Histochem Cell Biol (2006) 126: 399–406
DOI 10.1007/s00418-006-0175-4

ORIGINAL PAPER

Zilong Li · Nobuhiko Ohno · Nobuo Terada
Shinichi Ohno

Immunolocalization of serum proteins in living mouse glomeruli under various hemodynamic conditions by "in vivo cryotechnique"

Accepted: 10 March 2006 / Published online: 7 April 2006

Abstract Distribution of serum proteins in renal glomeruli is important for histopathology in medical and biological fields, but mechanisms of their passage through glomerular capillary loops (GCL) are still difficult to clarify. We have tried to visualize topographical changes of the serum proteins passing through GCL by "in vivo cryotechnique" in combination with immunohistochemistry. Albumin and immunoglobulin G (IgG), Ig kappa light chain and IgG1 heavy chain were mainly immunolocalized in GCL, but not colocalized with zonula occludens-1 (ZO-1) under normotensive condition. Under heart-arrest condition and in quick-frozen fresh tissues, albumin and kappa light chain were immunolocalized in Bowman's space, indicating their passage caused by the stoppage of blood supply. However, under acute hypertensive condition, they were more clearly immunolocalized along basement membranes and in the Bowman's space, indicating their increased passage through GCL. IgG was also more clearly localized in mesangial areas under acute hypertension, compared with that under the normotensive or heart-arrest condition. This study is the first direct visualization for glomerular passage of serum proteins under abnormal hemodynamic conditions by the "in vivo cryotechnique", and the experimental protocol will be useful for morphofunctional examination of living mouse GCL and immunohistochemical analyses of dynamically changing proteins.

Keywords In vivo cryotechnique · Acute hypertension · Mouse kidney · Immunohistochemistry · Serum proteins

Z. Li was a research fellow from the Department of Nephrology, First Hospital of China Medical University, while this work was in progress at the University of Yamanashi.

Z. Li · N. Ohno · N. Terada · S. Ohno (✉)
Department of Anatomy,
Interdisciplinary Graduate School of Medicine and Engineering,
University of Yamanashi, 1110 Shimokato,
Tamaho, Yamanashi 409-3898, Japan
E-mail: sohno@yamanashi.ac.jp
Tel.: +81-55-2736743
Fax: +81-55-2736743

Z. Li
Department of Nephrology,
First Hospital of China Medical University,
Shenyang, China

Introduction

It was reported that hemodynamic factors, such as blood pressure and flow, exerted an important influence on the native morphology of glomerular capillary loops (GCL) in living mouse kidneys (Ohno et al. 1996a, b, 2001). In addition, they have been supposed to affect glomerular leakage of serum proteins in animal kidneys, which leads to proteinuria under experimental and pathological states, and is also related to progression in human renal diseases (Olivetti et al. 1981; Remuzzi and Bertani 1998). In such abnormal states, hyperfiltrated serum proteins in Bowman's space are usually processed for increased reabsorption in renal proximal tubules of living mouse kidneys (Li et al. 2005). However, both experimental animal studies and clinical human cases have already suggested that excessive amounts of the leaked proteins are harmful to the proximal tubular epithelium, resulting in interstitial inflammation and fibrosis (Gansevoort et al. 1997; Jerums et al. 1997; Remuzzi 1995; Schreiner 1995; Thomas et al. 1999). This concept implicates that the leaked serum proteins may be an important factor for the histopathological progression of chronic renal diseases.

Glomerular filtration barriers are known to be composed of highly fenestrated endothelium, glomerular basement membranes (GBM) and foot processes of podocytes. They always play a role in charge- and/or size-selective barriers against serum proteins (Reiser et al. 2000; Rico et al. 2005). Under normal blood circulation, the main GBM filtration barrier for serum albumin was the central (lamina densa) and outer layers of GBM and that for immunoglobulin G (IgG) was the whole layers of GBM in rat kidneys (Fujigaki et al.

1993). After acute hypertension induced by angiotensin II, there was an increased immunointensity of albumin and IgG in the GBM and urinary space of rat kidneys (Olivetti et al. 1981). Such albumin and IgG were also immunolocalized in mesangial matrix areas (Fujigaki et al. 1993; Olivetti et al. 1981). However, their preparation methods using the in situ drip-fixation with 4% paraformaldehyde suffered from the drawback that the drip-fixation solution caused hemodynamic perturbation of GCL in living rat kidneys and the translocation of serum proteins might be easily induced by their leakage from the blood capillary during the fixation period. Therefore, the renal glomerular selectivity of permeable or non-permeable serum proteins has never been visualized on thin paraffin sections with a light microscope and a confocal laser scanning microscope (CLSM) because of such technical limitations of the specimen preparation.

The common fixatives in buffer solution, such as glutaraldehyde and paraformaldehyde, are mostly effective owing to their cross-linking properties for many substances in animal cells and tissues. However, it was already reported that morphological studies with the routine immersion or perfusion fixation were not able to clarify native structures of functioning kidneys in vivo under normal or abnormal blood circulation (Yu et al. 1998a, b). In addition, they usually need considerable time to finish cross-linking of soluble substances during the perfusion or immersion fixation, and some of them are usually washed out from the fixed specimens. An ultimate goal of our morphological studies is that all features of cells and tissues to be examined should reflect the physiological meaning during animal investigation. Therefore, the in situ preservation of cells and tissues in living animal organs is necessary for the histological studies to define their functioning structures in vivo.

