Endothelial Cell Apoptosis during Glomerular Capillary Lumen Formation \textit{In Vivo}

WOLFGANG FIERLBECK,* AILIAN LIU, † RALUCA COYLE,* and BARBARA J. BALLERMANN*

*Department of Medicine, Division of Nephrology, Albert Einstein College of Medicine, Bronx, New York; †Shanghai Second Medical University, People’s Republic of China.

Abstract. Transforming growth factor–β (TGF-β) stimulates endothelial cell apoptosis \textit{in vitro}, and inhibition of TGF-β1 leads to retention of undifferentiated endothelial cells in developing glomerular capillaries and reduced lumen formation \textit{in vivo}. This study explored the question whether glomerular capillary lumen formation \textit{in vivo} may involve TGF-β1–dependent endothelial cell apoptosis. Neutralizing anti-TGF-β1 or non-immune IgY were infused into the renal arteries of 3-d-old rats, and the kidneys were examined 2 d later. By transmission electron microscopy, endocapillary apoptotic cells were observed at a frequency of 0.10/loop in immature glomeruli of 3-d-old rats, and the kidneys were examined 2 d later. By transmission electron microscopy, endocapillary apoptotic cells were observed at a frequency of 0.10/loop in immature glomeruli of 3-d-old rat pups. In 5-d-old rat pups given neutralizing TGF-β1 antibody or control IgY, the frequency of endocapillary apoptotic cells was 0.03 and 0.09/loop, respectively (\(P < 0.001, \chi^2\)). Dual labeling with TUNEL and anti-von Willebrand factor (vWF) antibody showed that apoptotic cells in immature glomeruli of 5-d-old rat pups are endothelial cells. Quantitative analysis showed significantly fewer TUNEL/vWF–labeled cells in glomeruli after anti-TGF-β1 antibody infusion than in controls. No endocapillary apoptotic cells were observed in any group in C-shaped or S-shaped bodies, and the TUNEL assay revealed no glomerular apoptotic cells in kidneys from mature rats. These findings suggest that superfluous endothelial cells are cleared from immature glomerular capillaries by apoptosis, a process regulated by TGF-β1. Taken together with the previous finding, that TGF-β1 blockade blunts glomerular capillary lumen formation \textit{in vivo}, it is proposed that TGF-β1–dependent apoptosis serves to open capillary lumens in this vascular bed during glomerular development.

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Correspondence to Dr. Barbara J. Ballermann, Professor of Medicine, The Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461. Phone: 718-430-3252; Fax: 718-430-8963; E-mail: bjballer@accom.yu.edu

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Tissue Source

Tissue for TUNEL assay and transmission electron microscopy (TEM) micrographs for quantification of endocapillary apoptosis

During kidney morphogenesis, glomerular endothelial cells are initially very large, filling the lumen of glomerular capillaries. Progressive differentiation then takes place to produce an extremely flattened cell monolayer that is densely perforated by fenestrae. Fenestrated endothelial cells represent an important component of the glomerular barrier and permit the rapid flux of water and small solutes to form the primary filtrate (1–3). Some of the molecular mechanisms that lead to general vascular and glomerular capillary development have been elucidated. Basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), as well as Ephrins and their receptors have been identified as important mediators of endothelial cell proliferation, migration, and morphogenesis (4,5).

We have studied the role of transforming growth factor–β1 (TGF-β1) during renal capillary morphogenesis. Bovine glomerular endothelial cells in monolayer culture form capillaries in response to TGF-β1, a process associated with apoptosis of a subset of the cells. Both effects are abrogated by stably expressed dominant negative type II TGF-β receptors (6). Pollman et al. (7) similarly observe apoptosis and reorganization of endothelial cell capillary networks in response to TGF-β1 \textit{in vitro}. They find differences between the response of aortic endothelial cells and umbilical vein endothelial cells, suggesting that the TGF-β1–mediated effects may be restricted to specific endothelial cell subsets (7,8). \textit{In vivo}, intrarenal neutralizing TGF-β1 antibody infusion inhibits invasion of the capillary cleft in comma-shaped and S-shaped bodies by endothelial cells and delays differentiation of glomerular capillary endothelium (9).

