Ultrastructural Localization of Type V Collagen in Rat Kidney

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ABSTRACT

Antibodies specific for the a1 (V) chain and native collagen molecules containing the a1 (V) chain have been used in electron immunohistochemical studies of rat kidney to determine the ultrastructural distribution of this class of collagen molecules. In addition, antibodies against type I collagen and whole basement membrane were used as markers for interstitial collagen and authentic basement membranes. Our results indicate that type V collagen is present in the renal interstitium in different forms: in close apposition to interstitial collagen fibers; in the stromal aspect of vascular basement membranes; and as particulate material not bound to other structures. On the basis of these findings, we postulate a binding or connecting function for this collagen type.

Since the description of a new class of collagen molecules in 1976 (5, 6) much has been learned about the chemistry and biology of the type V collagen system (25). It has been shown that this collagen type may contain three distinct a chains: a1 (V), a2 (V), and a3 (V) (30). The composition of these chains resembles more closely that of basement membrane collagen, i.e., type IV (11, 17) than that of the interstitial collagens, i.e., types I, II and III. For this reason it was postulated that type V collagen could be derived from basement membranes (6). In this regard, type V collagen is relatively abundant in vascular tissues (1, 6, 30) yet may be isolated from avascular corneal stroma (32) and from hyaline cartilage (28). It appears, therefore, that the distribution of this collagen type is significantly different from that of type IV collagen.

From a morphological point of view, the light microscopic localization of this collagen type has been studied by immunofluorescence in a variety of organs (2, 12, 18, 19, 20) as well as in tissue culture (10, 12). It has become clear however, that light microscopy, although a desirable first approximation, lacks the resolution needed to define many of the connective tissue macromolecules (23, 29). The only electron microscopy localization available (29) was limited to renal glomerulus and tubules, with no description of the interstitium or the possible relationship to the interstitial collagens. We therefore undertook the present electron immunohistochemical study to define the ultrastructural distribution of type V collagen, and any possible interactions of this collagen class with other connective tissue components. Our results indicate that type V collagen is present in the kidney interstitium as individual fibrils, and that it seems to have significant interactions with the interstitial collagens and with vascular basement membranes. Based on these findings, a binding or connecting function is proposed for this collagen type.

MATERIALS AND METHODS

Tissue Preparation

Adult rat (Sprague-Dawley) kidneys were prepared as previously described (23, 24). Briefly, after heparinization the kidneys were perfused with phosphate-buffered saline (PBS) at 120 cm H2O of pressure for 1 min via the abdominal aorta. PBS was followed by 4% freshly made paraformaldehyde in 0.1 M phosphate buffer for 30 min. The kidneys were then cut into 5 × 5 × 3-mm blocks fixed in 4% paraformaldehyde at 4°C for an additional hour, washed in multiple changes of PBS with 4% sucrose at 4°C for 16 h, followed by a last 1-h wash in PBS, 4% sucrose, and 7% glycerol, and quickly frozen in methylbutane precooled at liquid nitrogen temperature. 8 μm thick cryostat sections were mounted on albumin-coated slides, air dried, and reacted with the different antibodies.

Antigens

Type I collagen was prepared from rat tail tendons as previously described (4). Whole basement membrane antigen (NBM) was prepared from the murine parietal yolk sac carcinoma as previously described (4, 24). Type V collagen was extracted by limited proteolysis, with pepsin from human placenta (28), followed by selective precipitation and chromatography, and purified by procedures described earlier (28, 32).

Antibodies

All primary antibodies were prepared in rabbits (N-Z white). For anti-type I collagen, anti-NBM, and normal rabbit serum (NRS) the gammaglobulins were purified by ammonium sulfate precipitation followed by chromatography on DEAE-Sephadex (8). As previously described (4), the titers and specificity of the antibodies were determined by ELISA and checkered-board dilution on kidney frozen sections. The second antibody, goat anti-rabbit IgG, was purified in an identical manner and the nonspecificity was established by immunoelectropho-
The peroxidase-anti-peroxidase complex (PAP) was prepared as previously described (4).

The anti-type V antibody was purified by immunoadsorption to and elution from native collagen molecules containing the α1(V) and α2(V) chains as described earlier (9, 13, 31). The purified antibodies showed high specificity for native type V collagen and no cross-reactivity with other collagen types or their constituent chains as evaluated by hemagglutination assay (9). To isolate α1(V) chain-specific antibodies, the aphereses were cross-adsorbed with isolated α2(V) chains followed by adsorption to and elution from isolated α1(V) chains. These affinity-purified antibodies were highly specific for antigenic determinants on denatured α1(V) chains as well as native molecules containing the α1(V) chains. Because both antibody preparations showed identical staining patterns in sections of vascular tissue as well as smooth muscle cell cultures, the results will be discussed in terms of the identification of native type V collagen containing the α1(V) chains.

