Immunohistochemical analyses on albumin and immunoglobulin in acute hypertensive mouse kidneys by “in vivo cryotechnique”

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Summary. The purpose of this study is to visualize topographical changes of serum proteins, albumin and immunoglobulin, passing through mouse glomerular capillary loops and their reabsorption in renal proximal tubules by immunohistochemistry in combination with our “in vivo cryotechnique”. The “in vivo cryotechnique” was performed on left mouse kidneys under normotensive, experimentally acute hypertensive and heart-arrest conditions. The cryofixed tissues by the technique were routinely processed for freeze-substitution. Serial deparaffinized sections were stained with hematoxylin-eosine and immunostained with anti-mouse albumin, immunoglobulin G (IgG), kappa or lambda light chain and IgG1 heavy chain antibodies. Under the normotensive and heart-arrest conditions, albumin and IgG were clearly immunolocalized in blood vessels and slightly in apical cytoplasmic parts of some proximal tubules. Under the acute hypertensive condition, the albumin and kappa or lambda light chains, but not IgG1 heavy chain, were strongly immunolocalized in the apical cytoplasm of almost all proximal tubules. This study is the first in vivo visualization for glomerular passage of serum proteins and their transtubular absorption. Thus, the “in vivo cryotechnique” with freeze-substitution can be used for clarifying not only the functional morphology of living animal cells, but also in situ immunohistochemical localization of their components.

Key words: In vivo cryotechnique, Acute hypertension, Mouse kidney, Immunohistochemistry, Serum proteins

Introduction

It is already known that hemodynamic factors, such as blood pressure and flow, exert an important influence on native morphology of glomerular capillary loops in living mouse kidneys (Ohno et al., 1996a,b, 2001). In addition, they have been supposed to affect abnormal leakage of serum proteins, which leads to proteinuria under experimental and pathological states in animal kidneys, and is also considered to be related to progression in human renal diseases. In such cases, hyperfiltrated serum proteins are usually processed for increased reabsorption in renal proximal tubules (Exaire et al., 1972). Both experimental animal studies and clinical human cases have already suggested that an excessive amount of the leaked proteins is toxic to the proximal tubular epithelium, resulting in a cause of interstitial inflammation and fibrosis (Remuzzi, 1995; Schreiner, 1995; Gansevoort et al., 1997; Jerums et al., 1997; Thomas et al., 1999). This idea implies that the tubular reabsorption of the leaked serum proteins may be an important factor for the histopathological progression of chronic renal diseases.

On the other hand, the renal glomerular selectivity for permeable serum solutes and transtubular absorption of filtered serum proteins have never been directly visualized on paraffin sections by light microscopy, because of technical limitations in preparing the specimens for the conventional immunohistochemistry. It was also reported that morphological studies with the routine immersion or perfusion fixation were not able to demonstrate native structures of functioning kidneys in vivo under normal or abnormal blood circulation (Yu et al., 1998a,b). The common fixatives in buffer solution, such as glutaraldehyde and paraformaldehyde, are well known to be most effective for many substances in animal cells and tissues owing to their cross-linking properties. However, they usually need considerable time for the final fixation to cross-link soluble substances during the routine perfusion or immersion
fixation, and many of them are usually washed out from the specimens. Moreover, an ultimate goal of our morphological studies is that all features of cells and tissues to be examined should reflect the physiological meaning under 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” (Ohno et al., 1996a,b, 2001; Terada et al., 1998; Xue et al., 1998; Takayama et al., 1999; Terada and Ohno, 2004; Terada et al., 2005), which is designed to arrest transiently dynamic structures in living animal organs by the combination of a cryoknife precooled in liquid nitrogen (-196°C) with another isopentane-propane cryogen (-193°C). All biological processes in the living animal organs were instantly stopped and embedded in the ice microenvironment, maintaining all their components in situ, followed by various preparation steps for morphological studies.