Cultured glomerular capillary endothelial cells respond to TGF-β1 with apoptosis, and large, undifferentiated glomerular endothelial cells are retained in the lumen of developing glomerular capillaries after neutralizing TGF-β1 antibody infusion; we therefore explored the question whether TGF-β1–induced apoptosis may occur during glomerular development \textit{in vivo}. We now show evidence for ongoing, TGF-β1–dependent apoptosis of glomerular capillary endothelial cells in immature glomeruli during rat renal development.

Material and Methods
were from the same animals prepared and reported in a previous study, where the handling and surgical procedures performed in developing rat pups were described in detail (9). Briefly, 8 μg of neutralizing chicken anti-TGF-β1 IgY (Collaborative Biomedical Products, Bedford, MA) or purified chicken IgY (Sigma, St. Louis, MO) were injected directly into the renal circulation of 3-d-old neonatal Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) while the infrarenal aorta was clamped (n = 8/group). Rat pups were allowed to awaken from surgery and then returned to their mothers. Kidneys were harvested 2 d later and processed for immunohistochemistry and transmission electron microscopy (TEM). Untreated 3-d-old rats (n = 5) served as controls.

Transmission Electron Microscopy

TEM studies were performed as described (9). Briefly, one fifth of each kidney from four rats per group were fixed in 2% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate-buffered saline, pH 7.2 (PBS; 4F-1G; Paragon, Baltimore, MD) at 4°C overnight and post-fixed in 1% osmium tetroxide in S-Collidine buffer. Thin sections (0.1 μm) were cut, stained with lead citrate and uranyl acetate, and examined at 60 kV with a JEOL 1200EX electron microscope. All glomeruli in a given section were then examined by TEM and photographed without selection by a technician who was blinded to the origin of the images (Table 1). A total of 39 and 47 glomeruli from IgY and anti-TGF-β1 antibody-infused groups. Capillary loops were defined as structures delimited by glomerular basement membrane and abluminal podocytes. Early apoptotic cells were defined as those containing nuclei with condensed chromatin along the nuclear membrane, dense cytoplasm, intracytoplasmic vacuoles, and dense bodies, as described previously by others (10,11).

Immunohistochemistry

Dual labeling for TUNEL and von Willebrand factor (vWF) was performed on formalin-fixed, paraffin-embedded kidney sections (5 μm) from 5-d-old rats. Sections were deparaffinized with xylene followed by rehydration through a graded series of ethanol and double distilled water in a standard manner. For the TUNEL assay, the in situ cell death detection kit (TMR red; Boehringer-Mannheim, Mannheim, Germany) was used. Tissue sections were incubated with proteinase K (50 μg/ml in 10 mM Tris/HCl, pH 7.4) for 15 min at room temperature (RT). The sections were rinsed twice with PBS and incubated with 50 μl of the TUNEL reaction solution for 60 min at 37°C in a humid chamber. Subsequent procedures were conducted in the dark. After performing the TUNEL assay, labeling of endothelial cells with vWF antibody was done by the avidin-biotin immunoperoxidase method Tyramide signal amplification. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 15 min at RT. Sections were then treated with 0.04% Pepsin (Sigma) in 0.01 N HCl for 30 min at 37°C and rinsed with PBS. Nonspecific binding was blocked with 1% normal goat serum (NGS) in PBS for 30 min at 37°C. Sections were then incubated with a rabbit polyclonal anti-vWF antibody (1:250; DAKO, Carpinteria, CA) in 1% NGS/PBS for 60 min at 37°C. The sections were then rinsed in PBS and incubated with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) for 30 min at RT, Sections were then incubated with the avidin-biotin complex (ABC Kit, Vector, Burlingame, CA, USA) for 30 min, also at RT, and rinsed with PBS. The incubation with biotinylated secondary antibody and avidin-biotin complex was repeated once for 10 min each at 37°C. Fluorescein staining was developed using the TSA fluorescence system (NEN Life Science Products Inc., Boston, MA). The Fluorophore Tyramide working solution was prepared following the manufacturer’s protocol and applied to the sections for 5 min. The sections were then rinsed with PBS and mounted under a cover slip using Prolong Antifade Kit (Molecular Probes, Eugene, OR). Immunocytochemistry with a rabbit polyclonal antibody directed against cleaved caspase 3 (Cell Signaling Technologies, Beverly, MA) was performed without tyramide amplification. The signal was visualized with diaminobenzimide. For negative controls, the primary antibody was omitted.

