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Identification of a 400-kd Protein in the Brush Borders of Human Kidney Tubules That Is Similar to gp330, the Nephritogenic Antigen of Rat Heymann Nephritis

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The nephritogenic antigen of Heymann nephritis (HN)—a well-studied experimental rat model disease of human membranous glomerulonephritis (MGN)—was recently shown to be a 330-kd glycoprotein (gp330) which is present in the membranes of both the rat tubular brush borders and of podocytes. Because the pathogenic antigen(s) of MGN are unknown, the authors have searched for a gp330-like molecule in human kidney and for its role in MGN. The authors here report that a membrane protein (apparent molecular weight 400 kd) is present in human kidney which is immunologically cross-reactive with rat gp330. By immunoelectron microscopy (using rabbit anti-rat gp330 IgG or a monoclonal anti-400-kd IgG) this molecule is similarly localized in human proximal tubules, but it is absent from the podocytes of human glomeruli. The 400-kd molecule is not detected in the glomerular immune deposits of 30 biopsies of MGN. It is proposed that this is due to the lack of the 400-kd protein in human glomeruli which prevents the formation of initial 400-kd anti-400-kd IgG immune complexes *in situ*. (Am J Pathol 1987, 129:183–191)

HEYMANN NEPHRITIS[1] (HN) in rats and membranous glomerulonephritis (MGN) in humans show similarities in their glomerular lesions and clinical symptoms. Both are characterized by granular immune deposits in the lamina rara externa of the glomerular basement membrane (GBM) and by severe proteinuria. Several attempts have been made to elucidate the molecular mechanisms of the pathogenesis of both diseases, and recently advances have been made in the case of HN.

HN was originally induced by immunization of rats with various preparations of rat kidney cortex containing brush border membranes of proximal tubules.[2,3] We have recently identified and isolated the pathogenic antigen of HN as a large membrane glycoprotein with an apparent molecular weight of 330 kd (gp330)[4] from microvillar fractions of rat kidneys and have further shown that gp330 directly participates in the formation of immune deposits.[4,5] These findings were subsequently confirmed by others.[6–13]

The antigens involved in the formation of immune deposits in human MGN are currently unknown. Because of the similarities of the human and the rat disease, it was speculated that the pathogenic antigen(s) involved in both species could be similar. A connection between both diseases was made by the finding that HN could be induced in rats by brush border preparations from human kidneys,[14] suggesting that the nephritogenic component for rats was also present in human kidneys.

In this study we have identified a 400-kd protein in human kidneys which resembles rat gp330 in its biochemical, immunochemical, and immunomorphologic characteristics. A major difference to gp330 in

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normal and HN rats is the finding that the 400-kd molecule is not present in human podocytes and in the immune deposits in human MGN.

Materials and Methods

Materials

^{125}I and ^{125}I-labeled protein A were from Amersham Corp. (Arlington Heights, Ill). Lactoperoxidase, glucose oxidase, and protease from Staphylococcus *griseus*, Type 24, were from Sigma Chemical Co. (St. Louis, Mo). Sodium dodecyl sulfate (SDS), acrylamide, and nitrocellulose paper were from BioRad Laboratories (Richmond, Calif). Sheep anti-rabbit and sheep anti-mouse Fab-peroxidase conjugates were from BioSys (Compiegne, France). Rabbit and mouse—peroxidase–antiperoxidase (PAP) complex were from Dakopatts (Copenhagen, Denmark).

Isolation of Human and Rat Tubular Microvillar Fractions

Microvilli were purified from two human kidneys (which were removed because of small hypernephroid carcinomas on one pole) by the method of Malathi et al.[15] Microvilli from rat kidneys were prepared as described previously.[4]

Preparation of Polyclonal and Monoclonal Antibodies

Polyclonal anti-gp330 IgG was obtained from a rabbit immunized against purified rat gp330 as described previously.[5,16] IgG was affinity-purified on a column containing isolated gp330 immobilized to cyanogenbromide-activated Sepharose CL-4B.[5] Monoclonal IgG against rat gp330 was prepared as described previously[5] by immunization of mice with purified rat gp330.

For the preparation of monoclonal anti-human 400-kd IgG (Clone 7), mice were immunized by intraperitoneal injection of 500 μg of isolated human microvilli, as described previously for rat microvilli.[5] Isolated spleen cells were fused with NS 1 myeloma cells (a gift from Dr. W. Woloszcuk, University of Vienna). The hybridoma supernatants were screened by indirect immunofluorescence and immunoperoxidase on paraffin sections of normal human kidneys and by immunoblotting on human microvillar proteins. The positive hybridomas were cloned two times by limiting dilutions. Supernatants were used for the work described below.

