Coxsackievirus and Adenovirus Receptor, a Tight Junction Membrane Protein, Is Expressed in Glomerular Podocytes in the Kidney

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SUMMARY: In nephrosis, filtration slits of podocytes are greatly narrowed, and slit diaphragms are displaced by junctions with close contact. Freeze-fracture studies have shown that the newly formed junctions consist of tight junctions and gap junctions. Several tight-junction proteins are known as integral membrane components, including occludin and claudins; but none of them have been found in podocytes. Coxsackievirus and adenovirus receptor (CAR) has recently been identified as a virus receptor that is a 46-kDa integral membrane protein with two Ig-like domains in the extracellular region. In polarized epithelial cells, CAR is expressed at the tight junction, where it associates with ZO-1 and plays a role in the barrier to the movement of macromolecules and ions. In the present study, we investigated the expression and localization of CAR in rat kidneys treated with puromycin aminonucleoside (PAN) and in rat kidneys perfused for 15 minutes with protamine sulfate (PS). Both the experimental models have been used to induce tight junctions in podocytes. Ribonuclease protection assay and Western blot analysis revealed a distinct increase of CAR transcript and protein in glomeruli during PAN nephrosis but no increase in glomeruli by PS perfusion. Immunohistochemistry revealed a significant increase in CAR staining intensity along the glomerular capillary wall in PAN nephrosis and after PS perfusion. Immunoelectron microscopy demonstrated in both the models that the immunogold particles for CAR along the capillary wall were found predominantly at close cell-cell contact sites of podocytes but were rarely found at slit diaphragms. In cultured podocytes, CAR was localized at cell-cell contact sites. CAR distribution was identical to that of ZO-1 and different from that of a gap junction protein, connexin43. These findings indicate that CAR is an integral membrane component of tight junction in podocytes and that CAR expression in podocytes is regulated at the transcriptional level and in the redistribution of protein. (*Lab Invest 2003, 83:901–911*).

*T*he nephrotic syndrome in humans and experimental animals is characterized by the retraction of foot processes of podocytes and the coincident alteration of filtration slits in the renal glomerulus (Caulfield et al, 1976; Farquhr et al, 1957; Pricam et al, 1975). Foot processes of podocytes are normally maintained wide open to facilitate passage of the glomerular filtrate, and they are held together by slit diaphragms that bridge the filtration slits. In the nephrotic condition, the filtration slits are greatly narrowed and the slit diaphragms are displaced by junctions with close contact. Freeze-fracture studies have revealed that the newly formed junctions consist of leaky tight junctions and gap junctions (Caulfield et al, 1976; Pricam et al, 1975; Ryan et al, 1975). The molecular components of slit diaphragms and their significant roles in glomerular filtration have been elucidated through studies of congenital nephrotic syndrome (Kerjaschki, 2001; Kestila et al, 1998; Miner, 2002). The components of gap junctions in podocytes are also becoming clear (Yaoita et al, 2002b). In contrast, the tight junctions remain obscure in their functions and components. ZO-1 is only one component that has been demonstrated in these junctions (Kurihara et al, 1992). ZO-1 is the first tight junction protein identified and localized at the cytoplasmic face of the tight junction. No transmembrane proteins, however, are known in the junctions of podocytes. In other epithelia, two distinct integral membrane proteins have been identified as components of tight junctions, namely occludin and claudins (Furuse et al, 1993, 1998). Neither occludin nor claudins have been

detected in podocytes (Kiuchi-Saishin et al, 2002; Saitou et al, 1997).

Puromycin aminonucleoside (PAN) nephrosis is widely used as a model of minimal change nephrotic syndrome. Although the pathogenesis of proteinuria is not clearly explained, it has been reported that oxygen radicals, which are produced during the metabolism of PAN, cause podocyte injury, resulting in nephrotic syndrome (Diamond et al, 1986; Nosaka et al, 1997). In recent immunostaining studies, we have discovered the striking change of the coxsackievirus and adenovirus receptor (CAR) in glomeruli in PAN nephrosis. The CAR, a 46-kDa transmembrane protein, has been identified as a common cellular receptor for both viruses (Bergelson et al, 1997; Tomko et al, 1997). Sequence analysis indicates that CAR cDNA encodes a typical Ig-like membrane protein with two Ig-like domains that interact with adenovirus fiber protein. In addition to its extracellular domain, CAR cDNA contains a 23-amino acid transmembrane domain and a 107-amino acid intracellular domain that has a putative tyrosine phosphorylation site. Expression of CAR is abundant in the fetal and perinatal periods, especially in the nervous system, but decreases rapidly after birth. Using RT-PCR, CAR expression has been detected in various tissues in the adult (Fechner et al, 1999). Recently, its biologic functions are coming to light. CAR promotes homotypic cell aggregation in cultured neuronal cells (Honda et al, 2000). In polarized epithelial cells, CAR is expressed at the tight junction, where it associates with ZO-1 and plays a role in the barrier to the movement of macromolecules and ions (Cohen et al, 2001b). Disrupting the tight junction integrity, a virus exploits CAR for two important steps in its life cycle: entry into host cells and escape across the epithelial barrier to the environment (Cohen et al, 2001b; Walters et al, 2002).

In the present study, we examined CAR expression and localization in rat glomeruli using two experimental models: PAN nephrosis and kidney perfusion with polycation protamine sulfate (PS). Both the models have been used to induce tight junctions in podocytes (Caulfield et al, 1976; Kerjaschki, 1978; Kurihara et al, 1992; Pricam et al, 1975; Seiler et al, 1977). In the latter model, perfusion of kidneys for 15 minutes with the polycation causes neutralization of negatively charged groups on the surface of podocytes, resulting in prompt retraction of foot processes and dislocation of slit diaphragms in addition to formation of tight junctions (Kerjaschki, 1978; Kurihara et al, 1992; Seiler et al, 1977). Based on the observations presented below, we conclude that CAR is a transmembrane component of tight junctions in podocytes.