We have already developed the "in vivo cryotechnique", which is designed to arrest transiently dynamic ultrastructures in living animal organs by the combination of a precooled cryoknife with isopentane–propane cryogen (Ohno et al. 1996b, 2004a; Terada et al. 2006). Using the "in vivo cryotechnique", all biological processes in the living animal organs were instantly stopped and embedded in the ice microenvironment, maintaining all their substances in situ (Terada et al. 2005). Then the "in vivo cryotechnique" can also be followed by various preparation steps for morphological analyses (Ohno et al. 2004b). The common freeze-substitution is supposed to cross-link some substances of the quickly frozen cells and tissues in non-aqueous organic solvents containing fixatives at low temperatures, in which diffusion of their soluble molecules would always be limited (Bridgman and Dailey 1989; Liao et al. 2006; Terada and Ohno 2004). The cryofixed and freeze-substituted specimens are then embedded in the paraffin wax, presumably leaving some antigenic sites exposed in the frozen cells and tissues due to the formation of tiny ice crystals (Ohno et al. 2005).

In the present study, topographical distributions of soluble serum proteins in living mouse renal glomeruli were immunohistochemically examined under different hemodynamic conditions by the "in vivo cryotechnique" because their leakage from the GCL and also following reabsorption in the proximal convoluted tubules were assumed to be strictly time-dependent processes under an acute hypertensive condition.

Materials and methods

"In vivo cryotechnique" for living mouse kidneys

Twelve adult C57BL/6 mice, weighing 20–30 g, were anesthetized by intraperitoneal injection of sodium pentobarbital. Then their abdominal cavities were opened, and left kidneys were detected under normal blood circulation, as a control group of four mice. As the first experimental group of four mice, to examine the immunolocalization of albumin and IgG passing through GCL, an experimental model with acute renal hypertension was prepared in the same way as described before (Li et al. 2005; Ohno et al. 1996b; Roman and Cowley 1985; Zhang et al. 1998). To examine the immunolocalization of serum proteins in GCL of mouse kidneys under heart-arrest condition as the second experimental group, blood flow into the kidneys was almost stopped by arresting their beating hearts with intraperitoneal injection of excessive amounts of the anesthetic, as described before (Ohno et al. 2001).

Then the "in vivo cryotechnique" was performed under the three blood flow conditions, as previously reported (Li et al. 2005). Briefly, a cryoknife edge precooled in liquid nitrogen (−196°C) was positioned over the left kidney of living mice. The kidney was immediately cut with the precooled cryoknife, and another liquid isopentane–propane cryogen (−193°C) was simultaneously poured over it with the assistance of the "in vivo cryoapparatus" (IV-11; Eiko Engineer Co., Ibaraki, Japan). The frozen kidneys were carefully trimmed out with a dental electrical drill in liquid nitrogen and then processed for the following freeze-substitution step, as reported before (Ohno et al. 1996a, b, 2001).

Quick-freezing of resected kidney tissues

Left kidney tissues of four living mice under the similar anesthesia were resected out with razor blades and quickly plunged into the isopentane–propane cryogen (−193°C) cooled in liquid nitrogen, as reported before (Zea-Aragon et al. 2004). The frozen specimens were then processed for the following freeze-substitution step.

Freeze-substitution fixation and paraffin embedding

Tissue pieces of the frozen kidneys were freeze-substituted in acetone containing 2% paraformaldehyde (PF) cooled with dry ice–acetone at −80°C for 48 h. They were then serially kept at −20, 4°C and room temperature for 2 h each. They were thoroughly washed in pure acetone at room temperature twice for 2 h each and

transferred into xylene. Finally, they were routinely embedded in the paraffin wax.

Immunostaining on de-paraffinized sections

The paraffin-embedded kidney tissues were cut at 4–5 μm thickness, and the thin sections were de-paraffinized with xylene and a graded series of ethanol. They were first incubated with 5% gelatin (Sigma Co., St Louis, MO, USA) in phosphate-buffered saline (PBS) for 1 h and then with goat anti-mouse albumin (at a dilution of 1:2,000; Bethyl Laboratories, Inc., Montgomery, TX, USA), rabbit anti-mouse IgG whole molecule (IgG; at a dilution of 1:2,000; Bethyl Laboratories, Inc.), goat anti-mouse kappa light chain, goat anti-mouse IgG1 heavy chain (at a dilution of 1:1,000 for both antibodies; Bethyl Laboratories, Inc.) and rabbit anti-zonula occludens-1 (ZO-1; at a dilution of 1:200; Zymed Laboratories, Inc., South San Francisco, CA, USA) antibodies at 4°C overnight. They were then incubated with donkey anti-rabbit IgG antibody coupled to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) at a dilution of 1:1,000 for 1 h. After being washed in PBS, they were also incubated with donkey anti-goat IgG antibody coupled to Alexa Fluor 546 and TO-PRO-3 (Molecular Probes) at a dilution of 1:1,000 each for 1 h. Finally, they were embedded in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) and examined by CLSM (FV1000, Olympus, Japan). Subsequently, the exactly same sections were stained with hematoxylin–eosine (HE) and observed again with a light microscope (BX-61, Olympus)

Results

HE staining with "in vivo cryotechnique"

The common HE staining was performed to examine the native morphology in the living mouse kidneys, as obtained by the "in vivo cryotechnique" (Fig. 1a–c). Well-preserved areas of renal cortical tissues under different hemodynamic conditions were usually obtained within 300–400 μm from the frozen tissue surface without visible ice crystals at a light microscopic level, as already reported (Li et al. 2005). To compare with the "in vivo cryotechnique", quick-freezing of resected kidney tissues followed by freeze-substitution was also performed, as described above (Fig. 1d). The GCL were kept open under normotensive condition (Fig. 1a, arrows), but almost collapsed under heart-arrest condition (Fig. 1b, arrows). On the contrary, they were widely open under acute hypertensive condition (Fig. 1c, arrows), as compared with that under normotensive condition. However, they were most severely collapsed in the conventional specimens prepared by tissue resection followed by quick-freezing (Fig. 1d, arrow). In addition, under acute hypertensive condition, the luminal spaces of proximal (Fig. 1c, P) and distal (Fig. 1c, D) tubules and Bowman's spaces (Fig. 1c, B) were the most clearly opened, as compared with those under the other hemodynamic conditions.