Immunoblotting and Immunoprecipitation

For immunoblotting the proteins of human or rat kidney microvilli were separated on 3.6–8% gradient SDS gels and then transferred onto nitrocellulose membranes for 4 hours at 20 C with 30 V, using a transfer-buffer containing 20 mM Tris and 150 mM glycine, pH 8.3. Immune overlays with monoclonal Clone 7 IgG were performed as described previously,[17] with the use of sheep anti-mouse Fab–horseradish peroxidase (HRP) conjugate, diluted 1 : 1000.

Microvillar fractions of human or rat kidneys were labeled with ^{125}I by the lactoperoxidase-glucose oxidase procedure.[4] They were solubilized in RIPA buffer, and immunoprecipitation with rabbit anti-rat gp330 IgG was performed as described previously.[4,5] The immunoprecipitates were separated on 3.6–8% gradient SDS gels and exposed for autoradiography.

Immunoelectron Microscopy

Kidney biopsies from 2 patients (8 and 26 years of age) with microhematuria of nonrenal origin were incubated in paraformaldehyde-lysine-periodate fixative (PLP)[18] for 6 hours at 20 C and processed for indirect immunoperoxidase as described previously.[5,19] Rabbit anti-rat gp330 IgG (20 μg/ml) and monoclonal Clone 7 IgG (10 μg/ml) were used as first antibodies, and sheep anti-rabbit or anti-mouse Fab–HRP conjugates (diluted 1 : 100) were used as second antibodies.

Small PLP-fixed pieces of these kidneys also were embedded in the acrylic resin LR-White, as described previously.[19,20] The ultrathin sections were incubated in Clone 7 IgG (10 μg/ml), followed by affinity-purified rabbit-anti mouse IgG (50 μg/ml) and 14 nm gold–protein A conjugate. As controls the first antibodies were omitted or replaced by irrelevant IgGs.

Immunohistochemical Localization of the 400-kd Protein in Normal Human Kidneys and in MGN

Paraffin sections of 4% formalin-fixed normal human and rat kidneys, of biopsies of human kidneys with various stages[21] of membranous nephritis (3 Stage I, 4 Stage II, 10 Stage III, 8 Stage IV), and of rats with active Heymann nephritis (12–18 weeks after immunization with 10 mg rat Fx1A) were deparaffinated, rehydrated, and digested with 0.1 mg/ml pronase for 3 minutes at 37 C (this time of digestion gave optimal results in preliminary experiments). The sections were then incubated with monoclonal Clone 7 IgG (5 μg/ml) or with rabbit anti-rat gp330 IgG (1

mg/ml), which were detected by the PAP procedure using diaminobenzidine (DAB), as described previously.[22] Controls consisted of paraffin sections of normal and nephritic human and rat kidneys which were similarly pretreated, but incubated with irrelevant first antibody, or alternatively the first antibody was omitted.

Cryostat sections of unfixed, N_2-frozen kidney biopsies of 5 patients with MGN were incubated with rabbit anti-gp330 IgG and fluorescein isothiocyanate-labeled (FITC) goat anti-rabbit IgG. Some of the sections were incubated in 1 M propionic acid (pH 2.9) or in 100 mM glycine–HCl buffer (pH 2.9) or in 100 mM glycine–NaOH buffer (pH 10.2) for 1 hour at 20 C in order to break antibody–antigen complexes and to enhance accessibility of the antigen(s) in the immune deposits. The extracted sections were processed for indirect immunofluorescence, as described above.

Results

Immunochemical Identification of a Human gp330-like Molecule

When the protein patterns of human and rat microvillar fractions were compared, several major proteins with similar electrophoretic mobility were observed (Figure 1). In rat microvilli, a major gp330 and weaker gp300 and a 280-kd band were visible (Figure 1, Lanes C and G), whereas in human microvilli bands with apparent molecular weight of 400 kd, 280 kd, and 260 kd are prominent (Figure 1, Lanes A and E).

When the proteins of human (Figure 1, Lane A) and of rat microvilli (Figure 1, Lane C) are separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose, and overlaid with monoclonal Clone 7 IgG, a single band with an apparent molecular weight of 400 kd is selectively labeled on human microvillar lysates (Figure 1, Lane B), whereas no binding of the antibody is obtained on rat microvilli (Figure 1, Lane D).

