Anti-perforin antibody treatment ameliorates experimental crescentic glomerulonephritis in WKY rats

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The depletion of $CD8^+$ cells has been shown to prevent the initiation and progression of antiglomerular basement membrane (GBM) crescentic glomerulonephritis (GN) in Wistar–Kyoto (WKY) rats. In this study, we asked whether $CD8^+$ cells produce their effects by perforin/granzyme-mediated or by Fas ligand (FasL)-mediated pathways. The glomerular mRNA expression of perforin and granzyme B corresponded with the number of $CD8^+$ cells, whereas that of granzyme A, Fas, and FasL did not. The enhanced mRNA level of perforin and granzyme B was not evident in $CD8^+$-depleted rats. The number of apoptotic cells in the glomeruli was significantly increased at day 3. Perforin mRNA was found in cells infiltrating the glomerulus by *in situ* hybridization and by using dual-staining immunohistochemistry perforin protein was found in glomerular $CD8^+$ cells. We found that perforin was readily visualized at the inner surface of the glomerular capillaries by immunoelectron microscopy. Based on these results, we treated animals with a perforin antibody *in vivo* and found that it significantly reduced the amount of proteinuria, frequency of crescentic glomeruli, and the number of glomerular monocytes and macrophages, although the number of glomerular $CD8^+$ cells was not changed. Our results suggest that $CD8^+$ cells play a role in glomerular injury as effector cells in part through a perforin/granzyme-mediated pathway in the anti-GBM WKY rat model of crescentic GN.

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In human crescentic glomerulonephritis (GN), both CD4- and CD8-positive T lymphocytes are observed in glomeruli,[1–3] often without deposition of humoral immune mediators.[4] In the pathogenesis of experimental crescentic GN, the pivotal roles for cell-mediated immunity, especially T helper 1 (Th1) immune responses have been strongly suggested.[5–7] In previous work, we showed a crucial role of $CD8^+$ cells ($CD8^+$) in the initiation and the progression of anti-glomerular basement membrane (GBM) crescentic GN in Wistar–Kyoto (WKY) rats, without significant involvement of humoral immune response and complement activation.[8] This CD8-dependent model is characterized by a rapid progression to renal failure with massive glomerular accumulation of monocytes and macrophages (Mo/Mϕ, ED-1^+ cells) and a high frequency of crescent formation. The unique features of this model may be due to the high susceptibility of WKY rats to anti-GBM antibody compared with other rat strains. Also in the accelerated form of anti-GBM GN in WKY rats, the important role for $CD8^+$ has been demonstrated by the suppressive effect of the anti-CD8 antibody administration for both the prevention and the treatment.[9] The $CD8^+$ have been considered to be involved in glomerular injury indirectly by accumulating monocyte/macrophages (Mo/Mϕ), as $CD8^+$ are shown to upregulate glomerular intercellular adhesion molecule-1 expression through induction of cytokines.[10] It has not been studied about the potency of $CD8^+$ to injure glomeruli directly as local effector cells.

In general, $CD8^+$ are known as cause target cell apoptosis due to their cytotoxic actions, via perforin/granzyme-mediated and/or Fas ligand (FasL)-mediated pathways,[11–15] and may contribute to inflammatory immune responses in some disease conditions. There are some *in vivo* studies showing the positive correlation between the histological severity and the increment of mRNA expressions of these cytotoxic molecules. For example, in acute renal allograft rejection, both mechanisms were operative and a direct correlation was demonstrated between the histological severity of the rejection and each of intrarenal mRNA

expressions of FasL, Fas, granzyme B, and perforin.[16] Also in other forms of kidney disease, glomerular CD8$^+$ accumulation along with the glomerular mRNA expressions of granzyme A and perforin was identified 8 weeks after the induction of active Heymann nephritis.[17] In the kidney of NZB/W F1 lupus mice, the perforin mRNA expression was significantly increased with age, which was ameliorated with methylpredonisolone.[18] In *in vitro* study, it was shown that T-cell clones that were cytotoxic to cultured proximal renal tubular epithelial cells expressed granzyme B and C, and that an antisense oligonucleotide against granzyme C inhibited the cytotoxicity.[19] However, none of these studies has presented the direct evidence of the perforin/granzyme-mediated or the FasL-mediated renal injury in crescentic GN by *in vivo* blocking of these cytotoxic molecules.