**Immunohistochemistry**

For tissue staining, the methods described by Kawari and Nakane (16) and Nakane (26, 27) with minor modifications (23) were used: tissue sections were treated with PBS containing 0.5% sodium borohydride and sequentially incubated with normal goat serum, primary rabbit antibodies, goat anti-rabbit IgG, and rabbit PAP. The peroxidase reaction was developed by incubating in diaminobenzidine solution (14) without H2O2 for 10 min, followed by 15-min incubation in diaminobenzidine solution containing H2O2. After washing in PBS for 15 min, the sections were reacted with 1% OsO4 for 1 h, dehydrated, and embedded as previously described (23). Ultra-thin sections were examined and photographed without any further staining.

**RESULTS**

Normal rabbit serum failed to react with any connective tissue structure. Only occasional lysosomes (intrinsic peroxidase activity) in renal tubular cells or leukocytes gave a positive reaction with this reagent (data not shown).

**Anti-NBM**

This antibody was used as a positive marker for basement membranes. As previously described (23), all authentic basement membranes in the kidney-endothelial, smooth muscle, tubular, glomerular, and Bowman's capsule—gave a positive reaction (Figs. 1, 4, and 7). All aspects of the basement membranes were equally positive, regardless of whether the basement membranes were lying between two cells or facing the stroma (Fig. 1).

**Anti-Type I**

This antibody was used as a positive marker for interstitial collagen. As previously described (23), most large collagen bundles (Fig. 5) and the renal capsule were positive. All
basement membranes, vascular-associated collagen (Fig. 2) and small collagen bundles were negative with this antibody.

**Anti-Type V**

Several structures gave a positive reaction with this antibody. The adventitial aspect (that in juxtaposition with the stroma) of the vascular smooth muscle cells was often positive (Figs. 3, 9, 10, 11 and 12). This localization corresponded with the smooth muscle basement membrane as demonstrated by staining with anti-NBM (Fig. 1). Not all the smooth muscle cell basement membrane was positive with anti-type V. Although the basement membrane completely surrounds each individual smooth muscle cell, those aspects of the smooth muscle basement membrane not directly facing the stroma were negative with anti-type V antibody (Figs. 3 and 10), yet they were positive with anti-NBM (Fig. 1). Pinocytotic vesicles in the adventitial aspect of the smooth muscle cells were also frequently positive with this antibody (Figs. 9, 10, 11, and 12). This was in contrast to pinocytotic vesicles in the endothelial aspect which were never positive. All smooth muscle cell organelles, including the rough endoplasmic reticulum and Golgi complex, were consistently negative. The basement membrane of the peritubular capillaries (completely in contact with the stroma) was consistently positive. All tubular basement membranes (Figs. 3, 6, 9 and 13), glomerulus basement membrane (Fig. 8), and Bowman's capsule (data not shown) were consistently negative.

Frequently, granular material was found to be positive with anti-type V antibody. The reaction product in these particles was found not only on the surface but evenly distributed throughout the granule (Figs. 11 and 13). This was in contrast to the interstitial cross-striated collagen fibers that reacted with anti-type I. With the latter antibody, the interstitial fibers gave exclusively regularly distributed reaction (Fig. 5) and (23). Furthermore, while the diameter of the interstitial collagen fibers was ~15 nm, the diameter of the particles containing the type V antigen was only 9 nm. Frequently, these type V granules could be seen juxtaposed to type I fibers (Figs. 11 and 13).

The third pattern of localization of the type V antigen was in between collagen bundles. This pattern was most dramatic in the renal capsule (Fig. 13), where antigen was so abundant as to produce an electron-dense matrix in which type I fibers seemed to be embedded, appearing as a "negative" image.

With anti-type V antibody, type I fibers in the interstitium frequently had surface staining at regular intervals (Figs. 6, 9, 11 and 13). However, not all interstitial collagen fibers were embedded in a matrix reacting with antibodies against type V collagen. Small cross-banded bundles of interstitial collagen
FIGURE 7  Anti-NBM: Renal glomerulus, peripheral capillary loop. The glomerular basement membrane (GBM) and mesangial matrix (Mm) are positive. Cap, capillary lumen; En, endothelial cell; P, podocyte. x 10,800.

FIGURE 8  Anti-type V collagen: Renal glomerulus, peripheral capillary loop. The glomerular basement membrane (GBM) and mesangial matrix (Mm) are negative. No glomerular structure was positive with this antibody. Cap, capillary lumen; En, endothelial cell; P, podocyte. x 8,000.

FIGURE 9  Anti-type V collagen: Portion of renal arteriole and interstitium. The endothelial basement membrane (EBM) is negative, the smooth muscle basement membrane (MBM) is positive, as is the matrix closely associated with it. Collagen bundles in cross and longitudinal section are embedded in a type V matrix (Coll V). Some collagen bundles are negative (Coll). Lu, arteriolar lumen; En, endothelial cell. x 23,300.
tended to be negative (Figs. 9 and 11).

In summary, type V collagen antigenic determinants in rat kidney are present in three major distributions: (a) as individual (9 nm) particles; (b) forming part of the matrix of large, dense collagen bundles and closely associated with the surface of individual type I collagen fibers; and (c) on the stromal surface of some vascular basement membranes. All other structures in the kidney were negative. Specifically, glomerular and tubular basement membranes were consistently negative.