Polyclonal rabbit anti-rat gp330 IgG immunoprecipitates specifically gp330 and gp300 from an RIPA buffer lysate of ^{125}I-labeled rat microvilli (Figure 1, Lane H), as reported previously.[23] This antibody immunoprecipitates a protein with an apparent molecular weight of 400 kd from ^{125}I-labeled human microvillar fractions (Figure 1, Lane F). In addition, a small amount of a 175-kd and traces of a 210-kd band are observed which could be degradation products of the 400-kd molecule.

This indicates that the human 400-kd protein shares epitopes with rat gp330, and that the Clone 7 IgG is specific only for the human molecule.

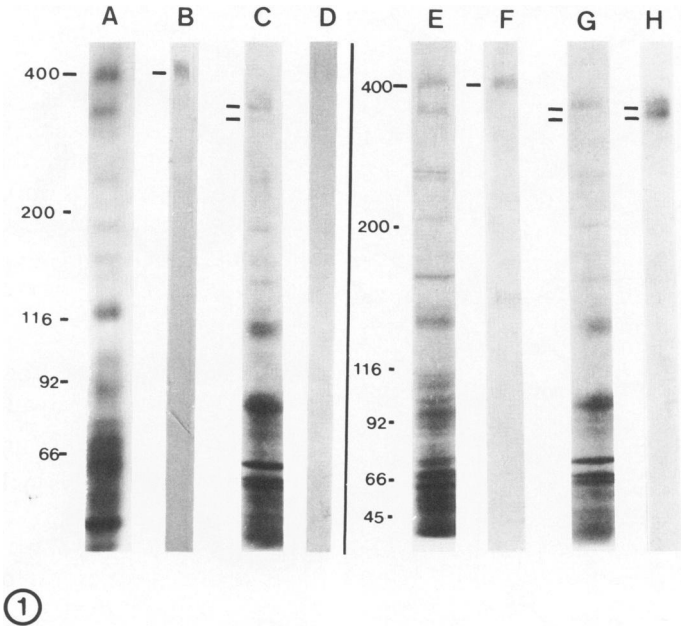


Figure 1—Immunochemical identification of the human 400-kd protein and gp330 in immune-overlays (Lanes A–D) and by immunoprecipitation (Lanes E–H). *Immune overlays*: The proteins of microvillar fractions of human (Lane A) and rat (Lane C) kidneys were separated on 3.6–8% gradient SDS gels, transferred onto nitrocellulose, and stained with Ponceau-S (Lanes A and C) or immuneoverlaid with monoclonal Clone 7 IgG (raised against human kidney microvilli). This antibody specifically binds to the human 400-kd protein (Lane B, *bar*), but not to rat gp330 or gp300 (Lane D; the position of gp330 and gp300 are indicated by bars in Lane C). *Immunoprecipitations*: Microvillar fractions of human (Lane E) and of rat (Lane G) kidneys were labeled with ^{125}I and the proteins separated by 3.6–8% gradient SDS-PAGE. The proteins were visualized by autoradiography. When human microvilli were incubated with rabbit anti-rat gp330 IgG, the 400-kd band was specifically immunoprecipitated (Lane F, *bar*). The same antibody binds gp330 and gp300 in rat microvillar lysates (Lane H, *bars*).

Immunocytochemical Localization of the Human 400-kd Molecule in Normal Human Kidneys