In this study, the role for perforin in CD8$^+$ was studied in anti-GBM crescentic GN in WKY rats. First, we examined the glomerular mRNA expressions of perforin and granzyme A, B, and the localization of perforin mRNA and protein in glomerular infiltrating cells. Next, *in vivo* blocking of perforin by antibody administration was carried out during the early stages of this GN in order to ascertain the role perforin plays in the glomerular injury.

RESULTS

Anti-GBM GN with or without anti-CD8 antibody administration

A marked infiltration of mononuclear cells was obvious at day 3 after anti-GBM antibody administration. At day 7, crescent formation was observed in most of the glomeruli. By immunofluorescence microscopy, a lot of ED-1$^+$ Mo/Mϕ were observed in glomeruli at day 3 and more at day 7, and the number of glomerular CD8$^+$ was slightly increased at day 1, peaked at day 3 and decreased at day 7 as we reported previously.[8,10] In MRC-OX8 administrated rats, no CD8$^+$ were detected in the glomeruli at 1 h and days 1 and 3, but only a few CD8$^+$ were counted at day 7. Rabbit immunoglobulin G (IgG) was localized along the GBM in a linear pattern intensely at 1 h, and the intensity of fluorescence was unchanged throughout the experiment. The intensity of rat IgG in glomeruli was negligible or faint in all time points.

Quantitation of glomerular mRNA for perforin, granzyme A, B, Fas, and FasL

By ribonuclease protection assay, perforin and granzyme B mRNAs were not detected in the normal rat glomerulus. Both of them were expressed slightly at day 1, greatly increased at day 3, and decreased at day 7, which corresponded with the number of glomerular CD8$^+$. The depletion of CD8$^+$ resulted in the complete suppression of perforin and granzyme B mRNAs at days 1 and 3 (Figure 1a), with small amounts of the mRNAs appearing at day 7 when the small number of CD8$^+$ was found. Granzyme A mRNA was not detected throughout the experimental period (data not shown). Fas and FasL mRNAs were also increased in the glomerulus after anti-GBM antibody administration, peaked at day 1 and decreased afterwards, which did not correspond with the number of glomerular CD8$^+$ (Figure 1b).

