M urea in phosphate buffer of 0.03 M disodium phosphate at pH 5.4 adjusted by addition of phosphoric acid. The sample solution was chromatographed on a phosphocellulose (P11) column. The elution was performed with a linear gradient of 0 to 0.2 M NaCl in the phosphate buffer at pH 5.4 and 3 M urea at room temperature.

Circular Dichroism Studies

Circular dichroism (CD) was examined using a Model J-500A JASCO spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo) in a water-jacketed cell with a 2-mm light path. The temperature was recorded with a thermistor inserted into the top of the sample chamber. To avoid ultraviolet light damage to the protein,[35] the slit in the instrument was intermittently closed, and the temperature was increased at about 60 C/hr.

Velocity Sedimentation

Velocity sedimentation was performed in a discontinuous series of sucrose concentration, 5%, 10%, 15% and 20% in 0.1 M Tris-HCl at pH 7.5 and 0.4 M NaCl. The centrifugation was done in a SW40 Spinco rotor at 40,000 rpm and 4 C.

Amino Acid Composition

Amino acid analysis was performed by high performance liquid chromatography using a Simazu model LC-3A analyzer and a ISC-07 column (Simazu, Tokyo) after hydrolysis with 6 M HCl in an evacuated, sealed tube at 110 C for 24 hours.

Preparation and Purification of Antibodies to Type IV and Type V Collagen

Lewis and Sprague–Dawley rats were immunized with human Type IV or Type V collagens. Intradermal injection of 0.8 mg of the collagen was given, dissolved in 5 mM acetic acid, 0.2 mg N-acetylmuramyl-L-alanyl-D-isoglutamine in distilled water and emulsified with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, Mich). The rats were bled at intervals of 1 or 2 weeks, and the presence of antibodies was monitored by passive hemagglutination tests. They were bled to sacrifice for the sera from the whole body about 60 days after the injection.

Immunoadsorbent columns were used for the purification of the anti-collagen antibodies.[4] Human Type I, Type II, Type III, Type IV, and Type V collagens (0.1%, 5–20 mg), dissolved in 0.2 M borate buffer, pH 8.0, containing 0.15 M NaCl, were coupled to activated CH-Sepharose 4B gels (1 ml/mg collagen). Antibody to Type IV or Type V collagen was successively passed through the columns packed with the other types of collagen and finally adsorbed and eluted from the Type IV or Type V collagen column, respectively. Reaction activities of antibodies were tested by passive hemagglutination assays as reported previously.[4]

SDS-Polyacrylamide Gel Electrophoresis and Immunoelectroblotting

SDS-polyacrylamide gel electrophoresis was carried out with the use of 3% stacking gel, 130 mM Tris-HCl, pH 6.8, and 5% separating gel, 380 mM Tris HCl, pH 8.8, in the presence of 3.6 M urea as reported previously.[36] Type I and Type IV collagens were electrophoresed after reduction with dithiothreitol. Immunoelectroblotting was performed as follows. Type IV and Type V collagens, which were electrophoresed on SDS–polyacrylamide gels separately, were transferred to a cellulose nitrate membrane filter electrophoretically in solution consisting of 50 mM Tris, 380 mM glycine, and 20% ethanol, pH 8.3. The membrane filter was soaked in PBS, pH 7.2, containing 1% bovine serum albumin and 0.02% NaN_3 at room temperature overnight. Then it was incubated with the rat antibodies to Type IV or Type V collagen diluted 1:2 with 0.15 M NaCl in 0.05 M Tris-HCl, pH 7.5, and FITC-labeled anti-rat IgG rabbit IgG (MBL Co., Nagoya), diluted 1:4 with the same buffer, at room temperature sequentially for 60 minutes. After being rinsed in several changes of PBS, pH 7.2, the membranes were observed under ultraviolet lamps (wave length, 254 nm).

Indirect Immunofluorescence Staining

Normal human cornea (54 years, female), normal human lung (50 years, male) and normal human skin (20 years, female; 43 years, male) tissues were obtained at autopsy or surgical operations and stored at −70 C until used. Six- to eight-micron sections of the sample tissues sliced in a cryostat were mounted on glass slides, air-dried, and pretreated with acetone for 10 minutes. The sections were incubated with antibody to human Type IV or Type V collagen, diluted 1:8 with 0.15 M NaCl in 0.05 M Tris-HCl, pH 7.5, in a moist chamber for 60 minutes at room temperature, then rinsed in several changes of PBS, pH 7.2. The sections were subsequently incubated with FITC-labeled anti-rat IgG rabbit IgG (MBL Co., Nagoya), diluted 1:16 with saline for 60 minutes, rinsed with PBS, and mounted in the same buffer containing 80% glycerol. The stained sections were observed in an Olympus BHF fluorescence microscope with a set of UG1 and L420 filters.

