Spontaneous apoptosis of podocytes in explanted glomeruli

**Technical Note**

YOSHIHISA ISHIKAWA and MASANORI KITAMURA

Glomerular Bioengineering Unit, Department of Medicine, University College London Medical School, London, England, United Kingdom

Spontaneous apoptosis of podocytes in explanted glomeruli. **Technical Note.** Despite the extensive use of isolated glomeruli for kidney research, little is understood about whether and how isolation and explantation affect the structure and function of the glomerulus. In this report, we investigate the incidence of apoptosis in explanted, normal rat glomeruli. By using ladder detection assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and histological analysis, the present study provides evidence that significant number of resident cells, especially podocytes, spontaneously undergo apoptosis immediately after explantation of glomeruli.

**METHODS**

**Isolation and explantation of glomeruli**

Normal glomeruli were isolated from 14 adult male Sprague-Dawley rats (200 to 280 g body wt) using the conventional sieving method [13]. In brief, kidneys were perfused through the renal artery with cold Dulbecco's modified Eagle's medium/Ham's F-12 (DME-F12; GIBCO BRL, Gaithersburg, MD, USA). In some experiments, kidneys without perfusion were used for glomerular isolation. The kidneys were minced well on ice and forced through sequential steel sieves, and glomeruli were collected using cold phosphate-buffered saline (PBS). Then, the glomeruli were washed and suspended in DME-F12 supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B and 1% fetal calf serum (FCS; GIBCO BRL). Incubation was performed in 24-well tissue culture plates at 37°C in a 5% CO₂ atmosphere for up to 16 hours. In some experiments, glomeruli were incubated in the presence of 5 to 20% FCS or human recombinant insulin-like growth factor-I (IGF-I, 100 ng/ml; Genzyme, Cambridge, MA, USA). After the incubation, glomeruli were washed twice with cold PBS and used for analyses, as described below.

**Ladder detection assay**

After ex vivo incubation, isolated glomeruli (2.5 to 5 × 10³) were lysed with 150 μl hypotonic lysis buffer (10 mM EDTA, 0.5% Triton X-100 in 10 mM Tris-HCl; pH 7.4) for 15 minutes on ice and precipitated with 2.5% polyethylene glycol and 1 mM NaCl for 15 minutes at 4°C. After centrifugation at 16,000 × g for 20 minutes at room temperature, the supernatant was incubated in the presence of proteinase K (300 μg/ml; Sigma Immunochemicals, St. Louis, MO, USA).
USA) at 37°C for one hour and precipitated with isopropanol at −20°C. After centrifugation, each pellet was dissolved in 10 μl of Tris-EDTA (pH 7.6) and electrophoresed on a 1.5% agarose gel containing ethidium bromide. Ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

**TUNEL assay**

TUNEL assay was performed using Apoptosis Detection System, Fluorescein (Promega, Madison, WI, USA). In brief, after incubation, glomeruli (5 × 10³) were fixed by 4% paraformaldehyde in PBS overnight at 4°C. Glomeruli were washed three times with PBS and permeabilized by 0.2% Triton X-100 in PBS for 15 minutes on ice. After washing twice, glomeruli were equilibrated at room temperature for 30 minutes in equilibration buffer (200 mM potassium cacodylate, 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 2.5 mM cobalt chloride in 25 mM Tris-HCl, pH 6.6) and then incubated in the presence of 5 μM fluorescein-12-dUTP, 10 μM dATP, 100 μM EDTA and terminal deoxynucleotidyl transferase at 37°C for 1.5 hours in dark. The tailing reaction was terminated by 2 × SSC. Glomeruli were washed three times with PBS and analyzed under a fluorescence microscope.

**Histological analysis**

For sectioning of isolated glomeruli, 1 × 10⁸ glomeruli fixed with 4% paraformaldehyde were embedded in collagen gel. In brief, type I collagen solution (450 μl) prepared from the tail tendon of rats [14] was mixed on ice with ice-cold reconstruction buffer (0.14 N NaOH - 0.26 N NaHCO₃; 50 μl). Glomeruli were suspended in this mixture and immediately transferred into a cold Eppendorf tube (0.5 ml) in which a small piece of filter paper was fixed on the bottom. After incubation for 30 minutes at 37°C, a small hole was made at the bottom of the tube, and the material was centrifuged gently. This allowed for condensation of the glomeruli-containing collagen gels. The condensed gels were then fixed with 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections prepared (3 to 5 μm) were stained with periodic acid-Schiff and hematoxylin and examined using a light microscope.