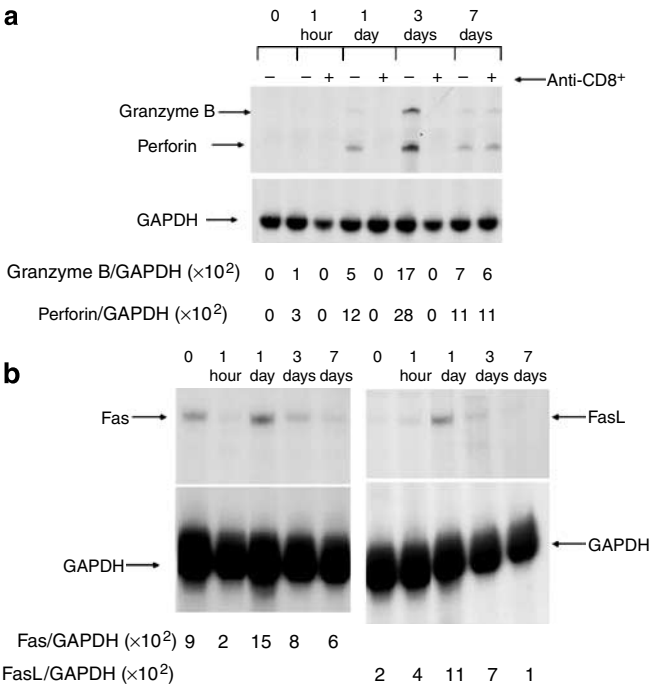

Figure 1 | Ribonuclease protection assay. (**a**) Representative glomerular mRNA expressions of perforin and granzyme B, (**b**) and Fas and FasL. (**a**) Both perforin and granzyme B mRNA expressions were not detected in normal glomeruli. Weak but significant expressions were found at day 1, intense expression at day 3, and weak expression at day 7. In CD8$^+$-depleted rats, both were completely suppressed at day 1 and day 3, and were only weakly detected at day 7 when a few CD8$^+$ appeared. (**b**) Intense expressions of Fas and FasL mRNA in glomeruli were found at day 1, and both were decreased afterwards, which did not correspond with the number of glomerular CD8$^+$.

Detection of apoptosis by TUNEL method

Although none or only a few terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick end labelling (TUNEL)-positive apoptotic cells were observed in normal glomeruli (0.10 ± 0.06 cells/glomerular cross-section (gcs)) and at day 1 (0.80 ± 0.18 cells/gcs) (Figure 2a), the number of apoptotic cells in glomeruli was significantly increased at day 3 (7.43 ± 0.74 cells/gcs, $P < 0.01$) (Figure 2b), and reduced at day 7 (3.00 ± 0.52 cells/gcs) (Figure 2c).

Localization of perforin mRNA and protein

Perforin protein and mRNA were demonstrated in glomeruli at day 3, by immunohistochemistry (Figure 3b) and *in situ* hybridization (Figure 3d), respectively. The protein staining and mRNA grains were most prominent on the cells that appeared to be infiltrating mononuclear cells. Immunohistochemistry using sequential serial sections for CD8$^+$ (Figure 4a) and perforin (Figure 4b) revealed that most of

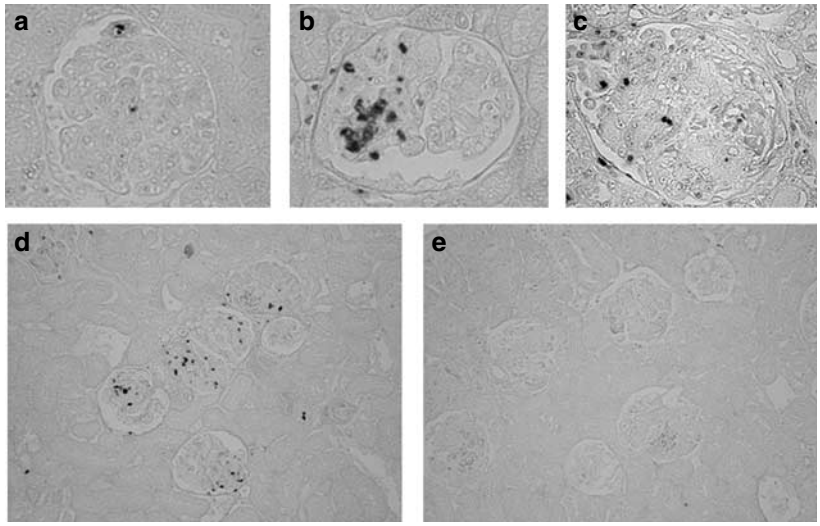

Figure 2 | Detection of apoptotic cells in glomeruli by TUNEL method. (**a**) Although no or only a few TUNEL-positive apoptotic cells were observed in glomeruli at day 1, (**b**) the number of apoptotic cells in glomeruli was significantly increased at day 3, (**c**) and reduced at day 7. (**d**) The positive result was confirmed (**e**) by omitting terminal deoxynucleotidyl transferase enzyme served as negative control. Original magnification: (**a**–**c**): $\times$ 200, (**d**, **e**): $\times$ 80.

