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Purification and localization of Tubulointerstitial Nephritis antigen (TIN-ag) in kidney and small intestine

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Tubulointerstitial Nephritis antigen (TIN-ag) is a novel recently described basement membrane component. It is the target of tubular basement membrane autoantibodies, which have been developed in patients with Tubular Interstitial Nephritis. In our study, TIN-ag was extracted and purified from rabbit kidney cortex basement membranes, by collagenase digestion and detected by Western blotting. The indirect avidin-biotin method and the immunogold and silver enhancement method were performed on thin and semi-thin sections from rabbit and human kidney cortices and small intestines, using the monoclonal antibody A8 and the polyclonal antibody Ab95. TIN-ag was detected on the tubular basement membrane (especially in proximal tubules), the parietal layer of Bowman's capsule (especially at the urinary pole) and on the basement membrane of the villi of the duodenum, jejunum and ileum and the crypts of Lieberkuhn as well. It was not detected on the glomerular basement membrane. Because of the localization of TIN-ag on the basement membranes under epithelial cells which are involved in active and extensive transport of many metabolites, it is tempting to speculate that TIN-ag has a specific role in the homeostasis of the body. Further molecular analysis will reveal the antigenic epitope, which is responsible for the development of the circulated autoantobodies in the blood of patients with tubulointerstitial nephritis.

Key words: Tubulointerstitial nephritis antigen, purification, immunohistochemical detection, photonic microscopy, electron microscopy.

INTRODUCTION

The Tubulointerstitial Nephritis antigen (TIN-ag) has been identified as a novel basement membrane macromolecule with limited tissue distribution (Yoshida *et al*., 1990, Nelson *et al*., 1998). It has been described as a 58-kDa basement membrane component recognized by human autoantibodies which circulate in the blood of patients with Tubulointerstitial Nephritis (TIN). It is responsible for the cause of acute renal failure and chronic renal damage (Yoshioka *et al*., 1986, Fliger *et al*., 1987, Miyazato *et al*., 1992, Nelson *et al*., 1997). Tubulointerstitial Nephritis is characterized by linear deposition of IgG and complement C3 along the renal Tubular Basement Membrane (TBM) (Butkowski *et al*., 1990, Crary *et al*., 1993). Anti-TBM antibodies are then isolated from the serum or renal eluates of the patients. Antibodies to TBM have also been identified in various types of glomerular nephropathies, recipients of renal allograft, uveitis and celiac desease (Burnier *et al*., 1986, Cacoub *et al*., 1989, Salu *et al*., 1990, Katz *et al*., 1997, Oda *et al*., 1998, Ivanyi *et al*., 1998). TIN coexists very rarely with membranous nephropathy and circulating anti-TBM antibodies, but in these cases the GFR and the clearance of creatinin have proved rather tubular lesions than glomerular ones (Ivanyi *et al*., 1998). Immunohistochemical studies on sections from normal human kidney using sera from TIN patients showed IgG binding of autoantibodies to the proximal TBM and Bowman's capsule, but not to the Glomerular Base-

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ment Membrane (GBM). Also the patients' serum reacts with the jejunal basement membranes. Animal models for TIN, based on immunization with heterologous TBM components have developed the disease (Wilson, 1991). Investigations on the molecular origin of TBM components, which react with the serum from patients with tubulointerstitial nephritis, have revealed various nephritogenic tubulointerstitial antigens with molecular weights ranging from 30 kDa to 70 kDa (Clayman *et al*., 1986, Nielson *et al*., 1991).

TIN may be either acute or chronic. Acute TIN is characterized by the presence of interstitial edema, interstitial leukocyte infiltration and focal tubular necrosis. Chronic TIN is characterized by mononuclear infiltration in the interstitium, focal interstitium fibrosis and extensive tubular atrophy. Deposition of autoantibodies and the C3 complement on the tubular basement membranes may modify the function of the tubules and finally leads to the last stage of renal disease (Wilson, 1991). Immunohistochemical studies have suggested that patients with nephronophthisis, a hereditary progressive tubulointerstitial disorder, had a defect in the molecule of TIN-ag (Cohen & Hoyer, 1986), which was analogous to type IV collagen in patients with Alport syndrome (Yoshioka *et al*., 1994). The molecular analysis of TIN-ag and the unique pattern of its limited distribution, indicate that this is a new molecule involving in specific functions of the basement membranes. During kidney embryogenesis, TIN-ag follows the expression of other structural extracellular matrix proteins, such as laminin and type IV collagen and leads to the conclusion that TIN-ag is not only a structural protein of the kidney, but it also involves in functional activities. Further research will clarify the specific role of this unique molecule in the pathology of the basement membrane-associated diseases of the kidneys.