Cultured Glomerular Endothelial Cells

To determine whether vWF remains associated with apoptotic bovine glomerular endothelial cells, they were cultured on collagen-coated glass cover slips as described previously. Apoptosis was induced by treating the cells with UV light for 5 min followed by incubation under the usual culture conditions for 2 h. Cells were fixed with 4% paraformaldehyde. The TUNEL assay was performed according to the manufacturers protocol followed by the immunostaining for vWF. Briefly, cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (Sigma) for 2 min on ice, washed twice with PBS, and incubated with the TUNEL reaction solution for 60 min at 37°C in a humid chamber in the dark. The cover slips were then washed twice with PBS for 5 min, and nonspecific binding was blocked with 1% NGS in PBS for 30 min at 37°C. The coverslips were incubated with a 1:25 dilution of the anti-vWF antibody (DAKO) for 1 h at 37°C, washed two times with PBS for 5 min, and then incubated with Alexa Fluor 488 (1:100; Molecular Probes, Eugene, OR) for 30 min at 37°C. After washing and mounting the cells were evaluated by confocal microscopy.

To define the ultrastructural appearance of apoptotic glomerular endothelial cells in culture, the cells were cultured on gelatin as

<p>| Table 1. Frequency of endocapillary apoptotic cells in developing rat kidney glomeruli* |
|-----------------------------|-----------------|---------------|-------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Group (n = 4 Rats Each)</th>
<th>Age (d)</th>
<th>Structure</th>
<th>Loops (n)</th>
<th>Endocapillary Apoptotic Cells (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3</td>
<td>S/C-shaped body</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Control IgY</td>
<td>5</td>
<td>S/C-shaped body</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Anti-TGF-β1 IgY</td>
<td>5</td>
<td>S/C-shaped body</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Untreated</td>
<td>3</td>
<td>Glomerulus</td>
<td>67</td>
<td>7 (10.4%)</td>
</tr>
<tr>
<td>Control IgY</td>
<td>5</td>
<td>Glomerulus</td>
<td>269</td>
<td>25 (9.3%)</td>
</tr>
<tr>
<td>Anti-TGF-β1 IgY</td>
<td>5</td>
<td>Glomerulus</td>
<td>380</td>
<td>11 (2.9%)‡</td>
</tr>
</tbody>
</table>

a 39 and 47 glomeruli from IgY and anti-TGF-β1 IgY-treated rats, respectively, were evaluated.

b P = 0.001 versus control IgY.
described previously (12). Cells were treated with UV light for 5 min and then returned to the usual culture conditions for 16 h, followed by harvesting. A separate plate of cells was treated with TGF-β1 (10 ng/ml) for 16 h, previously shown to induce apoptosis (6) and then harvested. Untreated cells served as controls. Apoptosis is associated with glomerular endothelial cell detachment. Therefore, cells in the culture supernatant were combined with trypsinized cells. The cells were then washed by centrifugation three times in ice-cold PBS. The final pellet was fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, 0.2 M sucrose in 0.1 M cacodylate buffer, and processed for transmission electron microscopy.

**Statistical Analyses**

Statistical evaluation of the apoptotic cell frequency was by χ² analysis. The critical value for P < 0.001 by χ² analysis is 10.83.

**Results**

**Endocapillary Apoptotic Cells in Developing Rat Glomeruli**

Representative photographs of endocapillary apoptotic cells in glomeruli of 5-d-old rat pups and of apoptotic cells in culture are shown in Figure 1. In these glomeruli, most capillary lumens are still filled with large cells. Three stages of apoptosis are shown for glomeruli in vivo (Figure 1, A to C and D to F.

**Figure 1.** Endocapillary cells undergoing apoptosis. Transmission EM of glomerular capillary loops showing intracapillary apoptosis at different stages. (A and D) Bold arrows point to cells with chromatin condensation. Nuclear chromatin is less dense in adjacent cells in the same field. (B and E) Cells apparently in the process of detaching from the GBM (*). Arrows point to regions where the cells are still attached to the GBM. Chromatin condensation is also observed in panels B and E. (C and F) Apoptotic bodies within glomerular capillaries (bold arrows). Panels A, C, and F are from control IgY-infused rats; Panels B, D, and E are from anti-TGF-β1-IgY infused rats. (G through J) Appearance of glomerular endothelial cells in culture. (G) Untreated normal cell. (H) Chromatin condensation and margination 16 h after UV exposure. (I) Chromatin condensation after 16 h of exposure to TGF-β1. (J) Apoptotic body observed after 16 h of exposure to TGF-β1.