Results

The Circular Dichroism Spectrum of Type IV and Type V Collagen

The solutions of Type IV and Type V collagens showed typical circular dichroism spectra which were similar to that of Type I collagen[35] with molar ellipticity at 221 nm about 3000 and 6000, respectively, indicating that the molecule was in triple-helical conformation.

Thermal Transition of Purified Type IV and Type V Collagen

The helix-to-coil transition of purified Type IV or Type V collagen was examined as a function of temperature. The protein was dissolved in 0.05 M acetic acid at a protein concentration of 100 μg/ml. The Type V collagen underwent a sharp thermal transition with a Tm of 39 C. The sharpness of the transition expressed by the range from 1/4 melting to 3/4 melting was 4 C, which was slightly larger than the range for Type I collagen melting temperature (1.5–2 C),[35] but the overall melting profile and Tm were very similar to those of interstitial collagens. The Type IV collagen showed a broader melting curve, a Tm of 44 C and a melting range of 8 C.

Sedimentation Velocity of Type V Collagen

When sucrose gradient centrifugation of Type I collagen and Type V collagen in separate tubes was

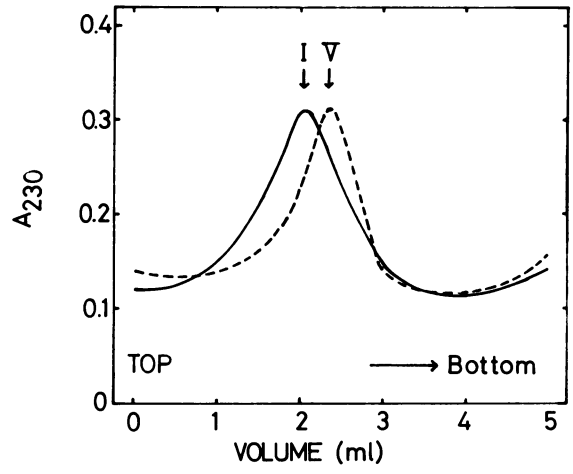


Figure 1 – Sucrose gradient centrifugation of Type I and/or Type V collagen from human placenta. The collagens were purified by differential salt fractionation and then sedimented for 20 hours on 5–20% sucrose gradients containing 0.1 M Tris-HCl (pH 7.5) and 0.4 M NaCl. Type I (———) and Type V (- - -) collagen are in separate tubes.

run, the Type V collagen sedimented to a position slightly further to the bottom than Type I collagen (Figure 1). A mixture of both Type I and Type V collagen was spun in the same centrifuge tube, and each collected fraction (total number of fractions, 14) was analyzed by SDS-gel electrophoresis. However, there was no significant difference in sedimented positions between the collagens (data not shown). From the apparent molecular weight of constituent polypeptides determined from the mobility on SDS gel (Figure 2) and the molar ellipticity of the triple-helical structure of Type V collagen by circular dichroism, the similar sedimentation values indicated that the overall molecular shape of Type V collagen was not different from that of Type I collagen, which was rodlike in shape.

Amino Acid Composition of Type IV and Type V Collagen

The amino acid composition of Type IV and Type V collagens was consistent with previous reports.[1,8,10] High ratios of hydroxyproline and hydroxylysine and a lower content of alanine are typical of Type IV and Type V collagen molecules.

Specificity of Antibodies to Type IV and Type V Collagen

The antibodies to Type IV and Type V collagens were confirmed to have type-specific high titers. No cross-reactions with the other types of collagen were detected by the passive hemagglutination assays. The titer values of anti-Type IV and Type V collagen antibodies were 2^{-12}.