In the present study TIN-ag was isolated and purified from rabbit kidney cortices and was detected immunohistochemicaly by photonic and electron microscopy in rabbit and human kidneys and intestines. The distribution of the molecule in the basement membranes was also ultrastructurally studied with specific and accurate methods such as colloidal gold and silver enhancement.

MATERIALS AND METHODS

TIN-ag Isolation

In this study TIN-ag was isolated from kidneys of New Zeland rabbits. The animals were anesthetized with a sodium phenobarbital injection and their kidneys were excised and immersed in 0.9% normal saline. In the saline, the cortices were removed from the kidneys in a thickness of 200-300 μm and collected in an inhibitor solution of 10 mM tris-HCl, 0.15 NaCl, (pH 7.4, 4°C) containing protease inhibitors (1 mM EDTA, 2 mM e- ACA and 2 mM NEM). The cortices were disrupted for 30 seconds with a Polytron in the presence of protease inhibitors. Disrupted tissues were passed through a #35 stainless steel sieve and the eluate was collected in a #250 sieve. The product was centrifuged for 10 min at 1500 × g and the pellet was washed by resuspending in 1M NaCl containing protease inhibitors. At this point the product contained a mixture of TBM and GBM (as confirmed by microscope observation) and then it was washed with 10 mM Tris, 1 M NaCl and inhibitors, centrifuged for 10 min at 2800 g and the pellet was resuspended in 50 mM Tris–HCl, 0.2 M NaCl, 2 mM $CaCl_2$, (pH 7.4). The product which mostly contained basement membranes was incubated for 16 hours at 37°C with collagenase (from *Clostridium histoliticum*, SIGMA C7926) 1 mg/10 ml of wet tissue and was brought to a volume of 40-100 ml with 50 mM Tris- HCl, 0.2 M NaCl and 2.0 mM $CaCl_2$, (pH 7.4). Following collagenase digestion, the sample was dialyzed against 2.0 M urea, 50 mM Tris-HCl, 0.2 M NaCl, 1.0 $CaCl_2$ (pH 7.4) and after centrifugation (10000 rpm for 10 min) and concentration it was applied to a Sephacryl S 300 gel filtration column (2.5 × 85 cm). The fraction containing TIN-ag was pooled, concentrated and dialyzed against 2.0 M urea, 50 mM Tris-HCl, 1.0 mM $CaCl_2$ (pH 6.8) and then further purified by cation – exchange chromatography on a 25 ml- S Sepharose column equilibrated in the same buffer. This column was eluated first with 0.1 M NaCl and then with 0.7 M NaCl in the column buffer. The purified TIN-ag in the 0.7 NaCl elution of the column was aliquoted and stored at –80°C. Purification steps were monitored by SDS PAGE analysis.

Samples from the aliquots were subjected to SDS-polyacrylamide gel electrophoresis in 10% polyacrylamide gel under reducing conditions. The protein was electrophoretically transferred (0.2 A for 2h) from the polyacrylamide gel to a nylon membrane, as described by Towbin *et al*., (1979) and the unoccupied sites on the nitrocellulose were blocked by incubating overnight at 4.0°C with PBS containing 3% skim milk. The blocked membrane was incubated for 1h at room temperature with the TIN-ag

monoclonal antibody (A80) diluted to 1:50. The membrane was washed with PBS and incubated for 30 min at room temperature with horseradish peroxidase-conjugated anti-mouse IgG antibody (SIGMA). After washing of the membranes with PBS, the bound secondary antibody was detected by enhanced chemiluminescence (Amersham Cor RPN 2106 kit). The MW of the markers, which were used were 200, 97.4, 68, 46, 31 and 20.1 kDa. (ECL Protein Mol Weight Markers, Amersham RPN 2107 kit).