Figures 1A and 1D show the initial condensation of nuclear chromatin for cells located within glomerular capillaries. Similar condensation and margination of chromatin at the nuclear envelopes is shown for UV-treated and TGF-β1–treated cells in Figures 1H and 1I, respectively. Figures 1B and 1F show apoptotic cells detaching from the GBM; the cell in Figure 1B showing condensed nuclear chromatin, and the cell in Figure 1F is still partially attached to the GBM. Finally, two apoptotic bodies located in the capillary lumen are shown in Figures 1C and 1F. Similar apoptotic bodies are observed in culture, both when cells are treated with UV light and TGF-β1, as shown in the inset of Figure 1J.

Representative glomerular loops of rats treated with either neutralizing TGF-β1 antibody (Figure 2A) or control IgY (Figure 2B) investigated on neonatal day 5 reveal capillary loops with retained, large endothelial cells (A) and differentiated, attenuated endothelial cells (B). We previously reported that the ratio of open capillary lumen area to total capillary loop area was significantly lower in rats given neutralizing TGF-β1 when compared with those given control IgY (9).

**Frequency of Endocapillary Apoptotic Cells in Developing Rat Kidney Glomeruli**

The frequency of apoptotic cells from four rat pups per group, as determined by electron microscopy, is shown in Table 1. No endocapillary apoptotic cells were observed in C-shaped or S-shaped bodies in any group. The frequency of endocapillary apoptotic cells in 5-d-old rats treated with neutralizing TGF-β1 antibody was significantly lower than that in littermates treated with control IgY (2.9 versus 9.3%; χ² = 12.2; P < 0.001). In untreated 3-d-old rats, 10.4% of glomerular capillary loops contained endocapillary apoptotic cells.

Intraglomerular apoptotic cells were also visualized using an antibody that detects cleaved caspase 3. A representative example is shown in Figure 3A, panel 1. To define whether
intraglomerular apoptic cells are endothelial cells, staining with the endothelial specific anti-vWF antibody (green) was used. The low-power image (Figure 3A, panel 2) shows strong vWF immunoreactivity in glomeruli and large blood vessels. Apoptotic cells were recognized by the TUNEL technique (red). Co-localization of vWF-positive and TUNEL-positive cells (Figure 3A, panels 5 and 6, open arrows) in glomeruli confirms the presence of apoptotic glomerular endothelial cells. The closed arrow (Figure 3A, panel 4) shows an extraglomerular non-endothelial apoptotic cell and a non-endothelial intraglomerular apoptotic cell is shown in Figure 3A, panel 3). In mature rat kidneys, apoptotic cells were exceedingly rare, and no apoptotic cells were observed in the glomeruli (data not shown).

In sections of IgY and anti-TGF-β1 IgY-treated animals (one section per rat; n = 8 rats per group), all glomeruli were evaluated for TUNEL-positive intraglomerular cells with or without dual labeling by vWF. A total of 734 and 731 glomeruli were evaluated in IgY and anti-TGF-β1 IgY-treated rats, respectively. In control rats, 63 TUNEL-positive cells were found, of which 44 were also labeled with vWF. In sections from anti-TGF-β1 IgY-treated rats, 35 TUNEL-positive cells were observed, of which 20 were also labeled by the vWF antibody (Figure 3B). The difference in the proportion of
glomeruli containing TUNEL/vWF dual-labeled cells between IgY and anti-TGF-β1 IgY-treated rats was highly significant ($\chi^2 = 154.62; P < 0.001$).

To determine whether vWF is retained by endothelial cells undergoing apoptosis, cultured glomerular endothelial cells were exposed to UV light, known to induce apoptosis, and then labeled with TUNEL and vWF antibody. Two hours after UV light treatment approximately 20% of cells were TUNEL-positive. vWF immunofluorescence was observed in all TUNEL-positive cells (Figure 3C).

