American Journal of Pathology, Vol. 151, No. 5, November 1997

Rare Glomerular Capillary Regeneration and Subsequent Capillary Regression with Endothelial Cell Apoptosis in Progressive Glomerulonephritis

Akira Shimizu, Hiroshi Kitamura, Yukinari Masuda, Masamichi Ishizaki, Yuichi Sugisaki, and Nobuaki Yamanaka

From the Department of Pathology, Nippon Medical School, Tokyo, Japan

Glomerulonephritis (GN) leading to glomerular sclerosis remains an important cause of renal failure. The glomerulus is a capillary network, but endothelial and vascular reactions during progressive GN are not well understood. We have, therefore, examined the morphological alterations of glomerular capillary network and endothelial cells during the progression of damaged glomeruli to glomerular sclerosis. A progressive model of anti-glomerular basement membrane (GBM) GN was induced in Wistar-Kyoto (WKY) rats with a single injection of anti-rat GBM antibody. Severe necrotizing glomerular injuries were observed between day 5 and week 3 with a reduction in the number of total glomerular endothelial cells and total glomerular capillary lumina per glomerular cross sections. In necrotizing lesions, the glomerular endothelial cells were lost with the destruction of the glomerular capillary network. Moreover, angiogenic capillary repair with proliferation of endothelial cells was rare in severely damaged regions of glomeruli. Subsequently, mesangial hypercellularity and marked mesangial matrix accumulation occurred with absence of the development of a capillary network, and the necrotizing lesions progressed to sclerotic scars until 8 weeks. Although active necrotizing lesions could not be seen in damaged glomeruli between week 4 and week 8, the number of apoptotic endothelial cells gradually increased in the glomerular capillaries (0.10 ± 0.01 apoptotic endothelial cells/glomerular cross section at week 8 *versus* 0.00 ± 0.00 control cells (mean ± SEM; $P < 0.05$) with the progression of glomerular sclerosis. Whereas the number of apoptotic endothelial cells increased in the damaged glomeruli, the number of total glomerular endothelial cells decreased (9.3 ± 3.0 cells/glomerular cross section at week 8 *versus* 24.8 ± 3.0 cells in control (mean ± SD); $P < 0.001$) with regression of glomerular capillaries (3.6 ± 2.5 capillary lumina/glomerular cross section at week 8 *versus* 35.0 ± 5.0 capillary lumina in control (mean ± SD); $P < 0.001$). Finally, glomerular endothelial cells could not be detected in the sclerotic lesions in progressive anti-GBM GN in WKY rats. These data indicate that the destruction of the capillary network of glomeruli and subsequent incomplete angiogenic capillary repair leads to glomerular sclerosis in progressive GN. Endothelial cell apoptosis with glomerular capillary regression may also contribute to the development of glomerular sclerosis. Injury of the glomerular capillary network with endothelial cell damage, including apoptosis and subsequent incomplete capillary repair, plays an important role in the progression of glomerular sclerosis during anti-GBM GN in WKY rats. *(Am J Pathol 1997, 151:1231–1239)*

Angiogenesis, the development of a new microvascular network, is an important phenomenon associated with both physiological conditions such as development of organs and pathological conditions including tumor vascularization and wound healing.[1–5] In the recovery models of experimental glomerulonephritis (GN), recent studies have demonstrated that angiogenic glomerular capillary repair can occur in damaged glomeruli.[6,7] In human GN, some may recover, allowing glomerular structure and function to return to normal. On the other hand, in the progressive form of GN, glomerular inflammation continues chronically and progresses to end-stage renal failure. The glomerulus is a well developed capillary network. However, damage of glomerular endothelial cells and the glomerular capillary network during progressive GN is still unclear. Moreover, the precise mechanisms that lead to sclerotic scar in GN remain to be clarified. Morphologically, GN is defined by an inflammation in glomeruli. In various organs, the apoptosis of endothelial cells has been seen during inflammation and wound healing.[8,9] However, apoptosis of endothelial cells in GN has not been well characterized. Recently, Wistar-Kyoto (WKY) rats have been shown to be very susceptible to anti-glomerular basement membrane (GBM) antibody, and a severe necrotizing and proliferative GN, which progresses to end-stage kidney disease and eventually to chronic renal failure can be induced by a single injection of anti-GBM antibody.[10–12] This study

Accepted for publication August 6, 1997.

Address reprint requests to Dr. Akira Shimizu, Department of Pathology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113 Japan.

investigated the destruction of the glomerular capillary network, capillary repair with glomerular endothelial cell proliferation, and glomerular capillary regression with endothelial cell apoptosis during the progression of glomerular sclerosis in anti-GBM GN in WKY rats. Endothelial cells were identified by immunostaining for thrombomodulin (TM) as a marker, which is known to be an endothelial cell surface glycoprotein.[6,13–16] Apoptosis was recognized morphologically, and nuclear DNA fragmentation of apoptosis was also detected in tissue sections using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method.

Materials and Methods

Induction of Anti-GBM GN

Inbred male WKY rats (Charles River Japan, Kanagawa, Japan) weighing 100 g were used for this study. In experimental groups, progressive GN was induced in WKY rats with a single injection of rabbit anti-rat GBM antibody (provided by Drs. Yasuhiro Natori and Naoyuki Nakao, International Medical center of Japan, Tokyo, Japan). An intravenous dose of 50 μg of IgG/100 g body weight was used to induce the disease.[12] Three rats were sacrificed on days 3, 5, 7, and 10 and 2, 3, 4, 6, and 8 weeks after the administration of anti-rat GBM antibody. Three uninjected WKY rats were sacrifices on day 0, week 4, and week 8 as controls.

Histological Examination

After removal of the kidney, tissue was fixed in 20% buffered formalin and embedded in paraffin for light microscopic examination. Hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and periodic acid-methenamine silver (PAM) stains were performed for histological examination.

To detect glomerular endothelial cells, tissue was stained with polyclonal rabbit anti-rat TM antibody[16] (provided by Dr. David Stern, Columbia University), which reacted with the surface of endothelial cells. This antibody has been used as a marker for endothelial cells.[6,13–16] Recent studies reported that TM expression is altered in renal disorders.[17–21] Confirmation of the glomerular endothelial cells displayed by TM was performed by staining tissue sections with the monoclonal antibody against a surface antigen expressed on all rat endothelial cells (RECA-1)[7,22] or *Griffonia (Baneirare) simplicifolia* lectin.[23] The results demonstrated similar endothelial cell patterns in glomeruli; neither was comparable in quality or specificity to TM (data not shown). This indicates that loss of TM activity in this model reflects a loss of glomerular endothelial cells, confirming data of a previous study.[6] TM was demonstrated by immunohistochemistry staining, using biotinylated anti-rat TM antibody. The antiserum was conjugated to biotin using a biotin labeling kit (Boehringer Mannheim, Mannheim, Germany). It is known that rat IgG deposits in the GBM during the autologous phase of the disease in this model. To show that the rabbit anti-TM antibody does not cross-react with rat IgG in the GBM, biotinylated nonimmune rabbit IgG was also prepared for control studies, using normal rabbit IgG (Vector Laboratories, Burlingame, CA) and a biotin labeling kit.