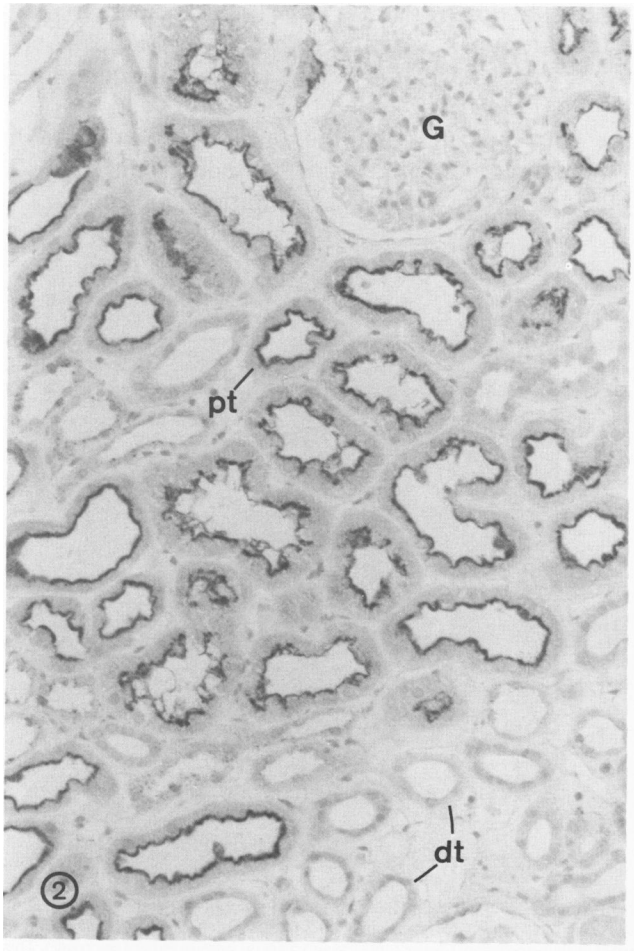
Rabbit anti-rat-gp330 IgG (affinity-purified on Sepharose 4B-linked isolated rat gp330[5]) binds in sections of paraffin-embedded human kidneys exclusively to the brush border region of proximal tubule epithelial cells (Figures 2 and 3). At higher magnification, the antigen is observed on the base of the microvilli, rather than on the microvilli proper (Figure 2B). No DAB reaction product was observed in glomeruli or epithelia other than those of the proximal tubules.

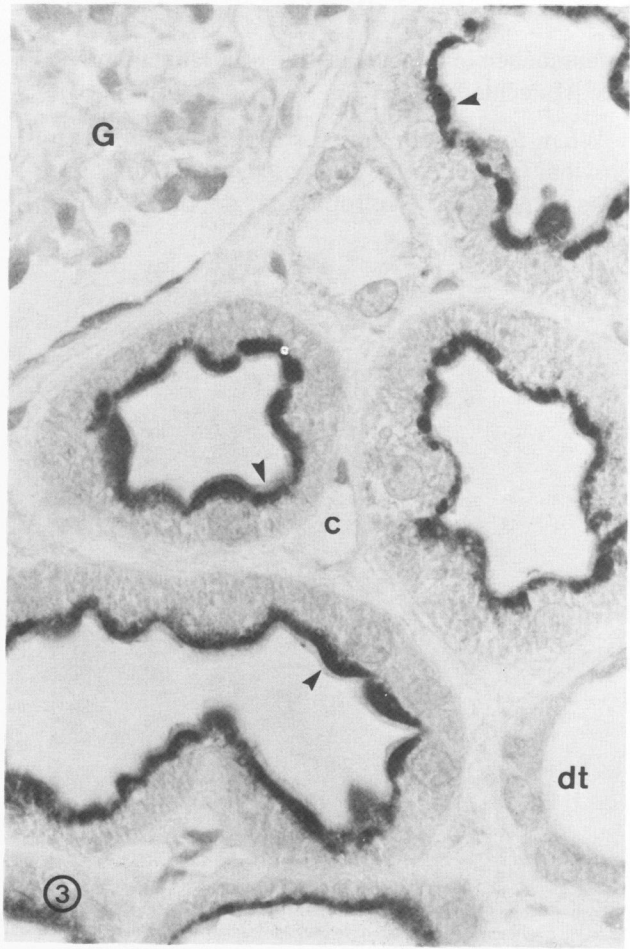
When the paraffin sections of normal kidney tissue are incubated with monoclonal Clone 7 IgG, an identical pattern of labeling results (not shown). No labeling was found in controls.