Immunolocalizations of albumin and ZO-1 in glomeruli

The ZO-1 is reported to be immunolocalized in epithelia of the nephron, but the glomerular epithelium is more intensely immunostained than any other epithelia (Fig. 2). So it can be a good tracer for the foot processes (Reiser et al. 2000; Schnabel et al. 1990). To identify whether the serum proteins, such as albumin and immunoglobulin, pass through the filtration barriers of GBM, we used the immunostaining of ZO-1 as a specific marker for the foot processes (Fig. 2b), which was followed by HE staining on the same sections under normotensive condition (Fig. 2a), prepared by the "in vivo cryotechnique". The immunoreactivity of ZO-1 was clearly detected at the sites of foot processes along the GCL (Fig. 2b), in which flowing erythrocytes were observed with HE staining (Fig. 2a). Albumin immunoreaction products were also examined with the double immunofluorescence staining for albumin and ZO-1 to be exclusively localized within or along the GCL under normotensive condition, respectively (Fig. 3a–c), as prepared by the "in vivo cryotechnique". Under heart-arrest condition, albumin was also immunolocalized in the Bowman's space and dotted immunolocalization patterns of ZO-1 were clearly seen along GCL (Fig. 3d). In addition, albumin immunolocalization in the specimens prepared by quick-freezing of resected kidney tissues was almost similarly detected (Fig. 3e). On the contrary, under acute hypertension, albumin immunolocalization was more clearly detected in the Bowman's space and proximal convoluted tubules by the double immunolabeling with ZO-1, indicating that such albumin proteins were passing through the foot process layers (Fig. 3f). We also counted the number of glomeruli where albumin leakage was definitely observed and found that the calculated percentage of leakage was 76.9, 80 and 87.5% of glomeruli under heart-arrest, quick-freezing followed by tissue resection and acute hypertensive conditions, respectively, although no albumin leakage could be observed under normotensive conditions.

Immunolocalizations of albumin and IgG in glomeruli

To directly examine the glomerular permeability to serum proteins, the double immunofluorescence staining for albumin and IgG was performed on paraffin sections of living mouse kidneys prepared by the "in vivo cryotechnique" (Fig. 4). The antibody against IgG detects both IgG whole molecules and free light chains in the kidney tissues. Under normotensive condition (Fig. 4a–c), immunoreaction products of both albumin and IgG were exclusively colocalized along or within the GCL, and only IgG immunolocalization was also seen in some mesangial areas (MA) (Fig. 4c, inset). Neither albumin nor IgG was immunolocalized in Bowman's space (Fig. 4a–c). On the contrary, under heart-

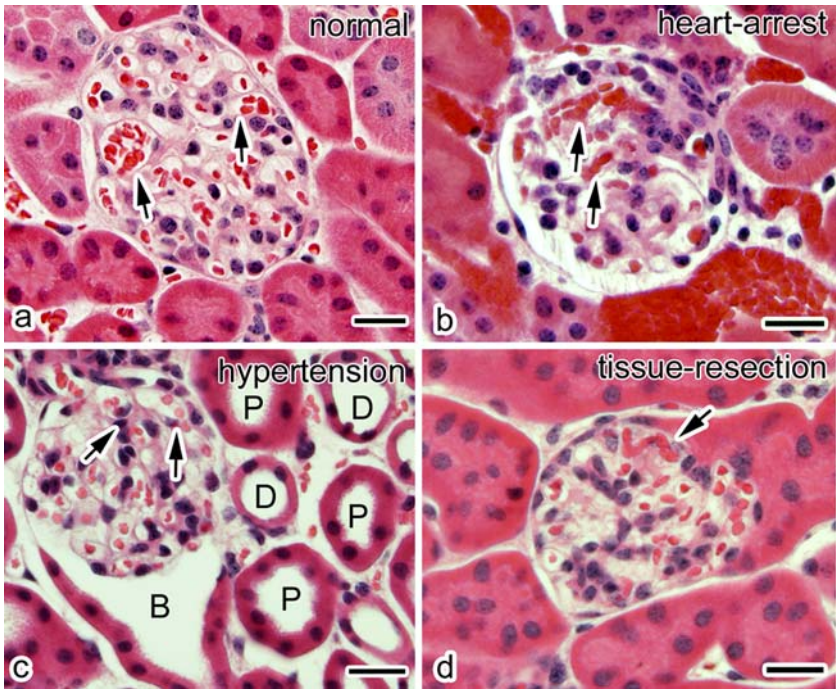

Fig. 1 Light micrographs of mouse renal cortical tissues prepared under various hemodynamic conditions; "in vivo cryotechnique" under normotensive (**a**; *normal*), heart-arrest (**b**; *heart-arrest*) and acute hypertensive (**c**; *hypertension*) conditions and tissue resection followed by quick-freezing (**d**; *tissue resection*). The glomerular capillary loops are kept open under normotensive condition (**a**, *arrows*), but almost collapsed under heart-arrest condition (**b**, *arrows*). They are widely open under acute hypertensive condition (**c**, *arrows*), as compared with those under normotensive condition. However, they are most severely collapsed in the specimens prepared by tissue resection followed by quick-freezing (**d**, *arrow*). The luminal spaces of proximal (**c**; *P*) and distal (**c**; *D*) tubules and Bowman's spaces (**c**; *B*) under acute hypertensive condition are more clearly opened, as compared with those under the other hemodynamic conditions (**a**, **b**, **d**). *Scale bars*: 20 μm