The common freeze-substitution is supposed to stabilize most components of the quickly frozen cells and tissues in non-aqueous microenvironment at low temperatures, in which diffusible movement of soluble components would always be limited (Bridgman and Dailey, 1989). 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, distributions of soluble serum proteins in mouse kidneys were immunohistochemically examined by the “in vivo cryotechnique” followed by the freeze-substitution, from the viewpoint of different hemodynamics, because their leakage across the glomerular capillary loops and following reabsorption in proximal convoluted tubules, more than one hundred times-concentrated under such various blood flow conditions, as described before (Ohno et al., 1996a). The cryotechnique was performed under such various blood flow conditions, as described in the previous paragraph. Briefly, a cryoknife edge precooled in liquid nitrogen (-196°C) was positioned over the left kidney (Fig. 1a). The mouse kidney was cut with the cryoknife edge, and liquid isopentane-propane cryogen (-193°C) was simultaneously poured over it with the assistance of the “in vivo cryoapparatus” (VI-11; Eiko Engineer Co. Ibaraki, Japan) (Fig. 1b), which had been already invented for the purpose of the “in vivo cryotechnique” (Zea-Aragon et al., 2004). The frozen kidneys were carefully trimmed out with a dental electrical drill in liquid nitrogen and then processed for the following freeze-substitution step (Fig. 1c), as reported before (Ohno et al., 1996a, 1996b, 2001).

Freeze-substitution fixation and paraffin-embedding

Some pieces of the frozen kidneys were freeze-substituted in pure acetone containing 2% paraformaldehyde (PF) cooled down in the dry ice-acetone at -80°C for 48h. They were afterward put in a deep-freezer at -20°C for 2h, and then in a refrigerator at 4°C for 2h, and finally raised up to room temperature. They were briefly washed in pure acetone twice and transferred into xylene. Finally, they were routinely embedded in the paraffin wax (Fig. 1c).

Immunostaining on de-paraffinized sections

The paraffin-embedded tissues were cut at 4-5 µm thickness, and the sections were de-paraffinized with xylene and a graded series of ethanol. Some sections were stained in common hematoxylin and eosine (HE) staining solution. For immunostaining, other sections were incubated with 1% hydrogen peroxide to block non-specific reactivity of endogenous peroxidase. After being washed in the phosphate buffer saline (PBS), they were incubated with 10% normal rabbit serum, avidin, and biotin for 1h each. As compared with conventionally fixed and dehydrated tissues (data not shown), to block endogenous biotin reaction especially in proximal tubules, more than one hundred times-concentrated blocking solution (Vector Laboratories Inc., Burlingame, CA, USA) was needed for our freeze-substituted specimens after the “in vivo cryotechnique”. The treated sections were then incubated with goat anti-mouse albumin antibody at a dilution of 1:5000 (Bethyl Laboratories Inc., Montgomery, TX, USA), kappa light chain, lambda light chain and IgG1 heavy chain antibodies (the dilution 1:2000 for kappa light and heavy chain antibodies; the dilution 1:200 for lambda light chain antibody) (Bethyl Laboratories Inc.) at 4°C overnight. After being washed with PBS, they were incubated with the biotinylated anti-goat IgG antibody (Nichirei, Tokyo, Japan) at room temperature for 1 h.

Materials and methods

“In vivo cryotechnique” for living mouse kidneys

The present animal experiment was approved by the University of Yamanashi Animal Care and Use Committee. Twelve adult C57BL/6 mice, weighing 20-30g, were anesthetized with sodium pentobarbital. Then their abdominal cavity of four mice was open, and left kidneys were detected under normal blood circulation, as a control group. As the first experimental group of four mice, to examine glomerular leakage and subsequent reabsorption of serum proteins in renal tubules, a mouse model with acute renal hypertension was prepared by ligation of abdominal aorta just below branching renal arteries for 10min, as described before (Roman and Cowley, 1985; Ohno et al., 1996a; Zhang et al., 1998) (Fig. 1). To demonstrate the immunolocalization of serum proteins in kidneys under the heart-arrest condition as the second experimental group of four mice, the blood flow into kidneys was stopped due to heart-arrest with overdoses of the injected anesthetic, as also described before (Ohno et al., 1996a).