Immunocytochemistry

Rabbit kidneys and small intestines were harvested and prepared for study with photonic and transmission microscopes. For the photonic microscope the tissues were fixed with 10% neutral formalin and embedded in paraffin. The avidin-biotin method was applied on 4-5 μm thick sections. The primary antibody was the mouse monoclonal anti TIN-ag (A8) at 1:100 dilution or the rabbit polyclonal (Ab95) at 1:2000. The detection kit was the EXTRA-2 (SIGMA Mouse extravidine peroxidase staining kit).

The tissues for electron microscopy were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in PBS buffer and embedded directly without dehydration in LR-White resin (London Resin Company). Colloidal gold particles 5nm in diameter were used for the Silver Enhancement Method. The silver Enhancement Method (Silver Enhancing Kit for Light and Electron Microscopy, Polysciences Inc.) was applied to semithin sections (0.5-1 μm in thickness).

Semithin sections were incubated overnight with blocking buffer TBS containing 1% BSA. The next day, the slides were incubated at room temperature with a normal goat serum at 1/5 dilution for 20 minutes and the sections without rinsing were incubated with the primary mouse monoclonal anti TIN-ag antibody (A8) for 2 h in a dilution of 1/200. The slides were washed with TBS plus 0.25% BSA and the sections were incubated with the secondary antibody which was the anti mouse IgG conjugated with 5 nm gold particles for 30 minutes in dilution of 1/50. After washing the slides with TBS plus 0.25% BSA, they were washed again thoroughly with deionized water. The slides were then incubated for 20 minutes in room temperature, (low illumination) with a mixture of 50% enhancer and 50% initiator (Polysciences Inc.) and observed with the light microscope. A color change was observed due to the reduction of silver in the solution. The specifically labeled sites were changed from invisible to light brown, then to dark brown and finally to deep black. In the case of weak staining, the procedure was continued with a second application of a fresh mixture of enhancer and initiator. A negative control slide by omitting the primary antibody was proceeded.

The immunogold method was applied to thin sections (100-200 nm), by using 10nm diameter immunogold particles which were conjugated to mouse IgG (SIGMA), if the mouse monoclonal (A8) was the primary antibody. The copper grids with the thin sections were incubated overnight at 4°C with TBS containing 1% BSA. The next day, the grids were incubated at room temperature for 2 h with the A8 antibody diluted to 1/100 in TBS plus 1% BSA. The grids were then washed 6 times for a total of 5 min with TBS plus 0.25% BSA and after that they were incubated for 5 min with TBS plus 1% BSA. The grids were covered for 2 h with a drop of the secondary antibody which was the anti-mouse IgG conjugated with gold particles 10 nm in diameter at a dilution of 1/5. The grids were washed 3 times with TBS plus 0.25% BSA, additionally 4 times with TBS plus 1% BSA and finally 4 times with distilled water. The grids ultimately were stained with 2% uranyl acetate for 5 min and lead citrate for 1 min, washed 4 times, with distilled water and observed with a JEOL 200 CX electron microscope. Grids incubated only with the secondary antibody were used as controls.

RESULTS

Western Blotting

The purified TIN-ag extracted from rabbit kidney cortices was detected with Western blotting. As shown in Fig. 1, under reducing conditions, a prominent immunoreactivity was observed at a molecular weight of 58 kDa using the monoclonal antibody A8. No other bands were detected. The reaction was specific and when the primary antibody was omitted, no reaction was observed.

Immunocytochemistry

Binding of A8 (monoclonal antibody to TIN-ag) and Ab95 (polyclonal antibody to TIN-ag) to kidney sections was evaluated by extravidin-peroxidase and immunogold methods. In Figs 2, 3 all TBMs in the renal cortex were stained positively, although the intensity was strong in proximal TBMs and weak in dis-

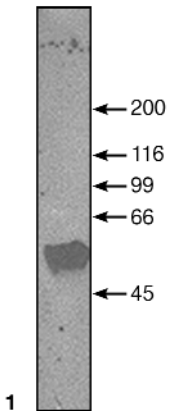

FIG. 1. Isolated rabbit TIN-ag extracted from rabbit kidney cortices and examined by Western blotting. Samples were electroforessed under reducing conditions through 10% SDS gel. One prominently stained band appears at a molecular weight of 58 kDa.

tal TBMs. The staining of the Bowman capsule varied depending on the location. Staining intensity was the greatest at the urinary pole, and it gradually decreased toward the vascular pole where it was minimal. No reactivity was detected on the Glomerular Basement Membranes. In Fig. 4 kidney medulla was slightly positive and immunoreactivity was detected on the collective tubules basement membranes. In Fig. 5, 5 mm immunogold particles conjugated to the secondary antibody and accumulated on the same areas, were visualized under light microscope. Particles