Results

CAR mRNA in the Kidney

Because CAR expression in the adult kidney has been reported only by RT-PCR using whole kidney RNA (Fechner et al, 1999), we evaluated the levels of CAR transcripts in the normal kidney compartments—glo-

meruli, cortex, and medulla—by ribonuclease protection assay using two probes corresponding to the extracellular and intracellular portions of CAR. The two probes produced the same results. Significant levels of CAR transcripts were detected in all of the samples. Although there were no obvious differences in the expression levels among the kidney compartments, the glomeruli tended to show more signals than the cortex (Fig. 1).

CAR mRNA in Glomeruli After PAN or PS Treatment

Measurement of 24-hour urinary protein levels revealed that a single intravenous injection of PAN
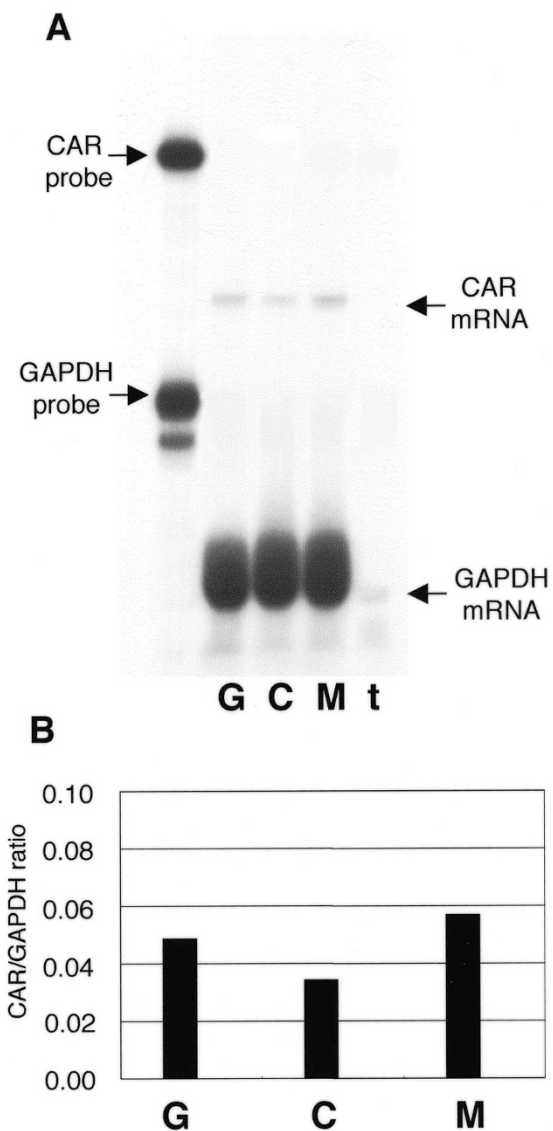
Figure 1.
Expression of coxsackievirus and adenovirus receptor (CAR) mRNA in the normal rat kidney compartments. A, Significant signals for CAR transcripts are detected in total RNA samples (10 μg each) from isolated glomeruli (*G*), cortices (*C*), and medullae (*M*) by ribonuclease protection assay. *t* = tRNA as negative control. B, CAR expression levels in A are quantified by molecular imager and shown as the ratio of the signal intensity for CAR to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

induced massive proteinuria at Day 6 (116 ± 53 mg/day) and at Day 10 (201 ± 39 mg/day). No increase or a slight increase in urinary protein was detected at 2 or 4 days in comparison with control rats (2.0 ± 1.0 mg/day).

After PAN injection, a striking increase of CAR transcripts was observed in mRNA from glomeruli (Fig. 2A). Molecular imager analysis revealed that CAR mRNA in glomeruli increased up to 2.7-fold at Day 1, 3.3-fold at Day 2, 2.6-fold at Day 4, 2.2-fold at Day 6, and 1.5-fold at Day 10 compared with the normal control level. In contrast, mRNA from the cortex and medulla did not show such significant changes (Fig. 2, B and C).

To examine whether 15-minute perfusion with PS changed CAR expression in glomeruli, glomerular mRNA from the perfused kidneys was compared with that from kidneys perfused with Hanks' balanced salt solution (HBSS) for 15 minutes. Ribonuclease protection assay detected no increase in CAR transcript by the PS treatment (data not shown).
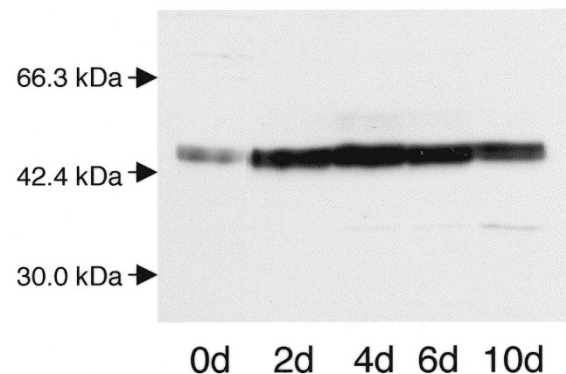
CAR Protein in Glomeruli in PAN Nephrosis

Western blot analysis was performed to quantify CAR protein in glomeruli. Anti-CAR antibody reacted with a protein with an apparent molecular mass of 46 kDa in samples of isolated glomeruli. In parallel with the transcript level, the intensity of signals for CAR in glomeruli distinctly increased to 2.4-fold at Day 2, 3.0-fold at Day 4, 2.6-fold at Day 6, and 2.0-fold at Day 10 of PAN nephrosis compared with the control level (Fig. 3).