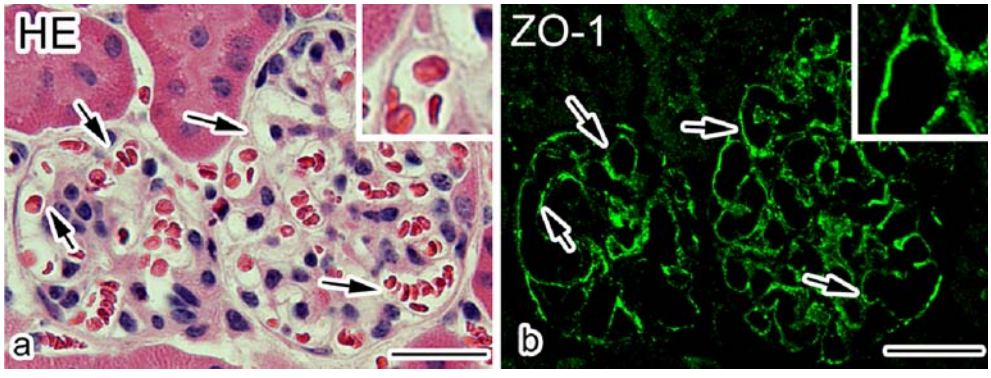

Fig. 2 Light micrographs, showing hematoxylin–eosine staining (*HE*; **a**) and immunofluorescence staining for zonula occludens-1 (*ZO-1*; **b**) on exactly the same section, prepared under normotensive condition by the "in vivo cryotechnique". The ZO-1 is clearly immunolocalized around the glomerular capillary loops (**b**, *arrows*), in which flowing erythrocytes are definitely observed with HE staining (**a**, *arrows*). *Scale bars*: 20 μm

arrest condition (Fig. 4d) and by quick-freezing after tissue resection (Fig. 4e), immunoreaction products of both albumin and IgG were found to be localized in the Bowman's space (Fig. 4d, e), indicating the rapid translocation of serum proteins under ischemia or anoxia. Under acute hypertensive condition (Fig. 4f), however, IgG immunostaining was more clearly detected in MA than that under normotensive and heart-arrest conditions. In addition, the immunostaining of both albumin and IgG was seen in some Bowman's spaces (Fig. 4f, right lower inset). We have also confirmed their reabsorption into the proximal convoluted tubules in a similar way (Fig. 4f), as previously reported (Li et al. 2005).

Immunolocalizations of ZO-1 and kappa light chain or IgG1 heavy chain in glomeruli

The antibody against kappa light chain detects IgG whole molecules and free kappa light chains, whereas another antibody against IgG1 heavy chain visualizes only IgG whole molecules. Under normotensive condition, both kappa light chain and IgG1 heavy chain were mostly immunolocalized within GCL and MA, as shown in Fig. 5a and b. To examine whether other IgG proteins with the full molecular weight were leaked out through GBM into Bowman's spaces under acute hypertensive condition, further immunostaining analyses for the