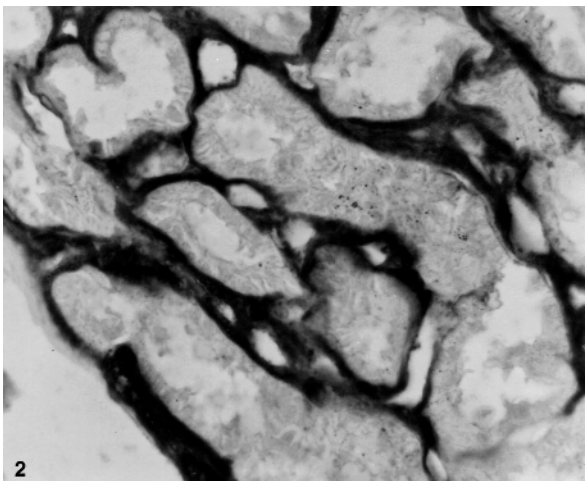

FIG. 2. Positive staining of the basement membrane of the convoluted tubules and interstitium with A8 antibody and avidin - biotin method. ×400.

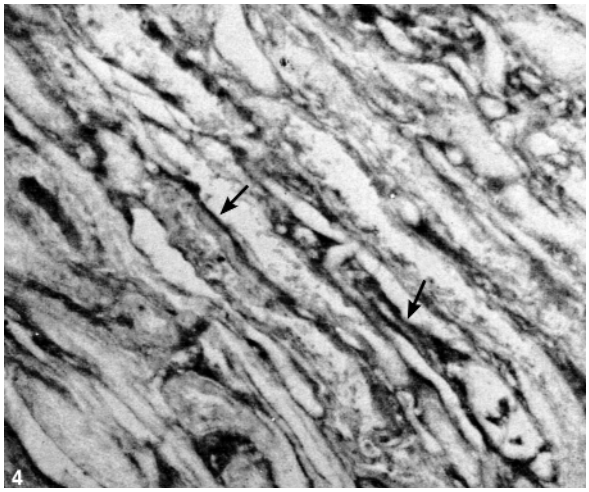

FIG. 4. Slight reaction of A8 and Ab95 at the collective tubules (arrows). ×100.

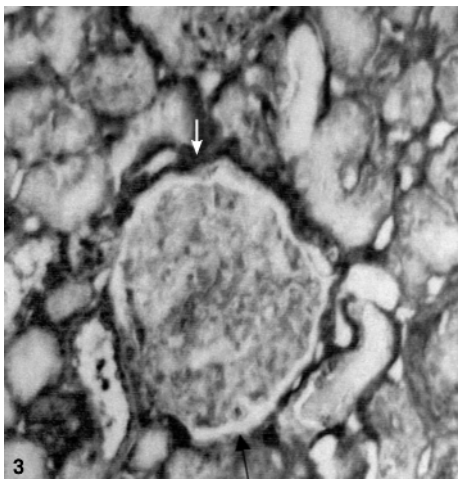

FIG. 3. Positive staining of the basement membrane of the tubules and the Bowman's capsule mostly on the urinary pole (white arrow). Staining intensity decreases toward the vascular pole. The vascular pole is almost negative (black arrow). ×100.

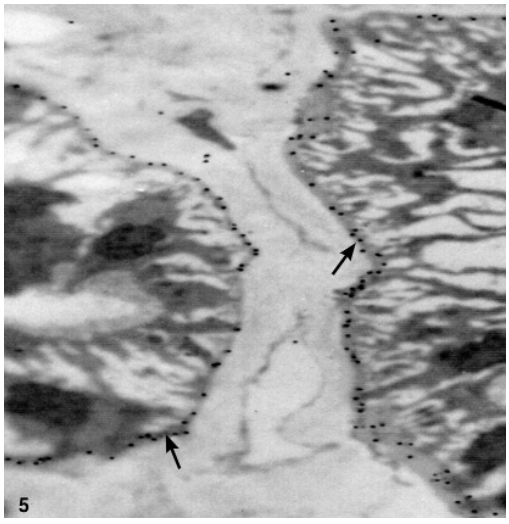

FIG. 5. Immunogold particles on the tubular basement membrane of two proximal convoluted tubules (arrows). Few particles occur on the interstitium. Silver Enhansement method. ×1000.

