Scanning electron microscopic study of the renal glomerulus by an in vivo cryotechnique combined with freeze-substitution

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ABSTRACT

The 3-dimensional ultrastructure of mouse renal glomeruli under normal haemodynamic conditions was studied by scanning electron microscopy using an in vivo cryotechnique followed by freeze-substitution, and compared with glomeruli prepared by conventional fixation methods. Mouse kidneys were frozen with a cryoknife apparatus and a liquid isopentane-propane mixture (− 193 °C). Surface areas of the frozen tissues were freeze-fractured with a scalpel in liquid nitrogen. The specimens were routinely freeze-substituted, freeze-dried, ion-sputtered, and then observed in a scanning electron microscope at an accelerating voltage of 5 kV. Renal glomeruli showed good ultrastructural preservation of the surface tissues. Podocytes with interdigitating foot processes covering capillary loops exhibited smooth surface contours and their cell surfaces were arranged more tightly than those seen by the conventional fixation method. Filtration slits between foot processes were found to be narrow. The internal structure of the glomerular tuft was seen in the freeze-fracture faces. The capillary lumen with variously shaped erythrocytes was kept open in frozen glomeruli under normal blood circulation conditions. The ultrastructure of renal glomeruli, as revealed by the in vivo cryotechnique with freeze-substitution, appears to be closer to that of the living state.

Key words: Renal glomerulus; foot process; in vivo cryotechnique; freeze-substitution.

INTRODUCTION

Scanning electron microscopy (SEM), which can provide 3-dimensional ultrastructural images, has often been used for morphological studies on biological tissues, including the kidney (Arakawa, 1970; Shirato et al. 1991; Hironaka et al. 1993a, b). Conventional glutaraldehyde and osmium tetroxide fixation methods, followed by critical-point drying, have been widely used for such morphological studies. The images of renal structures revealed by SEM have demonstrated that a glomerulus consists of intricate network of blood capillaries which are usually covered by podocytes with foot processes. Internal structures within the glomerular tuft, which are seen in the fracture faces on SEM, include capillary endothelial cells, glomerular basement membranes (GBM), and the mesangium in addition to the podocytes. The endothelial cells lining the capillary lumen are characteristic of abundant fenestrations. The GBM surrounded both such capillaries and mesangial matrix (Shirato et al. 1991). It is well known that podocytes of renal glomeruli have a unique foot structure, which appears to be specifically differentiated to allow blood filtration. The foot processes form an interdigitating pattern on the outer surface of glomerular capillaries, so that they establish a filtration slit, through which circulating blood can be filtered (Kriz et al. 1994). It has been also reported that the ultrastructure of such foot processes is easily changed after cessation of blood flow into the kidney (Griffith et al. 1967). Moreover, conventional chemical fixation methods have been assumed to alter glomerular ultrastructure and to extract some of their components (Yu et al. 1997). Thus it is difficult to elucidate the true ultrastructure of renal glomeruli by conventional chemical fixation methods. Improved methods are therefore needed for freezing animal tissues in vivo to obtain acceptable morphology in functioning organs (Chang et al. 1980).
Fig. 1. SEM micrographs of freeze-fractured glomerular tufts after conventional fixation methods. (a) The fracture face exhibits an outer surface of the glomerular tuft covered by podocytes (P) with primary or foot processes (F). The fracture faces of capillaries (Cap) are covered by abundant fenestrated endothelial cells. Bar, 1.0 µm. Inset: At higher magnification, filtration slits are widely open (arrowheads) and surfaces of foot processes show shrunken and irregular contours. Bar, 1.0 µm. (b) Inner surface of peripheral portions of capillary walls,
Recently, a new technique termed the ‘in vivo cryotechnique’ was developed for freezing tissues and cells in vivo without interruption of their blood supply (Ohno et al. 1996). This technique was used to examine functioning kidneys in vivo by transmission electron microscopy (TEM) and revealed the dynamic ultrastructural changes of glomeruli under different circulating blood conditions (Ohno et al. 1996). In the present study, we describe the 3-dimensional ultrastructure of functioning kidneys in vivo under normal blood circulation by using the in vivo cryotechnique followed by freeze-substitution for SEM. The ultrastructural appearances of renal glomeruli obtained differed from those seen by conventional perfusion fixation, indicating that the former images appear to be closer to those in the living state.

