Immunological Properties of Type IV Collagen from Mouse Kidney

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Some immunological properties of mouse kidney type IV collagen (MKIVC) were investigated. In enzyme-linked immunosorbent assay (ELISA), rabbit anti-MKIVC antiserum reacted strongly with MKIVC but not at all with type I collagen or laminin of mouse origin. Thus, the purified MKIVC preparation was not contaminated with detectable amounts of other constituents of the extracellular matrix and the basement membrane including interstitial collagens, as suggested previously (T. Oikawa *et al.*, *Chem. Pharm. Bull.*, 34, 789 (1986)). In ELISA inhibition assay, human kidney type IV collagen (HKIVC) only partially inhibited the binding of MKIVC to anti-MKIVC antiserum. Similarly, HKIVC showed a far weaker binding capacity to the antiserum compared to MKIVC in ELISA direct binding assay. After the anti-MKIVC antiserum was absorbed with acetone powder of human kidney, the resulting antiserum still possessed a considerable binding activity to MKIVC but did not react with HKIVC. In accord with these findings, anti-MKIVC antiserum immunostained both mouse and human renal basement membranes, while it stained the former but not the latter after being absorbed with human kidney. Taken together, these results suggest that MKIVC possesses at least two antigenic determinants; one but not the other cross-reacts with human kidney basement membrane collagen.

Keywords type IV collagen; enzyme-linked immunosorbent assay; species specificity; mouse kidney; human kidney

Type IV collagen exists exclusively in basement membranes which underlie epithelial and endothelial cells and regulate the interaction between epithelium and mesenchyme. The collagenous protein exhibits similar physicochemical properties including amino acid composition irrespective of its sources. Most if not all information on the molecular structure of the collagen and its organization in the basement membrane is derived from studies on type IV collagen from mouse EHS chondrosarcoma.[1] Recent studies have suggested the presence of two novel chains in basement membrane collagen in addition to the established collagen chains.[2–4]

Immunochemical studies on type IV collagen have been performed with polyclonal and/or monoclonal antibodies against collagen prepared from different species and tissues. Polyclonal antibodies reported so far include those against type IV collagen from EHS sarcoma,[5–7] human placenta,[6,8] human kidney,[9] bovine kidney,[9,10] and porcine kidney.[11] Monoclonal antibodies have also been produced against collagen from human placenta,[12–17] human chorioamniotic membrane[18] and human kidney.[19] Studies with these antibodies have shown that type IV collagen has no tissue-specificity, except for the basement membranes of the corneal epithelium and endothelium, or the posterior lens capsule.[19] These studies also provided different results concerning the immunological species specificity of type IV collagen. Namely, some antibodies against type IV collagen cross-reacted with the collagen from different species,[6,11,12,14,19] while some others did not.[9,10,13,16,18,19]

We recently reported that the antiserum against mouse kidney type IV collagen (MKIVC) reacted immunohistochemically with basement membranes of mouse and human kidneys, indicating that there are certain antigenic determinants shared by both species.[20] This paper describes the immunochemical characterization of MKIVC by enzyme-linked immunosorbent assay (ELISA). The present results suggest that MKIVC has at least two antigenic determinants; one shows no cross-reactivity with human kidney type IV collagen (HKIVC) and the other is shared by MKIVC and HKIVC.

Experimental

Chemicals Affinity-purified laminin from mouse EHS chondrosarcoma was obtained from E-Y Lab., microtiter plates (Plate H for ELISA) from Sumitomo Bakelite Co. and affinity-purified goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase from Bio-Rad.

Preparation of Type IV Collagen and Its Antiserum MKIVC was purified and used to raise antiserum in rabbits as described previously.[20] HKIVC was isolated by the method used to purify MKIVC as described previously.[21,22] Type I collagen was purified from mouse tail tendon by the method of Miller and Rhodes.[23]

ELISA Titration Assay Wells of microtiter plates were coated with 0.2 ml of various concentrations of MKIVC in 50 mM Tris–HCl buffer, pH 7.4 containing 0.9% NaCl and 0.02% NaN_3 for 16 h at 4 °C. The wells were washed three times with a mixture of 0.9% NaCl and 0.05% Tween 20 (solution A), aliquots (0.2 ml) of 5% bovine serum albumin (BSA) in 10 mM Tris–HCl buffer, pH 7.4 prepared with solution A (solution B) were added, and the plates were allowed to stand for 16 h at 4 °C. The wells were washed with solution A as described above, then various dilutions (0.2 ml) of anti-MKIVC antiserum in solution B containing 1% BSA were added and the plates were incubated for 3 h at 25 °C. The wells were washed with solution A, and aliquots (0.2 ml) of goat anti-rabbit IgG labelled with horseradish peroxidase (1 : 1000 dilution) were added. Following a 1-h incubation at 37 °C, the wells were washed and enzyme substrate solution (0.2 ml), composed of 10 mg of *o*-phenylenediamine and 0.03% H_2O_2 in 100 ml of distilled water, was added. After incubation for the indicated times, the enzymatic reaction was stopped by adding 0.05 ml of 2 M H_2SO_4. The enzymatic product was measured with a MICROELISA AUTO-READER MR 580 (Dynatech Lab.; Alexandria, U.S.A.) equipped with a 490-nm filter. Control experiments were performed in wells not coated with the antigen. All points indicated are the averages of triplicate values.

