Histol Histopathol (2007) 22: 137-145

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Immunohistochemical analyses on serum proteins in nephrons of protein-overload mice by "in vivo cryotechnique"

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Summary. Immunohistochemical analyses on local distributions of serum proteins in living mouse kidneys are usually difficult to examine with conventional preparation methods. By using our "in vivo cryotechnique" combined with freeze-substitution, we have checked immunolocalizations of the serum proteins in nephrons of bovine serum albumin (BSA)-overload mice, and compared them with those obtained by the conventional preparation methods. In two days of daily BSA-injected mice, the immunolocalization of BSA could be observed in Bowman's space and urinary tubules with their overt proteinuria, where another endogenous mouse albumin was similarly immunolocalized. The leakage of BSA and mouse albumin in Bowman's space and their reabsorption into proximal tubules were detected in 55% of nephrons, where no leakage of immunoglobulin G1 (IgG1) was detected. However, the leakage of IgG1, in addition to BSA and mouse albumin, was detected in the other nephrons. By carefully examining immunolocalizations of BSA and IgG1, they were obviously different from those obtained by the conventional preparation methods without normal blood circulation into the kidneys. The immunolocalizations of both BSA and mouse serum proteins could be directly analyzed with the "in vivo cryotechnique", suggesting that functional damage to glomerular filtration barriers are different at early stages of the BSA-overload mouse model, depending on each nephron of living mice.

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Key words: Immunohistochemistry, In vivo cryotechnique, Bovine serum albumin, Protein-overload mouse, Proteinuria

Introduction

The proteinuria of clinical patients is well known to be a common feature of chronic renal diseases. Under physiological conditions, only a small fraction of intermediate-molecular-weight (IMW) proteins (molecular weight >40kD and <100kD), such as albumin, can pass through the glomerular filtration barrier and temporarily localize in Bowman's space (Chang et al., 1975a,b). Then, such IMW or lower molecular-weight (LMW, molecular weight <40kD) proteins are almost completely reabsorbed in the proximal tubular epithelial cells (Tojo and Endou, 1992). Under pathological conditions, a moderately increased permeability of glomerular barrier functions probably causes more increased transglomerular passage of IMW proteins, including albumin, which is often not accompanied with that of higher molecular-weight (HMW) proteins, such as immunoglobulin G (IgG). When the structural damage of glomerular filtration barriers progresses under severely pathological conditions, the HMW proteins also leak out and reach the proximal tubular lumen, resulting in their obvious excretion in the urine (D'Amico and Bazzi, 2003). Although the pathophysiology of proteinuria has been elaborated in many published experiments, immunohistochemical analyses on the in situ distributions of various serum proteins in proteinuric animal kidneys are difficult to make by the conventional preparation methods, because of their common technical

problems, such as immersion- or perfusion- fixation.

The morphology and molecular localization obtained by conventional preparation methods always contain some inevitable technical artifacts caused by resection for immersion-fixation, perfusion with fixatives and dehydration with organic solvents (Ohno et al., 2004a). On the contrary, the "in vivo cryotechnique" followed by freeze-substitution can directly cryofix living animal organs, and stabilize soluble proteins of cells and tissues for preservation of their antigenicity (Terada and Ohno, 2004; Ohno et al., 2005a; Liao et al., 2006; Terada et al., 2006a,b). With the "in vivo cryotechnique", morphological analyses of glomerular capillary loops and immunohistochemical analyses of soluble serum proteins have already been performed in living mouse kidneys under various hemodynamic conditions (Ohno et al., 1996; Li et al., 2005).

It has been reported that bovine serum albumin (BSA)-overload animal models can easily show proteinuria within a few days of its administration, and some morphological changes of glomerular epithelial cells, but not immune-complex deposition in glomeruli, are related to the severe proteinuria in these animal models (Simpson and Shand, 1983; Davies et al., 1985; Weening et al., 1987; Eddy, 1989). Although some protein reabsorption droplets of albumin and IgG were described in the tubular epithelial cells of BSA-overload rats (Weening et al., 1987; Eddy, 1989), there has been no in situ examination about immunolocalizations of various serum proteins in such proteinuric animal models. By applying the "in vivo cryotechnique" followed by freeze-substitution, we have analyzed the immunohistochemical distributions of exogenous BSA or endogenous serum proteins in the BSA-overload mouse model, compared with those obtained by conventional preparation methods.

Materials and methods

BSA-overload mouse model

Twenty 8-week-old female C57BL/6 mice, weighing 20~30g, were used in the present study. Twelve experimental mice received two injections of bovine serum albumin (low endotoxin BSA: Sigma, St. Louis, MO) at a dose of 20mg/g body weight (Suzuki et al., 2001), once daily for two days. The BSA was routinely dissolved in half saline (400mg/ml), and then directly injected into the peritoneal cavity, as reported before (Eddy, 1989). Other control mice were treated with an intraperitoneal injection of an equivalent volume of half saline. They were all sacrificed 5~6 hours after the last injection on the second day.

Semiquantitive measurement of urinary protein

Spot mouse urine samples were collected always before the first injection and just before the sacrifice time. The concentrations of urinary protein were semiquantitively determined with urine checking strips commercially available, according to the instructions of the manufacturer (#413-90201, Wako, Osaka, Japan), and graded from (-) to (4+), including their intermediates.