To detect proliferating cells, tissue sections were stained with mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (PC10, DAKO, Glostrup, Denmark). The epitope recognized by PC10 is very sensitive to the type of fixative used and the length of the fixation time.[24] Therefore, the tissues for immunohistochemistry studies were fixed uniformly for a period of 16 to 18 hours in 20% buffered formalin. Recently, Lan et al[25] demonstrated that the combination of microwave treatment and a reduced concentration of PC10 (1/1000 dilution) produce optimal and reliable detection of PCNA. We also confirmed the method of Lan et al for detecting PCNA-positive cells using microwave treatment before performing immunohistochemistry staining. This alternative procedure using the microwave technique demonstrated a similar pattern and frequency of PCNA-positive cells.

After deparaffinization, the 2.5-μm-thick sections were treated with 0.3% H_2O_2 in methanol for 30 minutes and then incubated for 60 minutes with a biotinylated anti-rat TM antibody, at a dilution 1:100, or a mouse anti-PCNA antibody, at a dilution of 1:300. Subsequently, the tissue sections for TM were incubated with avidin-biotin peroxidase complex (DAKO) for 60 minutes and were visualized by using H_2O_2 containing 3,3′-diaminobenzidine (DAB) in 0.05 mol/L Tris buffer. The tissue sections for PCNA were incubated with a peroxidase-conjugated goat anti-mouse IgG antibody (DAKO), at a dilution of 1:100 for 60 minutes, and were visualized by using H_2O_2- and NiCl-containing DAB in buffer (DAB substrate kit for peroxidase, Vector). Double immunostaining with PCNA and TM for the identification of the proliferating endothelial cells was performed, using a color modification method of DAB precipitation by NiCl, which changes the DAB color from brown to black.[26] Sections were incubated with PCNA followed by a peroxidase-conjugated goat anti-mouse IgG, and H_2O_2- and NiCl-containing DAB. Sections were then incubated with biotinylated rabbit anti-rat TM antibody, avidin-biotin peroxidase complex followed by H_2O_2-containing DAB. Staining controls include the substitution of the anti-PCNA antibody with an irrelevant murine monoclonal antibody, substitution of the biotinylated anti-rat TM antibody with the biotinylated normal rabbit IgG, and the use of anti-TM before anti-PCNA or *vice versa*.

In electron microscopic studies, the kidney tissue was fixed in 2.5% glutaraldehyde solution in phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with a Hitachi H7100 electron microscope.

Identification of Apoptosis

Apoptosis was recognized morphologically, using light and electron microscopy. In histological sections, frag-

mented nuclear DNA associated with apoptosis was labeled by the TUNEL method.[27] The 2.5-μm-thick sections were deparaffinized and incubated with proteinase K (100 μg/ml) for 15 minutes at room temperature. After blocking endogenous peroxidase by immersion in distilled water containing 2% H_2O_2, sections were rinsed in TdT buffer (30 mmol/L Tris/HCl buffer, pH 7.2, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride) and incubated with TdT 1:100 and biotinylated dUTP 1:200 in TdT buffer for 60 minutes at 37°C. The biotinylated nuclei were detected with avidin-peroxidase and H_2O_2- and NiCl-containing DAB. Apoptotic endothelial cells were detected morphologically and by double staining using the TUNEL method and staining for TM. Double staining was performed by first staining the sections with the TUNEL method (color reagent H_2O_2-, NiCl-containing DAB), followed by blocking free residues of biotin using 0.1% avidin (AVIDIN D, Vector) in PBS and 0.01% biotin (d-BIOTIN, Sigma Chemical Co., St. Louis, MO) in PBS for 20 minutes, respectively.[28] Sections were then incubated with biotinylated rabbit anti-rat TM antibody and avidin-biotin peroxidase complex, followed by H_2O_2-containing DAB. Negative controls consisted of omission of the dUTP or TdT in the TUNEL method, substitution of the biotinylated anti-TM antibody with the biotinylated normal rabbit IgG, and the use of anti-TM before the TUNEL procedure.

Quantification of Histological Findings

Morphometric studies were performed to determine the total number of glomerular endothelial cells, the total number of proliferating endothelial cells, and the total number of apoptotic endothelial cells per glomerular cross section. The total number of glomerular capillary lumina per glomerular cross section was also determined. In each kidney sample, more than 30 cross sections of glomeruli were examined sequentially for the following: 1) total number of endothelial cells, ie, the mean number of TM-positive cells per glomerular cross section, 2) proliferating endothelial cells, ie, the mean number of both PCNA- and TM-positive cells per glomerular cross section, 3) glomerular capillary regression, ie, the mean number of glomerular capillary lumina surrounded by TM-positive cells per glomerular cross section, and 4) incidence of apoptosis in endothelial cells, ie, the mean number of both TM- and TUNEL-labeled cells per glomerular cross section. We also examined the number of TM-positive apoptotic cells in TM-stained sections, and the result was a very small number of TM-positive apoptotic cells in glomerular cross sections. This suggests the loss of the cell surface protein (TM) from most of the affected cells, particularly in the late process of apoptosis in endothelial cells or bodies that we were able to identify by light microscopy. The TUNEL method can detect the early phase of apoptotic cells on which TM remained, and it is a more sensitive method for the detection of TM-positive apoptotic endothelial cells in this model. Excluded from the analysis were glomerular cross sections that contained only a small portion of the glomerular tuft. These results were expressed as the mean ± SD or SEM, and statistical analysis was performed using the Student's *t*-test.