ELISA Inhibition Assay Aliquots of anti-MKIVC antiserum (1 : 1000 dilution) were preincubated at 4 °C for 16 h with an equal volume of various inhibitors dissolved in solution B containing 1% BSA. The mixture (0.2 ml) was added to the wells of the microtiter plates coated with 1 μg/ml of MKIVC and incubated at 25 °C for 15 to 60 min. The wells were washed 3 times with solution A, then treated in the manner described for the ELISA titration assay mentioned above.

ELISA Direct Binding Assay The wells coated with various concentrations of HKIVC were incubated with the anti-MKIVC antiserum previously unabsorbed or absorbed with acetone powder of human kidney (see below) as described above. Subsequently, the wells were processed as described for the ELISA titration assay.

Immunohistology Indirect immunofluorescence studies with frozen sections (5-μm thickness) of kidney tissues were performed as described previously.[20] Absorption of anti-MKIVC antiserum with acetone powder of human kidney was performed as follows. The antiserum was diluted with 3 volumes of phosphate-buffered saline, then 100 mg of the acetone powder per ml of the diluted antiserum was added, and the mixture was incubated at 37 °C with occasional shaking. After a 1-h incubation, the mixture was centrifuged at 20000 × *g* for 30 min at 4 °C. The supernatant obtained was further absorbed one or more times with 50 mg or less of the acetone powder per ml as mentioned above.

Quantitation of Type IV Collagen by ELISA Lyophilized mouse kidneys (5 mg/ml) were homogenized in 0.5 M acetic acid and treated with various concentrations of pepsin (2500 unit/mg; Boehringer) in a total volume of 25 ml at 4 °C. Aliquots of the reaction mixture were collected at the indicated times and ultracentrifuged at 160000 × *g* for 30 min at 4 °C. The solubilized type IV collagen was determined by using the ELISA inhibition assay system.

Protein Determination Protein contents of standard mouse and human collagens were determined on a dry weight basis after dialysis against 0.1 M acetic acid by the method of Yurchenco and Furthmayr.[24] Laminin was dissolved as described in the product information for use.

Results

ELISA Assays ELISA titration curves of anti-MKIVC antiserum are shown in Fig. 1. Similar titration curves were obtained using wells coated with 1 μg/ml or more antigen. Subsequent ELISA assays were, therefore, performed in wells coated with 1 μg/ml of type IV collagen. The 50% titer of the antiserum was approximately 1:3000 under the present conditions.

As shown in Fig. 2, MKIVC entirely inhibited the reactivity of anti-MKIVC antiserum with the MKIVC coating the wells, whereas neither laminin nor type I collagen from mice had any influence on it. In contrast, the reactivity was partially inhibited by HKIVC used as an inhibitor (Fig. 3). The concentrations causing the half-maximum inhibition were 0.08 and 100 μg/ml for MKIVC and HKIVC, respectively.

To clarify further the immunological relationship between MKIVC and HKIVC, ELISA direct binding assays were performed. Reactivity of anti-MKIVC antiserum with HKIVC was dependent on the concentrations of both the antiserum and the collagens used for coating. HKIVC showed a far lower affinity to the antiserum than MKIVC (Fig. 4).

Absorption of anti-MKIVC antiserum with human kidney tissue resulted in complete abrogation of its reactivity with HKIVC (Fig. 5). In contrast, the absorbed antiserum still maintained a considerable reactivity with MKIVC.

Immunohistology In immunofluorescence staining, anti-MKIVC antiserum exhibited reactivity with both mouse and human kidney basement membranes (Fig. 6A and B) in accord with our previous observation.[20] Treatment of the

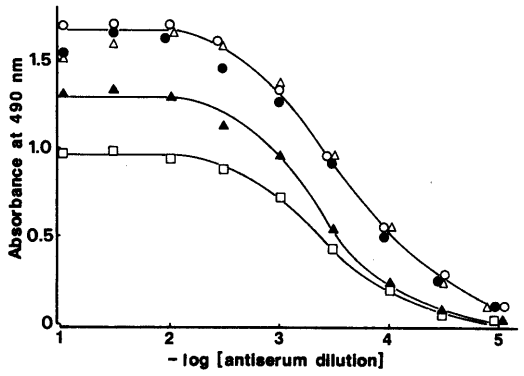

Fig. 1. ELISA Titration Assay with Anti-MKIVC Antiserum

The wells were coated with various concentrations (μg/ml) of MKIVC: ○, 10; ●, 3; △, 1; ▲, 0.3; □, 0.1.