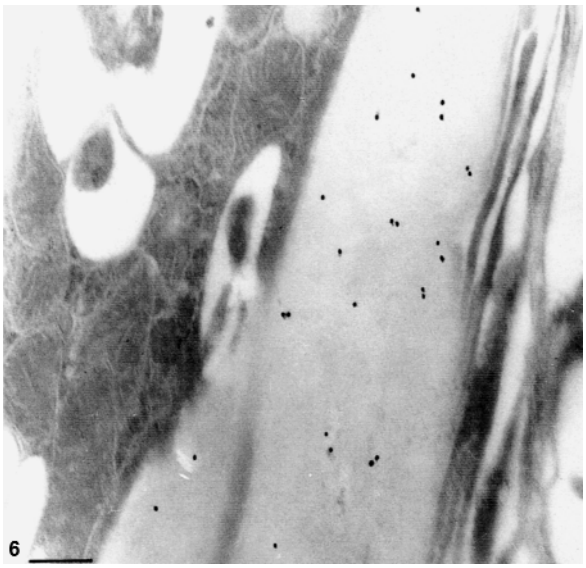

FIG. 6. Immunogold particles on the proximal tubule basement membrane. Immunogold particles are distributed throughout the lamina lucida and lamina densa. Immunogold method. Bar: 500 nm.

were clearly detected on the TBMs and rarely on the interstitium and the peritubular capillary basement membranes. No particles were detected inside the glomerulus. The distribution of the 5 nm particles was the same with the extravidin peroxidase complex, but the presence of the particles reveals that the basement membranes and not the interstitium occur the natural localization sites of TIN-ag. In Fig. 6, 10 nm particles conjugated to the secondary antibody were detected with the electron microscope. These particles had the same pattern of distribution as the 5 nm ones and the extravidin peroxidase complex, but they recognized the exact position of TIN-ag molecules through the layers of the Tubular Basement Membranes. There was no special pattern of distribution of TIN-ag. The latter was distributed throughout the basement membrane, with no difference in the lamina lucida and lamina densa. The use of A8 and Ab95 antibodies showed the same distribution pattern with TIN-ag.

By using the same methods, was also tested the binding of the above antibodies to the intestine sections.

In Figs 7, 8 by using both A8 and Ab95 antibodies, TIN–ag was localized on the basement membrane of the columnar epithelium and the central lacteal of the villi of the small intestine with the same intensity. The basement membrane of the Lieberkuhn's glands shown only focal and weak staining. TIN-ag was detected with the same intensity along the basement membranes of duodenum, jejunum and ileum. The intensity of staining in the intestine was certainly weaker than that of the kidney tubules and it was similar to that of the distal convoluted tubules. Also, the stained area along the basement membranes of the intestine was 2 fold thinner compared to that of the corresponding area across the

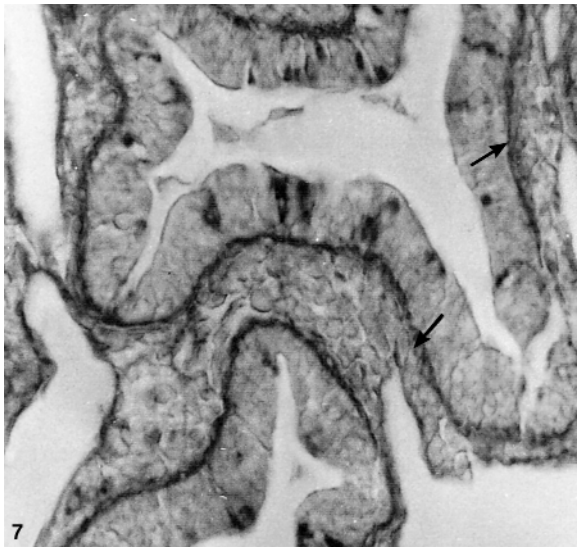

FIG. 7. Positive staining with Ab95 of the villi basement membrane of jejunum. The intensity of staining of this membrane is weaker and the thickness of the stained area is smaller, compared to the tubular basement membrane (arrows). Avidin-biotin method. ×400.