Results

Destruction of Glomerular Capillary Network in Progressive GN

A severe necrotizing GN with crescent formation was induced in WKY rats by a single injection of anti-rat GBM antibody. This model is characterized by early infiltration of CD8-positive lymphocytes and adhesion of these cells to glomerular endothelial cells through the LFA-1/ICAM-1 pathway, which is crucial for the initiation and subsequent progression of anti-GBM GN.[10,11] Our results in this study also demonstrated that leukocytes infiltrated the glomeruli on day 3, and subsequent severe necrotizing and mesangiolytic glomerular damage was observed with massive exudative changes on day 5 to day 7 (Figure 1A). TM-positive endothelial cells were lost in the necrotizing and mesangiolytic lesions with the destruction of the glomerular capillary network (Figure 2A). Glomerular endothelial cells were reduced markedly with the destruction of capillary network in severely damaged areas as detected by electron microscopy (Figure 3A), which is consistent with the observation of the immunohistochemistry results for TM. Numerous proliferating endothelial cells ($PCNA^+$ TM^+) were found among the mildly damaged regions of glomeruli in the early phase (Figures 4 and 5B). However, rare proliferating endothelial cells were present in severely damaged necrotizing areas (Figure 4). Necrotizing GN continued between day 5 and week 3, and the number of total endothelial cells and the number of glomerular capillary lumina per glomerular cross section gradually decreased with the destruction of the glomerular capillary network (Figure 5, A and C). After glomerular damage, glomerular cell proliferation and mesangial hypercellularity with mononuclear cell infiltration was observed in mesangiolytic and necrotizing regions (Figures 2B and 3B). Subsequently, glomerular inflammation progressed, and mesangial matrix accumulation advanced with the development of glomerular sclerosis. Proteinuria and renal dysfunction gradually developed. The process of the development of glomerular sclerosis with renal dysfunction was demonstrated in our previous study.[12] Although active necrotizing GN resolved between week 4 and week 8, damaged glomeruli progressed to global glomerular sclerosis. During the progression of proliferative lesions to sclerotic scars, capillary regeneration and proliferation of glomerular endothelial cells were rare in markedly damaged and subsequent proliferative regions (Figures 2C and 3B). Moreover, in accordance with the development of glomerular sclerosis between week 3 and week 8, the number of glomerular capillary lumina per glomerular cross section gradually decreased with a reduction of glomerular endothelial cells in number (Figures 2D and 5, A and C). This suggested that glomerular capillaries regressed during the progression of glomerular sclerosis. Finally, most

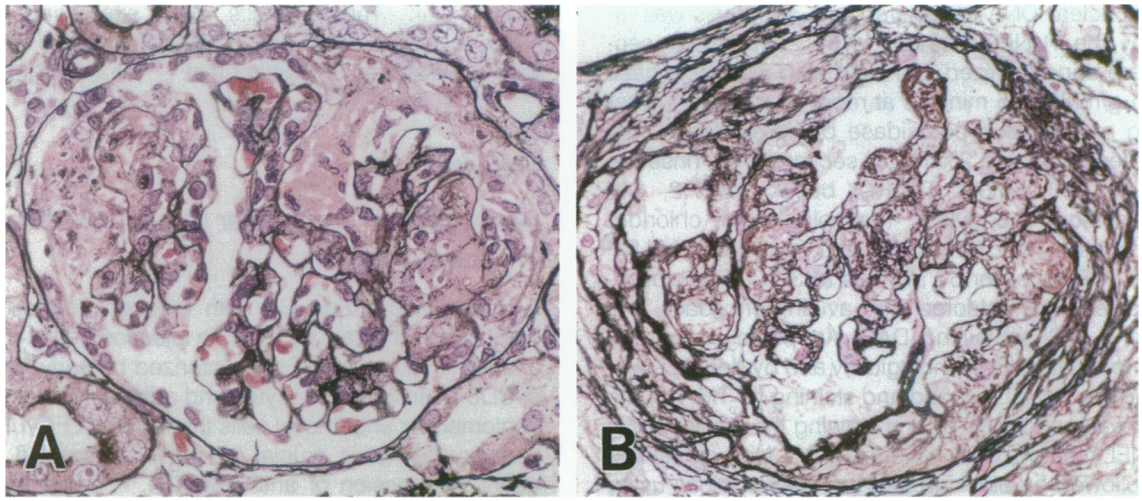

Figure 1. Morphological alteration of glomerulus in anti-GBM GN in WKY rats. **A:** Five days after disease induction, segmental necrotizing and mesangiolytic lesions occur with exudative change. **B:** Eight weeks after disease induction, glomerulus have become sclerotic, and glomerular capillary as well as endothelial cells are reduced in sclerotic lesions. PAM stain; magnification, ×600.