Figure 2 shows SDS-polyacrylamide-gel electrophoresis of Type I, and Type IV, and Type V collagens (Figure 2, *1*, *2*, and *3*) and immunoelectroblotting of Type IV and Type V collagens incubated with anti-Type IV collagen antibody (Figure 2, *4* and *5*) and with anti-Type V collagen antibody (Figure 2, *6* and *7*). Anti-Type IV collagen antibody reacted with the subchains presumably derived from Type IV collagen (Figure 2, *4*), in that the bands did not correspond to the other types of collagen and they were sensitive to purified bacterial collagenase. Anti-Type V collagen antibody reacted with only (and all) Type V collagen-derived polypeptides; $\alpha 1$, $\alpha 2$, $\alpha 3$, and β(V) chains. The latter were presumed to be β(V), because the mobility corresponded to the dimers of $[\alpha 1\ (V)]_2$ and α1(V) and α2(V). Because it is not clear in Figure 2, *7* whether α3(V) chain reacted or not, the immunoelectroblotting was performed in the absence of urea where the α3(V) chain migrated distinctly from α2(V) and α1(V) chains (Hayashi et al, unpublished data). All three bands reacted with the anti-Type V collagen antibody (data not shown). These antibodies showed no reactions with α-chains of the other types of collagen (I–IV).

Indirect Immunofluorescent staining

The tissue distribution of Type IV and Type V collagens in normal human cornea (Figure 3A and D), lung (Figure 3B and E) and skin (Figure 3C and F) was examined. Anti-Type IV collagen antibody reacted along a sharp delineation of subepithelial layer in cornea (Figure 3A), or epidermo-dermo junction in skin, and of capillary walls (Figure 3C). Alveolar septa were also stained by anti-Type IV collagen antibody in a linear fashion (Figure 3B). However, epithelium of cornea, Bowman's membrane, corneal stroma, epidermis, and dermis were not stained by antibody to Type IV collagen. The results are consistent with the previous reports in that Type IV collagen was a component of basement membrane.[3]

Stroma and Bowman's membrane in cornea were well stained diffusely with anti-Type V collagen antibody (Figure 3D). The staining in lung with anti-Type V collagen antibody showed two typical appearances: the delineation of fine fibers and of discontinuous membranous constituents (Figure 3E). The dermis was stained diffusely with the anti-type V collagen antibody. The papillary layer and interstitium around capillaries were especially well stained (Figure 3F). The staining locations were very close to the basement membrane, if not in it. At the indirect immunofluorescence microscopic level, it is difficult to distinguish the basement membrane from fine fibrils associated