**Northern blot analysis**

Expression of a podocyte marker, glomerular epithelial protein 1 (GLEPP1) [15], was examined by Northern blot analysis, as described previously [16]. In brief, isolated normal glomeruli were incubated in the presence of 1% FCS for 24 hours. Total RNA was extracted by a single-step method, and RNA samples were electrophoresed on 1.2% agarose gels and transferred onto nitrocellulose membranes. For hybridization, a rabbit GLEPP1 cDNA (a gift from Dr. R.C. Wiggins, University of Michigan, MI, USA) was labeled with ³²P-dCTP using the random priming method. As a loading control, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. The membranes were hybridized with probes at 65°C overnight in a solution containing 4 × SSC (600 mM sodium chloride, 60 mM sodium citrate), 5× Denhardt’s solution, 10% dextran sulfate, 50 μg/ml herring sperm DNA and 50 μg/ml poly(A), washed at 50°C and exposed to Kodak XAR films at −80°C.

**RESULTS**

Cleavage of chromatin into oligonucleosome-sized fragments is regarded as a biochemical hallmark of apoptosis. To examine whether glomerular cells undergo apoptosis after glomerular explantation, isolated rat glomeruli were subjected to a ladder detection assay before or after ex vivo incubation. Immediately after isolation, oligonucleosomal fragments of DNA were not detectable in isolated glomeruli. However, after ex vivo incubation for one hour, obvious DNA fragmentation (characteristic “ladders” on agarose gel electrophoresis) was observed (Fig. 1A). The fragmented DNA was not derived from blood-borne cells but from resident cells, since blood-free glomeruli from perfused kidneys similarly exhibited DNA fragmentation (Fig. 1B). The spontaneous induction of apoptosis in isolated glomeruli was not inhibited even in the presence of FCS (5 to 20%) or IGF-I (100 ng/ml) (Fig. 1C), both of which are regarded as putative survival factors for glomerular cells [17]. Time-course studies revealed that the apoptosis occurred immediately after explantation. Within 15 minutes, the DNA fragmentation was detectable (Fig. 1D). Substantial ladder formation was observed after 30 minutes, which was peaked at two hours and attenuated thereafter. After 16 hours, only a modest level of laddering was detectable.

Apoptosis in glomeruli isolated from perfused kidneys was further examined by a TUNEL method. We applied the one-step TUNEL assay to whole glomeruli. Without incubation, TUNEL-positive nuclei were hardly detectable in isolated normal glomeruli (Fig. 2A). However, after ex vivo incubation of glomeruli for two hours, a number of resident cells became positive for TUNEL (Fig. 2B). At this stage, entire nuclei were strongly positive. Consistent with the results of the ladder detection assay, neither FCS nor IGF-I attenuated the glomerular positiveness for TUNEL (data not shown). After 16 hours, the nuclei of glomerular cells were still TUNEL-positive, but the intensity was much weaker than that at two hours (Fig. 2C).

To identify the cell type undergoing apoptosis, we attempted to section the isolated glomeruli. After ex vivo incubation, blood-free glomeruli were fixed and embedded in collagen gel. The whole materials were then embedded in paraffin, sectioned and stained using conventional processing. The structure of isolated glomeruli embedded in collagen gels was well preserved. Light microscopic analysis revealed that a number of podocytes showed condensation of nuclear chromatin that is typical of apoptotic cells (Fig.
Using 5 μm sections, apoptotic podocytes were detectable in 93% of glomeruli. The mean number of apoptotic podocytes on each glomerular section was 3.1 cells per glomerulus. About 80% of the total podocytes were apoptotic. Similarly, apoptosis of Bowman’s epithelial cells was evident (Fig. 3B). Although the incidence was much less, apoptosis of endothelial cells was also observed. Detached and retracted endothelial cells with chromatin condensation or apoptotic bodies were occasionally observed within capillary lumens (Fig. 3C-E). A small population of glomeruli contained clusters of apoptotic bodies in a segmental manner (Fig. 3F, G). In these glomeruli, cellular components of some tufts were completely replaced with apoptotic bodies (Fig. 3G), implying that mesangial cells also undergo spontaneous apoptosis in isolated glomeruli.

To further confirm physical and/or functional ablation of podocytes in explanted glomeruli, the expression of GLEPP1, a marker of podocytes, was examined by Northern blot analysis. Immediately after isolation, substantial expression of GLEPP1 mRNA was dramatically down-regulated (Fig. 4). Expression of a housekeeping gene GAPDH was constantly maintained during the ex vivo incubation of isolated glomeruli.

**DISCUSSION**

During the past decades, isolated glomeruli have been widely utilized to investigate biochemical properties of the glomerulus. In contrast, little attention has been paid to structural aspects of explanted glomeruli. It is largely unknown whether and how the histological integrity of the glomerulus is affected during ex vivo incubation. A previous
study has shown that, after explantation, glomerular podocytes became mitogenic in response to a high concentration of serum [18]. However, currently, there are no reports that examined cellular fate in isolated glomeruli. In this report, we provide novel evidence that resident cells, especially podocytes, spontaneously undergo apoptosis immediately after explantation of glomeruli. We found that the apoptotic process was rapid and self-limiting; that is, following explantation, apoptosis was induced within 15 minutes, peaked at two hours and subsided thereafter. Based on the results from agarose gel electrophoresis and TUNEL, massive apoptosis did not occur after this initial phase. Histological analysis and Northern blot analysis evidenced that the major cell type undergoing apoptosis was the podocyte. Apoptosis of endothelial cells and mesangial cells was also occasionally observed.