Dot blot analyses of mouse urine samples

The mouse urine samples and BSA or mouse albumin solution (FITC-conjugated; Inter-cell technologies, Jupiter, FL, USA) were spotted on a piece of nitrocellulose membrane (Millipore, Billerica, MA, USA). Following the wash in PBS containing 0.05% Tween 20 (PBS-T), the membrane was blocked with 1.5% cold fish gelatin (Sigma Co, St. Louis, MO, USA) in PBS-T for 1 hour, and incubated with a specific antibody obtained from sheep against BSA (Bethyl Laboratories, Montgomery, TX, USA) for 1 hour. The antibody was visualized by incubation in biotin-conjugated donkey anti-sheep IgG antibody (Jackson Immunoresearch laboratories, West Grave, PA, USA) for 1 hour and horse-radish-peroxidase (HRP)-conjugaed streptavidin (Pierce, Rockford, IL, USA) for 1 hour, and following reaction with an enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA). Subsequently, to detect mouse albumin in each urine sample, the signals were removed by incubation in Re-Blot Plus Western Blot Recycling Kit (Chemicon, Temecula, CA, USA) for 15 minutes, and reblotted with rabbit anti-mouse albumin antibody (Bethyl Laboratories, Montgomery, TX, USA), an HRP-conjugated goat anti-rabbit IgG antibody (Vector Laboratories Inc., Burlingame, CA, USA) and the enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA).

The "in vivo cryotechnique" followed by freeze-substitution

Experimental or control mice were anesthetized with intraperitoneal injection of sodium pentobarbital, and kidney specimens of the living mice were obtained under the "in vivo cryoapparatus" (IV-11, Eiko Engineering, Ibaraki, Japan) by the "in vivo cryotechnique", as previously described (Ohno et al., 2004a). Briefly, while their hearts were normally beating, exposed left kidneys were cut with a cryoknife precooled in liquid nitrogen (-196°C) as quickly as possible and simultaneously poured with another isopentane-propane cryogen (-193°C). Then the frozen kidney tissues were trimmed out with a dental electrical drill in liquid nitrogen and processed for the following freeze-substitution.

The frozen specimens were freeze-substituted in acetone containing 2% paraformaldehyde at about –80°C for 24 hours. The 2% paraformaldehyde in acetone solution was prepared, as described before (Ohno et al., 2004c). Then the freeze-substitution solution with the specimens were kept at –20°C for 2 hours, at –4°C for 2 hours, and finally at room temperature for 2 hours. They were washed in pure acetone, transferred into xylene,

and routinely embedded in paraffin wax, as previously described (Li et al., 2005).

Resection of kidney tissues for immersion-fixation or quick-freezing

Fresh kidney tissues of anesthetized mice were partially resected out with razor blades. Some specimens were immersed with 2% paraformaldehyde in 0.1M phosphate buffer, pH7.4, at 4°C overnight, dehydrated with a graded series of ethanol, and routinely embedded in the paraffin wax, as described before (Ohno et al., 2004b). Others were quickly frozen by plunging into the isopentane-propane cryogen (-193°C) as described before (Ohno et al., 2004c), routinely freeze-substituted, and similarly embedded.

Perfusion-fixation followed by immersion-fixation or quick-freezing

The anesthetized mice were transcardially perfused with 2% paraformaldehyde in 0.1M phosphate buffer, pH7.4, and then the fixed kidney tissues were resected with razor blades. Some specimens were additionally immersed in the same fixative at 4°C for 1 hour, dehydrated with a graded series of ethanol and embedded in the paraffin wax, as described above. Others were quickly frozen, similarly freeze-substituted and embedded, as described above.

Peroxidase-immunostaining with avidin-biotin complex

The paraffin-embedded kidney tissues were cut at 4-μm thickness and mounted on glass slides. After deparaffinizing with xylene and ethanol, some thin sections were routinely stained with hematoxylin-eosine (HE). Others were treated with 1% hydrogen peroxide in phosphate buffer saline (PBS) to block the activity of endogenous peroxidase for 1 hour. After washing in PBS, they were incubated with 1% gelatin (Sigma Co, St. Louis, MO, USA) in PBS and treated with an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) to reduce the non-specific background staining, as described before (Li et al., 2005). Subsequently, they were immunostained with various primary antibodies at 4°C overnight. The primary antibodies were as follows: sheep anti-bovine albumin antibody, goat anti-mouse albumin antibody not cross-reacted with BSA, goat anti-mouse immunoglobulin G1 heavy chain (IgG1) antibody and goat anti-mouse kappa light chain (Kappa) antibody. All the primary antibodies in peroxidase-immunostaining were purchased from Bethyl Laboratories (Montgomery, TX, USA). After washing in PBS, the immunostained sections were incubated with biotin-conjugated donkey anti-sheep IgG (Jackson Immunoresearch laboratories, West Grave, PA, USA) or biotin-conjugated rabbit anti-goat IgG (Vector Laboratories Inc., Burlingame, CA, USA) at room temperature for 1 hour. After washing in PBS, the immunostained sections were visualized with peroxidase-DAB method and postfixed, as described before (Ohno et al., 2005b). They were counter-stained with methyl green, coverslipped with glycerol and observed with a light microscope (BX-61, Olympus, Tokyo, Japan). For statistical analyses, glomeruli and proximal tubules with immunoreaction DAB-products were counted, as shown with percentage, on the image at a magnification of x400.