### Immunolocalization of CAR in Normal, PAN-, and PS-Treated Kidneys

Compared with the negative control, faint but significant immunostaining for CAR was detected along some parts of the glomerular capillary wall in the normal kidney (Fig. 4, A and D). Parietal epithelial cells of Bowman's capsule, distal tubular epithelial cells, and collecting ducts were more intensely labeled with the antibody than the glomerular capillary wall. In PAN nephrosis, distinctly enhanced staining was detected along the glomerular capillary wall, which contrasted with no change in staining intensity in parietal cells of Bowman's capsule at Day 4 (Fig. 4, B and E) and Day 10 (Fig. 4, C and F). The change was consistent with those at the mRNA and protein levels in isolated glomeruli. The positive cells in glomeruli were likely to be podocytes based on their morphology and location in glomeruli. The intensity of staining in podocytes was apparently weaker on Day 10 (Fig. 4, C and F) than on Day 4 (Fig. 4, B and F). In the tubular epithelial cells, CAR was seen uniformly along the entire length of the basolateral membrane and seemingly on the apical membrane (Figs. 4, D to F). However, as shown by the immunoelectron microscopy as described below, the
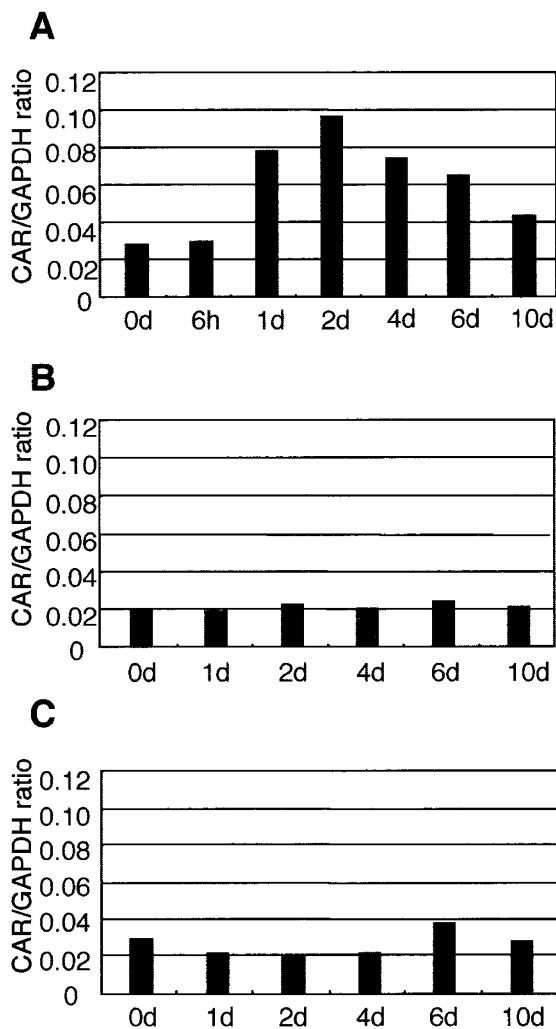
Figure 2.
Expression of CAR mRNA in glomeruli (A), cortices (B), and medullae (C) in puromycin aminonucleoside (PAN) nephrosis detected by ribonuclease protection assay. Total RNA samples (10 μg each) were obtained from isolated glomeruli, cortices, and medullae during different stages of PAN nephrosis from Day 0 (0d) to Day 10 (10d). CAR expression levels were quantified by molecular imager and shown as the ratio of the signal intensity for CAR to that for GAPDH. Significant increase of CAR mRNA during PAN nephrosis is seen in glomeruli but in not cortices or medullae.