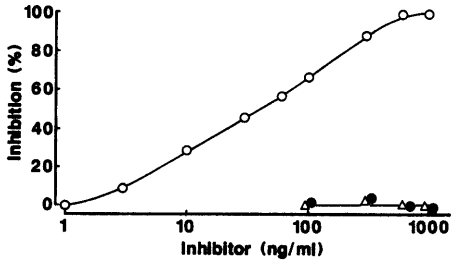

Fig. 2. ELISA Inhibition of Binding of Anti-MKIVC Antiserum to MKIVC

Inhibitors: ○, MKIVC; ●, laminin; △, type I collagen from mouse tail tendon.

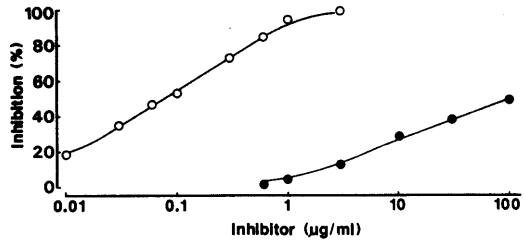

Fig. 3. ELISA Inhibition of Reactivity of Anti-MKIVC Antiserum with MKIVC

Inhibitors: ○, MKIVC; ●, HKIVC.

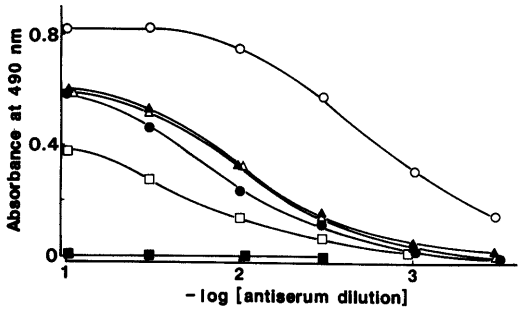

Fig. 4. ELISA Direct Binding Assay of HKIVC with Anti-MKIVC Antiserum

The wells were coated with HKIVC (▲, 10; △, 3; ●, 1; □, 0.3 μg/ml), MKIVC (○, 1 μg/ml) or type I collagen from mouse tail tendon (■, 10 μg/ml).

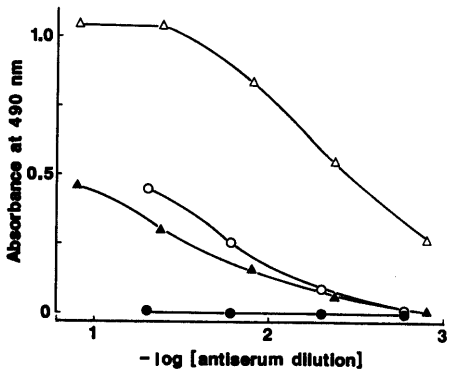

Fig. 5. ELISA Direct Binding Assay of MKIVC (△, ○) and HKIVC (▲, ●) with Anti-MKIVC Antiserum Absorbed (○, ●) or Unabsorbed (△, ▲) with Acetone Powder of Human Kidney

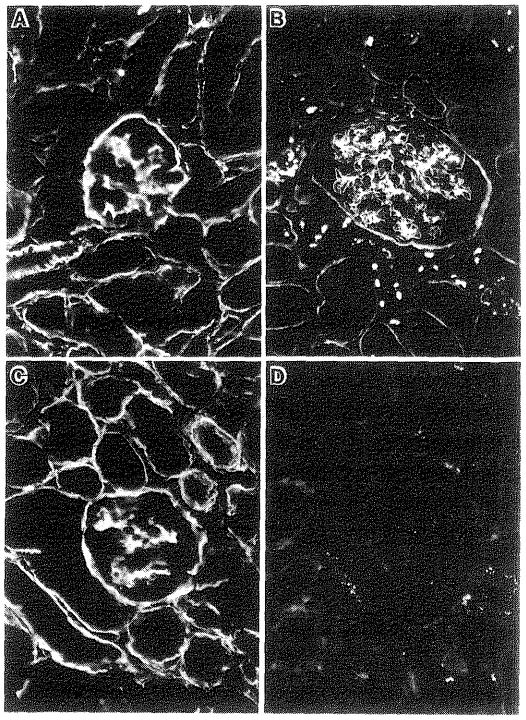

Fig. 6. **Indirect Immunofluorescence Staining of Mouse (A and C) and Human (B and D) Kidneys**

The tissue sections were incubated with anti-MKIVC antiserum unabsorbed (A and B) or absorbed (C and D) with human kidney tissue; magnification, ×400 (A and C) or ×200 (B and D).