Localization of the Human 400-kd Protein by Immunoelectron Microscopy

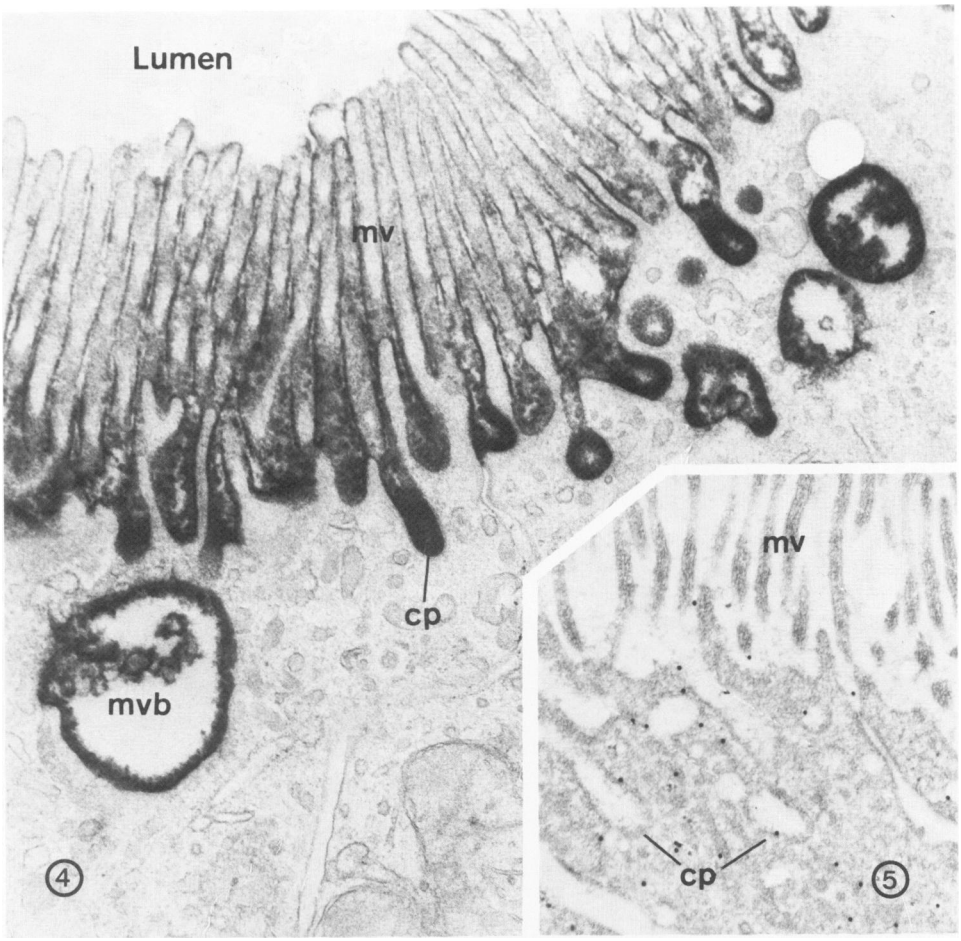
When PLP-fixed sections of normal human kidney tissue are incubated with affinity-purified rabbit anti-rat gp330 IgG (Figure 4), or with monoclonal Clone 7 IgG (not shown), the 400-kd antigen is detected exclusively in the bursh border region of proximal tubule cells, where it is confined to the clathrin-coated intermicrovillar invaginations (Figure 4) and occasionally also to apical vacuoles and multivesicular bodies. DAB reaction product is virtually absent from the membranes of the microvilli with both antibodies. A similar localization of the 400-kd antigen is also revealed in sections of LR-White-embedded human kidney tissue by an indirect immunogold surface technique[19,20] (Figure 5).

Human podocytes fail to show any DAB reaction product in coated pits, elsewhere on their cell surfaces, or in their biosynthetic intracellular compartments (ie, the endoplasmic reticulum and the Golgi apparatus) (Figures 6 and 8–10). This is in contrast to the localization of gp330 rat glomeruli, which was found previously both on the cell surfaces and the endoplasmic reticulum of podocytes.[5,10,11] The human mesangial and endothelial cells are also de-





Figures 2 and 3—Localization of the 400-kd protein in paraffin sections of normal human kidney, using affinity-purified rabbit anti-rat gp330 IgG in an indirect immunoperoxidase procedure. DAB reaction product is seen exclusively in proximal tubules (*pt*) and is absent from glomeruli (*G*), distal tubules (*dt*) and intertubular capillaries (*c*). At higher magnification in **Figure 3** the DAB reaction product is localized at the base of the microvilli (*arrowheads*). (**Figure 2**, ×180; **Figure 3**, ×750)



Figures 4 and 5—Localization of the 400-kd protein in proximal tubules of human kidneys by immunoelectron microscopy, using polyclonal affinity-purified anti-rat gp330 IgG in an indirect immunoperoxidase procedure (**Figure 4**) or monoclonal Clone 7 IgG on acrylic resin-embedded tissue in an indirect immunogold surface method (**Figure 5**). In both instances, the 400-kd protein is found concentrated in the intermicrovillar coated membrane regions (*cp*) and in some multivesicular bodies and is absent from the membranes of microvilli (*mv*). (**Figure 4,** ×38,000; **Figure 5,** ×32,000)

void of the 400-kd protein. The parietal epithelial cells lining the Bowman's capsules, however, contain reaction product in most of their coated pits, both at their luminal and—less frequently—also at their abluminal sides (Figures 6 and 7). The same results were obtained with rabbit anti-rat gp330 IgG and with monoclonal Clone 7 IgG.