Figure 3.
Immunoblot analysis of CAR in glomeruli during different stages of PAN nephrosis from Day 0 to Day 10. Ten micrograms of total protein from isolated glomeruli was separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and blotted with anti-CAR antibody. A significant increase of CAR protein is seen during PAN nephrosis.
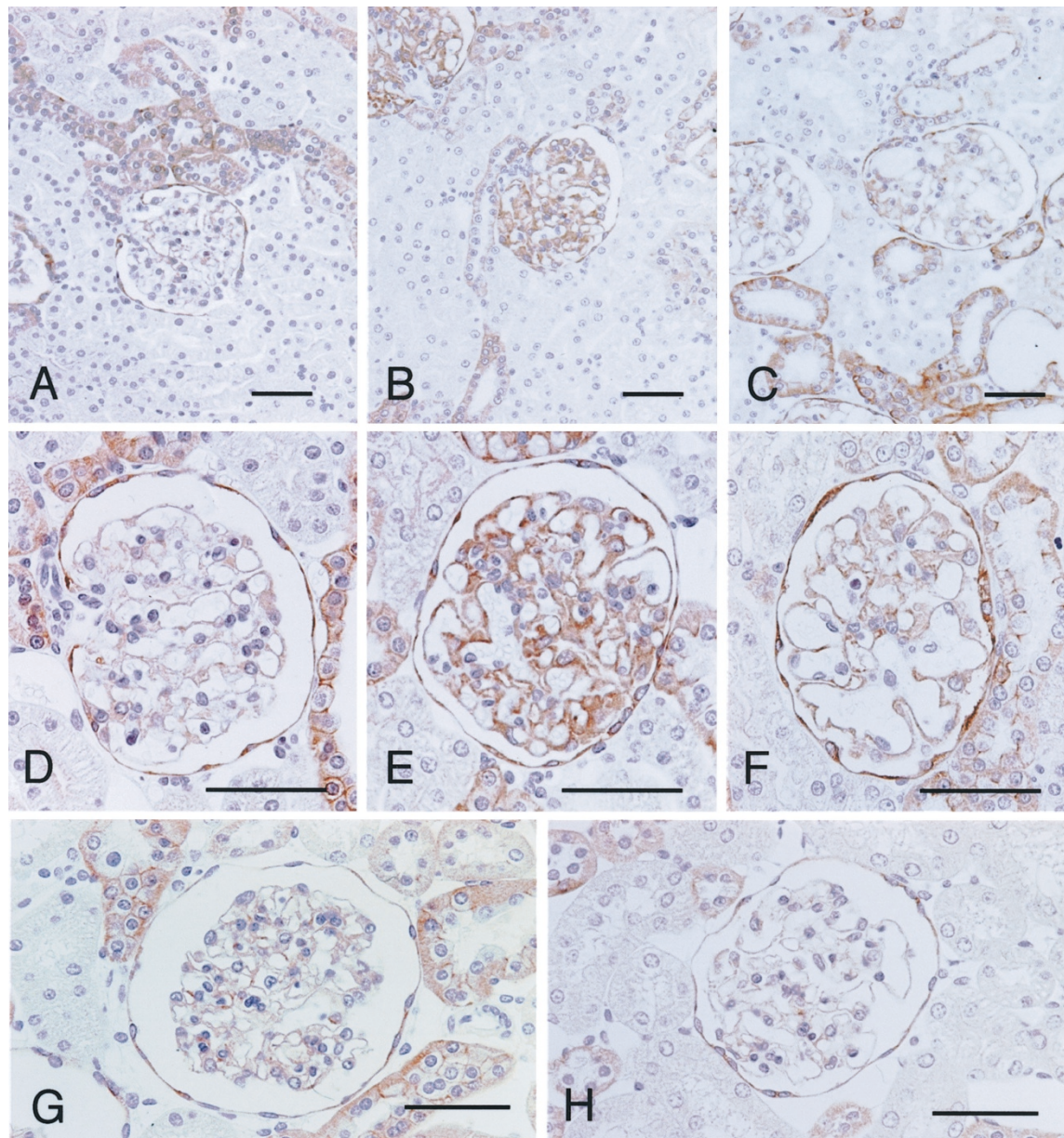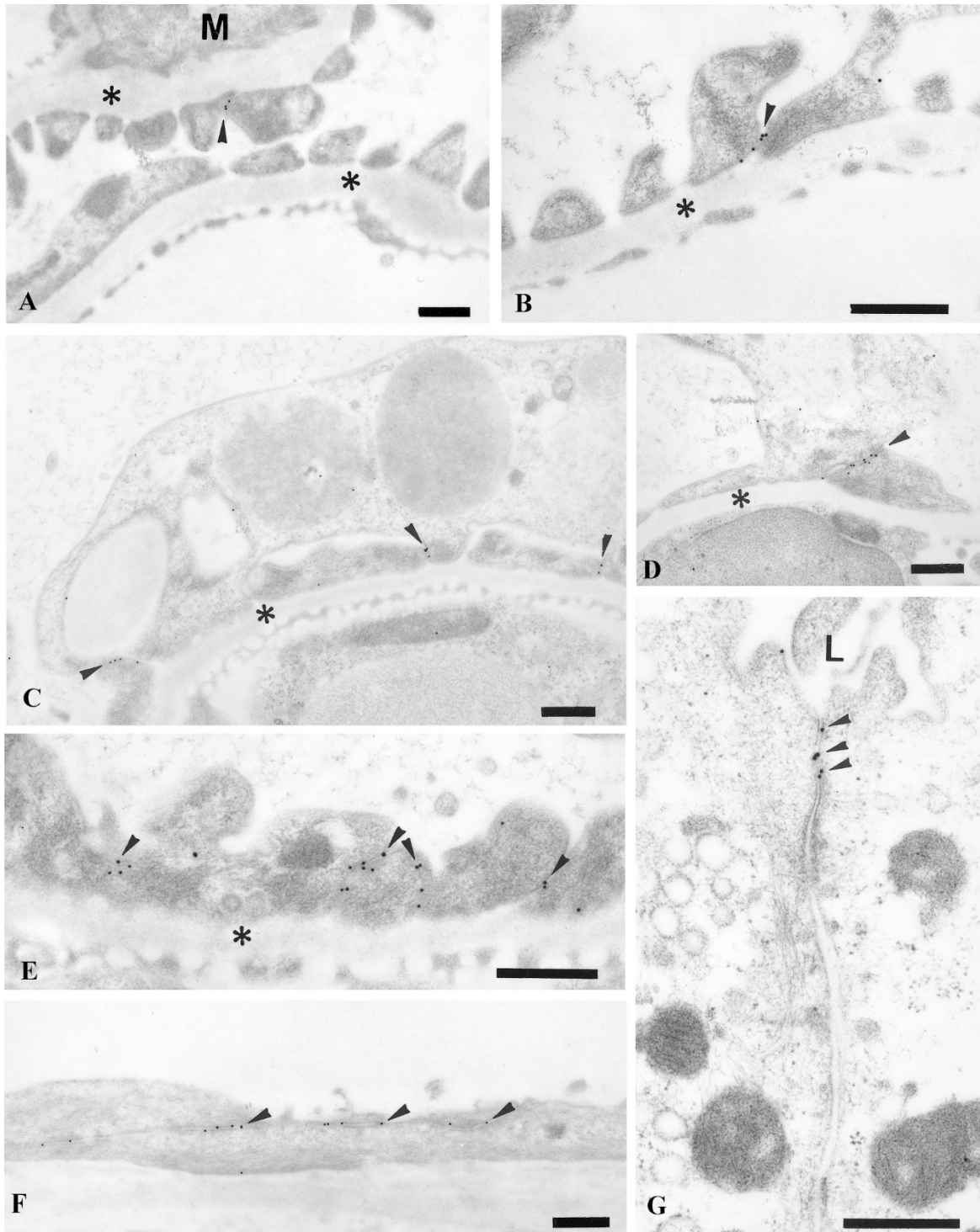
Figure 4.
Immunohistochemistry for CAR in the cortex of the normal (A and D), PAN-treated (B, C, E, and F), protamine sulfate (PS)-perfused (G), and HBSS-perfused (H) kidneys. CAR staining is observed in glomeruli, distal tubules, and collecting ducts, but not in proximal tubules (A to C). Weak but significant staining for CAR is seen along the glomerular capillary wall in normal glomeruli (D). Staining intensity for CAR is enhanced in podocytes along the capillary wall at Day 4 (B and E) and Day 10 (C and F) of PAN nephrosis. The change is apparent when compared with the unchanged staining of parietal epithelial cells of Bowman's capsule (D to F). Kidney perfusion with PS also enhances CAR staining intensity along the capillary wall (G), whereas enhanced staining is not detected in the HBSS-perfused kidney (H). Bar, 50 μm.