Double immunofluorescence staining and PAS-staining combined with immunofluorescence staining

Some sections for PAS staining were deparaffinized and then routinely rinsed in 0.5% periodic acid solution, Schiff's reagent (Muto Pure Chemical Co., Tokyo, Japan) and 0.5% sodium pyrosulfite solution for PAS-staining. After washing in PBS, they were additionally immunostained with the sheep anti-bovine albumin antibody at 4°C overnight. After washing again, they were incubated with the biotin-conjugated donkey anti-sheep IgG followed by Alexa-488 conjugated streptavidin (Invitrogen Corp., Carlsbad, CA, USA). The other sections for double immunofluorescence staining were deparaffinized, treated with the avidin-biotin blocking kit, blocked with 1% gelatin, and incubated in a mixture of the anti-BSA antibody (Bethyl Laboratories, Montgomery, TX, USA) and a rabbit polyclonal anti-ZO-1 antibody (Zymed Laboratories, South San Francisco, CA, USA). The antibodies were visualized with incubation in biotin-conjugated donkey anti-sheep IgG antibody (Jackson Immunoresearch laboratories, West Grave, PA, USA), and following incubation in an Alexa488-conjugated donkey anti-rabbit IgG antibody and Alexa546-conjugated streptavidin. The nuclei were counter-stained with TO-PRO-3 (Invitrogen Corp., Carlsbad, CA, USA). The immunostained sections were coverslipped with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Specific fluorescent red-color signals of PAS-staining and green fluorescence of BSA immunostaining were observed under the FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Results

Determination of urinary protein

As shown in Figure 1a, concentrations of mouse urinary proteins, which were checked with urine checking strips before injection, were (+)~(2+) grades, and almost similar to those in the previous report (Chen et al., 1995). In the experimental BSA-overload mice, the urinary proteins were increased from the grades to (3+)~(4+) and most of the increased grades were two scales or more. Thus, overt proteinuria occurred in the C57BL/6 mice after 2 days of daily BSA-injection. In the control mice, the urinary proteins were unchanged or slightly decreased due to the injection of half saline (Fig.

1a). The dot blot analyses were used to confirm the appearance of BSA in the mouse urine. As shown in Figure 1b~d, BSA could be detected in the urine samples of BSA-injected mice, but not in those of control mice. On the contrary, mouse albumin could be detected in all examined urine samples (Fig. 1d).

BSA immunolocalizations in nephrons of living mice

To elucidate the in situ immunolocalizations of exogenous BSA in the living mouse kidneys, serial thin sections of the experimental BSA-overload mouse kidney tissues, as prepared with the "in vivo cryotechnique", were either stained with HE (Fig. 2a) or immunostained for BSA (Fig. 2b,d). Many flowing erythrocytes could be seen inside the well-preserved glomerular capillary loops and interstitial blood vessels of living mouse kidneys, in which the exogenous BSA was clearly immunostained (Fig. 2a). The basal striation areas of proximal convoluted tubules were also clearly immunostained (Fig. 2b,c, large arrows). Moreover, BSA was slightly detected in Bowman's space (Fig. 2b,d, arrowheads), along the apical cytoplasm of proximal convoluted tubules and also in tubular lumens (Fig. 2b,c, small arrows). There was no positive immunostaining of BSA in the prepared specimens of control mice without the BSA injection (Fig. 2a,b, insets), indicating that the BSA immunostaining was specific and not cross-reacted with the mouse albumin, as expected. In the double fluorescence analyses (Fig. 3), the red fluorescence signals of PAS-staining clearly visualized the PAS-positive glomerular basement membrane (Fig. 3a) (unpublished data; Li et al., manuscript in preparation). The green immunofluoresence staining for BSA was partially detected not only in blood capillaries but also in Bowman's space (Fig. 3c), demonstrating the passage of BSA through the glomerular basement membrane. The BSA immunoreactivity in Bowman's space was not colocalized with that of ZO-1 (Fig. 3d, arrows).

Immunolocalizations of various serum proteins

We have performed the immunostaining analyses of BSA, mouse albumin, IgG1 heavy chain (IgG1) and kappa light chain (Kappa) on serial thin sections and examined different distributions in vivo of the various molecular weight proteins in the exactly identical