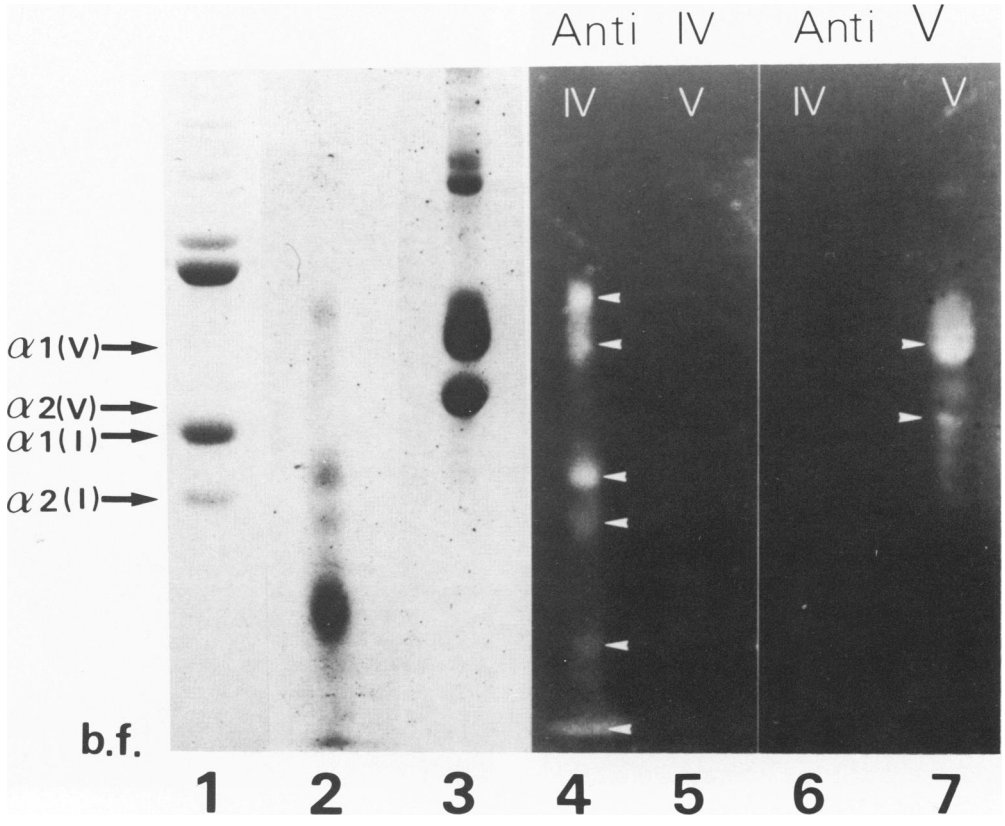


Figure 2 – SDS-polyacrylamide gel electrophoresis in 380 mM Tris-HCl, pH 8.8, 5% gel in the presence of 3.6 M urea (*1–3*) and immunoelectroblotting (*4–7*). Type I collagen (*1*), Type IV collagen (*2, 4,* and *6*), and Type V collagen (*3, 5,* and *7*) were electrophoresed. Type I and IV collagens were reduced with dithiothreitol. Staining was performed with Coomassie brilliant blue R-250 (*1–3*) and anti-Type IV collagen antibody (*4* and *5*) and anti-Type V collagen antibody (*6* and *7*), followed by incubation with FITC-labeled anti-rat IgG rabbit IgG. (*4–7*). Anti-Type IV collagen antibody reacted with subchains of Type IV collagen (*4, arrowheads*) and anti-Type V collagen antibody with α1(V) and α2(V) chains of Type V collagen (*7, arrowheads*). *b.f.*, buffer front.

with the basement membrane. Epithelium of cornea and epidermis of skin were not stained by anti-Type V collagen antibody (Figure 3D and F). At the resolution level of the fluorescence immunohistochemical observation the distribution of Type V collagen could clearly be distinguished from that of Type IV collagen.

Discussion

The present results clearly indicate that the distribution of the Type V collagen in human cornea, lung, and skin tissues was distinct from that of the Type IV collagen. While the distribution of the Type IV collagen was confined only to the locations where basement membrane was either found or was expected to exist, the distribution of the Type V collagen showed two different appearances in these tissues. One was the diffuse staining in the interstitium, and the other was discontinuous, linear staining, which was obviously distinct from that of Type IV collagen. The latter location may be adjacent to or on some localized sites of basement membrane.

Three different tissues (cornea, lung, and skin) were examined for distinguishing the localization of Type IV and Type V collagens. Distributions of Type V collagen in various tissues, including lung,[17,18] liver,[24,37] kidney,[23,27] and cornea,[12,28,29] have been reported. The apparent discrepancy in the conclusions may be due to technical differences and a direct comparison of the data is not possible. We thought that one way to generalize the localizations of Type IV and Type V collagen in tissues in terms of the type-specific aggregate structures, if at all possible, was to examine tissues with different organizations. The tissues examined here have very different microscopic morphologic characteristics and tissue scaffolds. Therefore, the locations of Type IV and Type V collagen would have to be ascribed to type-specific aggregate structures which may commonly be found in many different tissues.

Collagen types can be combined in two groups: interstitial (Types I, II, and III) and membranous (Type IV). Type V collagen is an intermediate type of collagen between the interstitial and membranous types in