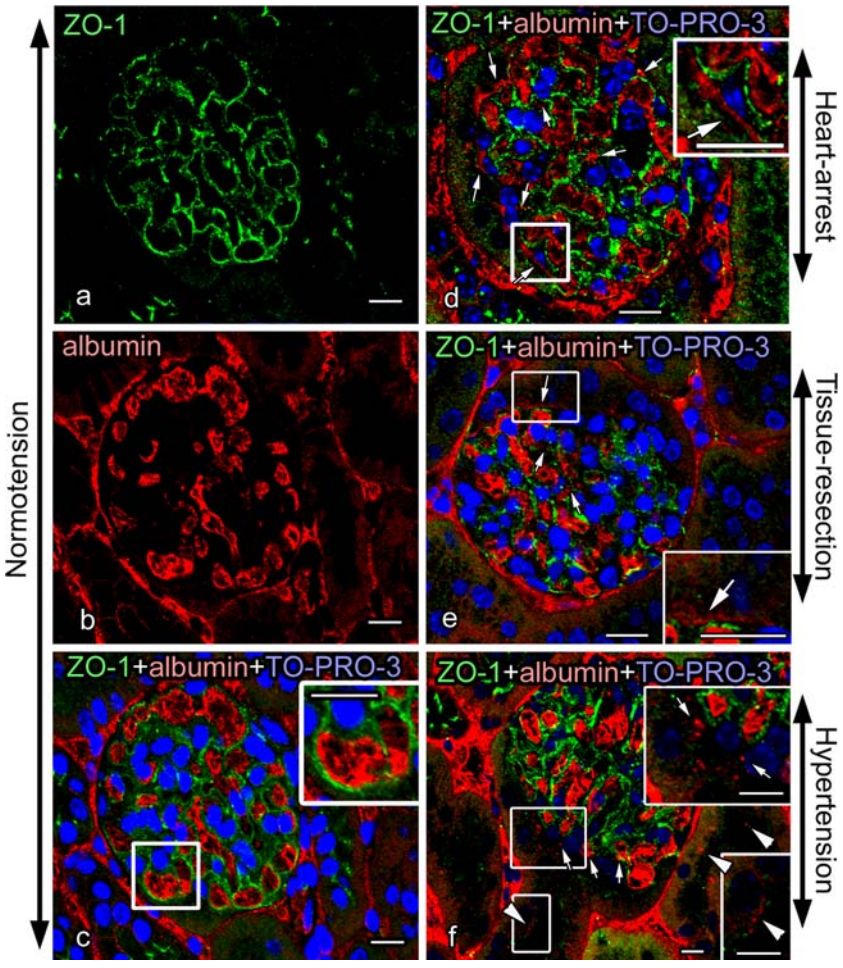

Fig. 3 Confocal laser scanning micrographs, demonstrating double-fluorescence immunolocalizations of zonula occludens-1 (*ZO-1*; *green color*) and albumin (*albumin*; *red color*). Under normotensive condition (**a**–**c**; *normotension*), albumin immunolocalization is primarily restricted within the glomerular capillary loops (**b**), and its immunoreactivity could not be detected in the Bowman's space nor did it overlap with the linear immunostaining of ZO-1 (**c**). Under heart-arrest condition (**d**; *heart-arrest*) and in the kidney tissues quickly frozen after tissue resection (**e**; *tissue resection*), albumin is also immunolocalized in the Bowman's space (**d** and **e**, *arrows*). Under acute hypertension (**f**; *hypertension*), dotted immunoreaction products of albumin in the Bowman's space are also confirmed by the immunolabeling of ZO-1 (**f**, *arrows*). The TO-PRO-3 (*blue color*) is used for nuclear fluorescence staining. *Scale bars*: 10 μm

kappa light chain or the IgG1 heavy chain were performed on serial paraffin sections (Fig. 5c, d). Alternative immunoreaction products of both kappa light chain and ZO-1 were clearly seen along some GCL (Fig. 5c, insets), but not those of IgG1 and ZO-1 (Fig. 5d). The kappa light chain was also found to be immunolocalized in Bowman's spaces (data not shown) and apical cytoplasm of almost all proximal tubules, as already reported (Li et al. 2005). Additionally, the immunoreactivity of kappa light chain was observed in MA (Fig. 5c, arrows). On the contrary, the immunoreactivity of IgG1 heavy chain was detected exclusively within the GCL and MA, but not in the Bowman's space. These findings indicate that immunoglobulin light chains, but not IgG with the full molecular length, were more easily leaked out under acute hypertensive condition.

Discussion

The main purpose of "in vivo cryotechnique" is to promptly immobilize all biological components of living animal organs in tiny ice crystals, which are followed by various analyses on the frozen samples, demonstrating the native morphology of functioning cells and tissues (Ohno et al. 2004a; Terada et al. 2006). In the present study, we have shown that the "in vivo cryotechnique" followed by freeze-substitution could visualize not only the morphology of living mouse kidneys under various hemodynamic conditions, but also the immunolocalization of serum proteins in their glomeruli. The "in vivo cryotechnique" is quick enough to arrest transient physiological processes of the living mouse kidneys under various hemodynamic conditions, which can overcome the technical problems of conventional fixation processes. In addition, the "in vivo cryotechnique" in combination with immunofluorescence microscopy has become a powerful tool to visualize the immunolocalization of various soluble molecules in the living mouse glomeruli. The fluorescence-labeled antibodies for the common CLSM are often used for obtaining high-resolution images of molecular localizations (Matsuzaki et al. 1997). In the present study, the usage of TO-PRO-3 for nuclear staining and anti-ZO-1 antibody to obtain the images with triple-fluorescence colors could clearly show the dynamically changing immunodistribution of serum proteins in GCL, MA and Bowman's spaces under various hemodynamic conditions.