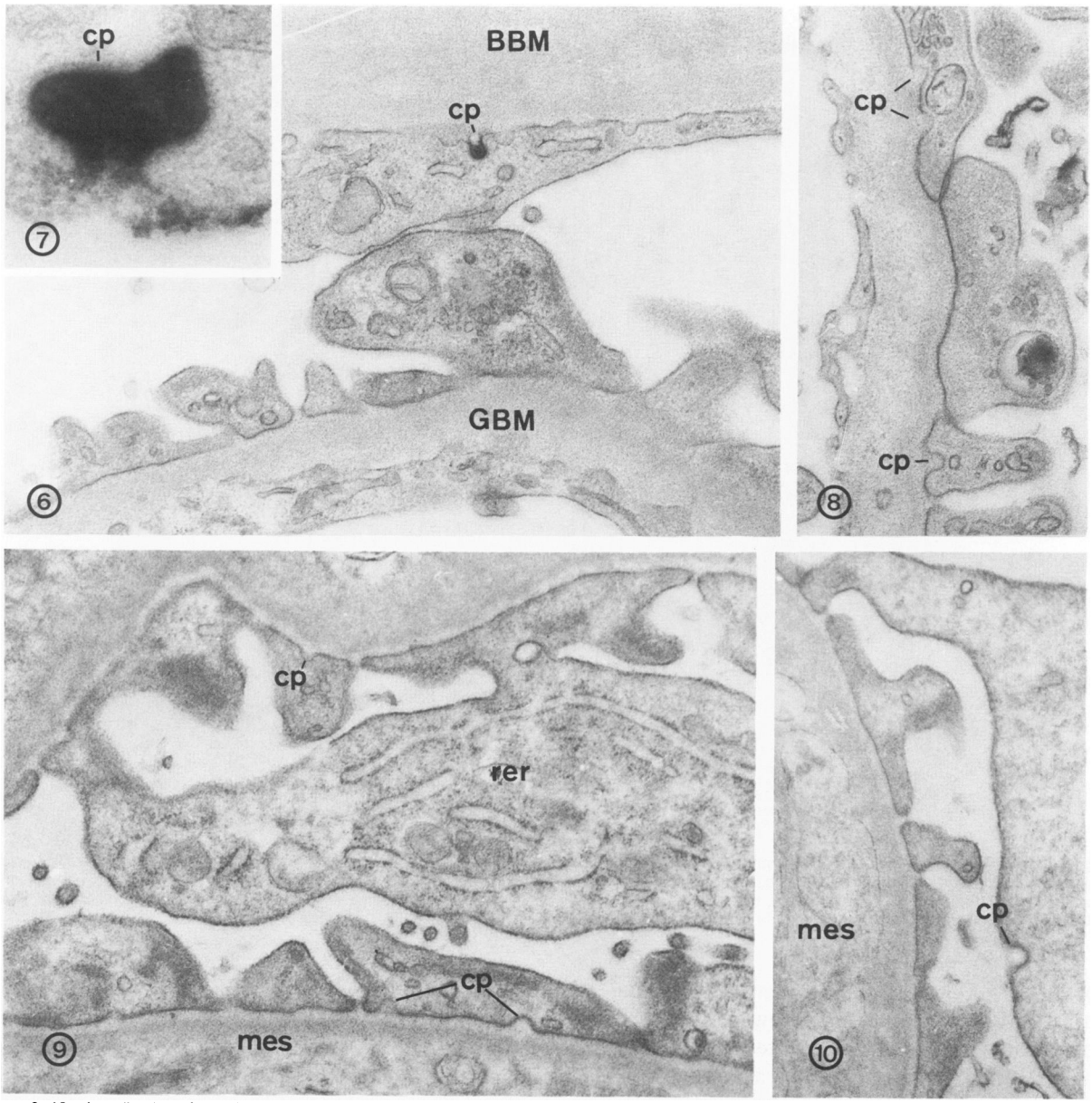
Immunocytochemical Localization of the 400-kd Human Protein in Kidneys From Patients With Membranous Glomerulonephritis

The localization of the 400-kd molecule was examined in paraffin sections of kidney biopsies of 25 patients with various stages of MGN by an indirect immunoperoxidase procedure, using both monoclonal Clone 7 IgG and affinity-purified rabbit anti-rat gp330 IgG (Figures 11 and 12). In all sections intense staining of the proximal tubule's brush border region was observed, but the glomeruli were constantly found negative in all 25 cases studied, even when the time of digestion of the sections with pronase was shortened or extended.