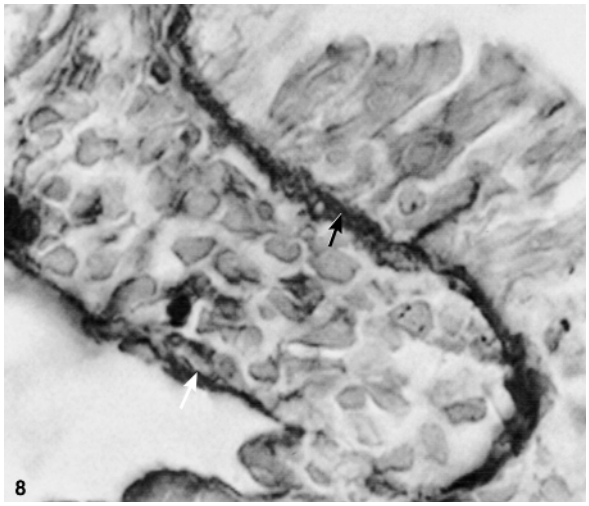

FIG. 8. Positive staining with Ab95 of the columnar epithelium basement membrane (black arrow) and the central lacteal basement membrane (white arrow) of ileum. Avidin- biotin method. ×1000.

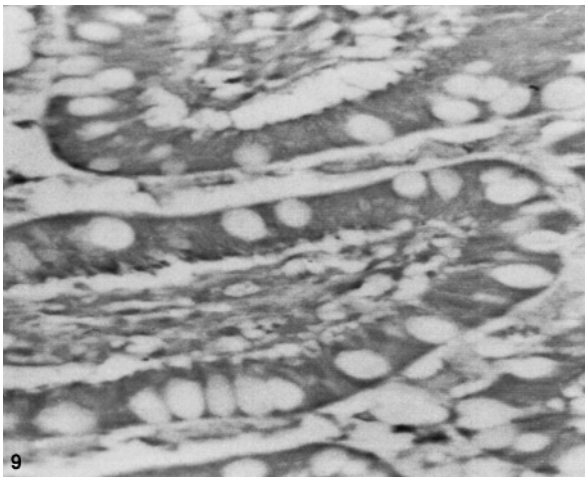

FIG. 9. Negative staining of ileum control section, by omitting the primary antibody. ×400.

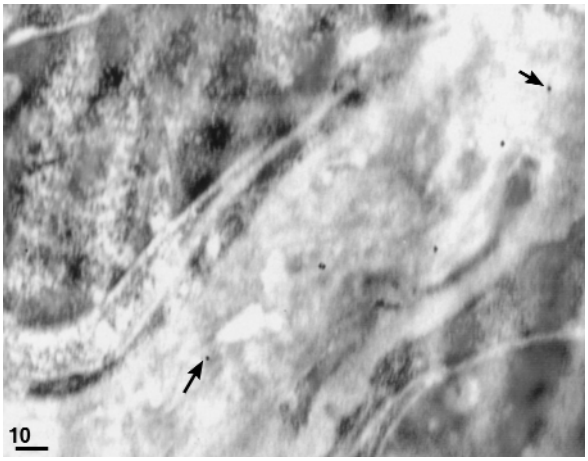

FIG. 10. Electron micrograph of ileum. A few immunogold particles are deposited on the basement membrane (arrows). Bar: 500 nm.

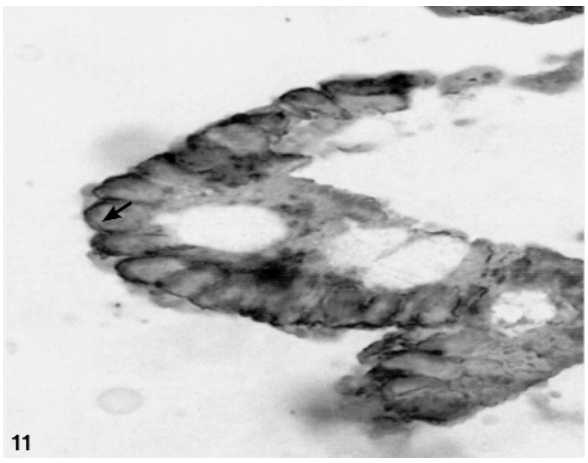

FIG. 11. Positive staining of the duodenum basement membrane (arrow). The section was taken from a biopsy of a patient examined for chronic duodenal ulcer. Avidin-biotin method. ×400.