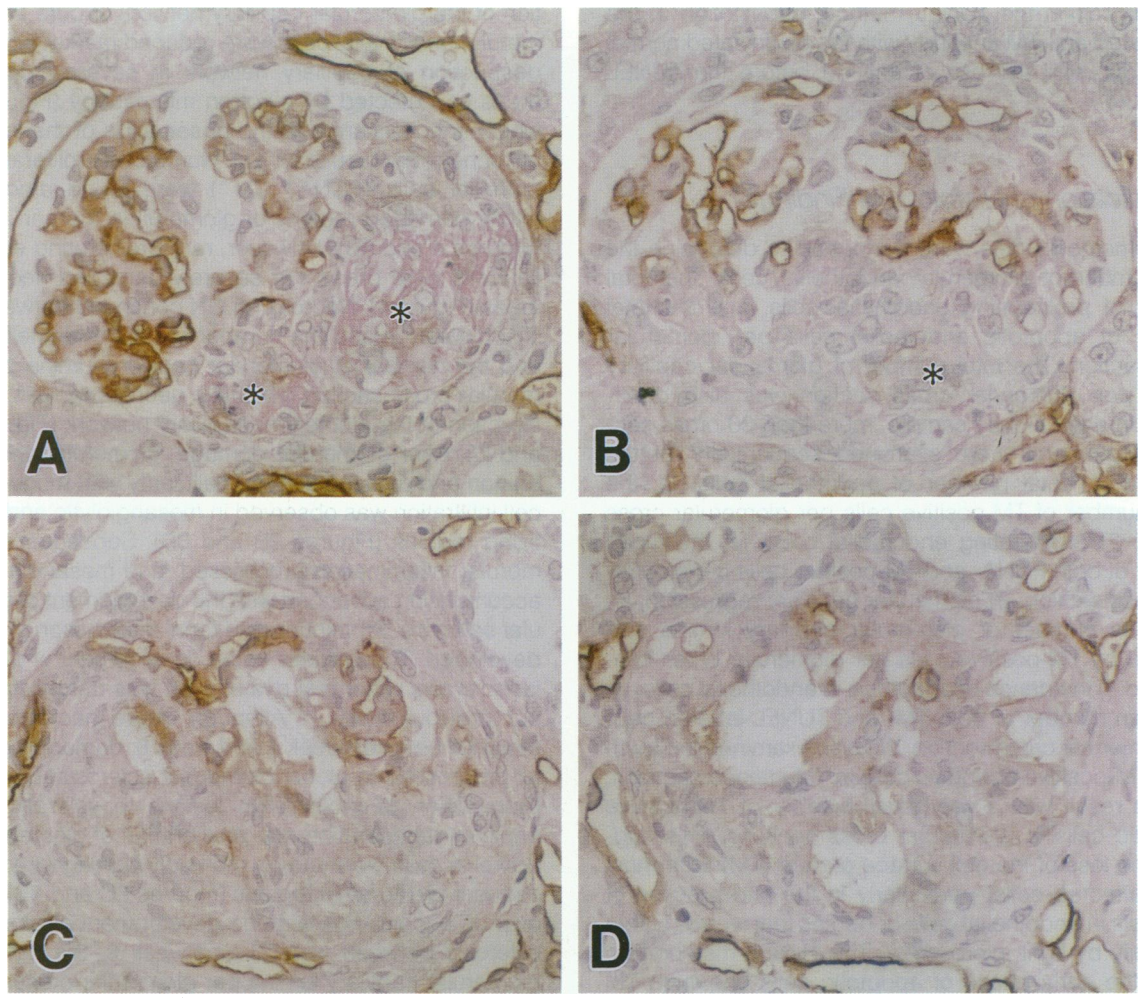

Figure 2. Capillary alteration in anti-GBM GN in WKY rats. **A:** Seven days after injection, the TM-positive glomerular endothelial cells disappear in the segmental necrotizing and mesangiolytic lesions (*). **B:** Two weeks after injection, segmental proliferative lesions develop with cellular crescent. Capillary regeneration is rare in areas of proliferation (**asterisk**). **C:** Six weeks after injection, the proliferative GN continues without regeneration of the glomerular capillary network. **D:** Eight weeks after injection, the number of TM-positive endothelial cells decreases in glomeruli with regression of the capillary network. Immunohistochemistry for TM; magnification, ×500.

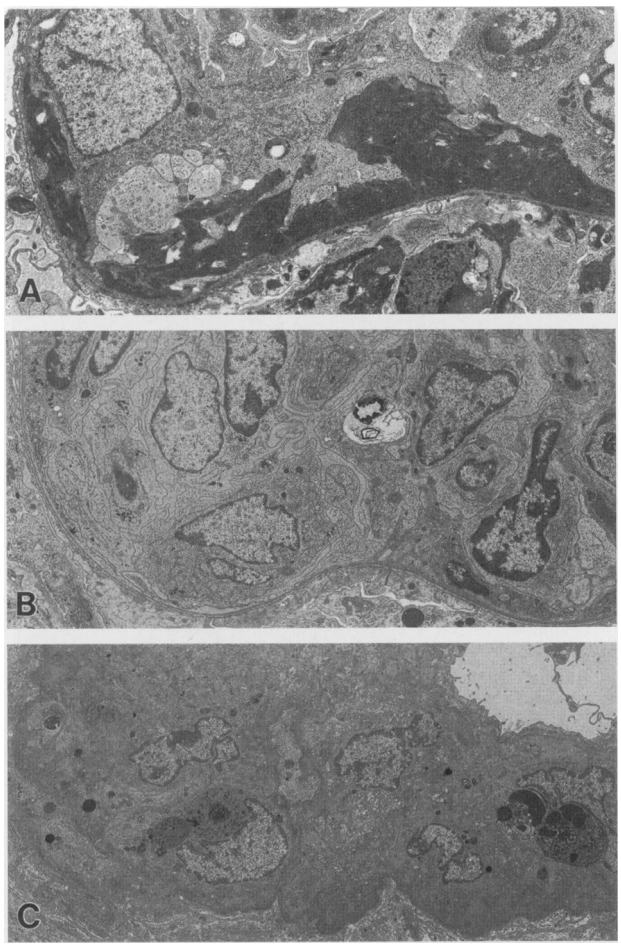

Figure 3. Progression of necrotizing lesions to sclerotic lesions. **A:** Five days after anti-GBM antibody administration. The glomerular capillary network is destroyed with a loss of endothelial cells and fibrin exudation. Magnification, ×4500. **B:** Two weeks after injection. Segmental proliferative lesions are found with marked infiltration of macrophages. The glomerular capillary network does not develop in the proliferative lesions. Magnification, ×3500. **C:** Eight weeks after injection. The glomerular sclerosis is advanced, and no glomerular capillary structure can be detected in sclerotic lesions. Magnification, ×3500.

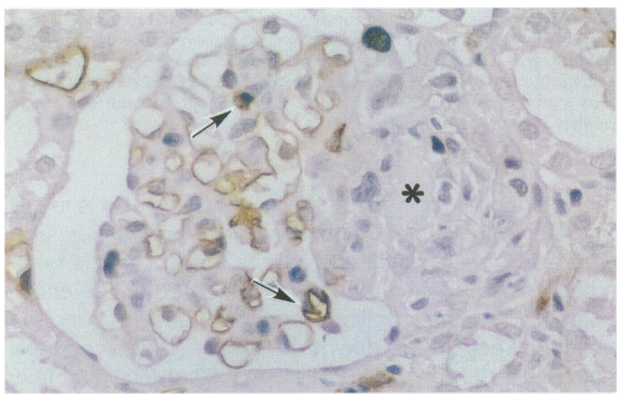

Figure 4. Endothelial cell proliferation in the damaged glomeruli. This is the damaged glomerulus with double immunostain for both PCNA (black) and TM (brown) at day 7. Double-labeled proliferating endothelial cells (**arrow**) are found in the mildly damaged lesions. However, no proliferating endothelial cells are present in mesangiolytic and proliferative areas (**asterisk**). Magnification, ×600.

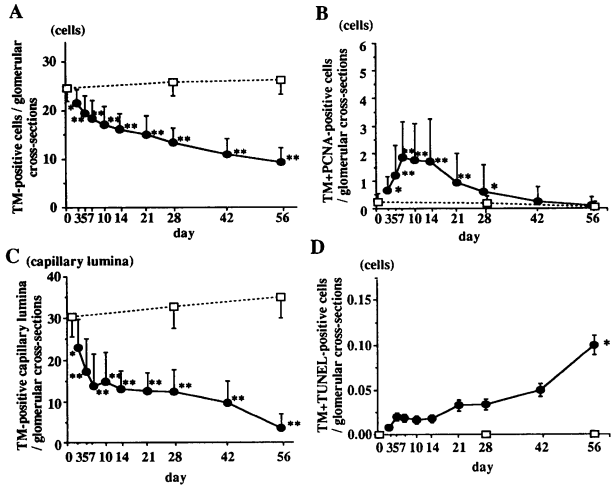

Figure 5. This is the correlation between the number of TM-positive total endothelial cells (**A**), PCNA- and TM-positive proliferating endothelial cells (**B**), TM-positive glomerular capillary lumina (**C**), and TUNEL- and TM-positive apoptotic endothelial cells (**D**) per glomerular cross section during the entire experiment. ● and □, number in experimental and control groups, respectively. $^{*}P < 0.05$; $^{**}P < 0.001$ when compared with control. In **A** to **C**, values are expressed as mean ± SD; in **D**, values are expressed as mean ± SEM.

of the glomeruli became globally sclerotic, and as a whole, the kidney appeared end-stage with tubulo-interstitial changes on week 8 with very little capillary network and very few endothelial cells remaining in the sclerotic glomeruli (Figures 1B and 3C).