**Materials and Methods**

A total of 8 female BALB/c mice, weighing 20–30 g, were used. They were kept in an air-conditioned room and fed on commercial diet and water ad libitum. They were divided into 2 groups to assess the different preparation procedures. Kidneys of 4 mice were processed by conventional fixation methods and the left kidneys of the other 4 mice were processed for the in vivo cryotechnique followed by freeze-substitution. Those specimens were prepared as follows.

**Conventional fixation method**

Under anaesthesia with sodium pentobarbital, the kidneys were perfused with 0.9% physiological saline at 150 cmH₂O pressure for 10 min and then with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.3, for 10 min via the heart. The perfused kidneys were removed and cortical tissues were cut into small pieces with razor blades and washed with 0.1 M PB. They were fixed with the same fixative for a further 2 h, and then washed with 0.1 M PB. They were postfixixed with 1% osmium tetroxide in 0.1 M PB for 2 h and routinely dehydrated in a graded series of ethanol (from 50% to 100%) at room temperature. The dehydrated specimens were quickly plunged into a liquid isopentane-propane mixture (−193 °C) cooled in liquid nitrogen. Then surface areas of the frozen tissues were freeze-fractured with a scalpel in liquid nitrogen. The specimens were transferred into 100% ethanol, again at room temperature for 15 min.

**In vivo cryotechnique followed by freeze-substitution**

The in vivo cryotechnique is a series of processes with a combination of the metal contact freezing method and the liquid cryogen plunging method (Ohno et al. 1996). Briefly the left kidney was cut into by the cryoknife edge, precooled in liquid nitrogen (−196 °C), over which liquid isopentane-propane mixture (−193 °C) was immediately poured. In addition, liquid nitrogen (−196 °C) was poured over the frozen kidney tissue to avoid rewarming. Two separated frozen specimens were removed from the abdominal cavity and their surfaces were freeze-fractured with a scalpel in liquid nitrogen. They were then freeze-substituted in absolute acetone containing 2% osmium tetroxide at −80 °C for 20 h. After the freeze-substitution fixation, the samples were kept at −20 °C for 2 h and then at 4 °C for 2 h. Finally, they were raised to room temperature and washed in absolute acetone several times.

**Preparation for SEM**

Specimens prepared both with the conventional fixation method and with the in vivo cryotechnique followed by freeze-substitution were transferred into t-butyl alcohol and freeze-dried at −5 °C in a Hitachi ES-2030 apparatus. The dried specimens were then mounted on aluminium stages, evaporated with platinum/palladium (10–15 nm) and observed in a Hitachi S-4500 scanning electron microscope at an accelerating voltage of 5 kV.

**Conventional preparation for TEM**

Some dehydrated specimens after the conventional fixation method were embedded in Quetol-812. Ultrathin sections were cut with an ultramicrotome, mounted on copper grids and contrasted with uranyl acetate and lead citrate. They were examined in a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV.

**Results**

The ultrastructures of glomeruli, as revealed with the conventional fixation method or by the in vivo cryotechnique followed by freeze-substitution, were observed by SEM (Figs 1–4). Some freeze-fractured...
Fig. 2. SEM micrographs of freeze-fractured glomerular tufts after the in vivo cryotechnique. (a) Normally functioning kidneys are seen to have many erythrocytes (RBC) with various shapes, in the capillary (Cap) networks. Bar, 10 µm. (b) Interdigitating foot processes cover outer capillary surfaces. Bar, 10 µm. P, podocytes; F, foot processes; U, urinary space. Inset: At higher magnification, the surface contours of foot processes (F) are smooth and tightly apposed each other. Filtration slits are also narrow (arrowheads). Bar, 1.0 µm.
tissue faces in scattered glomeruli of the renal cortices were visualised by both preparation methods. The inside of blood capillaries was observed in freeze-fractured glomeruli, while the podocytes were observed from the Bowman’s capsular space in non-freeze-fractured areas (Fig. 1a). By the conventional fixation method, the external surface of the glomerulus was covered by foot processes of podocytes, which were shown to be shrunken, and the filtration slits between foot processes were widely open (Fig. 1a, inset). Widely-opened clefts between foot processes, glomerular basement membranes with the typical 3 layers, and endothelial cells showing abundant fenestration were observed in transverse sections of capillaries (Fig. 1b). Such morphological features were similar to those seen in ultrathin sections viewed by TEM (Fig. 1b, inset).