Tubule Basement Membranes. In Fig. 9 control section by omitting the primary antibody was also negative. In Fig. 10 a few immunogold particles were detected on the basement membrane and lamina propria, fewer than that of the TBMs. The staining with Ab97 was more intense compared to that with A8, which was very weak. TIN-ag was not detected in the large intestine that appeared absolutely negative. In Fig. 11, TIN-ag was also identified in the basement membrane of duodenum with both antibodies from biopsies of patients undergoing checking for chronic gastritis.

DISCUSSION

TIN-ag was extracted from rabbit kidney TBMs at a molecular weight of 58 kDa and was immunodetected by Western blotting using the A8 monoclonal antibody as one very wide and intense band corresponding to a 58 kDa molecular weight.

More precisely immunostaining results showed that TBMs and villi basement membranes were the areas mostly associated with TIN-ag. The fact that the proximal TBMs and the Bowman's capsule were intensely reactive, whereas the distal TBMs appeared less reactive, suggests variability in tissue concentration of TIN antigen in the same organ. The phenomenon was also observed in the intestine where small intestine was intensely stained, whereas the large was totally negative. Thus a selective distribution of TIN-ag exists in both kidney and small intestine. The intensity of staining in TBMs was higher than that in intestine and so was the width of the stained area across the membranes. Although immunocytochemistry is not a quantitative method, we attempted to compare with each other the partial results of staining, because the experiments were contacted in parallel under the same conditions. As concerns immunocytochemistry, the total amount of TIN-ag in kidney was much higher than that in intestine. The distribution of TIN-ag in kidney showed a gradual decrease from the cortex to the medulla. The staining of the glomerulus and the capsule of Bowman at the vascular pole was negative, but toward to the urinary pole it was positive. This can be explained by the fact that TIN-ag selectively regulates tubulogenesis and has no effect on glomerulogenesis during renal development (Kumar *et al*., 1997, Kanwar *et al*., 2001). The proximal tubules showed the highest intensity, which was gradually decreased to the collective tubules. The intensity of staining through the small intestine basement mem-

branes was the same and only the Lieberkuhn's glands showed a weaker reaction. The presence of immunogold particles mostly on the basement membranes and their limited distribution on the interstitium reveal that TIN-ag is a basement membrane component, which participates in the basement membrane functions. The thickness of staining across the tubule basement membranes, as it was detected with the avidin-viotin method, is in contradiction with the distribution of immunogold particles across the basement membranes, which obviously is much more thinner. The high amount of TIN-ag in TBMs, the diffusion of the extarvidin-peroxidase complex, the size of the avidin-biotin peroxidase complex, and the over 100 nm resolution of the microscope, are facts that give the impression that TIN-ag is distributed both to the basement membrane and the interstitium. The small size and electron opacity of the gold particles are two important factors, which permit the exact localization of TIN-ag. Immunogold particles occupy the total thickness of the basement membranes, and thus there is not a specific pattern of TIN-ag distribution through the basement membranes. Very few epithelial cells of the tubules and the columnar epithelium of the villi showed reactivity, evaluated as nonspecific. According to Butkowski *et al*. (1991) and our unpublished data, kidney cortex first and villi secondly are the two positions with the highest distribution of TIN-ag. However other organs with limited distribution, such as skin and eye, had also been detected. It is important to note that TIN-ag was not detected in lungs, liver, gall bladder, prostate, skeletal and cardiac muscle (our unpublished data). Both A8 and Ab95 antibodies recognize antigenic epitopes which are located on the TIN-ag molecule. Immunocytochemistry is unable to recognize whether TIN-ag is exactly the same molecule in the kidney and intestine. It is possible the difference in staining intensity between kidney and intestine is not due to the amount of TIN-ag, but to the presence of different but relative molecules which share the same antigenic epitopes. Zhou *et al*. (2000) have identified two forms of TIN-ag from two human TIN-ag mRNA species referred as TIN 1 and TIN 2. The two proteins are the final products of the alternative splicing of TIN-ag primary gene transcript, which results in two mRNAs coding for the two different forms. It has been verified by Nelson *et al*. (1995) that a full length rabbit TIN-ag clone, which is equivalent to human TIN 1 codes for the 58 kDa form and perhaps TIN 2 may code for the 50 kDa. Several tubulointerstitial antigens associated with TIN have been reported (Yoshida *et al*., 1990, Butkowski *et al*., 1991, Miyazato *et al*., 1992, Fliger *et al*., 1987, Nelson *et al*., 1998). The molecular weights of these antigens vary depending the purification way (Bonthron *et al*., 1986). It is unknown also whether they share the same reactive epitope. But most of these molecules have similar distribution of reactivity within kidney. Recently, biomedical studies and purification of potential target antigens from basement membranes of different animals, have led to the detection of multiple protein forms ranging from 27 to 300 kDa. Cloning of these proteins has shown an exclusive expression on TBM (Masaru *et al*., 2000). TIN-ag is the target of raising autoantibodies in tubulointerstitial nephritis, various types of glomerular nephropathies and renal allograft recipients (Butkowski *et al*., 1990). Anti-TBM antibodies eluted from a patient's kidney have been reported to react with jejuna and tubule basement membranes (Wilson, 1989). Furthermore, anti-TBM antibodies have been recognized in a patient with celiac disease and nephritic syndrome and in two patients with villous atrophy of the small intestine (Wilson, 1989). Another study has reported the presence of anti-TBM antibodies in a human renal allograft reactive with basement membranes of peritubular capillaries and with distal and collective tubules (Klassen *et al*., 1973). Persistence of anti-TBM antibodies has been found in two patients with recurrent membranous nephropathy in the transplanted kidney and both conditions seemed to be immunologically associated. The presence of HLA B7 and DR W8 antigens in these patients is an indication that TIN is an autoimmunity disorder associated with HLA (Katz *et al*., 1997). Other investigators have reported absence of HLA-associated antigens in patients with membranous nephropathy and circulating anti-TBM antibodies (Ivanyi *et al*., 1998). Coexistence of TIN and uveitis has been also reported and TIN-ag could be the common link in these cases (Burnier *et al*., 1986, Cacoub *et al*., 1989, Salu *et al*., 1990).