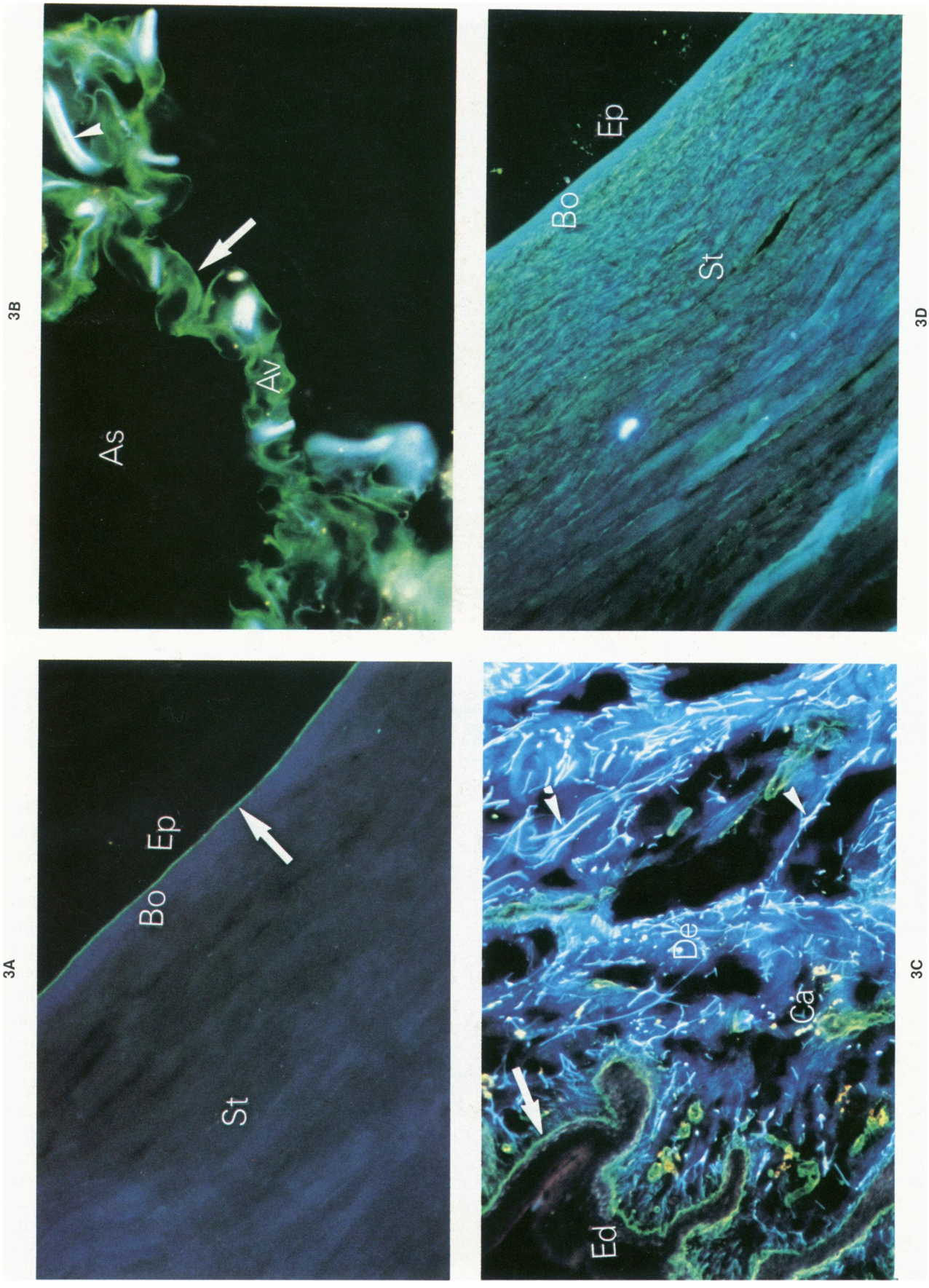
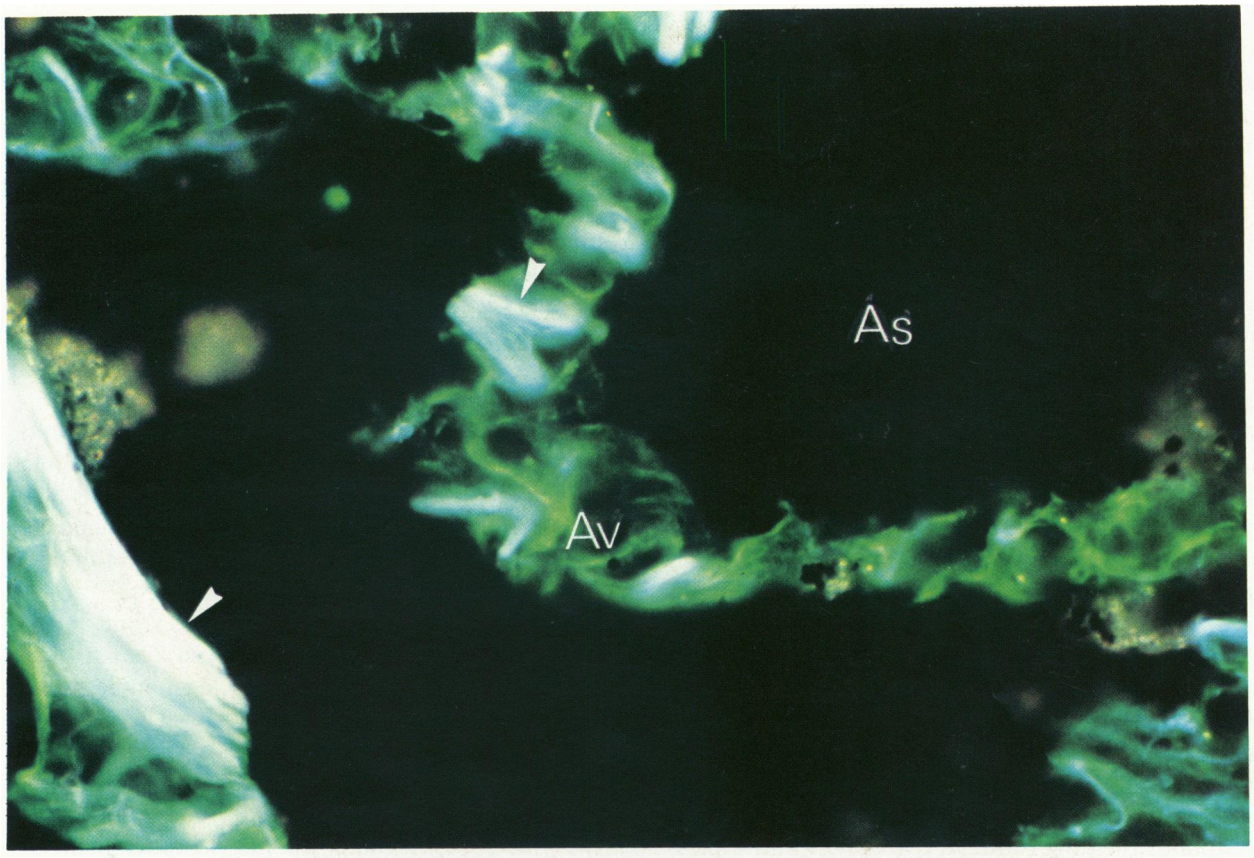