DISCUSSION

The tissue distribution of type V collagen has been studied by light microscopy (2, 12, 18, 19, 20); however, the dimensions of most connective tissue macromolecules are well beyond the resolving power of light microscopy (23). For this reason, we undertook the present electron immunohistochemical study to define the ultrastructural distribution of this class of collagen molecules in rat kidney. In addition to using nonimmune rabbit IgG, we decided to use antibodies against type I collagen and whole basement membrane as additional controls. The use of different antibodies has distinct advantages; first of all, each antibody serves as an additional control for the other antibodies, therefore obviating questions about penetration, diffusion, intrinsic activity, etc. Furthermore, since in electron immunohistochemistry some ultrastructural preservation has to be sacrificed to maintain antigenicity, each well-characterized antibody can be used to define specific structures, i.e. anti-type I defines large interstitial collagen fibers and anti-NBM defines classical basement membranes, and therefore provide evidence of the specificity of the reaction.

The present study provides a clear picture of the ultrastructural distribution of type V collagen in the rat kidney. First of all, it provides evidence of the existence of type V collagen particles of 9-nm Diam (Figs. 11 and 13). To our knowledge, this is the first identification of "free" type V collagen antigen. In addition, it is clear that type V antigens are present not only in the matrix (Figs. 5, 9, and 13) but also in close association with the surface of type I collagen fibers (Figs. 6, 9, 11, and 13). This particular distribution (and only this one) of type V antigen is somewhat similar to the ultrastructural localization of fibronectin in the connective tissue matrix that has been recently described (23). Nevertheless, there are significant differences between the localization of type V collagen and that of fibronectin. Fibronectin fibrils are significantly smaller; fibronectin is more abundantly distributed in the renal interstitium, it is abundant in the mesangial matrix, and it is not present in the vascular basement membranes. Based on the localization of the type V antigen, it is possible to envision the
connective tissue matrices as collections of interstitial collagens (type I and III) held together and connected to surrounding cells (fibroblasts) and other structures (vascular basement membranes) by either fibronectin and or type V collagen. In other words, one function of type V collagen may be the binding of interstitial collagens to surrounding structures.

The unique localization of type V collagen on one particular aspect of the smooth muscle basement membrane (Figs. 3 and 10) deserves further consideration. It is known that smooth muscle cells in tissue culture synthesize type V collagen (9, 31) and that this collagen is distributed around these cells in such a way as to resemble an exocytoskeleton (10). However, in tissue sections the antigen is found exclusively on that aspect of the basement membrane that is directly in contact with the stroma. The obvious question is whether or not type V collagen is an integral component of vascular smooth muscle basement membrane. Clearly, this is a question that is better answered by biochemical and biosynthetic studies rather than by immunohistochemical studies, but several points should be made. First, in this study all epithelial and glomerular basement membranes were consistently negative. This clearly establishes that type V collagen or at least forms of type V which contain the α1(V) chain are not an integral component of all basement membranes. Second, even in smooth muscle cells, parts of the basement membrane (the intercellular and endothelial aspect) are negative. In other words, type V collagen is not a general component of basement membranes; however, in certain locations it appears to be intimately associated with them. Therefore it is easy to visualize type V collagen as an anchoring system binding together the vascular basement membrane and the interstitial collagens (Fig. 11).

An interesting point is the presence of type V antigen within pinocytotic vesicles in smooth muscle cells (Figs. 9-12). The antigen is never present in vesicles close to the endothelial aspect of the smooth muscle cell but is present within vesicles far enough from the cell surface to exclude the possibility of all of its being extracellular and artifactually appearing intracellularly by tangential sectioning. Obviously, the presence of the antigen within intracellular vesicles can be an indication of either synthesis or catabolism. The lack of demonstrable antigen within the rough endoplasmic reticulum (15, 24) or Golgi complex would suggest catabolism rather than synthesis.

Our findings in the glomerular and tubular basement membranes are not in agreement with those of Roll et al. (29). These authors found a complete co-distribution of collagen types IV and V in the glomerular and tubular basement membranes. The reasons for this discrepancy are not completely clear. However, the differences may be due to the dissimilar techniques: ultrathin frozen sections and ferritin labeling vs perfusion fixation and peroxidase labeling. Also, the methods of purification of the antigen by Roll et al. (29) were less complete, in that the antigen was obtained by salt precipitation without subsequent ion-exchange chromatography on DEAE cellulose (11) followed by CM cellulose (31) to remove noncollagenous contaminants. Furthermore, it is clear that our antibody localization is more selective than that of Roll et al. (29).

In summary, this study provides a picture of type V collagen as free particles (often granular, occasionally fibrillar) widely distributed in the renal interstitium. Its presence adjacent to some interstitial collagen fibers, and on the stromal aspect of vascular basement membranes, suggests a binding function for this class of collagen molecules. Our findings are not compatible with type V collagen's being an integral component of
either tubular or glomerular basement membranes.

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