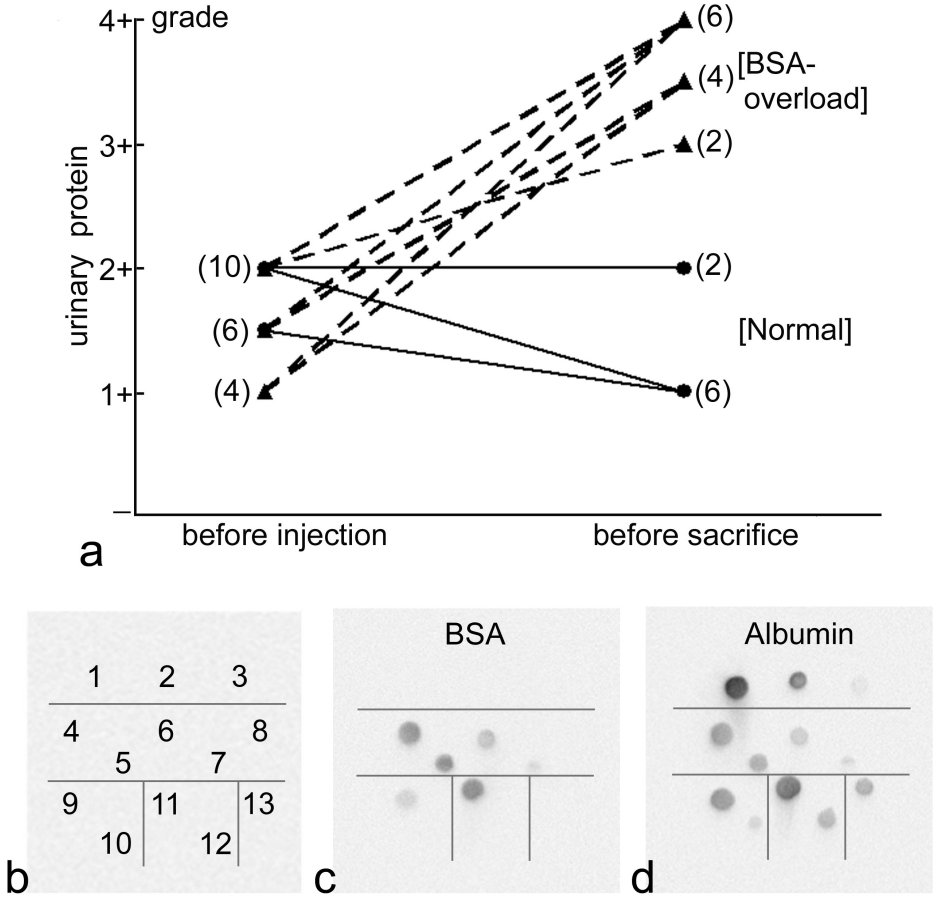

Fig. 1.a. The graded concentrations of urinary proteins in the experimental BSA-overload mice (dotted lines) and control mice (solid lines) before the BSA or half saline injection and just before their sacrifice. Note that the protein concentrations of each mouse urine are clearly increased in the BSA-overload mice, whereas they are unchanged or slightly decreased in the control mice. The figures in parentheses mean the number of mice at each grade. **b-d.** Dot blot analyses of mouse urine samples obtained from BSA-overload or control mice, using the antibody specific for BSA **(c)** or with potential cross-reactivity **(d)**. Numbers shown in **(b)** represent where each sample was spotted; mouse albumin 1mg/ml (1), 0.01mg/ml (2) or 0.0001mg/ml (3) in PBS; BSA 1mg/ml (4), 0.1mg/ml (5), 0.01mg/ml (6) or 0.001mg/ml (7) in PBS; no BSA (8); Urine samples from one BSA-injected mouse (9, 10), another BSA-injected mouse (11, 12) and a control mouse (13). The samples, No. 9, 11 and 13, are obtained 5hrs after the final injection, whereas the samples, No. 10 and 12, were obtained before the injection from the same mice. Note that BSA is detected in the urine of BSA-overload mice after the injection (c, No. 9 and 11), whereas mouse albumin is detected in all urine samples (d, No. 9~13). The spots of BSA are visualized with the anti-mouse albumin antibody because of the potential cross-reactivity.

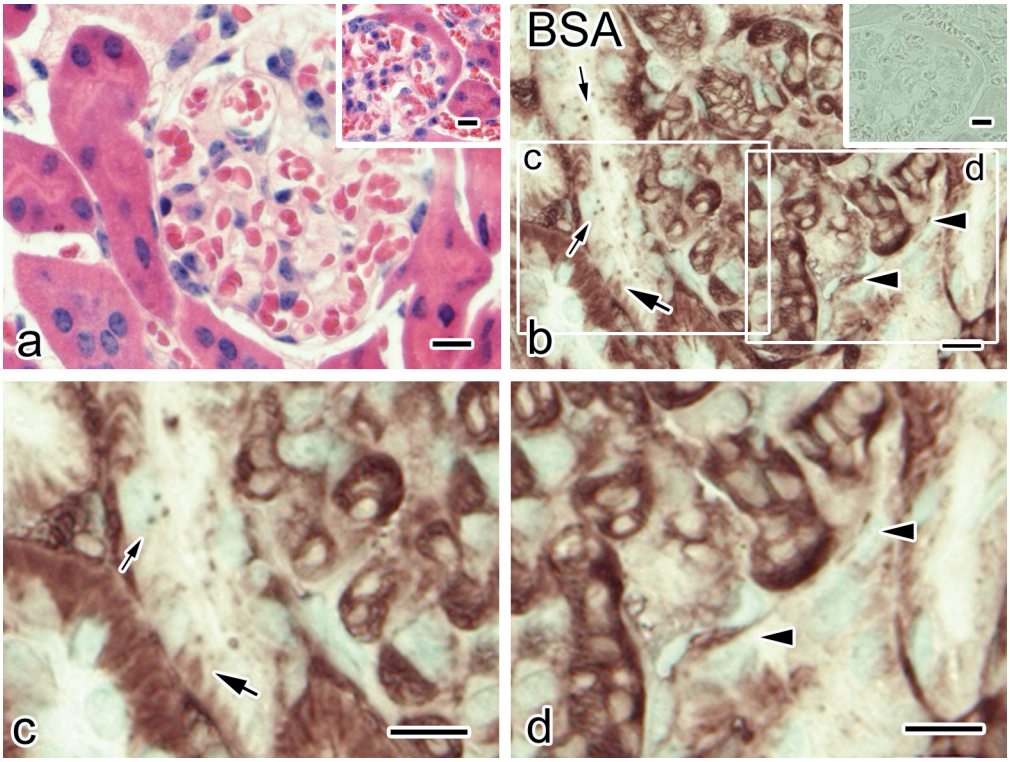

Fig. 2. Hematoxylin-eosine (HE) staining (**a**) and immunostaining for bovine serum albumin (BSA; **b-d**) in kidney tissues of the BSA-overload mice (**a-d**) and the control mice (**a, b, insets**), which were prepared with the "in vivo cryotechnique". Two glomerular and tubular areas marked with rectangles in **b** are magnified in **c** and **d**. The morphology of glomeruli and urinary tubules is well preserved, and the specific immunostaining for BSA can be clearly detected in glomerular capillary loops of the BSA-overload mice (**b**). Note that immunoreaction products of BSA are also localized in Bowman's space (**b, d**, arrowheads) and proximal convoluted tubules (**b, c**, small arrows). Large arrows: basal striation portions. Scale bars: 10 μm.