The 400-kd protein was localized in the brush border region of unfixed cryostat sections of 5 cases of MGN by immunofluorescence (not shown). Glomerular immune deposits were not reactive with the polyclonal anti-rat gp330 antibody, even after preincubation of the sections in extreme pH buffers (pH 2.9 or 10.2) in an attempt to "unmask" the antigen(s) in the immune deposits.

Localization of gp330 in the Kidneys of Heymann Rats

When paraffin sections of kidneys of rats with active Heymann nephritis were incubated in rabbit anti-gp330 IgG under the same conditions, intense labeling of the immune deposits in the glomeruli was observed, along with staining of the proximal tubule brush borders (Figures 13 and 14).



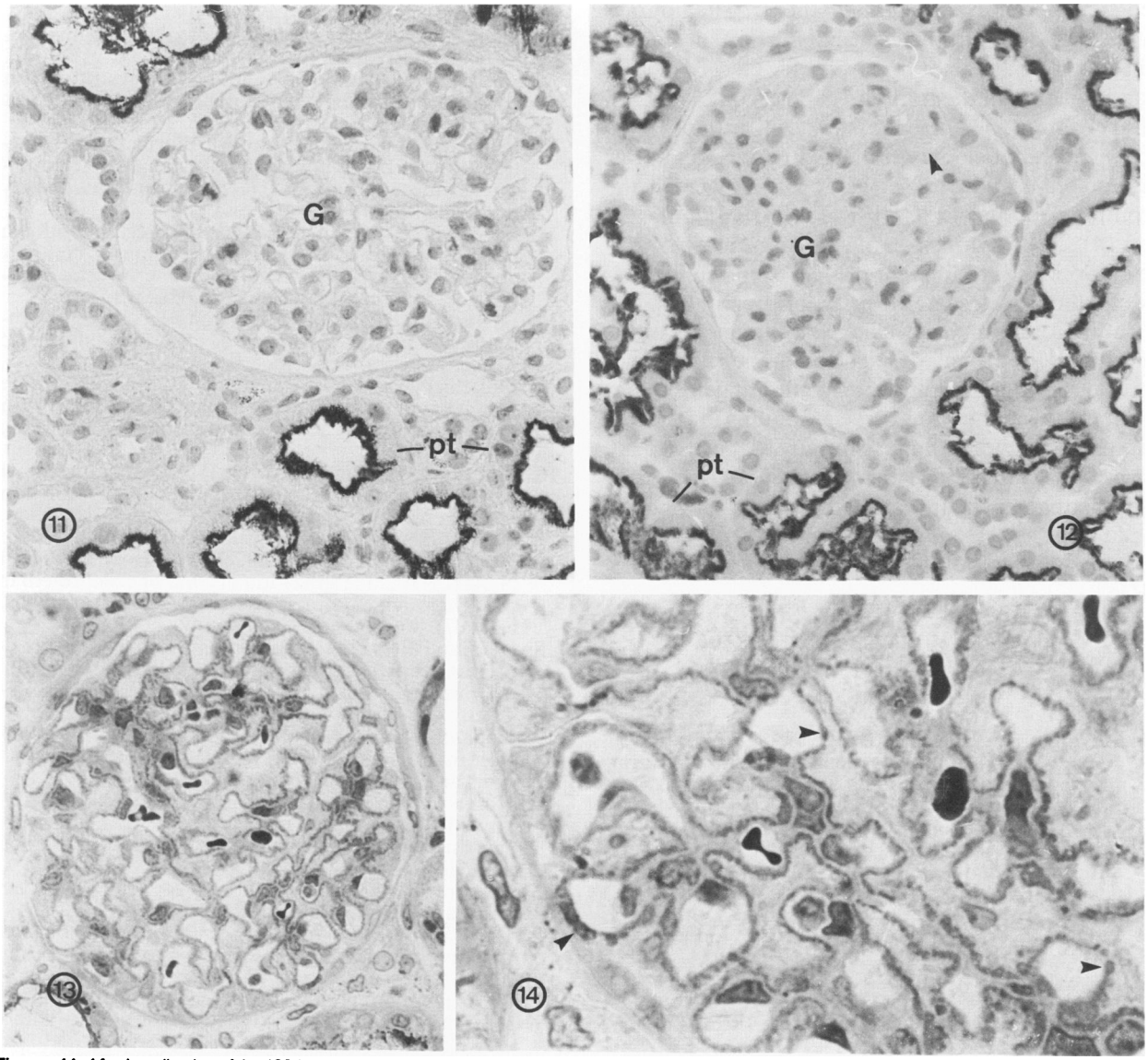
Figures 6–10—Localization of the 400-kd protein in human glomeruli, using affinity-purified rabbit anti-rat gp330 (**Figures 6–9**) **and monoclonal Clone 7 IgG (Figure 10)** in an indirect immunoperoxidase reaction. The antigen is not found in the coated pits (*cp*) or the endoplasmic reticulum (*rer*) in human podocytes, in contrast to gp330 in rats. Coated pits of the epithelial cells of Bowman's capsule (*cp* in **Figures 6 and 7**) contain DAB-reaction product. *mes*, mesangium. (**Figure 6**, ×42,000; **Figure 7**, ×72,000; **Figure 8**, ×42,000; **Figures 9 and 10**, ×36,000)