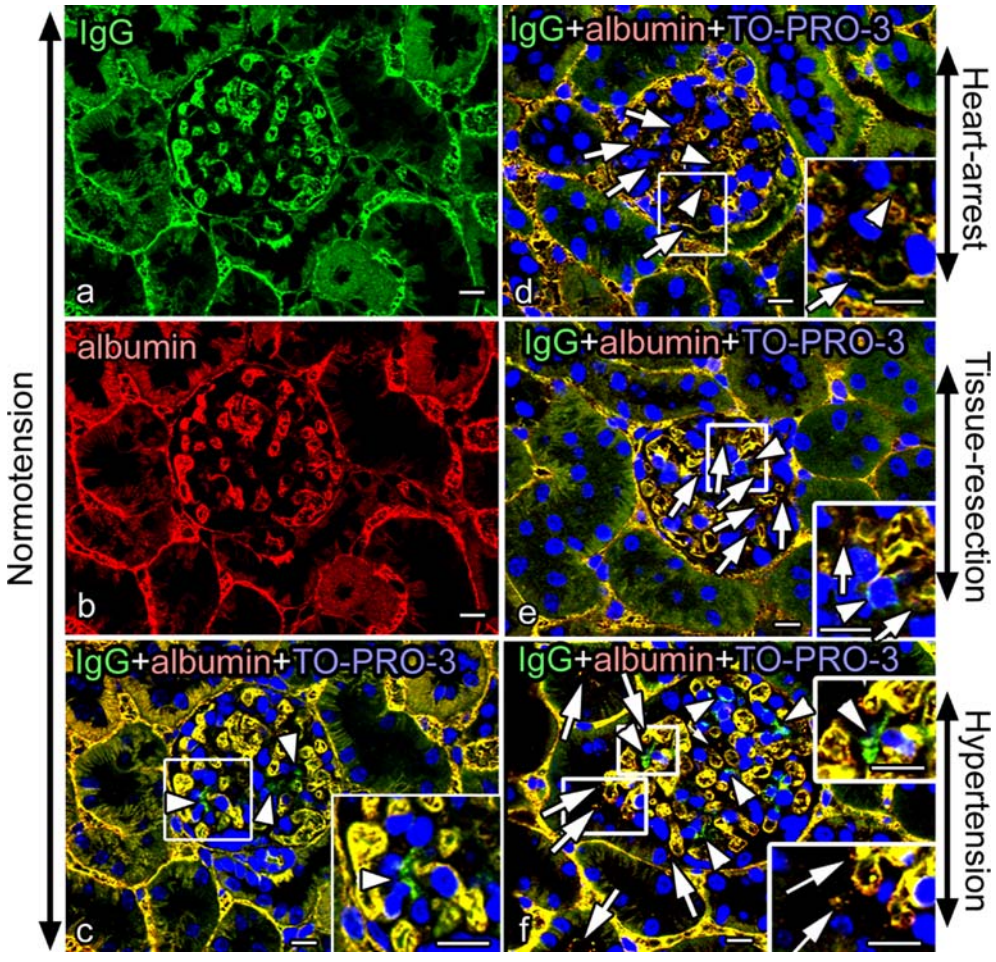

Fig. 4 Confocal laser scanning micrographs, showing immunolocalizations of IgG with whole molecular weight (**a**; *IgG*; *green color*) and albumin (**b**; *red color*) in the renal glomeruli, obtained by the double immunofluorescence staining. Under normotensive condition (**a**–**c**; *normotension*), both IgG and albumin immunolocalizations are detected within the glomerular capillary loops, and only IgG is also immunolocalized in the mesangial areas (MA) (**c**, *arrowheads*). Under heart-arrest condition (**d**; *heart-arrest*) and tissue resection followed by the quick-freezing method (**e**; *tissue resection*), the immunoreaction products of both IgG and albumin are also found in the Bowman's space (**d** and **e**, *arrows*), and only IgG is slightly immunolocalized in MA (**d** and **e**, *arrowheads*). Under acute hypertensive condition (**f**; *hypertension*), large clumped immunoreaction products of IgG are more clearly seen in MA (**f**, *arrowheads*). The rapid translocation of both albumin and IgG is detected in some Bowman's spaces, and their reabsorption is also detected in the proximal convoluted tubules (**f**, *arrows*). *Scale bars*: 10 μm

The present study demonstrated that serum albumin and IgG were usually kept within GCL under normal hemodynamic condition, except for the IgG in some MA, and neither albumin nor IgG was immunolocalized in the Bowman's space of the living mouse glomeruli. However, under acute hypertensive condition, the immunolocalization of IgG1 heavy chain was more clearly detected in MA than that under normal hemodynamic condition, and the increased immunoreactivity of IgG in MA might be attributed to the acutely increased pressure in GCL by ligation of aorta at a distal point of branching renal arteries, as reported before (Li et al. 2005). These findings are different from the previous observation with the conventional preparation methods that the mesangial matrix contains small amounts of albumin and IgG, which might be due to the conventional fixation procedures (Olivetti et al. 1981; Ryan et al. 1976; Ryan and Karnovsky 1976).

The leakage of albumin and kappa light chain into the Bowman's space was increased under acute hypertensive condition, as also suggested by their reabsorption into the proximal tubules (Li et al. 2005). Moreover, their immunolocalization changes in the glomeruli were also due to the increased pressures in GCL, easily causing their passage through GBM. From our findings, the serum proteins' leakage and their reabsorption usually occurred within 10 min under the experimentally acute hypertension because higher pressures in the GCL would mechanically change the barrier structures of molecular sieves in the GBM, as already examined at an electron microscopic level by the "in vivo cryotechnique" (Ohno et al. 1996a, b, 2001). Thus, higher pressures would impair the size-selective barrier functions of GBM and slit diaphragm, and then we could detect the leakage of serum proteins with low molecular weights, such as albumin and kappa light chain, which in turn caused their reabsorption in the proximal tubules (Batuman et al. 1990; Batuman and Guan 1997; Birn et al. 2000; Brunskill 2000; Khamlichi et al. 1995; Li et al. 2005; Olivetti et al. 1981; Remuzzi and Bertani 1998; Ryan et al. 1976; Ryan and Karnovsky 1976). Some IgGs with whole molecular weight were reported to be extensively trapped in the GBM under pathological conditions, such as membranoproliferative glomerulonephritis, membranous nephropathy and lupus nephritis (Bijl et al. 2002; Imai et al. 1997). In such cases, they may be hardly leaked out into Bowman's spaces, because of large molecular sizes, conformational changes and immune complex formation.

The ischemia or hypoxia, such as the hypotensive condition, was reported to induce some changes in glomerular