Endothelial Cell Apoptosis in Progressive GN

Typical apoptotic cells containing condensed nuclear fragments were found in capillaries among sclerotic regions (Figure 6, A and B). These cells also expressed TM. Moreover, individual cells in capillaries were positive for both TUNEL method and TM (Figure 7, A and B). These data indicated that numerous apoptotic cells in capillaries around and among sclerotic lesions were identified as having endothelial cell origin. A few apoptotic cells in capillaries were not stained by TM, and these cells were considered to be infiltrating mononuclear cells. In electron microscopic studies, the various types of apoptotic cells and bodies were present in glomerular capillaries among sclerotic regions. Scattered individual endothelial cells in glomerular capillaries among sclerotic lesions underwent early apoptosis, which was characterized by

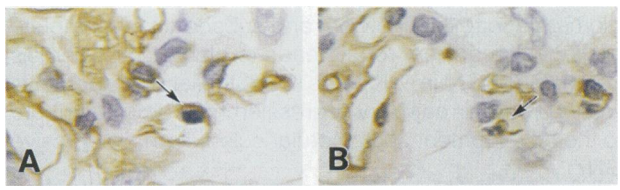

Figure 6. Apoptotic endothelial cells in glomerular capillaries six weeks (**A**) and 8 weeks (**B**) after disease induction. Typical apoptotic cells or apoptotic bodies (**arrow**) with condensed nuclei are seen in the glomerular capillaries. These apoptotic cells or apoptotic bodies are also expressing TM, indicating that these apoptotic cells are of endothelial origin. Immunohistochemistry for TM; magnification, ×800.

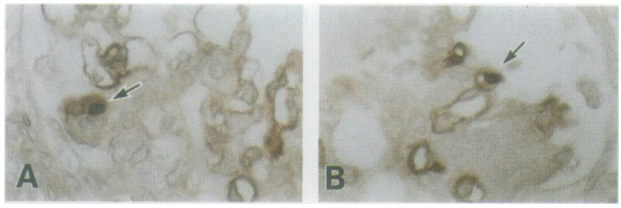

Figure 7. An apoptotic endothelial cell with double stain for both TUNEL method (black) and TM (brown) at week 6 (**A**) and week 8 (**B**). A double-labeled apoptotic endothelial cell (**arrow**) is found in capillaries around and among sclerotic lesions. Magnification, ×800.

condensation of the nucleus (Figure 8A). It has been known that apoptotic cells are removed through phagocytosis either by macrophages or by neighboring cells. The typical apoptotic bodies with dense nuclear and cytoplasmic fragments were found in infiltrating macrophages or surviving endothelial cells in glomerular capillaries (Figure 8, B and C).

On the other hand, one possibility still remained that morphological TM-positive apoptotic cells or both TM- and TUNEL-labeled cells could be due to the phagocytosis of nonendothelial cells by TM-positive endothelial cells. Therefore, we examined the correlation between the incidence of both TM and TUNEL-labeled cells in glomeruli and the number of TM-positive glomerular endothelial cells or TM-positive glomerular capillary lumina. In the control kidneys, no apoptotic cells were identified in the glomerular endothelial cells (Figure 5D). In the GN-induced kidneys, a small number of apoptotic endothelial cells (which were also demonstrated by double staining with the TUNEL method and TM) appeared in necrotizing and subsequent proliferative regions in the early phase. Thereafter, necrotizing lesions could not be found in damaged glomeruli on week 4 to week 8, and the number of apoptotic endothelial cells gradually increased with the progression of glomerular sclerosis (Figure 5D). Although the apoptotic endothelial cells were prominent in glomerular capillaries, the total number of endothelial cells and capillary lumina per glomerular cross section gradually decreased with progression of glomerular sclerosis (Figure 5, A and C). Then, a significant number of apoptotic endothelial cells appeared on week 8, and these cells were observed in glomerular capillaries among sclerotic lesions.