Cloning of TIN-ag revealed the presence of follistatin motifs, which are known as novel autoantigen in systemic rheumatic diseases (Tanaka *et al*., 1998). It is possible, the presence of follistatin modules on the TIN-ag molecule is the target to which immune response was developed in TIN.

Molecular cloning of TIN-ag has revealed a 30% homology to cathepsin B-like proteases, but TIN-ag

is lacking of a proteolytic activity (Masaru *et al*., 2000). On the basis of structural features, searching for novel cathepsin-related proteins, a new family of proteins has described, the TIN-ag Related Proteins (TIN-ag-RP) (Bromme *et al*., 2000, Wex *et al*., 2001). These proteins are expressed not only in the kidneys, but also in the vascular smooth muscle cells, the cardiac muscle fibers, the enterocytes of small intestine and the placenta. It is worthy to note that the TIN-ag-RP have a significant expression in the collecting tubules, in the kidney medulla and cortex and also in the renal corpuscles. The latter are totally opposite to the distribution of TIN-ag. The possibility that the polyclonal antibody raised against TIN-ag-RP showed some cross reactivity with the convoluted tubules specific TIN-ag cannot be excluded. In our experiments, both monoclonal and polyclonal anti-TIN antibodies showed a similar distribution. Cloning and sequence comparison of TIN-ag-RP revealed that this protein is more closely related to TIN-ag than to cathepsin B-like proteases (Bromme *et al*., 2000). The localization of the human TIN-ag-RP gene on the 1p34.2-3 chromosome showed that the gene locus is far away than the TIN-ag gene, but very close to the genes of other extracellular matrix proteins (Wenzel *et al*., 1998, Gervais *et al*., 1999). This gene locus is mainly related to human tumors (Tsukamoto *et al*., 1998), dystrophies (Cormand *et al*., 1999) and deafness (Van Hauwe *et al*., 1999).

TIN-ag is a unique molecule with limited distribution in extracellular matrices, which are involved in the creation and maintenance of a physiological environment for the body. It is also a target of developing autoantibodies in immunologically mediated human nephropathies, which lead in some cases to the final stage of renal diseases. Further molecular analysis of the follistatin motifs of TIN-ag will reveal the antigenic epitope, which is responsible for the development of autoantibodies in the TIN. This knowledge will lead to the developing of advanced methods to remove the circulating autoantibodies from the patient's serum. This method has already been successfully established for the Goodpasture syndrome.

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