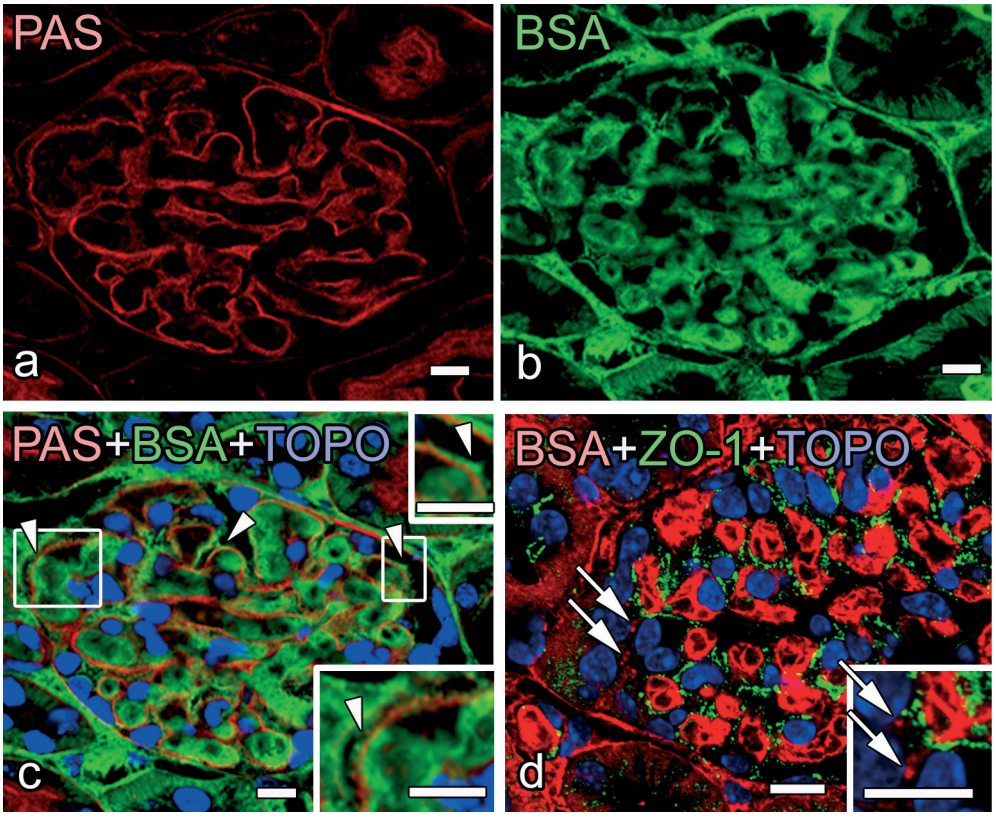

Fig. 3. Fluorescent signals of PAS-staining (**a**, **c**, red color) and immunofluorescence staining for BSA (**b**, **c**, green color; d, red color) and ZO-1 (**d**, green color) in the BSA-overload mouse kidneys prepared with the "in vivo cryotechnique". Nuclei were counter-stained with TO-PRO-3 (**c**, **d**, blue color). The merged image often demonstrates the BSA passage into Bowman's space (**c**, arrowheads) through glomerular capillary walls. The BSA immunoreactivity in Bowman's space is not seen to colocalize with that of ZO-1 (**d**, arrows). **Inset in c and d**: higher magnified view. Scale bars: 10 μm.

nephrons of the BSA-overload mice (Fig. 4). The immunoreaction products of IgG1 indicated the localization of intact IgG1 whole molecules, and those of Kappa showed all types of immunoglobulins and also free kappa light chains. In nine nephrons out of twenty one, BSA, mouse albumin and Kappa were immunolocalized in Bowman's space of their glomeruli (Fig. 4b-d) and in the apical cytoplasm and tubular lumens of proximal convoluted tubules (Fig. 4g-i), but IgG1 were not (Fig. 4e,j). On the other hand, in the other nephrons, immunoreaction products of BSA, mouse albumin, Kappa and IgG1 could all be localized in the Bowman's spaces and proximal convoluted tubules (Fig. 4l,m,o,p; data of BSA and Kappa are not shown). The immunolocalizations of both BSA and mouse albumin were similarly detected throughout the identical nephrons of kidney tissues. In control mice, such immunolocalization of IgG1 could not be observed in the Bowman's spaces and urinary tubules (data not shown). Although both albumin and Kappa immunoreaction products could be observed in most nephrons of the BSA-injected mice, albumin was immunolocalized in seven glomeruli and five urinary tubules out of twenty nephrons, and Kappa was