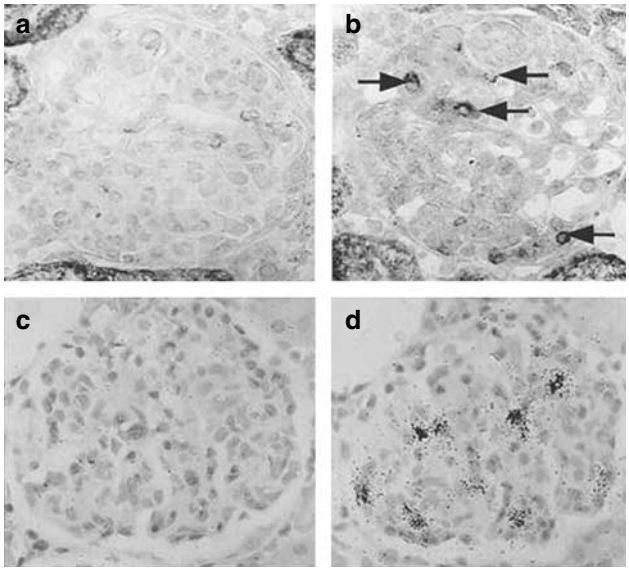

Figure 3 | Perforin protein and mRNA localization in glomerulus. (**a**, **b**) Immunohistochemistry for perforin protein. (**a**) control rabbit IgG, (**b**) anti-perforin antibody. Positive staining cells (arrows) appear to be infiltrating cells. (**c**, **d**) *In situ* hybridization for perforin mRNA. (**c**) Sense probe, (**d**) antisense probe. Original magnification: (**a**–**d**): $\times$ 320.

the perforin-positive cells in glomeruli were considered to be $CD8^+$. And Dual-staining immunohistochemistry (Figure 4e) confirmed that the perforin protein was localized in the $CD8^+$. Immunoelectron microscopy (Figure 5) revealed that perforin was localized in the cytoplasmic granules of glomerular infiltrating lymphocyte (square-1). Also, perforin released outside was demonstrated at the inner surface of the glomerular capillaries (square-2), where the structure of endothelial cell was obscure.

In vivo blocking of perforin in anti-GBM GN

Urinary protein excretion. All rats that received anti-GBM antibody excreted significant amount of urinary protein. The amount was significantly decreased from 23.0 ± 7.0 mg/day (day 3), 61.0 ± 3.0 mg/day (day 5), and 45.0 ± 1.2 mg/day (day 7) in control group, to 6.2 ± 1.4 mg/day (day 3, $P < 0.01$), 6.6 ± 0.4 mg/day (day 5, $P < 0.01$), and 4.2 ± 0.2 mg/day (day 7, $P < 0.01$) in the anti-perforin antibody group, respectively. The amounts at day 3, 5, 7 in anti-perforin antibody group were all comparable with the amount of normal rats (5.4 ± 2.0 mg/day, NS).

Histology. The frequency of crescentic glomeruli was dramatically decreased at day 7 from $85.0 \pm 5.0\%$ (preimmune serum-injected group, Figure 6a) to $15.0 \pm 3.0\%$ (anti-perforin antibody-treated group, Figure 6b) ($P < 0.01$). The number of glomerular $ED\text{-}1^+$ Mo/Mϕ was apparently reduced by the anti-perforin antibody treatment, from 26.3 ± 3.1 cells/gcs (preimmune serum group, Figure 6c) to 7.5 ± 1.2 cells/gcs (anti-perforin antibody group, Figure 6d) ($P < 0.01$). In contrast, the number of glomerular $CD8^+$ was not changed by the anti-perforin antibody treatment; 7.7 ± 1.4 cells/gcs (preimmune serum-injected group) and 7.5 ± 1.6 cells/gcs (anti-perforin antibody-treated group) (NS). Rabbit IgG was localized along the GBM in a linear pattern intensely throughout the experiment, and the intensity was not changed by the administration of anti-perforin antibody in all time points. And the intensity of rat IgG in glomeruli was negligible or faint also in rats with anti-perforin antibody.