In contrast, the kidney tissues showed well-preserved ultrastructure in the frozen surface tissues prepared by the in vivo cryotechnique (Fig. 2a). Interdigitating foot processes, which covered outer capillary surfaces, reflected the in vivo arrangement, as revealed by this technique (Fig. 2b). The foot processes were arranged at various angles with respect to the capillary axis, and their surfaces were smooth. They were tightly apposed each other, and the filtration slits were also seen to be narrow (Fig. 2b, inset). By freeze-fracturing the frozen tissues after the in vivo cryotechnique procedure, the structure of capillary networks under normal circulatory conditions was also revealed (Fig. 3a). In these capillaries, erythrocytes showed various shapes, which were not typical biconcave discs, under normal blood flow conditions. In a capillary that was freeze-fractured along its longitudinal axis, with foot processes aligned on both sides of the endothelial cells, variously shaped erythrocytes were observed within the lumen (Fig. 3b). Such ultrastructural appearances of glomeruli were very similar to those seen by TEM after the in vivo cryotechnique, as reported before (Ohno et al. 1996). As compared with the ultrastructure of capillary loops seen by the conventional fixation method (Fig. 4a), in tissues prepared by the in vivo cryotechnique, interdigitating foot processes were more tightly apposed to each other, the slits between foot processes were narrower, and surface contours of podocytes were smooth (Fig. 4b).

DISCUSSION

Scanning electron microscopy provides significant information regarding the 3-dimensional architecture of the renal glomerulus (Mitoshi et al. 1971; Shirato et al. 1991; Inokuchi et al. 1993). The processing of kidney tissues for SEM study has been designed to preserve them under a condition approximating to the living state. Although conventional fixation methods are widely used, chemical fixation, such as with glutaraldehyde and osmium tetroxide, has been known to denature tissues and cells, and occasionally disturb their ultrastructure (Elder, 1989). The next step, such as rapid dehydration in organic solvents at room temperature, has been found to produce osmotic changes and induce shrinkage (Hayat, 1970). Moreover, the critical-point drying method, which is often used for drying biological materials, is accompanied by inevitable artifact problems (Boye & Wood, 1969). When kidney tissues are prepared according to such conventional procedures, shrinkage and loss of cytoplasmic matrix occur, similar to those described in previous studies (Hayat, 1970; Boyde & Wood, 1969; Chan et al. 1993). In contrast, the cryotechnique followed by freeze-substitution has greatly improved the ultrastructural preservation without such artifacts (Ohno et al. 1996).

A promising approach is a quick-freezing method (Harreveld & Crowell, 1964; Nicolas, 1991). There is a wealth of information on cryotechniques, which include plunging tissues into liquid cryogen, jetting liquid cryogen against specimens or slamming them against a precooled metal surface (Harreveld & Crowell, 1964; Greene & Walsh, 1992; Ohno et al. 1996; Yu et al. 1997, 1998). It means a time resolution for dynamic morphology, quick enough to fix ultrastructural appearances (Nicolas, 1991; Greene & Walsh, 1992). It is also accepted that cryofixation is the best way to preserve both antigenicity and chemical components of tissues, and morphological changes produced by the chemical fixation and dehydration steps are relatively reduced (Ohno et al. 1992, 1996; Chan et al. 1993; Hippe-Sanwald, 1993; Yu et al. 1997).