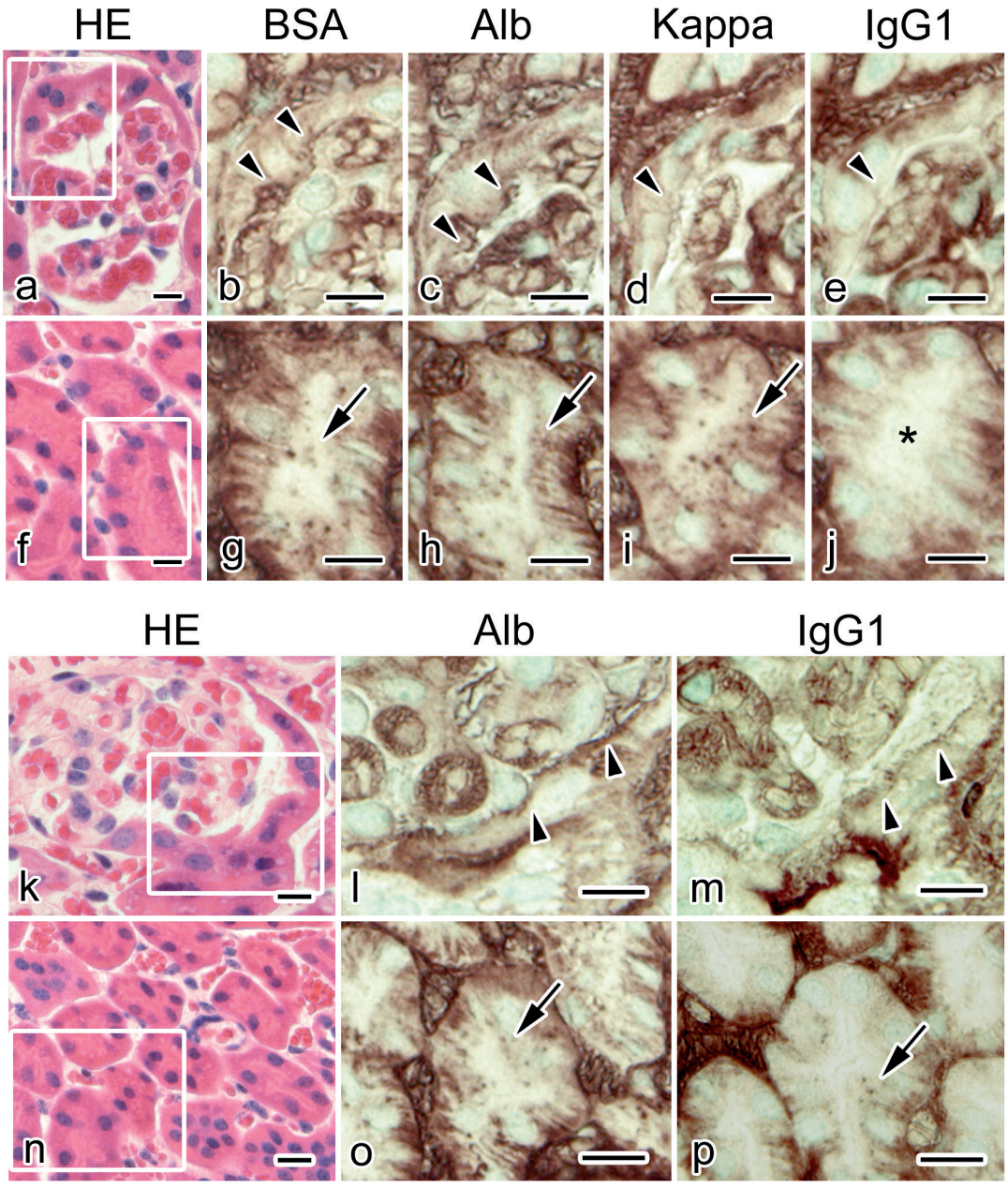

Fig. 4. Hematoxylin-eosine (HE) staining (**a, f, k, n**) and immunostaining for bovine serum albumin (BSA; **b, g**), mouse albumin (Alb; **c, h, l, o**), mouse immunoglobulin kappa light chain (Kappa; **d, i**) and mouse immunoglobulin G1 heavy chain (IgG1; **e, j, m, p**) on serial thin sections of the BSA-overload mouse kidneys prepared with the "in vivo cryotechnique". Some glomerular and tubular areas marked with rectangles in **a, f, k** and **n** are immunostained for each serum protein on the serial sections and magnified in **b-e, g-j, l-m** and **o-p**, respectively. Immunoreaction products of BSA, Alb and Kappa, not accompanied by those of IgG1 (e, arrowhead; j, asterisk), are localized in Bowman's space (**b-d**, arrowheads) and proximal convoluted tubules (**g-i**, arrows) in some nephrons. In other nephrons, however, immunoreaction products of IgG1 are localized in the Bowman's space (**m**, arrowheads) and proximal convoluted tubules (**p**, arrow), together with those of Alb (**l**, arrowheads; **o**, arrow). Scale bars: 10 μm.

immunolocalized in five glomeruli and six urinary tubules. Neither glomeruli nor urinary tubules of IgG1 immunolocalization were seen in nephrons of the BSA-overload mice.

BSA immunolocalizations with the conventional preparation methods

In the kidney specimens prepared by the routine tissue-resection immediately followed by the conventional immersion-fixation (Fig. 5a), the faint immunostaining for BSA was found to be remained inside the collapsed glomerular capillary loops (Fig. 5a, small arrow), though more BSA was immunolocalized in the Bowman's space (Fig. 5a, large arrow). The BSA-immunopositive clump patterns were seen in some tubular lumens (Fig. 5a, arrowhead). There was little immunostaining for BSA in the basal striation portions of urinary tubules.