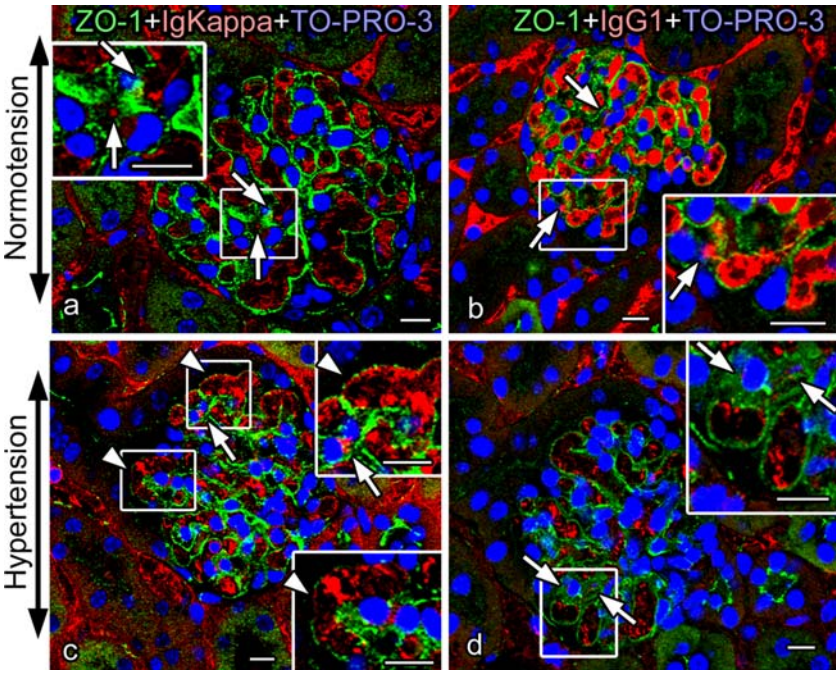

Fig. 5 Light micrographs, showing double immunofluorescence stainings of zonula occludens-1 (*ZO-1*; *green color*) and kappa light chain (*IgKappa*; **a** and **c**; *red color*) or IgG1 heavy chain (*IgG1*; **b** and **d**; *red color*) in the kidney sections under normotensive (**a** and **b**; *normotension*) or hypertensive (**c** and **d**; *hypertension*) conditions prepared by the "in vivo cryotechnique". Under normotensive condition, both kappa light chain and IgG1 heavy chain are exclusively immunolocalized within the glomerular capillary loops (GCL) and mesanginal areas (MA) (**a** and **b**, *arrows*). Under acute hypertensive condition, the immunostainings for both kappa light chain and IgG1 are observed in MA (**c** and **d**, *arrows*), but the immunoreaction products of only kappa light chain are seen between the dotted immunoreaction products of ZO-1 lining around GCL (**c**, *arrowheads*). *Insets*: highly magnified views of some GCL. **c** and **d** were obtained from two serial sections of the same glomerulus. *Scale bars*: 10 μm

structures and also damages of renal microvascular cell–cell junctions, which then contribute to the increased vascular permeability and local interstitial edema (Griffith et al. 1967; Pagtalunan et al. 1999; Sutton et al. 2003). In our study, the leakage of both albumin and IgG from the GCL into Bowman's spaces could be detected under heart-arrest condition. In addition, their immunolocalization in the conventional specimens prepared by quick-freezing of the resected kidney tissues was almost similar to that observed under heart-arrest condition. The rapid translocation of serum proteins from the GCL into Bowman's spaces may be caused by lower blood pressures, ischemia and/or serious hypoxia before quick-freezing (Fujigaki et al. 1993; Zea-Aragon et al. 2004).

In conclusion, the "in vivo cryotechnique" followed by the freeze-substitution method would be a reliable tool to keep soluble serum proteins in situ and provide transient images of functioning glomeruli in the living mice. In addition, it also allowed us to examine the precise immunodistribution of serum proteins leaked out of the glomeruli and their reabsorption into the renal proximal tubules under various hemodynamic conditions.

Acknowledgements We would like to thank Drs. T. Baba and Y. Fujii, Department of Anatomy, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, for their constructive comments and technical support in this work.

References

Batuman V, Guan S (1997) Receptor-mediated endocytosis of immunoglobulin light chains by renal proximal tubule cells. Am J Physiol 272:F521–F530

Batuman V, Dreisbach AW, Cyran J (1990) Light-chain binding sites on renal brush-border membranes. Am J Physiol 258:F1259–F1265

Bijl M, Dijstelbloem HM, Oost WW, Bootsma H, Derksen RH, Aten J, Limburg PC, Kallenberg CG (2002) IgG subclass distribution of autoantibodies differs between renal and extra-renal relapses in patients with systemic lupus erythematosus. Rheumatology 41:62–67

Birn H, Fyfe JC, Jacobsen C, Mounier F, Verroust PJ, Orskov H, Willnow TE, Moestrup SK, Christensen EI (2000) Cubilin is an albumin binding protein important for renal tubular albumin reabsorption. J Clin Invest 105:1353–1361

Bridgman PC, Dailey ME (1989) The organization of myosin and actin in rapid frozen nerve growth cones. J Cell Biol 108:95–109

Brunskill NJ (2000) Albumin and proximal tubular cells—beyond endocytosis. Nephrol Dial Transplant 15:1732–1734

Fujigaki Y, Nagase M, Kobayasi S, Hidaka S, Shimomura M, Hishida A (1993) Intra-GBM site of the functional filtration barrier for endogenous proteins in rats. Kidney Int 43:567–574

Gansevoort RT, Navis GJ, Wapstra FH, de Jong PE, de Zeeuw D (1997) Proteinuria and progression of renal disease: therapeutic implications. Curr Opin Nephrol Hypertens 6:133–140

Griffith LD, Bulger RE, Trum BF (1967) The ultrastructure of the functioning kidney. Lab Invest 16:220–246

Imai H, Hamai K, Komatsuda A, Ohtani H, Miura AB (1997) IgG subclasses in patients with membranoproliferative glomerulonephritis, membranous nephropathy, and lupus nephritis. Kidney Int 51:270–276

Jerums G, Panagiotopoulos S, Tsalamandris C, Allen TJ, Gilbert RE, Comper WD (1997) Why is proteinuria such an important risk factor for progression in clinical trials? Kidney Int Suppl 63:S87–S92

Khamlichi AA, Rocca A, Touchard G, Aucouturier P, Preud'homme JL, Cogne M (1995) Role of light chain variable region in myeloma with light chain deposition disease: evidence from an experimental model. Blood 86:3655–3659

Li Z, Terada N, Ohno N, Ohno S (2005) Immunohistochemical analyses on albumin and immunoglobulin in acute hypertensive mouse kidneys by "in vivo cryotechnique". Histol Histopathol 20:807–816