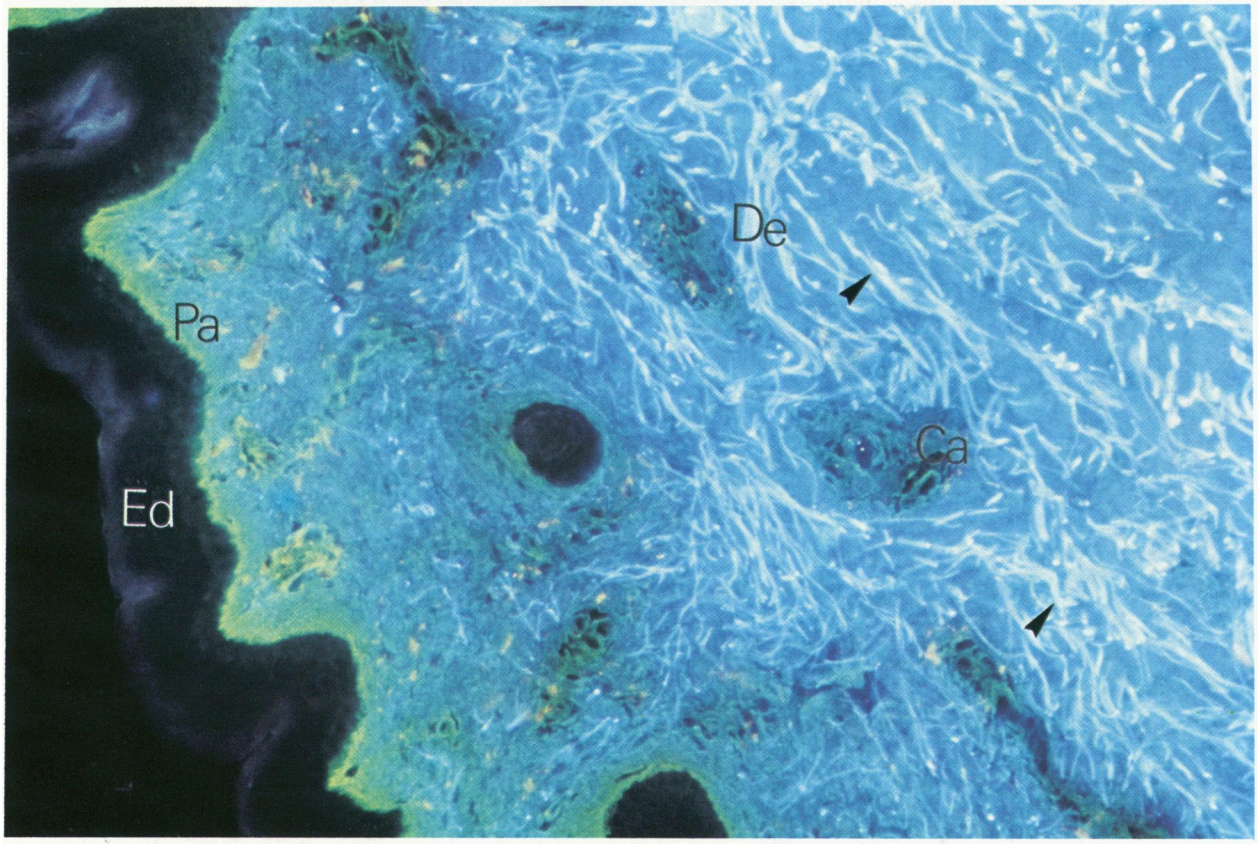