In the kidney specimens prepared by perfusion-fixation followed by immersion-fixation (Fig. 5b), lumens of glomerular capillary loops were mostly open, and a few BSA immunoreaction products still remained in glomerular or interstitial blood capillaries (Fig. 5b, large arrows). Some immunoreaction products of BSA were also localized in Bowman's space (Fig. 5b, small arrow) and proximal tubular lumens (Fig. 5b, arrowhead). No BSA immunostaining was detected in the basal striation portions of the urinary tubules. Similar immunostaining patterns were found in the specimens prepared by perfusion-fixation followed by quick-freezing (Fig. 5c). Although the glomerular capillary loops were not as small in the kidney specimens prepared by the tissue-resection followed by quick-freezing (Fig. 5d,e) as those in the specimens prepared by the tissue-resection followed by conventional immersion-fixation (Fig. 5a), they appeared to be collapsed to some extent, as compared with those in the kidney specimens prepared with the "in vivo cryotechnique" (Fig. 2b). The immunostaining for BSA

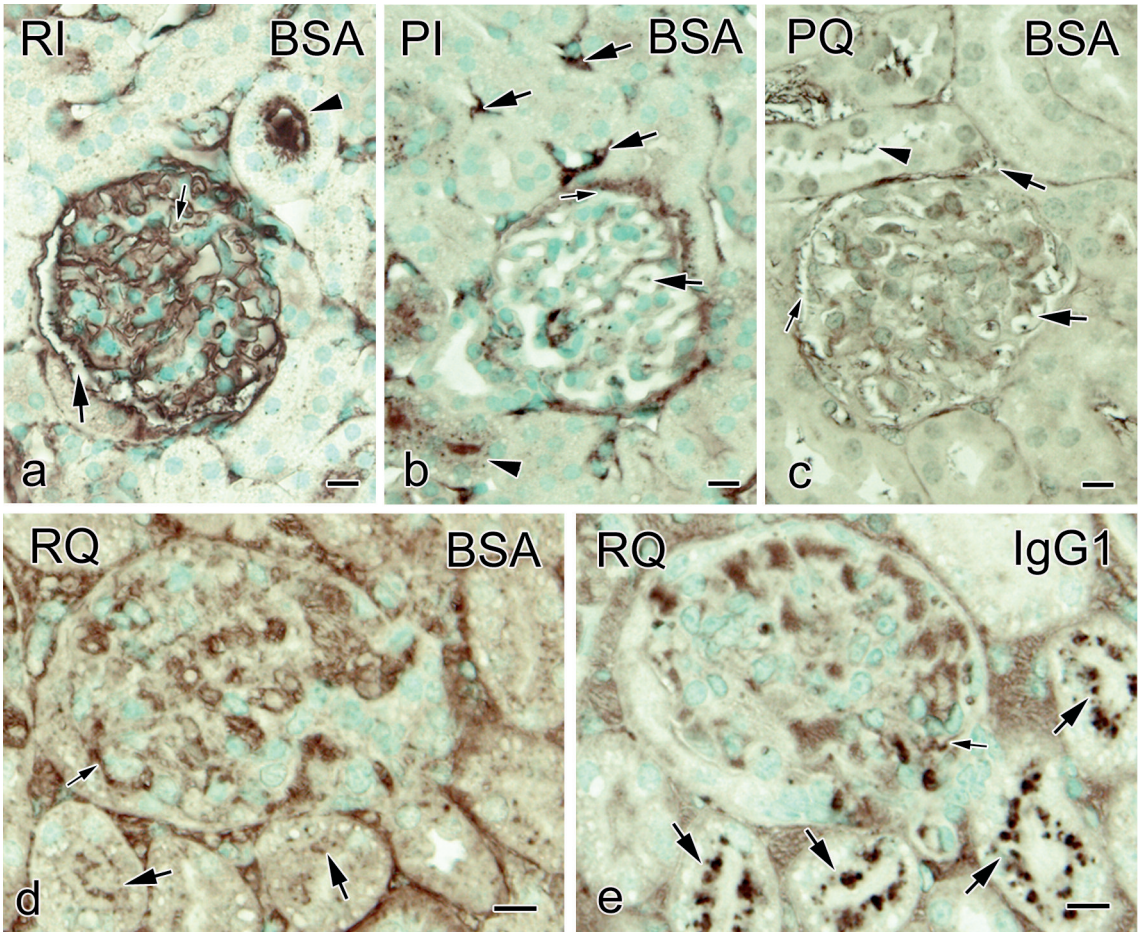

Fig. 5. a-c. Immunostaining for bovine serum albumin (BSA) in the BSA-overload mouse kidney tissues prepared with tissue-resection immediately followed by immersion-fixation (RI; a), perfusion-fixation followed by immersion-fixation (PI; b) and perfusion-fixation followed by quick-freezing (PQ; c). Note the definitely changed immunolocalizations of BSA in the glomerular capillaries (**a**, small arrow; **b, c,** large arrows), Bowman's spaces (**a**, large arrow; **b, c,** small arrows) and urinary tubules (**a-c**, arrowheads), depending on different preparation methods. **d-e.** Immunostaining for BSA **(d)** and mouse immunoglobulin G1 heavy chain (IgG1; **e**) in the BSA-overload mouse kidney tissues prepared with tissue-resection followed by quick-freezing (RQ). The passage of BSA into the Bowman's space (**d**, small arrow) and urinary tubules (**d**, large arrows) can be detected with the RQ, and dotted immunoreaction products of IgG1 are also localized in the Bowman's space (**e**, small arrow) and urinary tubules (**e**, large arrows) with the RQ. Scale bars: 10 μm

could be seen at some basal striation portions in the kidney specimens prepared by the tissue-resection followed by quick-freezing (Fig. 5d). In addition, the IgG1 showed large immunoreaction products in Bowman's space (Fig. 5e, small arrow) and along the apical cytoplasm of tubular epithelial cells (Fig. 5e, large arrows). The immunohistochemical findings about both BSA and IgG1 obtained were clearly different from those in the kidney specimens of living mice prepared with the "in vivo cryotechnique" (Figs. 2~4).