Then the “in vivo cryotechnique” was performed under such various blood flow conditions, as described in the previous paragraph. Briefly, a cryoknife edge precooled in liquid nitrogen (-196°C) was positioned over the left kidney (Fig. 1a). The mouse kidney was cut with the cryoknife edge, and liquid isopentane-propane cryogen (-193°C) was simultaneously poured over it with the assistance of the “in vivo cryoapparatus” (VI-11; Eiko Engineer Co. Ibaraki, Japan) (Fig. 1b), which had been already invented for the purpose of the “in vivo cryotechnique” (Zea-Aragon et al., 2004). The frozen kidneys were carefully trimmed out with a dental electrical drill in liquid nitrogen and then processed for the following freeze-substitution step (Fig. 1c), as reported before (Ohno et al., 1996a, 1996b, 2001).

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They were incubated with horseradish peroxidase (HRP)-conjugated avidin-biotin complex (ABC) for 1h and visualized with metal-enhanced 3,3’-diaminobenzidine (DAB) (Pierce, Rockford, IL, USA) for 5 min (ABC-DAB method). Finally, they were incubated in 0.04% osmium tetroxide solution for 2 min.

To examine another immunohistochemical localization of IgG in the mouse kidneys, de-paraffinized sections were blocked with the same process as described in the albumin immunostaining, and then incubated with biotinylated anti-mouse IgG antibody (Nichirei, Tokyo, Japan) at room temperature for 1h. They were finally visualized by the ABC-DAB method, as described above. For the double labeling study, some paraffin sections were routinely de-paraffinized and incubated with 10% normal rabbit serum for 1h. Subsequently, they were incubated with fluorescein isothiocyanate (FITC)-conjugated *Lotus tetragonolobus* agglutinin (LTA), a proximal tubule-specific marker, at a concentration of 10 µg/ml (Sigma, Saint Louis, MO, USA), and then immunostained for albumin by the ABC-DAB method, as described in the previous paragraph.

**Immunostaining on conventional paraffin sections**

Three adult C57BL/6 mice under anesthesia were routinely perfused with 2% PF via the heart. They were dehydrated in a graded series of methanol and embedded in the paraffin wax. Serial paraffin sections were cut and first immunostained with the primary antibody (goat anti-mouse albumin antibody) overnight, as described above. Subsequently, they were immunostained with the secondary antibodies: Alexa Fluor 594-conjugated

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**Fig. 1.** a. A schematic drawing of “in vivo cryotechnique” for mouse kidneys under acute hypertensive condition. The abdominal aorta is ligated by thin thread just below both renal arteries. A left kidney is directly frozen in vivo with a cryoknife, and the isopentane-propane cryogen (-193°C) precooled in liquid nitrogen is simultaneously poured over the cryocut kidney under the “in vivo cryoapparatus”, as shown in (b). c. After the “in vivo cryotechnique”, the frozen specimen is processed for immunohistochemistry, as following in the flowchart.
donkey anti-goat IgG antibody for albumin and the Alexa Fluor 488-conjugated anti-mouse IgG antibody (Molecular Probes; Eugene, OR, USA) for mouse IgG.

Results

Hematoxylin-Eosine (HE) staining with “in vivo cryotechnique”

To examine native morphology in the mouse kidneys, as obtained by the “in vivo cryotechnique”, the routine HE-staining was first performed for the de-paraffinized sections. Some well-preserved areas of renal cortices under different blood circulation conditions, such as normotension (Fig. 2a), acute hypertension (Fig. 2b) and heart-arrest (Fig. 2c), could be obtained within 300-400 µm from the frozen surface tissues without visible ice crystals at a light microscopic level, as already discussed before (Zea-Aragon et al., 2004; Ohno et al., 2004). The renal interstitium between tubules under the normotensive condition was observed to have widely open peritubular spaces (Fig. 2a). However, some luminal spaces in the proximal tubules in vivo were too narrow to be detected, because of overlapping brush borders near apical parts of the proximal tubules (Fig. 2a, arrows). Under the acute hypertensive condition, the Bowman’s space (Fig. 2b, asterisk) and luminal spaces of the proximal (Fig. 2b, arrowheads) or the distal tubules (Fig. 2b, arrows) became more widely opened in the renal cortices than those seen under the normotensive (Fig. 2a) or heart-arrest (Fig. 2c) condition. On the contrary, neither Bowman’s spaces nor luminal spaces of the proximal tubules were clearly opened under the heart-arrest condition (Fig. 2c, arrows).