Figure 3—Indirect immunofluorescence staining of human cornea (**A** and **D**), lung (**B** and **E**), and skin (**C** and **F**) reacted with antibodies to Type IV (**A, B,** and **C**) and Type V (**D, E,** and **F**) collagens (**A**, ×225; **B**, ×450; **C** and **D**, ×110; **E**, ×640; **F**, ×160) **A**: *Ep*, epithelium; *Bo*, Bowman's membrane; *St*, Stroma. **B**: *As*, alveolar sac. **C**: *Ed*, epidermis; *De*, dermis. **D**: *Ep*, epithelium. **E**: *As*, alveolar sac. **F**: *Ed*, epidermis. The subepithelial layer of cornea (**A**, †), alveolar septum (*Av*) of lung (**B**, †), epidermo-dermo junction of skin (**C**, †), and capillary walls (**C**, *Ca*) were sharply stained



3E



3F

by anti-Type IV collagen antibody in a linear fashion. Anti-Type V collagen antibody stained as follows (**D**, **E**, and **F**): corneal stroma (*St*) and Bowman's membrane (*Bo*) were stained diffusely (**D**). Staining of alveolar septa (*Av*) was revealed to show two different appearances: one was discontinuous and linear, and the other was a delineation of fine fibers (**E**). The dermis (*De*) was stained diffusely, and the papillary layer (*Pa*) and areas around capillaries (*Ca*) were stained well (**F**). The *arrowheads* indicate white autofluorescence of elastic fibers (**B**, **C**, **E**, and **F**). The dermis has blue autofluorescence which is easily distinguished from FITC-specific fluorescence (**C** and **F**).

terms of chemical characterization. Its velocity sedimentation behaviour resembles that of interstitial types of collagen, which are rodlike in structure (Figure 1). Bachinger et al[38] observed the Type V procollagen molecule with the electron microscope and suggested that the length of the helical portion of the molecule was similar to an interstitial collagen but different from Type IV collagen. On the other hand, its amino acid composition was more similar to that of Type IV collagen. Immunohistochemical observations suggest that Type V collagen in tissues has two features, codistribution with interstitial fibrils and location close to membranous structure. The bimodal features can also be explained by more than one subtype of collagen.[20-22] It may well be that one of the subtypes of Type V collagen is associated with the fibril organization of interstitial collagens and the other subtype with the basement membrane organization.

Our findings concerning the localization of Type V collagen were consistent with some of the previous reports.[12,27-30] The α3(V) chain was more abundant in placental villi.[21] Whether or not the discontinuous, linear staining close to basement membrane in alveolar septa of the lung is due to reaction of anti-Type V collagen antibody with the α3(V) chain is an interesting problem and is under investigation. However, the observations with anti-Type V collagen antibody were not in agreement with those of Madri et al[17] and Roll et al.[23] They described the codistribution of Type V collagen and Type IV collagen in basement membrane. The discrepancy might be due to preparations of Type V collagen and the antibodies, staining techniques, and the state of the specimens examined. In our studies with indirect immunofluorescence microscopy, anti-Type V collagen antibody reacted also with basement membrane, but only partially. Furthermore, the staining is not continuous or linear, as is the case with anti-Type IV collagen antibody staining. Therefore, Type V collagen may have affinities with certain localized sites of basement membrane, but not all of the basement membrane. It is difficult at the fluorescence microscopic level to distinguish the staining of the basement membrane from that of fine fibrils associated with the basement membrane.