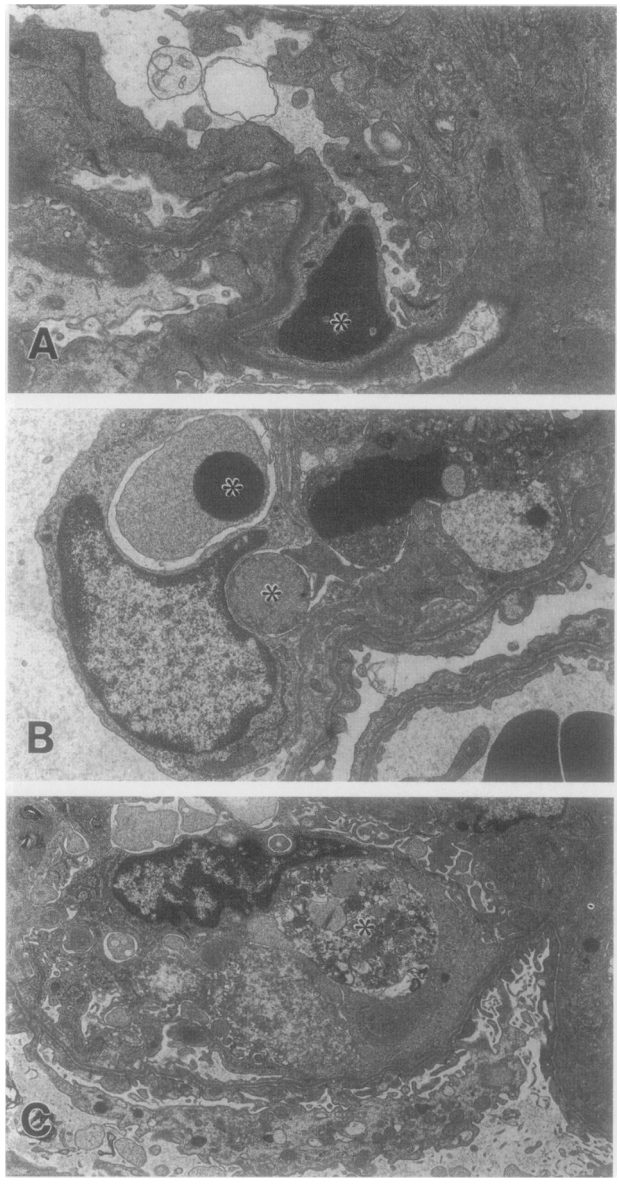

Figure 8. Various types of apoptotic endothelial cells and bodies in sclerotic lesions. **A:** An endothelial cell undergoing early apoptosis (**asterisk**) is present in glomerular capillaries on week 8, characterized by condensation of the nucleus. Magnification, ×5000. **B:** On week 6, typical apoptotic bodies (**asterisk**) that have been ingested by a viable endothelial cell are observed. Magnification, ×8000. **C:** On week 6, an apoptotic body (**asterisk**) that has been ingested by an infiltrating macrophage is found in the glomerular capillary lumen. Magnification, ×6000.

Discussion

The present study demonstrates that the destruction of glomerular capillary network and subsequent incomplete angiogenic capillary repair leads to glomerular sclerosis in anti-GBM GN in WKY rats. Endothelial cell apoptosis with glomerular capillary regression may also contribute to the development of glomerular sclerosis in progressive GN.

Endothelial cells were identified by electron microscopy and by immunohistochemistry for thrombomodulin (TM), which is expressed on the surface of endothelial cells and which inhibits the procoagulant activities of thrombin.[13] It has been known that TM is an excellent specific marker for the endothelial cell,[14] and TM is found on the endothelial cells in arteries, veins, capillaries, and lymphatics in all tissues and organs, except for the brain in humans.[15] In rat glomeruli, TM is located on the endothelial cell of the capillary loop and absent from the mesangial cell and podocyte.[16] Recent studies reported that TM expression is altered in renal disorders.[17–21] In *in vitro* assays, TM activity in rat and human isolated glomeruli was down-regulated by tumor necrosis factor-α and lipopolysaccharide.[17] In *in vivo* studies, although decreased TM expression was observed in human renal allografts during rejection, glomerular TM expression was

not decreased in rejecting grafts.[18] Moreover, increased TM expression was described in endothelial areas in human idiopathic or lupus-related proliferative glomerulonephritis.[19,20] Even in the glomerular thrombotic microangiopathy, TM expression in glomeruli does not change in a septic rat model.[21] Recently, Kitamura et al[6] demonstrated the presence of TM along the exposed surface of the endothelial cells in not only normal glomerular capillaries but also regenerating capillaries in the rat Habu-snake-venom-induced GN. In agreement with previous observations, the present study also showed that TM was found on the surface of endothelial cells in glomerular capillaries of both control and experimental rats. Moreover, TM-positive cells disappeared in necrotizing and subsequent sclerotic lesions in anti-GBM GN, and endothelial cells were reduced markedly in these regions as described by electron microscopy.

Progressive GN and Rare Capillary Regeneration

In progressive GN, damaged glomeruli progress to sclerotic scar glomeruli after glomerular inflammation,[12,29,30] and GN leading to glomerular sclerosis remains an important cause of renal failure. The renal glomerulus is a well developed network of capillaries. Therefore, when considering the mechanisms of the progression of damaged glomeruli to sclerotic scar, it is probable that the injury of glomerular capillary network is the most important factor for pathological glomerular healing with sclerotic scar formation, but this has never been demonstrated.

It has been known in the progressive form of anti-GBM GN that severe glomerular injury, including necrotizing and mesangiolytic glomerular damage, disintegration and exfoliation of damaged endothelial cells, and denudation of GBM, occur during the early phase of GN.[31,32] Our results also indicated that glomerular endothelial cells were reduced in the severely damaged lesions with the destruction of the glomerular capillary network in the early phase in this model. By contrast, if the glomerular capillary network is destroyed in experimental recovery models of GN, such as Habu-snake-venom-induced GN or Thy-1 GN, many endothelial cells remain in damaged lesions.[6,7] Furthermore, subsequent angiogenic glomerular capillary repair can occur in areas that had marked destruction of the capillary network. Then, damaged glomeruli recover to their normal structure with the development of the capillary network in the recovery models of GN.[6,7] However, in anti-GBM GN in WKY rats, rare proliferating endothelial cells were present in severely damaged necrotizing lesions. Angiogenic capillary repair was also rare in damaged glomeruli after the inflammatory injury, and subsequently damaged glomeruli progressed to glomerular sclerosis. Therefore, we concluded that angiogenic glomerular capillary repair is necessary for recovery of damaged glomeruli after the destruction of the capillary network.

Angiogenesis is crucial in wound healing.[1–5] Vessel growth is controlled by the local actions of chemical mediators, the extracellular matrix, metabolic gradients, and physical angiogenic or angiogenesis-inhibitory factors.[1–4] A number of substances important for angiogenesis, including peptide growth factors such as fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, platelet-derived growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor, have been identified in the embryonic kidney.[5] It is apparent that similar angiogenic factors have been implicated as important mediators in both embryogenesis and wound healing, suggesting that the angiogenesis occurring in wound healing recapitulates some aspects of embryonic angiogenesis. Although the regulating mechanisms of capillary repair in the progression of glomerular sclerosis in GN have not been fully investigated, it is probable that these angiogenic factors may not be precisely regulated during the incomplete repair of the capillary network in this model. Indeed, basic fibroblast growth factor cannot enhance after glomerular damage in this model.[33] Furthermore, vascular endothelial growth factor/vascular permeability factor and its receptors (Flk-1, Flt-1, and Flt-4) on endothelial cells are down-regulated during anti-GBM GN.[34] Angiogenic capillary repair is an integral process in glomerular healing, and additional work is important to clarify the regulatory mechanisms of capillary repair during GN. Stimulation of angiogenesis has been shown to accelerate healing of various wounds.[2] Therefore, understanding the mechanisms that mediate the glomerular capillary repair may provide new approaches to the treatment of GN.