Liao X, Terada N, Ohno N, Li Z, Fujii Y, Baba T, Ohno S (2006) Immunohistochemical study of serum albumin in normal and cadmium-treated mouse testis organs by "in vivo cryotechnique". Histol Histopathol 21:35–40

Matsuzaki T, Suzuki T, Fujikura K, Takata K (1997) Nuclear staining for laser confocal microscopy. Acta Histochem Cytochem 30:309–314

Ohno S, Baba T, Terada N, Fujii Y, Ueda H (1996a) Cell biology of kidney glomerulus. Int Rev Cytol 166:181–230

Ohno S, Terada N, Fujii Y, Ueda H, Takayama I (1996b) Dynamic structure of glomerular capillary loop as revealed by an in vivo cryotechnique. Virchows Arch 427:519–527

Ohno S, Kato Y, Xiang T, Terada N, Takayama I, Fujii Y, Baba T (2001) Ultrastructural study of mouse renal glomeruli under various hemodynamic conditions by an "in vivo cryotechnique". Ital J Anat Embryol 106:431–438

Ohno N, Terada N, Fujii Y, Baba T, Ohno S (2004a) "In vivo cryotechnique" for paradigm shift to "living morphology" of animal organs. Biomed Rev 15:1–19

Ohno N, Terada N, Ohno S (2004b) Advanced application of the in vivo cryotechnique to immunohistochemistry for animal organs. Acta Histochem Cytochem 37:357–364

Ohno N, Terada N, Murata S, Katoh R, Ohno S (2005) Application of cryotechniques with freeze-substitution for the immunohistochemical demonstration of intranuclear pCREB and chromosome territory. J Histochem Cytochem 53:55–62

Olivetti G, Kithier K, Giacomelli F, Wiener J (1981) Glomerular permeability to endogenous proteins in the rat: effects of acute hypertension. Lab Invest 44:127–137

Pagtalunan ME, Olson JL, Tilney NL, Meyer TW (1999) Late consequences of acute ischemic injury to a solitary kidney. J Am Soc Nephrol 10:366–373

Reiser J, Kriz W, Kretzler M, Mundel P (2000) The glomerular slit diaphragm is a modified adherens junction. J Am Soc Nephrol 11:1–8

Remuzzi G (1995) Abnormal protein traffic through the glomerular barrier induces proximal tubular cell dysfunction and causes renal injury. Curr Opin Nephrol Hypertens 4:339–342

Remuzzi G, Bertani T (1998) Pathophysiology of progressive nephropathies. N Engl J Med 339:1448–1456

Rico M, Mukherjee A, Konieczkowski M, Bruggeman LA, Miller RT, Khan S, Schelling JR, Sedor JR (2005) WT1-interacting protein and ZO-1 translocate into podocyte nuclei after puromycin aminonucleoside treatment. Am J Physiol Renal Physiol 289:F431–F441

Roman RJ, Cowley AW Jr (1985) Characterization of a new model for the study of pressure-natriuresis in the rat. Am J Physiol 248:F190–F198

Ryan GB, Karnovsky MJ (1976) Distribution of endogenous albumin in the rat glomerulus: role of hemodynamic factors in glomerular barrier function. Kidney Int 9:36–45

Ryan GB, Hein SJ, Karnovsky MJ (1976) Glomerular permeability to proteins. Effects of hemodynamic factors on the distribution of endogenous immunoglobulin G and exogenous catalase in the rat glomerulus. Lab Invest 34:415–427

Schnabel E, Anderson JM, Farquhar MG (1990) The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium. J Cell Biol 111:1255–1263

Schreiner GF (1995) Renal toxicity of albumin and other lipoproteins. Curr Opin Nephrol Hypertens 4:369–373

Sutton TA, Mang HE, Campos SB, Sandoval RM, Yoder MC, Molitoris BA (2003) Injury of the renal microvascular endothelium alters barrier function after ischemia. Am J Physiol Renal Physiol 285:F191–F198

Terada N, Ohno S (2004) Immunohistochemical application of cryotechniques to native morphology of cells and tissues. Acta Histochem Cytochem 37:339–345

Terada N, Ohno N, Li Z, Fujii Y, Baba T, Ohno S (2005) Detection of injected fluorescence-conjugated IgG in living mouse organs using "in vivo cryotechnique" with freeze-substitution. Microsc Res Tech 66:173–178

Terada N, Ohno N, Li Z, Fujii Y, Baba T, Ohno S (2006) Application of in vivo cryotechnique to the examination of cells and tissues in living animal organs. Histol Histopathol 21:265–272

Thomas ME, Brunskill NJ, Harris KP, Bailey E, Pringle JH, Furness PN, Walls J (1999) Proteinuria induces tubular cell turnover: a potential mechanism for tubular atrophy. Kidney Int 55:890–898

Yu Y, Leng CG, Kato Y, Terada N, Fujii Y, Ohno S (1998a) Ultrastructural study of anionic sites in glomerular basement membranes at different perfusion pressures by quick-freezing and deep-etching method. Nephron 78:88–95

Yu Y, Leng CG, Terada N, Ohno S (1998b) Scanning electron microscopic study of the renal glomerulus by an in vivo cryotechnique combined with freeze-substitution. J Anat 192:595–603

Zea-Aragon Z, Terada N, Ohno N, Fujii Y, Baba T, Ohno S (2004) Effects of anoxia on serum immunoglobulin and albumin leakage through blood–brain barrier in mouse cerebellum as revealed by cryotechniques. J Neurosci Methods 138:89–95

Zhang Y, Magyar CE, Norian JM, Holstein-Rathlou NH, Mircheff AK, McDonough AA (1998) Reversible effects of acute hypertension on proximal tubule sodium transporters. Am J Physiol 274:C1090–C1100