Discussion

In this study we have shown that a 400-kd protein in human kidney brush border fractions is similar to gp330—the pathogenic antigen of HN in rats. This molecule is, however, absent from normal human glomeruli and from those of patients with MGN. We conclude that the 400-kd glycoprotein is not a pathogenic antigen in human MGN.

The identification of the 400-kd molecule in humans as a relative of rat gp330 was based on the detection of common epitopes that were specifically recognized by a polyclonal anti-rat gp330 IgG. In support of these findings, a molecule with an apparent molecular weight of 440 kd was recently identified as the HN-inducing antigen in human Fx1A fractions (Y. Natori, personal communication). Monoclonal antibodies against rat gp330 and the human 400-kd proteins were found to be strictly species-specific. Therefore, gp330 and the 400-kd protein obviously are derived from one or more similar genes in which sequence homologies are preserved among several species.

The search for a human gp330-related molecule in



Figures 11–14—Localization of the 400-kd protein in paraffin-embedded kidney biopsies from patients with MGN Stage II (**Figure 11**) and Stage III (**Figure 12**), and of gp330 in paraffin sections of rats with HN (**Figures 13 and 14**). In the human tissue monoclonal Clone 7 IgG was used. Note the absence of DAB reaction product from the immune deposits of the glomeruli (*G*). By contrast, gp330 is readily detected in the immune deposits in HN (*arrowheads* in **Figure 14**) with the use of affinity-purified polyclonal anti-rat gp330 IgG. (**Figures 11–13**, ×480; **Figure 14**, ×750)

human glomeruli is of interest for the pathogenesis of MGN, because the presence of gp330 in glomerular epithelial cells was shown to be a prerequisite for the formation of immune deposits in HN.[4–13,24–26] Initially, the immune deposits in passive HN (and presumably also in active HN) are caused by binding of anti-gp330 IgG to the gp330 antigen which is located in the coated pits of the basal aspects of the cell membranes of podocytes.[5,16,27] This step is followed by shedding of the immune complexes,[5,28] and rapid attachment to the GBM.[27] Here we have shown by immunoelectron microscopy that the 400-kd protein is not expressed inside or on the surface of human podocytes. In this respect, human kidneys resemble those of mice[29] and dogs,[12] in which the gp330-related molecules are confined to the proximal tubules but are absent from glomeruli; consequently, immune deposits cannot be induced in these species by anti-gp330 IgG.[12,29]

The participation of tubular brush border antigen(s) in human MGN has been controversial. While in a group of Japanese patients[30] and in individual other cases[31,32] brush border antigens were detected in immune deposits, most other studies fail to support these findings.[24,33–35] These divergent data could be caused by the use of polyspecific antibodies, or by

genetic differences among the patients. The findings in this study support the view that the human equivalent of rat gp330 is not involved in MGN, because it was not found in the immune deposits in 25 cases of MGN by immunocytochemistry, using monospecific poly- and monoclonal antibodies under conditions that preserve and expose the gp330 antigen in the immune deposits of rats with active HN (Figures 13 and 14). The examination of a larger number of kidney biopsies from different sources with more sensitive methods is needed to verify this hypothesis.

Several other molecules have been detected in the immune deposits in MGN, such as hepatitis virus antigens[36] and the carcinoembryonic antigen,[37] but it is not clear whether they serve as a nidus for the formation of epimembranous immune deposits or are unspecifically trapped. The identification of the pathogenic antigen(s) in human MGN will reveal whether the findings on HN are relevant for the pathogenesis and probably also for the specific treatment of human MGN.

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