apparent apical staining was not present on the apical membrane but at the apex of the lateral membrane. No significant change was observed in the tubular staining during the course of PAN nephrosis (Fig. 4, A to F). Compared with kidneys perfused with HBSS, CAR staining was significantly enhanced in glomeruli after 15-minute perfusion with PS (Fig. 4, G and H). Normal rabbit IgG did not react to the glomerular capillary wall in the PS-perfused kidneys.

The precise localization of CAR in the glomerular capillary wall was examined by immunoelectron microscopy. In the normal kidney, plasma membranes of adjacent podocytes are widely spaced at the slit pore and are bridged by the slit diaphragm. On occasion, this wide space appears completely obliterated with focal regions of contact between apposing plasma membranes in or near the plane of the slit diaphragm (Rodewald and Karnovsky, 1974). Significant accumulation of immunogold particles for CAR was restricted to the focal regions of contact (Fig. 5, A and B). CAR labeling at slit diaphragms was very rare. In PAN-treated animals, foot process effacement and narrow filtration slits had already appeared at Day 2 and became distinct at Day 10. Many of the cell mem-

Figure 5.

Immunogold localization of CAR in podocytes (A to E), parietal epithelial cells of Bowman's capsule (F), and distal epithelial cells (G). A and B, In normal glomeruli, accumulation of CAR immunogold particles (*arrowheads*) is seen at the site of cell-cell contact of podocytes, but not in slit diaphragms. C to E, At Day 4 of PAN nephrosis, gold particles are frequently observed at newly formed cell contact sites (*arrowheads*). F, CAR immunogold particles are extensively distributed along intercellular junctions of parietal epithelial cells. G, Significant accumulation of gold particles (*arrowheads*) is observed at tight junctions of distal tubular cells. * = glomerular basement membrane; *M* = mesangium; *L* = lumen in tubules. Bar, 0.5 μm.

branes between adjacent podocytes formed close intercellular junctions, which are known to be composed of leaky tight junctions and gap junctions as shown by freeze-fracture studies (Caulfield et al, 1976; Pricam et al, 1975; Ryan et al, 1975). The immunogold particles were detected frequently in podocytes of PAN-treated rats. The newly formed junctions with close contact were well marked with the particles, whereas dislocated or remaining slit diaphragms were rarely labeled (Fig. 5, C to E). No significant gold

particles were observed in endothelial cells or mesangial cells. Immunogold labeling was also found at cell-cell contact sites of parietal cells of Bowman's capsule, distal tubular epithelial cells, and collecting ducts but not proximal tubules (Fig. 5, F and G). The particles were concentrated at tight junctions of tubular cells but not on the apical membrane (Fig. 5G).

Within 15 minutes after perfusion with PS, changes of podocytes apparently typical of those in PAN nephrosis were seen: reduction of foot process number and filtration slits, formation of tight junction-like structures, and dislocation of slit diaphragms above the newly formed junctions. By freeze-fracture study, the junctions have been shown to consist of tight junctions and gap junctions (Kerjaschki, 1978). Immunogold labeling along the capillary wall significantly increased in the PS-perfused kidney in comparison with the HBSS-perfused one (Fig. 6, A to C). The newly formed cell contact sites between podocytes were marked with immunogold particles, although the particle density was lower than that in PAN nephrosis.
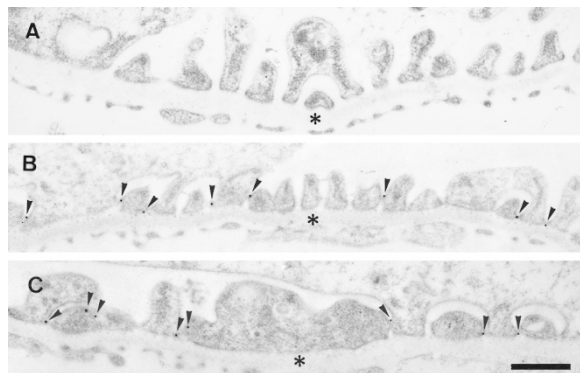
CAR in Cultured Podocytes

Immunoelectron microscopy demonstrated that CAR was concentrated at the newly formed junctions, but did not clarify the distinction between CAR at tight junctions or gap junctions. Podocytes in the primary culture exhibit extreme hypertrophy, which facilitate molecular localization in the cell (Yaoita et al, 2001). Thus, CAR distribution was compared in cultured podocytes with that of connexin43, a gap junction component (Yaoita et al, 2002b). Double-label immunofluorescence microscopy was performed using rabbit antibodies against CAR or connexin43 in combination with murine monoclonal anti-ZO-1 antibody. Immunofluorescence for CAR was observed at cell-cell contact sites of cultured cells and was exactly colocalized with ZO-1 staining (Fig. 7). Connexin43 also located at cell-cell contact sites, but their location