**Discussion**

During embryogenesis, newly formed glomerular capillaries are initially filled with large, non-fenestrated endothelial cells (9,13) (Figures 1 and 2). During maturation, lumen-formation occurs and glomerular endothelial cells differentiate into flattened, highly fenestrated cells lining the capillary loops. The mechanisms involved in the formation of the glomerular capillary lumen are not fully understood. We now show that there are abundant apoptotic endothelial cells within glomerular capillaries of immature glomeruli. Neutralizing TGF-β1 antibody infusion significantly reduces the frequency of these apoptotic glomerular endothelial cells during glomerular maturation. Taken together with our previous finding that TGF-β1 is necessary for formation of open capillary lumens during development (9), we interpret the data to indicate that TGF-β1–mediated apoptosis serves to remove redundant glomerular capillary cells and thus facilitates lumen formation during glomerular capillary development.

Apoptosis is a general mechanism for the clearance of redundant cells during embryogenesis and has been described for different organ system such as developing kidney (14), immune system (15), and nervous system (16). The development of the limb into a distal extremity with separated digits (17,18) and the involution of the Xenopus tadpole’s tail during metamorphosis (19) are prototypical examples for developmental apoptosis. With regard to lumen formation, apoptosis of interstitial cells plays a role in the remodeling mechanisms of the developing fetal lung to achieve a mature alveolar structure (20). Apoptosis has also been proposed as a mechanism of ureteral lumen formation and maturation (21). Lumen formation by mammary acinar cells in culture similarly requires apoptosis; the apoptotic cells appear in the lumen, unattached to matrix, while the surrounding, surviving cells produce and attach to extracellular matrix (22). In the present study, apoptotic bodies were seen within the glomerular capillary lumen (Figure 1, C and F) and cells that appeared to be sloughing from the basement membrane were observed (Figure 1, B and E). Although proof that the apoptotic bodies are endothelial cells was not obtained by EM, it seems likely that apoptotic endothelial cells are shed into the capillary lumen.

There are also several reports showing apoptosis of endothelial cells during embryogenesis. However, in those studies, apoptosis was observed during capillary and vascular network regression rather than capillary formation. Mitchell et al. (23) reported extensive apoptosis of endothelial cells during the regression of the hyloid vascular system, a transient vascular network of the developing eye. In addition, apoptosis of endothelial cells has been described in regression of the microvascular bed of the Xenopus tadpole at the height of metamorphic climax (24) and during corpus luteum regression (25).

Capillary endothelial cell apoptosis in renal glomeruli has previously been observed in several models of glomerular injury. For instance, in the remnant kidney model glomerular endothelial cell apoptosis was associated with progressive loss of capillaries and glomerular sclerosis (26,27). Also, in the model of progressive glomerular sclerosis that follows anti-GBM–mediated glomerulonephritis, endothelial cell apoptosis was observed (26). In the recovery phase of the anti-Thy-1 model of mesangial proliferative glomerulonephritis, glomerular capillary repair involved proliferation of endothelial cells and new glomerular capillary growth from the glomerular vascular poles. During the repair process, apoptosis was observed (28) and postulated to be necessary in regulating the number of intrinsic endothelial cells.

This laboratory first reported TGF-β1–induced endothelial cell apoptosis associated with capillary formation in vitro (6), a phenomenon also observed by Pollman et al. (7,8). Until now, it was unclear whether TGF-β1–mediated apoptosis has any functional relevance in vivo. This study provides evidence for TGF-β1–dependent apoptosis of glomerular endothelial cells during glomerular capillary maturation in vivo. We observed apoptotic cells within the glomerular capillary lumen of developing, but not mature, rat glomeruli by transmission electron microscopy, and their abundance was significantly reduced in rat pups treated with neutralizing TGF-β1 antibody, Co-localization of TUNEL staining with vWF, the latter a specific marker of endothelial cells in vivo, shows that a significant number of these cells are endothelial cells. Co-localization of TUNEL and vWF was also observed in cultured glomerular endothelial cells stimulated to undergo apoptosis with UV light, indicating that vWF is retained during apoptosis. Furthermore, quantification of the dual-labeled cells in glomeruli in vivo was consistent with the electron microscopy data, in that significantly fewer TUNEL/vWF–positive cells were observed in glomeruli of developing rat kidneys treated with neutralizing TGF-β1 antibody rather than in glomeruli from control rats. Taken together with our previous observation that inhibition of TGF-β1 is associated with reduced glomerular capillary lumen formation (9), we propose that TGF-β1–dependent endothelial cell apoptosis is necessary to open glomerular capillary lumens during development. Whether this mechanism also applies in other capillary beds or is unique to glomerular capillaries is unknown.

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References