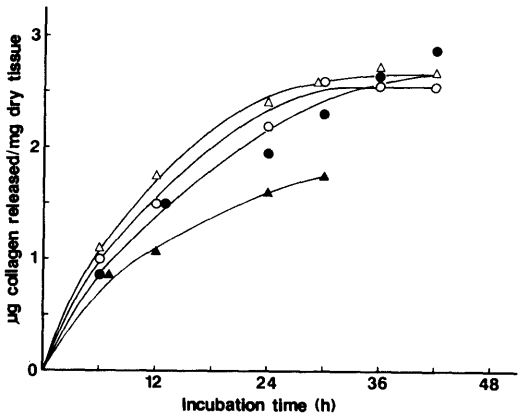

Fig. 7. Time Course of Solubilization of Type IV Collagen from Mouse Kidney Tissue by Pepsin

The lyophilized mouse kidney tissues were solubilized with various doses of pepsin. The weight ratio of pepsin to kidney: ○, 1:5; ●, 1:10; △, 1:20; ▲, 1:40.

antiserum with human kidney powder had little or no effect on the intensity of immunofluorescence staining of mouse kidney basement membranes (Fig. 6C) but completely cleared the specific fluorescence from human kidney basement membranes (Fig. 6D).

Type IV Collagen Quantitation Unlike interstitial collagens, type IV collagen is not extractable with dilute acid alone.[23] Therefore, solubilization of the collagen with proteolytic enzymes such as pepsin was a prerequisite for quantitation of type IV collagen in tissues. Mouse kidneys, used as a standard tissue, were treated with various amounts of pepsin and the solubilized type IV collagen was determined by the ELISA inhibition assay mentioned above. As shown in Fig. 7, when the weight ratio of pepsin to kidney was 1:20 or higher, solubilization of type IV collagen reached a plateau at 30-h incubation; longer incubation did not cause further solubilization of the collagen. Thus, the combination of ELISA inhibition assay and solubilization of collagens with pepsin may enable quantitation of structural type IV collagen in tissues.

Discussion

Immunochemical tissue-specificity and species-specificity of type IV collagens have been studied with different antibodies, polyclonal and monoclonal. Polyclonal antibodies against bovine kidney type IV collagen did not cross-react with basement membranes of the human kidney.[9,10] Monoclonal antibodies to human chorioamniotic membranes,[18] placenta[13,16] and kidney[19] type IV collagens showed no cross-reactivity with basement membrane collagens from other species. On the other hand, several antibodies, monoclonal and polyclonal, against type IV collagens from EHS sarcoma,[6] human placenta[6,12,14] and human,[19] porcine[11] and mouse[20] kidneys have been reported to show an interspecies reactivity.

The antibody against MKIVC exhibited a strong binding to MKIVC but did not react with other extracellular matrix components such as type I collagen and laminin (Figs. 1 and 2), suggesting that our MKIVC preparation used as an immunogen contained no other interstitial and basement membrane constituents. This view is supported by the complete digestion of MKIVC with bacterial collagenase.[20]

We demonstrated the presence of a common antigenic determinant between mouse and human kidney basement membrane collagens by immunohistology.[20] This was confirmed by ELISA in this study; the anti-MKIVC antiserum showed distinct cross-reactivity with HKIVC that was completely eliminated by absorption with human kidney (Figs. 3 and 4). In addition, the absorbed antiserum still exhibited a considerable reactivity with MKIVC but did not react at all with HKIVC in ELISA direct binding assay (Fig. 5). The results were supported by immunofluorescence staining (Fig. 6). Therefore, this study clearly points to the existence of at least two distinct antigenic determinants in MKIVC, one but not the other being cross-reactive with HKIVC. Risteli *et al.* also demonstrated such antigenic determinants in type IV collagen from EHS sarcoma.[25]

Type IV collagen is one of the major constituents of basement membranes and has been shown to undergo functional and structural alterations in various disorders.[26,27] More recently, Dulbecco *et al.*[28] analyzed immunohistochemically the behavior of basal lamina components including type IV collagen using rat mammary carcinomas as a model system for elucidating the mechanism of tumor progression. They suggested that the progressive decrease of these components in basal lamina was due to the progressive alteration of their gene expression rather than to their enzymatic degradation. The assay method of type IV collagen in tissues by ELISA established in the present study (Fig. 7) may contribute to a further understanding of the role of basement membrane collagen in tumor progression. A study along this line is in progress using a transplantable pregnancy-dependent mouse mammary tumor line and its autonomous sublines established in DDD mice[29] as a model.

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