Figure 6.
Immunogold localization of CAR in the glomerular capillary wall in HBSS-perfused (A) and PS-perfused (B and C) kidneys. A, HBSS perfusion does not affect wide filtration slits between foot processes, where marking with immunogold particles is very rare. B and C, By PS perfusion, filtration slits are narrowed and junctions between foot processes are newly formed. The junctions are labeled with CAR immunogold particles (*arrowheads*). * = glomerular basement membrane. Bar, 0.5 μm.

did not coincide with that of ZO-1 (Fig. 7). The findings suggest that CAR distribution is related to tight junctions but not to gap junctions in cultured podocytes.
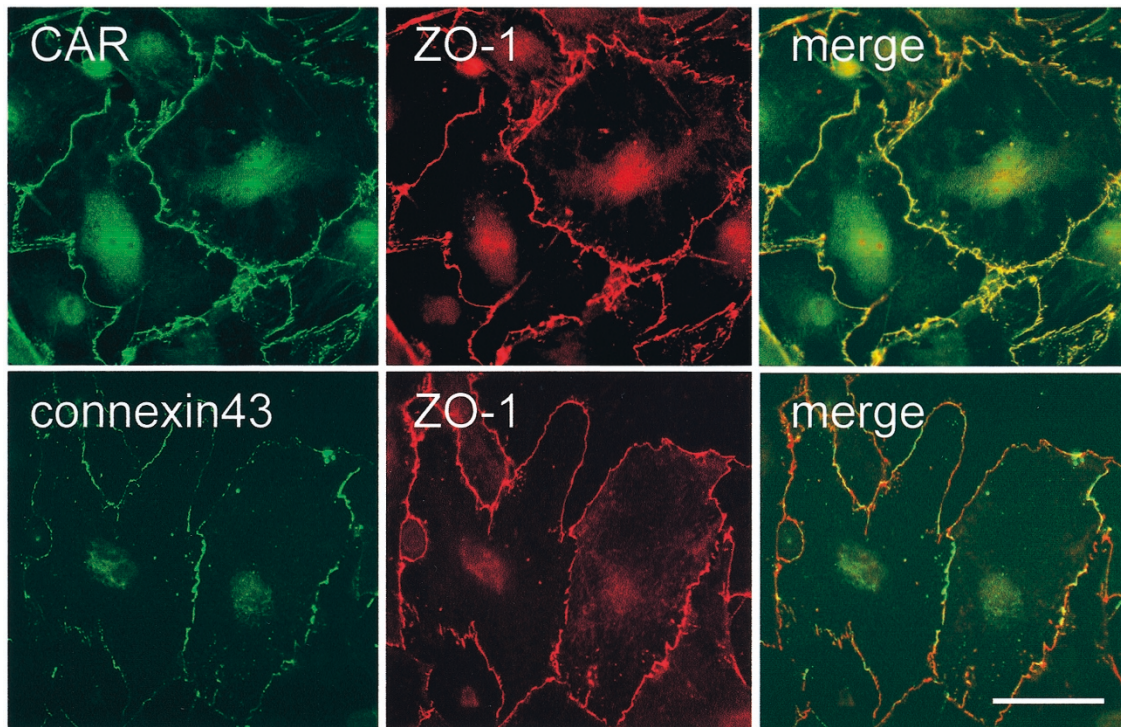
Discussion

In the present study, CAR expression in podocytes was reported for the first time. The immunolocalization study demonstrated that CAR was concentrated at intercellular junctions of podocytes where opposed plasma membranes were in close contact. The junctions with close contact were occasionally encountered in normal podocytes and were newly emerged and increased in number in PAN-treated and PS-perfused kidneys. Based on morphologic observations on routine Epon-embedded sections and freeze-fracture preparations, it has long been suspected that the junctions are composed of tight junctions and gap junctions (Caulfield et al, 1976; Kerjaschki, 1978; Pricam et al, 1975; Ryan et al, 1975; Seiler et al, 1977). This idea has been corroborated by immunoelectron microscopy using antibodies against a tight junction protein, ZO-1, and a gap junction protein, connexin43 (Kurihara et al, 1992; Yaoita et al, 2002b). These findings do not rule out the possibility that the junctional complex contains other types of junctions, such as adherens junction, because it has been shown in many cell lines that the formation of the epithelial junctional complex requires adherens junction proteins including cadherins and catenins (Gumbiner et al, 1988; Meyer et al, 1992). However, the newly formed junctions with close contact in PAN nephrosis did not react with any antibodies against cadherins or catenins (Usui et al, 2003; Yaoita et al, 2002a). CAR is unlikely to be associated with gap junctions because of different distribution of CAR compared with connexin43 in cultured podocytes. Taken together, the data show that CAR is localized at tight junctions in podocytes as an integral membrane component.

PAN and PS affect podocytes through different mechanisms, namely, production of oxygen radicals and neutralization of the cell surface polyanion with polycation, respectively. Both the treatments, however, cause similar morphologic changes of podocytes: the number of filtration slits and foot processes is greatly reduced, and many of the membranes between adjacent foot processes are in close contact. In PAN nephrosis, the increase of the junctions with close contact in podocytes is likely to be attributable to increased synthesis of CAR, because the change of CAR mRNA and protein levels in isolated glomeruli is well correlated with that of staining intensity for CAR in podocytes in the kidney sections. This does not pertain to PS-treated glomeruli, in which significant new CAR synthesis is unlikely because the effect occurs within 15 minutes after PS perfusion. In fact, we could not detect an increase of CAR transcript in PS-treated glomeruli by ribonuclease protection assay. Nevertheless, signals for CAR in podocytes clearly increased after PS treatment as shown by immunohistochemistry and immunoelectron microscopy in this study. Kerjaschki (1978) reported that the formation of tight