Immunolocalization of albumin and IgG in renal proximal tubules

To examine the glomerular leakage and reabsorption of serum proteins in renal proximal tubules, the immunohistochemistry for albumin and IgG was performed on paraffin sections prepared by the “in vivo cryotechnique” (Figs. 3-5). Under the normotensive condition (Fig. 3a,b for albumin; Fig. 3c,d for IgG) and the heart-arrest condition (Fig. 4a,b for albumin; Fig. 4c,d for IgG), immunoreaction products of both albumin and IgG were detected in blood vessels and also at the interstitium between renal tubules, extending along their

Fig. 2. Light micrographs of mouse renal cortices under different blood circulation conditions; normotensive (normal; a), acute hypertensive (ligation; b) and heart-arrest (arrest; c) conditions. Under the acute hypertension (b), the luminal spaces of both proximal (arrowheads) and distal tubules (arrows) and also Bowman’s spaces (asterisk) are more widely open than those seen in the normotensive (a, arrows) and heart-arrest (c, arrows) conditions. Bar: 20 µm.
In vivo localization of albumin and IgG

Fig. 3. Immunohistochemical localization of albumin (a, b) and IgG (c, d) in mouse kidneys under the normotensive condition, as revealed by the "in vivo cryotechnique" (a and c, HE staining; b and d, peroxidase-DAB; insets, higher magnified images). The immunolocalization of both albumin and IgG is seen not only inside blood vessels, but also in the interstitium, extending along basolateral cell membranes of renal tubules. Some immunolocalization is slightly detected in the cytoplasm of proximal tubules under the normotensive condition (b, d insets, arrows). Bar: 20 µm.

Fig. 4. Immunohistochemical localization of albumin (a, b) and IgG (c, d) in mouse kidneys under the heart-arrest condition, as revealed by the "in vivo cryotechnique" (a and c, HE staining; b and d, peroxidase-DAB; insets, higher magnified images). Some immunolocalization of albumin and IgG is still detected in the apical cytoplasm (b and d, insets; arrows) and their immunolocalization is seen along the basolateral cell membranes of the renal tubules (b, d). Bar: 20 µm.
basolateral cell membranes (Fig. 3b,d, 4b,d). These immunohistochemical images could be confirmed in corresponding serial sections with the HE-staining (Fig. 3a, 4a). Another immunolocalization for albumin and IgG was found partially in some apical parts of the proximal tubules under the normotensive condition (Fig. 3b.d, insets). Under the acute hypertensive condition, however, in addition to the similar immunolocalization under the normotension, both albumin (Fig. 5b,c) and IgG (Fig. 5e,f) were clearly immunolocalized along cell membranes containing apical brush borders and also in the apical cytoplasm of most proximal tubules (Fig. 5).
5c,f). Those proteins’ localizations in the proximal tubules were also confirmed by the double labeling with LTA lectin specific for the proximal tubule and immunostaining for IgG (Fig. 6a-c) or albumin (Fig. 6d,e).

Immunolocalization of immunoglobulin light or heavy chain in proximal tubules

To examine whether immunoglobulins with the full molecular length were leaked out through glomerular capillary loops under the acute hypertensive condition, another immunostaining analysis for the light or heavy chain was performed respectively on serial paraffin sections (Fig. 7). In the cortex of mouse kidneys under such acute hypertension, both kappa (Fig. 7b) and lambda (data not shown) light chains’ immunolabeling was clearly detected along the apical cell membranes of almost all proximal tubules. On the contrary, the antibody against the IgG1 heavy chain was not immunoreacted in such proximal tubular areas, but in peritubular capillaries and the interstitium (Fig. 7c), indicating that no IgGs with the full molecular length were leaked out under this experimental condition.