Discussion

The *in situ* morphology of living animal kidneys and the distribution of serum proteins, including HMW proteins, would be changed with the conventional preparation methods, because of ischemia with tissue-resection and extrinsic artificial pressures of perfusion-fixation, as already reported (Ohno et al., 2004a; Li et al., 2005). The distributions of soluble serum proteins revealed by the conventional preparation methods make it difficult to examine their in situ functional significance. On the contrary, one of the distinct merits with the "in vivo cryotechnique" is that living animal kidneys can be immediately cryofixed under normal blood circulation with fewer morphological artifacts. It is also assumed that the soluble serum proteins hardly diffuse in acetone containing fixatives used during the freeze-substitution step at about -80°C. Moreover, the antigenicity of such proteins is reported to be preserved by the cryotechniques, because structural components are less compactly cross-linked (Terada et al., 2005). For these reasons, the immunolocalizations of both exogenous BSA and endogenous mouse serum proteins could be precisely analyzed by the "in vivo cryotechnique" with immunohistochemistry in the present study.

Although an earlier morphological study already reported a variability of individual nephron responses to the injected exogenous albumin, the reported immunohistochemical results indicated only different degrees of total protein deposits in nephrons (Simpson and Shand, 1983). However, in the present study, different immunolocalizations of various molecular weight proteins were detected in nephrons of the BSA-overload mouse kidneys. In our findings, the in vivo passage of both BSA and mouse albumin, but not the IgG1, was found to be induced in some nephrons under living states, while in the others, both serum albumin and IgG1 were leaked out through the glomerular capillary walls and reabsorbed into the tubular epithelial cells. The concomitant size-barrier and charge-barrier defects, which were already proposed in this animal model, might explain these differences among nephrons (Lemley, 1993). It was also reported that the glomerular filtration barrier structures may behave like filter-membranes with many functional small pores and a very limited number of large pores with charge-selectivity (Blouch et al., 1997). The slight damage to the filtration barrier structures, which would firstly impair their function as a charge-barrier, leads to the markedly increased passage of serum albumin through the functional small pores. This impairment of the charge-barrier would be induced firstly in some nephrons, and the functional heterogeneity among nephrons would cause discrepancies about structural modification of glomerular capillary walls under pathological conditions. Although the histochemical distribution of anionic sites was found to be unaltered in the glomerular basement membranes (Davies et al., 1985; Weening et al., 1987), the loss of glomerular anions was also reported in the similar BSA-overload rat model (Bliss and Brewer, 1985). On the other hand, when the barrier structures are more severely damaged and the size-barrier function against serum proteins is also impaired, lots of HMW proteins, including IgG whole molecules, pass through the increased large pores. The significant defect of size selectivity has already been demonstrated in the BSA-overload animal model, by measuring the fractional clearances of neutral dextrans after six twice-a-day injections of BSA into rats (Lemley, 1993). The detection of leaked IgG1 in the present study suggests that the glomerular size-barrier impairment was already induced in some nephrons of living mice even after two days of the daily BSA injection. Therefore, we consider that many nephrons are temporarily and heterogenously damaged in the BSA-overload mice and that serum proteins with various molecular weights exhibit their different immunolocalizations in nephrons of the living mice, corresponding to the different degrees of functional impairment in each nephron.

The distributions of BSA and IgG1 were different among the preparation methods used in the present study. The immunostaining of serum proteins obtained at basolateral areas in the urinary tubules was difficult to explain with non-specific background, usually induced by the DAB reaction, because there is no immunoreaction product for BSA without the BSA-injection (Fig. 2b), and the similar staining image could be obtained by another immunofluorescence technique (Fig. 3) (Li et al., 2006). We already reported that both ischemia and anoxia after common resection of tissues altered the morphology and molecular distributions of kidney tissues, and the serum proteins were translocated into urinary space under heart-arrest condition or after tissue-resection (Li et al., 2006). The BSA and IgG1 were leaked out into the urinary spaces under heart-arrest condition, and so some amounts of both BSA and IgG1 might be attributed to the ischemic stimuli after tissue-resection. In addition, the distribution of serum proteins would be significantly affected by perfusion-fixation, because the artificial pressures of fixatives would wash them out into urinary spaces before their fixation was completed (Hippe-Sanwald, 1993; Shiurba, 2001; Li et al., 2005). This could be also confirmed by the results shown in Figure 5, because the immunoreactivity of BSA and IgG1 in blood vessels appeared to be partially weak after the perfusion-

fixation, as compared with other preparation methods.

Acknowledgements. The authors would like to thank Drs. T. Baba and Y. Fujii from The Department of Anatomy, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, for their constructive comments on this work.

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Accepted August 16, 2006