Endothelial Cell Apoptosis with Capillary Regression in Progressive GN

Recently, apoptosis has been widely recognized as a precisely controlled mode of cell death, and the various roles of apoptosis have been reported.[35,36] Apoptosis in vascular endothelial cells has been described in various physiological and pathological conditions, such as endothelial cell injury or activation,[37–40] survival factors deprivation,[41,42] resolution of inflammation,[8] the process of wound healing,[9] and the regression of capillaries.[43,44]

The WKY strain demonstrates increased activity of natural killer cells and antibody-dependent cell-mediated cytotoxicity in anti-GBM GN.[45] In addition, binding of CD8-positive lymphocytes to glomerular endothelial cells through the intercellular adhesion molecules is crucial for the initiation of glomerular injury and subsequent progression of anti-GBM GN.[10,11] It has been known that cytotoxic lymphocytes and natural killer cells can kill target cells by multiple mechanisms, including cytolysis and apoptosis.[46,47] Our morphometric study demonstrated that apoptosis of endothelial cells were found in necrotizing lesions on day 5, and a small number of apoptotic endothelial cells persisted in necrotizing and proliferative lesions between day 5 and week 3. More interestingly, although active necrotizing lesions could not be observed in damaged glomeruli on week 4 to week 8, endothelial cell apoptosis apparently increased

during the progression of proliferative regions to sclerotic scars. One of the roles of apoptosis has been described as a mechanism of deletion of endothelial cells during capillary regression.[9] Our results showed that the number of capillary lumina and endothelial cells per glomerular cross section gradually decreased during the increase in number of apoptotic endothelial cells and the progression of glomerular sclerosis, suggesting that during the development of glomerular sclerosis, glomerular capillary regression and obsolescence occurred with endothelial cell apoptosis. Glomerular capillary obsolescence with subsequent glomerular sclerosis is a common finding in most progressive glomerular disease. Therefore, we conclude that the regression and obsolescence of glomerular capillaries with apoptosis of glomerular endothelial cells may accelerate to the development of glomerular sclerosis. Although the mechanisms for apoptosis induction in glomerular endothelial cells during the development of glomerular sclerosis have not been fully investigated, a possible explanation for the apoptosis of glomerular endothelial cells could be due to glomerular hyperfiltration. It is established that the progression of glomerular sclerosis in GN results in extensive loss of renal mass, consistent with experimental renal ablation models.[48,49] It has been clarified that one of the central pathogenic events in the progression of glomerular sclerosis in renal ablation models is the rapid increase in pressure as well as shear stress in the glomerular capillaries.[48,49] Recent studies in a model of hypertensive small vessel injury have identified that increases in pressure within glomerular capillaries can cause endothelial cell injury.[48–50] Recent reports also indicate that apoptosis is regulated by external signals such as various receptor-ligand interactions, by alterations of extracellular matrix and survival factors, and by endogenous proto-oncogenes as well as gene products including bcl-2, bcl-x, BAX, and interleukin-1β-converting enzyme.[51–53] Additional work is necessary to identify the stimulus factors that lead to apoptosis, but it appears that apoptosis may be the mechanism by which regulation of the number of endothelial cells occurs in regressing capillaries in sclerotic regions. The regulation of the apoptotic phenomenon in glomerular endothelial cells may be important in the progression of glomerular sclerosis during GN.

We conclude that the destruction of the glomerular capillary network and subsequent incomplete angiogenic capillary repair leads to glomerular sclerosis in this model. Endothelial cell apoptosis with glomerular capillary regression may also contribute to the development of glomerular sclerosis. Injury of the glomerular capillaries with endothelial cell damage including apoptosis and subsequent incomplete capillary repair plays an important role in the progression of glomerular sclerosis during anti-GBM GN in WKY rats.

Acknowledgments

We thank Drs. David Stern and Yukio Yuzawa (Columbia University) for providing the anti-TM antibody, Drs. Yasuhiro Natori and Naoyuki Nakao (Research Institute, International Medical Center of Japan, Tokyo, Japan) for providing the anti-GBM antibody, Mr. Bernard A. Collins (Department of Immunopathology, Massachusetts General Hospital, Boston, MA) for his advice and critical review of the manuscript, and Mr. Takashi Arai, Ms. Mitsue Kataoka, and Arimi Ishikawa for expect technical assistance.

References

1. Folkmann J, Shing Y: Angiogenesis. J Biol Chem 1992, 267:10931–10934
2. Folkmann J: Clinical applications of research on angiogenesis. N Engl J Med 1995, 333:1757–1763
3. Klagsburn M, D'Amore PA: Regulators of angiogenesis. Annu Rev Physiol 1991, 53:217–239
4. Arnold F, West DC: Angiogenesis in wound healing. Pharmacol Ther 1991, 52:407–422
5. Hyink DP, Abrahamson DR: Origin of the glomerular vasculature in the developing kidney. Semin Nephrol 1995, 15:300–314
6. Kitamura H, Sugisaki Y, Yamanaka N: Endothelial regeneration during the repair process following Habu-snake venom induced glomerular injury. Virchows Arch 1995, 427:195–204
7. Iruela-Arispe L, Gordon K, Hugo C, Duijvestijn AM, Claffey KP, Reilly M, Couser WG, Alpers CE, Johnson RJ: Participation of glomerular endothelial cells in the capillary repair of glomerulonephritis. Am J Pathol 1995, 147:1715–1727
8. Polunovsky VA, Chen B, Henke C, Snover D, Wendt C, Ingbar DH, Bitterman PB: Role of mesenchymal cell death in lung remodeling after injury. J Clin Invest 1993, 92:388–397
9. Desmoulière A, Redard M, Darby I, Gabbiani G: Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am J Pathol 1995, 146:56–66
10. Kawasaki K, Yaoita E, Yamamoto T, Kihara I: Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. Kidney Int 1992, 41:1517–1526
11. Kawasaki K, Yaoita E, Yamamoto T, Tamatani T, Miyasaka M, Kihara I: Antibodies against intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 prevent glomerular injury in rat experimental crescentic glomerulonephritis. J Immunol 1993, 150: 1074–1083
12. Shimizu A, Kitamura H, Masuda Y, Ishizaki M, Sugisaki Y, Yamanaka N: Apoptosis in progressive crescentic glomerulonephritis. Lab Invest 1996, 74:941–951
13. Esmon CT, Owen WG: Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc Natl Acad Sci USA 1981, 78:2249–2252
14. De Bault LE, Esmon N, Olson JR, Esmon CT: Distribution of the thrombomodulin antigen in the rabbit vasculature. Lab Invest 1986, 54:172–178
15. Maruyama I, Bell CE, Majerus PW: Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics and on syncytiotrophoblast of human placenta. J Cell Biol 1985, 101:363–371
16. Horvat R, Palade GE: Thrombomodulin and thrombin localization on the vasculature endothelium: their internalization and transcytosis by plasmalemmal vesicles. Eur J Cell Biol 1993, 61:299–313
17. He CJ, Kanfer A: Quantification and modulation of thrombomodulin activity in isolated rat and human glomeruli. Kidney Int 1992, 41: 1170–1174
18. Tsuxhida A, Salem H, Thomson N, Hancock WW: Tumor necrosis factor production during human renal allograft rejection is associated with depression of plasma protein C and free protein S levels and decreased intragraft thrombomodulin expression. J Exp Med 1992, 175:81–90
19. Mizutani M, Yuzawa Y, Maruyama I, Sakamoto N, Matsuo S: Glomerular localization of thrombomodulin in human glomerulonephritis. Lab Invest 1993, 69:193–202
20. Tomura S, Deguchi Y, Marumo F, Aoki N: Enhanced presence of thrombomodulin in the glomeruli of lupus glomerulonephritis. Clin Nephrol 1994, 41:205–210