Figure 7.
Double-labeled immunofluorescence photographs of cultured podocytes incubated with rabbit antibodies against CAR or connexin43 in combination with murine monoclonal anti-ZO-1 antibody. Cells growing out from decapsulated glomeruli are used as cultured podocytes. CAR and connexin43 are stained with FITC-conjugated secondary antibody (green), and ZO-1 is stained with tetramethylrhodamine isothiocyanate-conjugated antibody (red). Colocalization of CAR and ZO-1 is shown by yellow color in the merged image (*merge*). In contrast, connexin43 does not coincide with ZO-1 as shown by the presence of green and red colors in the merged image. Bar, 50 μm.

junctions was not suppressed by PS perfusion in cold temperature (5–6° C). This is also a strong argument against de novo synthesis of tight junction components. Consequently, it has been assumed that in the normal glomerulus, there is a pool of unassembled junctional membrane proteins either already present in the plasma membrane or stored in vesicles in the cytoplasm of podocytes (Kerjaschki, 1978; Kurihara et al, 1992). CAR staining was faint along the glomerular capillary wall in the normal glomerulus. Unfortunately, immunoelectron microscopy did not detect significant accumulation of CAR immunogold particles in the podocytes, except in the intercellular junctions with close contact. In polarized epithelial cells, CAR is localized exclusively to the basolateral membrane as observed in some tubular epithelial cells in this study and as reported in cultured epithelial cells (Cohen et al, 2001a; Pickles et al, 1998). The information required for basolateral sorting is present within the highly conserved region of the cytoplasmic domain (Cohen et al, 2001a). The pool of unassembled CAR proteins in normal podocytes may be diffusely present on the basolateral membrane at so low density as to be undetectable by immunoelectron microscopy.

The reduction of filtration slits and foot processes by perfusion with polycations such as PS has led to the conclusion that the filtration slits are normally maintained open by electrostatic repulsion as a result of the high net negative charge found on the cell surface of podocytes (Kerjaschki, 1978; Seiler et al,

1977). On neutralization of the cell surface charge by PS treatment, the adjoining cell membrane of podocytes immediately come into close opposition. Transfection experiments have demonstrated that CAR mediates homotypic cell adhesion in cultured neuronal cells and Chinese hamster ovary cells (Cohen et al, 2001b; Honda et al, 2000). It is plausible that CAR molecules on the adjacent cell membrane of podocytes form homodimers and facilitate tight junction formation (van Raaij et al, 2000). A similar sequence of events presumably occurs in PAN nephrotic rats, in which a decrease in the negatively charged podocyte polyanion has been shown by colloidal ion and Alcian Blue stains (Michael et al, 1970). A main candidate for the molecule responsible for maintenance of the negative charge on podocytes is podocalyxin, which is the major sialoprotein of the glomerulus (Kerjaschki et al, 1984). Considerable evidence suggests that podocalyxin plays a key role in holding filtration slits open (Gelberg et al, 1996). A reduction in the sialic acid content of podocalyxin has been detected in PAN nephrosis (Kerjaschki et al, 1985). Thus, it is conceivable that CAR expression in PAN nephrosis is regulated by the redistribution of CAR molecules from the pool in addition to up-regulation of transcription.

Typical tight junctions provide continuous circumferential intercellular contact at the apical poles of lateral cell membrane. They function as a barrier that regulates the paracellular transit of water and solutes, and they define the boundary of apical and basolateral

plasma membrane domains. In contrast, tight junctions in podocytes are known to be composed of discontinuous ridges characteristic of leaky tight junctions as shown by freeze-fracture studies (Caulfield et al, 1976; Pricam et al, 1975; Ryan et al, 1975). It is not certain that the primary function of the junctions in podocytes involves barriers to the movement of paracellular solute or plasma membrane proteins. Recently, it has been observed that CAR expression leads to modulation of the cell cycle regulatory proteins p21 and Rb and inhibits the growth of bladder carcinoma cells, effects that are prevented by antibody that recognizes the CAR extracellular domain (Okegawa et al, 2001). Mature podocytes rarely undergo cell division even after subtotal nephrectomy (Fries et al, 1989; Pabst and Sterzel, 1983; Rasch and Nørgaard, 1983). There is increasing evidence that several negative cell regulatory proteins have a critical role in the inability of podocytes to replicate (Nagata et al, 1998; Shankland and Wolf, 2000). p21 is one of the regulators in podocytes as demonstrated in experimental membranous nephropathy and p21 knockout mice (Kim et al, 1999; Shankland et al, 1997). Homotypic CAR interactions forming tight junctions may thus increase signals contributing to contact-dependent inhibition of podocyte proliferation in response to injury.

Materials and Methods

Animals

Female Wistar-Kyoto rats weighing 150 to 200 gm were obtained Charles River Japan Inc. (Atsugi, Kanagawa, Japan).

Induction of PAN Nephrosis

PAN nephrosis was induced by a single intravenous injection of PAN (Sigma, St. Louis, Missouri) at a dose of 5 mg/100 gm of body weight in PBS. Control animals received an identical volume of PBS. The rats were housed in individual metabolic cages, and 24-hour urine specimens were collected before injection and at 2, 4, 6, and 10 days after injection of PAN. Rats were killed under diethyl ether anesthesia 6 hours and 2, 4, 6, and 10 days after PAN injection, and kidneys were removed and processed for immunohistochemical analysis, Western blotting, or ribonuclease protection assay. Glomeruli isolated from four or six kidneys at each time point were mixed and used as one sample of glomerular protein or RNA.

Perfusion with PS

Rats were anesthetized with diethylether, and the left kidneys were perfused in situ with PS (500 μg/ml in HBSS) for 15 minutes as described by Kerjaschki (1978).