The apoptosis of podocytes in isolated glomeruli may be supported by some previous observations. Norgaard reported that foot processes of podocytes spontaneously retracted during culture of isolated glomeruli [19]. Norgaard also prepared single cell suspension from isolated glomeruli and performed cytological analysis [18]. Although the author did not mention it, the cell suspension contained significant number of apoptotic bodies and apoptotic cells.

What is the trigger for spontaneous apoptosis in isolated glomeruli? Several possibilities may be postulated. The first possibility is deprivation of survival factors that might be continuously supplied via the circulation. Using cultured mesangial cells, Mooney et al. reported that FCS and IGF-I inhibited mesangial cell apoptosis induced by serum deprivation [17]. We tested the protective effects of FCS and IGF-I, but neither factor prevented glomeruli from the spontaneous apoptosis. Normally, glomerular cells are continuously exposed to hemodynamic pressure and tension. As the second possibility, a lack of hemodynamic forces in isolated glomeruli may lead to spontaneous apoptosis, as has been reported in vascular endothelial cells [20]. The third possibility is that during the sieving process, mechanical stress might have triggered apoptosis of glomerular cells. Although, in general, mechanical damage causes necrosis, a recent report has shown that excessive mechanical stress induced apoptosis of myocytes [21]. The fourth possibility is release of death mediators within glomeruli. Tumor necrosis factor-α (TNF-α) is widely recognized as an inducer of apoptosis [22]. It has been shown that isolated, intact glomeruli spontaneously release TNF-α [23]. Endogenous cytokines including TNF-α could play a role in the induction of apoptosis in isolated glomeruli.

Histologic changes of explanted glomeruli during ex vivo incubation are poorly understood. This is mainly due to the difficulty in sectioning of isolated glomeruli. A few reports addressed methods for preparing glomerular sections [18, 24, 25], but none of those have been applied for paraffin sections. In the present study, we embedded isolated glomeruli in collagen gels, and the whole materials were subjected to conventional processing for paraffin sections. This method preserved the glomerular structure and allowed for
histological examination on isolated glomeruli. This simple approach would be useful for histochemical and immuno-histological analyses of isolated glomeruli to examine their ex vivo responses to certain stimuli.

In summary, we demonstrated that spontaneous apoptosis is observed in explanted glomeruli. Elucidation of molecular mechanisms involved in this process may lead to a better understanding of the podocyte biology and to a better explantation system that preserves glomerular integrity in vitro. Further investigation should be focused on the trigger of apoptosis, intracellular signaling and modulators that attenuate the spontaneous apoptotic event.

Fig. 3. Detection of apoptotic cells on paraffin sections of isolated glomeruli. Blood-free glomeruli were fixed and embedded in type I collagen gel. The whole materials were embedded in paraffin, sectioned and stained with periodic acid-Schiff and hematoxylin. An apoptotic podocyte with condensed nuclear chromatin (A), an apoptotic Bowman’s epithelial cell (B), detached and retracted endothelial cells with chromatin condensation (C), apoptotic bodies within capillary lumens (D, E), a glomerular tuft replaced with apoptotic bodies (F), and high power view of the cluster of apoptotic bodies (G). Light microscopy (×400 - ×1000).
Fig. 4. Expression of glomerular epithelial protein 1 (GLEPP1) in explanted glomeruli. Isolated glomeruli were incubated in the presence of 1% FCS for 24 hours, and Northern blot analysis was performed on the expression of GLEPP1. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control. Position of ribosomal RNAs is indicated on the right. A typical result from independently performed four experiments is shown.

ACKNOWLEDGMENTS

We thank Dr. R.C. Wiggins for the kind gift of GLEPP1 cDNA. This work was supported by grants from Baxter Healthcare Corporation (Extramural Grant Program), Wellcome Trust and National Kidney Research Fund to M. Kitamura.

APPENDIX

Abbreviations used in this article are: DME-F12, Dulbecco’s modified Eagle’s medium/Ham’s F-12; FCS, fetal calf serum; GLEPP1, glomerular epithelial protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor-I; PBS, phosphate buffered saline; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Reprint requests to Dr. M. Kitamura, Glomerular Bioengineering Unit, Department of Medicine, University College London Medical School, The Rayne Institute, 5 University Street, London WC1E 6JJ, England, United Kingdom. E-mail: m.kitamura@medicine.ucl.ac.uk

REFERENCES