In the present study, the in vivo cryotechnique is a series of processes which combine the metal contact freezing method with the liquid cryogen freezing method (Hora et al. 1990; Furukawa et al. 1991; Ohno et al. 1996). It was directly performed in the mouse and quick enough to arrest transient physiological processes in the kidneys. All living processes in the kidney organs were instantly arrested and their components were maintained in situ. Moreover, the morphological changes created by stopping blood circulation were avoided in the outermost tissues due to the simultaneity of cutting and freezing (Zglinicki et al. 1986). In the present study, the filtration slits of glomeruli were found to be narrow, and the surface
Fig. 3. (a) SEM micrograph of a freeze-fractured glomerular capillary (Cap), which was preserved by the in vivo cryotechnique. Podocytes (P) and foot processes (F) with a tight arrangement are seen (arrowheads). Erythrocytes (RBC) are seen with various shapes in the capillary, and not the typical biconcave discoid shapes observed in the normal blood circulation. P, podocytes; F, foot processes. Bar, 1.0 μm. (b) SEM micrograph of well preserved freeze-fractured foot processes (F) tightly arranged (arrowheads) on both sides of a capillary (Cap). The tissue
Fig. 4. SEM micrographs of freeze-fractured glomerular capillaries (Cap) after conventional fixation methods (a) and by the in vivo cryotechnique (b). As compared with foot processes in the conventional fixation group (a), the arrangement of interdigitating foot processes (F) after the in vivo cryotechnique (b) are more tightly apposed each other. The slits between foot processes are narrower (b, arrowheads) and surface contours of foot processes are smoother in the in vivo cryotechnique group at the same magnification. BM, basement membrane; P, podocytes; Cap, capillary; RBC, erythrocyte. Bars, 1.0 µm. Insets: Higher magnification views. Bars, 1.0 µm.

contours of foot processes to be smooth after the in vivo cryotechnique (Fig. 2). However, they were widely open and there were irregular shrunken surface contours after the conventional fixation method (Fig. 1). After arresting the blood circulation, glomerular ultrastructure is rapidly changed (Griffith et al. 1967), in addition to the tissue shrinkage due to the chemical fixation and ischaemic influences on the excised tissues (Ryan & Karnovsky, 1976).

Freeze-substitution after cryofixation results in a slow and gentle dehydration. The cryofixation and freeze-substitution sequence thus generally preserves fragile ultrastructure, which yields an image closer to the living state than conventional processing (Elder, 1989). Such ultrastructural changes were also minimised by the in vivo cryotechnique followed by freeze-substitution. It is apparent, during normal blood flow, that glomerular ultrastructure differs from that revealed in conventionally fixed kidneys, as shown in Figure 2. A functional interpretation of glomerular morphology is valid only if haemodynamic factors are taken into consideration (Ohno et al. 1996). In the present study, the glomerular ultrastructural features shown by the in vivo cryotechnique...
are similar to those described in our previous study by using this technique for TEM (Furukawa et al. 1991; Ohno et al. 1996). In addition, various erythrocyte shapes were well preserved. The typical biconcave discoid shape was not observed under normal blood flow condition. It appears that erythrocytes in glomerular capillaries have no uniform shape in their living state.

In the present study, the t-butyl alcohol freeze-drying method was used which resulted in good preservation of cell surface structures equivalent to that obtained by the critical-point drying method. However, for intracellular structures, the lamellar arrangement of the endoplasmic reticulum has been reported to be better preserved by the freeze-drying method than the critical-point drying method. As was pointed out in an earlier work, t-butyl alcohol usage causes less shrinkage during specimen dehydration than other dehydration agents (Boyle & Wood, 1969; Inoue & Osatake, 1988). The quality of ultrastructural preservation by the cryofixation method depends in part upon destructive artifacts induced by ice crystals. In poorly frozen cells, large ice crystals could easily be identified as large spaces. However, it is difficult to detect damage induced by tiny ice crystals (Terracio et al. 1981; Bridgman & Reese, 1984). Our current criteria for evaluating the quality of freezing levels were based on morphological comparisons with specimens prepared by the conventional method (Zglinicki et al. 1986). In conclusion, using the in vivo cryotechnique followed by freeze-substitution, the important aspect was specimen preparation under lower temperature conditions. The findings suggested that the ultrastructure of renal glomeruli was well preserved and appeared to be closer to that in the living state, in comparison with that obtained by conventional fixation methods for scanning electron microscopy.

REFERENCES