Ribonuclease Protection Assay

Ribonuclease protection assay was performed as described previously (Feng et al, 1993; Sambrook et al, 1989; Yamamoto et al, 1997). Antisense cRNA probes for mRNA of CAR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were ^{32}P-labeled by in vitro transcription, using the plasmids inserted with each cDNA: 241 bp corresponding to bp 68–308 at the N-terminus of rat CAR 1 (AF109644), 184 bp corresponding to bp 907–1090 at the C-terminus of rat CAR 1, and 114 bp corresponding to bp 674–787 of GAPDH as a housekeeping gene. Total RNA (10 μg each) from rat glomeruli, cortex, and medulla was hybridized with the cRNA probes at 45° C overnight in hybridization buffer (80% formamide, 40 mM 1,4-piperazinediethanesulfonic acid, 0.4 M NaCl, 1 mM ethylenediaminetetra-acetic acid). Unhybridized probes were digested with ribonuclease A (4 μg/ml) and ribonuclease T1 (120 U/ml) for 60 minutes at 30° C, and then ribonucleases were digested with proteinase K (0.5 μg/ml) at 37° C for 30 minutes. After phenol/chloroform extraction, the hybridized probes were precipitated with ethanol and heat denatured for electrophoresis on 6% polyacrylamide gels. Detection and analysis of bands were performed by phosphor imaging techniques using Molecular Imager FX (Bio-Rad Laboratories, Hercules, California). The results of ribonuclease protection assay are represented as the ratio of CAR to GAPDH.

Western Blotting

To prepare anti-CAR antibody, a peptide (KTQYN-QVPSEDFERAPQ) corresponding to a partial sequence of the cytoplasmic domain of CAR was synthesized and used to immunize white rabbits as described previously (Honda et al, 2000). Renal cortex, medulla, and glomeruli were homogenized in lysis buffer (8 M urea, 0.2% SDS, 0.8% Triton X-100, 3% 2-mercaptoethanol) on ice. The homogenates were centrifuged at 20000 $\times g$ to remove insoluble debris. The protein in samples was quantified by Lowry's method after precipitation by trichloroacetate with sodium deoxycholate (Peterson, 1997). Western blotting was performed as described previously (Funaki et al, 1998). Briefly, a sample of 10 μg of each protein was loaded on a 10% SDS-polyacrylamide gel, and the bands were transferred to a polyvinylidene difluoride membrane. The membranes were preincubated with 5% nonfat milk in PBS for 1 hour, incubated with the anti-CAR antibody overnight, and washed in 0.05% Tween 20 in PBS. Then they were incubated with a 1:2000 diluted goat anti-rabbit immunoglobulin-conjugated peroxidase-labeled dextran polymer (rabbit EnVision; DAKO, Carpinteria, California), and the immunoreactivity was visualized by an "ECL plus Western blotting detection system" (Amersham Pharmacia Biotech). Densitometric analysis was performed with NIH image software (version 1.62, National Institutes of Health).

Immunohistochemistry

Immunostaining was performed by a procedure as described previously (Nihei et al, 2001; Yaoita et al,

2001). Normal, PAN-treated, or PS-perfused kidneys were fixed in methyl-Carnoy's solution overnight at 4° C and embedded in paraffin. They were sectioned at 2-μm thickness, deparaffinized, and incubated with anti-CAR antibody (20 ng/μl) for 1 hour at room temperature. The sections of control rat kidney incubated with PBS or normal rabbit sera were used as negative controls. They were then incubated with EnVision of anti-rabbit IgG and colored with 3' diaminobenzidine tetra-hydrochloride substrate for peroxidase. The sections were counterstained with hematoxylin.

Immunoelectron Microscopy

Immunoelectron microscopic observations of kidneys from PAN-treated, PS-treated, and control rats were performed as reported previously (Goto et al, 1998). Briefly, the tissues were fixed with periodate-lysine-paraformaldehyde fixative by perfusion and immersion, and then embedded in glycol methacrylate. Ultrathin sections of the glycol methacrylate-embedded tissues were collected on nickel grids and incubated with 5% nonfat milk for 1 hour and then with the anti-CAR antibody (5 μg/ml) for 3 hours. After washes with PBS, the sections were incubated with gold (15 nm)-labeled anti-rabbit IgG (Amersham Life Sciences, Buckinghamshire, United Kingdom) for 1 hour, washed in PBS several times, and then counterstained with 5% aqueous uranyl acetate and 1% lead citrate for observation by electron microscope (H-600A; Hitachi, Tokyo, Japan).

Primary Culture of Podocytes

Glomeruli were isolated from rat kidneys by the method described previously (Yaoita et al, 2001). Decapsulated glomeruli were selected under an inverted tissue culture microscope with phase-contrast optics and cultured on type I collagen-coated culture dishes in DMEM nutrient mixture F-12 HAM (Sigma), supplemented with ITS liquid media supplement (Sigma), 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell outgrowths from decapsulated glomeruli were used as cultured podocytes. Podocytes cultured on 8-well glass chamber slides were fixed in 2% paraformaldehyde in PBS for 15 minutes, permeabilized with 0.3% Triton X-100 in PBS for 3 minutes, and stained with antibodies. For double-label immunofluorescence microscopy, rabbit anti-connexin43 antibody (Sigma) or anti-CAR antibody in combination with murine mAb against ZO-1 (Zymed Laboratories, South San Francisco, California) were simultaneously applied as primary antibodies. After washing with PBS, the sections were stained with FITC-conjugated anti-rabbit IgG, rewashed with PBS, and subsequently reacted with tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG. Immunofluorescence of the sections and cultured cells were observed with an Olympus microscope (BX50; Tokyo, Japan) equipped with epi-illumination optics and appropriate filters.

Acknowledgements

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