On the basis of these results by immunohistochemical observation and by biochemical characterization, we postulate that Type V collagen is an intermediate collagen between fibril-forming, interstitial collagens (Types I, II, and III) and membranous collagen (Type IV). The structure and function of Type V collagen may well be binding or connecting these two different architectural components of tissues.

Because the location of Type V collagen is controversial and the method is usually based on the anti-type V collagen antibodies, some technical comments may be worthwhile.[39] Passive hemagglutination assay,[4] radioimmunoassay,[40] and enzyme-linked immunoassay[41] can be used as quantitative tests (titers) for antibodies. Without confirmation by the qualitative analysis, we could not preclude the possibility that some of the polyclonal antibodies may have reacted with unexpected substances in the antigen fraction. Immunoelectroblotting may detect such a problem in the quantitative characterization of antibodies. The combined characterization of antibodies by such quantitative and qualitative methods may help to clarify some of the observed discrepancies with different preparations of antibodies to the same type of collagen.

The type-specific antibodies to Type IV and Type V collagen in our preparations were revealed to react with native Type IV and Type V collagen molecules in triple-helical structures, respectively. They had no cross-reactions with the other types of collagen by the passive hemagglutination assay. They were also demonstrated to have affinity with the corresponding denatured collagen chains but not the other chains by immunoelectroblotting (Figure 2). The antibody preparation contains the immunoglobulins which recognize both the native and the denatured forms of collagen molecules. Furthermore, the anti-Type V collagen antibody reacted with the α3(V) chain, which was present as a minor component in a preparation of Type V collagen (data not shown). No reports have noted affinity of anti-Type V collagen antibody preparations with the α3(V) chain. In our case, the Type V collagen preparation used as the immunizing antigen contained several percents α3 (V) chain. The reaction with the α3(V) chain of our anti-Type V collagen antibody imposes the possibility that the polymorphic distribution of Type V collagen was due to two different species of Type V collagen,[20,21] one containing α3(V) and the other containing no α3(V).

The antibodies to Type IV and Type V collagen prepared in this report had high antibody titers. The antibodies with high titers seemed particularly important for Type V collagen. In this report, the anti-Type V collagen antibody was used for indirect immunofluorescent staining at a dilution of 1:8. However, when the anti-Type V collagen antibody was diluted to 1:16–1:32 (the resecting passive hemagglutination assay titers were 2^{-7}–2^{-8}), the staining was much less clear. The fact that the antibodies to the other types of collagens with the titer values of 2^{-7}–2^{-8} sufficiently reacted with the tissue sections[4] suggests

that the major portion of the anti-Type V collagen antibody used was against Type V collagen antigenic determinants masked in the aggregates of the tissues.

Linsenmayer et al[29] and von der Mark and Ocalan[28] reported the necessity of pretreatment of tissues with acetic acid or pepsin for immunofluorescence studies of Type V collagen using monoclonal antibody. Our anti-Type V collagen antibody reacted without such pretreatment. Instead, we performed pretreatment of tissues with acetone for 10 minutes. Our anti-Type V collagen antibody was a mixture of polyclonal antibodies and contained the antibody which may have recognized antigenic domain(s) of Type V collagen exposed in tissues. In a few reports pretreatment with acetone for immunofluorescent study was described. Good staining reactions in our study may also have come from the pretreatment with acetone. Because of our observation that the staining ability of the anti-Type V collagen antibody decreased rapidly with dilution and the observation that staining with two different monoclonal antibodies became possible only after pretreatment of the tissue with acetic acid[29] or pepsin,[28] one may surmise that the antigenic determinant sites of Type V collagen molecule may tend to be masked in the aggregates in tissues.

Autofluorescence was also always seen, particularly in the human tissues. We do not know what materials in human tissues cause autofluorescence. Since most of the animal tissues examined were much less autofluorescent, the autofluorescent material may be specific for the human tissues. An alternative possibility is that the autofluorescent materials depend on the age of the animals. That is, the human tissues examined were more than 20 years old, and animal tissues usually examined were younger.

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