21. Laszik Z, Carson CW, Nadasdy T, Johnson LD, Lerner MR, Brackett DJ, Esmon CT, Silva FG: Lack of suppressed renal thrombomodulin expression in a septic rat model with glomerular thrombotic microangiopathy. Lab Invest 1994, 70:862–867
22. Duijvestijn AM, Van Goor H, Klatter F, Van Bussel E, Van Breda Vriesman PCJ: Antibodies defining rat endothelial cells: RECA-1, a pan-endothelial cell-specific monoclonal antibody. Lab Invest 1992, 66:459–466
23. Roux F, Durieu-Trautmann O, Chaverot N, Claire M, Mailly P, Bourre JM, Strosberg AD, Couraud PO: Regulation of γ-glutanyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. J Cell Physiol 1994, 159:101–113
24. Coltrera MD, Skelly M, Gown AM. Anti-PCNA antibody PC10 yields unreliable proliferation indexes in routinely processed, deparaffinized, formalin-fixed tissue. Appl Immunohistochem 1993, 1:193–200
25. Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC: A novel, simple, reliable, and sensitive method of multiple immunoenzymic staining: use of microwave oven heating to block antibody cross-reactivity and retrieve antigens. J Histochem Cytochem 1995, 43:97–102
26. Hsu SM, Soban E: Color modification of diaminobenzidine (DAB) precipitation by metallic irons and its application for double immunohistochemistry. J Histochem Cytochem 1982, 30:1079–1082
27. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992, 119:493–501
28. Wood GS, Warnke R: Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. J Histochem Cytochem 1981, 29:1196–1204
29. Jones DB: Inflammation and repair of the glomerulus. Am J Pathol 1951, 27:991–1009
30. Savill J, Johnson RJ: Glomerular remodeling after inflammatory injury. Exp Nephrol 1995, 3:149–158
31. Shigematsu H, Kobayashi Y: The distortion and disorganization of the glomerulus in progressive Masugi nephritis in the rat. Virchows Arch B 1973, 14:313–323
32. Kondo Y, Shigematsu H, Kobayashi Y: Cellular aspects of rabbit Masugi nephritis. II. Progressive glomerular injuries with crescent formation. Lab Invest 1972, 27:620–631
33. Feng L, Tang WW, Xia Y, Wilson CB: Growth factor, extracellular matrix, and protease inhibitor mRNA expression in anti-GBM antibody associated glomerulonephritis. J Am Soc Nephrol 1994, 5:468
34. Tang WW, Qi M, Farrell K, Scully S, Gabbai F, Wang G, Lyons D: Vascular endothelial growth factor/vascular permeability factor and its receptors (Flk-1, Flt-1 and Flt-4) are downregulated in experimental GN. J Am Soc Nephrol 1995, 6:855
35. Ellis RE, Yuan J, Horvitz HR: Mechanisms and functions of cell death. Annu Rev Cell Biol 1991, 7:663–698
36. Savill J: Apoptosis and the kidney. J Am Soc Nephrol 1994, 5:12–21
37. Robaye B, Mosselmans R, Fiers W, Dumont JE, Galand P: Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells *in vitro*. Am J Pathol 1991, 138:447–453
38. Yang JJ, Kettritz R, Falk RJ, Jennette JC, Gaido M: Apoptosis of endothelial cells induced by the neutrophil serine proteases proteinase 3 and elastase. Am J Pathol 1996, 149:1617–1626
39. Lizard G, Deckert V, Dubrez L, Moissant M, Gambert P, Lagrost L: Induction of apoptosis in endothelial cells treated with cholesterol oxides. Am J Pathol 1996, 148:1625–1638
40. Araki S, Ishida T, Yamamoto T, Kaji K, Hayashi H: Induction of apoptosis by hemorrhagic snake venom in vascular endothelial cells. Biochem Biophys Res Commun 1993, 190:148–153
41. Araki S, Shimada Y, Kaji K, Hayashi H: Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. Biochem Biophys Res Commun 1990, 168:1194–1200
42. Araki S, Shimada Y, Kaji K, Hayashi H: Role of protein kinase C in the inhibition by fibroblast growth factor of apoptosis in serum-depleted endothelial cells. Biochem Biophys Res Commun 1990, 172:1081–1085
43. Azmi TI, O'Shea JD: Mechanism of deletion of endothelial cells during regression of the corpus luteum. Lab Invest 1984, 51:206–217
44. Latker CH, Feinberg RN, Beebe DC: Localized vascular regression during limb morphogenesis in the chicken embryo. II. Morphological changes in the vasculature. Anat Rec 1986, 214:410–417
45. Granados R, Mendrick DL, Rennke HG: Antibody-induced crescent formation in WKY rats: potential role of antibody-dependent cell cytotoxicity (ADCC) in vivo. Kidney Int 1990, 37:414
46. Berke G: Unlocking the secrets of CTL and NK cells. Immunol Today 1995, 16:343–346
47. Kawakami A, Tian Q, Duan X, Streuli M, Schlossman SF, Anderson P: Identification and functional characterization of a TIA-1-related nucleolysin. Proc Natl Acad USA 1992, 89:8681–8685
48. Meyer TW, Brenner BM: The contribution of glomerular hemodynamic alterations to progressive renal disease. The Progressive Nature of Renal Disease. Edited by Mitch WE, Brenner BM, Stein JH. New York, Churchill Livingstone, 1986, pp 1–16
49. Diamond JR, Karnovsky MJ: Focal and segmental glomerulosclerosis: analogies to atherosclerosis. Kidney Int 1988, 33:917–924
50. Reidy M, Schwartz SM: A technique to investigate surface morphology and endothelial cell replication of small arteries: a study in acute angiotensin-induced hypertensive rats. Microvasc Res 1982, 24:158–167
51. Williams GT, Smith CA: Molecular regulation of apoptosis: genetic controls on cell death. Cell 1993, 74:777–779
52. Steller H: Mechanisms and genes of cellular suicide. Science 1995, 267:1445–1449
53. Vaux DL: Toward an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci USA 1993, 90:786–789