Immunolocalization of albumin and IgG by conventional fixation

To examine the immunolocalization of albumin and IgG in the kidney at the artificial state washing out all of the circulating proteins, the conventional perfusion fixation method followed by alcohol dehydration was performed. By double immunofluorescence method, both albumin (Fig. 8b) and IgG (Fig. 8c) were immunolocalized mainly in Bowman’s spaces and along apical cell membranes of some proximal tubules. In addition, the serum proteins, albumin and IgG, in both interstitial blood vessels and glomerular blood capillaries were partially washed out and weakly immunostained due to the perfusion fixation (Fig. 8d). The immunolocalizations of albumin and IgG stained by the ABC-DAB method were similar to those by the immunofluorescence method (data not shown).

Discussion

In the present study, we have presented that our “in vivo cryotechnique” followed by freeze-substitution method is a simple and reliable technique to visualize not only the in vivo morphology of living mouse kidneys under various blood circulation conditions, as shown in Figure 2 and summarized in Figure 9, but also the immunolocalization changes of serum proteins in the similar renal tissues at the same time, as shown in Figures 3-5. The application of routine immunohistochemistry to detecting the in vivo localization of soluble serum proteins in cells and tissues has been limited, because they are easily redistributed during the conventional chemical fixation step. As shown in Figure 8, by the conventional perfusion fixation method, both albumin and IgG were strongly immunostained in Bowman’s spaces and along apical cell membranes in
most of proximal tubules, presumably because of artificial permeability by the perfusion pressure against glomerular capillary loops. On the contrary, the “in vivo cryotechnique” is quick enough to arrest transient physiological processes under various blood circulation conditions in the living mouse kidney, then allowing us to describe the in vivo morphological or immunohistochemical study of renal tubules and glomerular capillary loops, as already reported at an electron microscopic level (Ohno et al., 1996a, 2001; Yu et al., 1998a).

Moreover, in the present study, the luminal space of proximal tubules was obviously enlarged under the acute hypertensive condition, and the reabsorption of leaked albumin and IgG through glomerular capillary loops was clearly increased in the proximal tubules, as shown in Figure 5 and summarized in Figure 9. The different immunolocalizations of both albumin and IgG in the proximal tubules under the hypertensive condition from those under the normotensive condition were probably due to hemodynamic changes, causing the glomerular protein leakage and transtubular reabsorption. From our results, the leakage of albumin and their reabsorption occurred within 10 minutes at an early stage of experimentally acute hypertension. Under such an acute hypertensive condition, a high glomerular blood capillary pressure mechanically changed the passage barriers of molecular sieves in the glomerular basement membrane, as already observed at an electron microscopic level by the “in vivo cryotechnique” (Ohno et al., 1996a,b, 2001). This high pressure of glomerular blood capillaries impaired the size-selective barrier function of the slit diaphragm and glomerular basement membrane, so that serum protein contents in the glomerular filtrate were probably increased, which in turn caused the active reabsorption of such filtered serum proteins, as albumin and light chains of IgG, in proximal tubular epithelial cells, shown in Figure 5 (Remuzzi and Bertani, 1998; Birn et al., 2000; Brunskill, 2000).

We have also demonstrated the different immunolocalization between kappa or lambda light chains and IgG1 heavy chains in the proximal tubules of living mouse kidneys under the hypertensive condition, as shown in Figure 7. The kappa and lambda light chains were clearly immunolocalized in the apical cytoplasm of almost all proximal tubules, but no heavy chain of IgG immunostaining was observed in such areas (Fig. 7c). These findings indicate that low molecular weight proteins, such as light chains of immuoglobulin and serum albumin, easily leak in vivo through the glomerular basement membrane and also slit diaphragms, and then are reabsorbed in the proximal tubules under the acute hypertensive condition (Batuman et al., 1990; Batuman and Guan, 1997). Some IgGs with the full molecular length are reported to be extensively trapped in the glomerular basement membranes under some pathological conditions, such as membrano-proliferative glomerulonephritis, membranous...
nephropathy and lupus nephritis (Imai et al., 1997; Bijl et al., 2002). In such cases, they may be difficult to be leaked out into Bowman’s spaces, because of their large molecular chain structure and immune complexes.

In conclusion, the present “in vivo cryotechnique” followed by the freeze-substitution method can provide the in vivo stopped images of functioning renal tissues in living mice, and also allow us to examine the in vivo immunodistribution of filtered serum proteins and their reabsorption in the renal proximal tubules, as summarized in Figure 9.

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