DISCUSSION

This is the first study demonstrating that the blocking of perforin by antibody administration greatly suppresses glomerular injury in anti-GBM GN in WKY rats. And

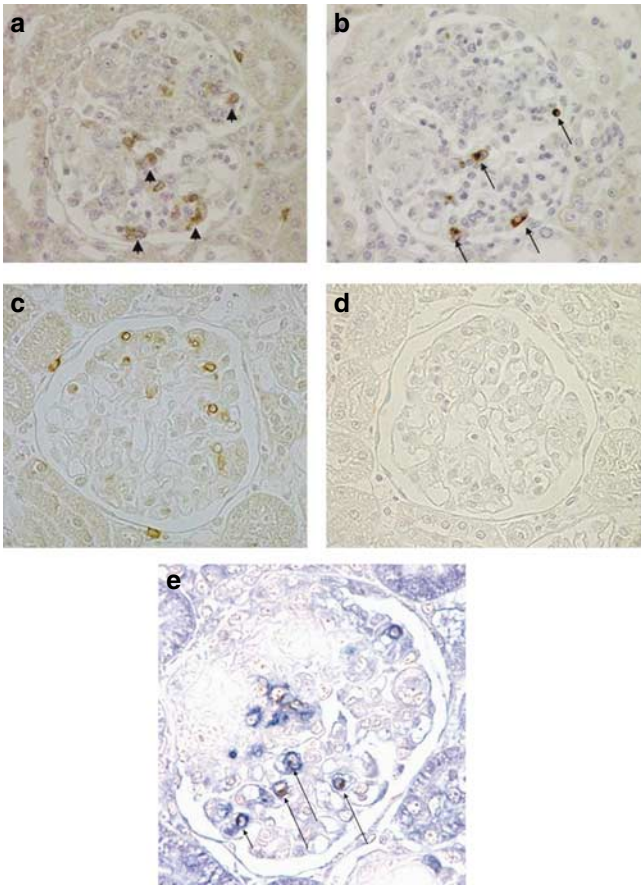

Figure 4 | Perforin protein localization in glomerulus. Immunohistochemistry using sequential serial sections for (**a**) $CD8^+$ and (**b**) perforin revealed that most of the perforin-positive cells in glomeruli were considered to be $CD8^+$. The result of immunohistochemistry for $CD8^+$ (**c**) was confirmed by using isotype-matched control mouse IgG, (**d**) served as negative control. (**e**) And Dual-staining immunohistochemistry for perforin (brown) and $CD8^+$ (blue) confirmed that the perforin protein (arrow) localized in the $CD8^+$. Original magnification: (**a**–**d**): $\times$ 200, (**e**): $\times$ 300.

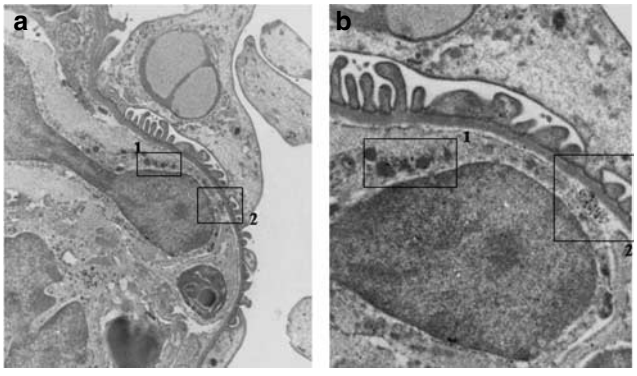

Figure 5 | Immunoelectron microscopy for perforin. Perforin granules was demonstrated in the cytoplasmic granules of glomerular infiltrating lymphocyte (shown by square-1). Also, perforin released outside was demonstrated at the inner surface of the glomerular capillaries (shown by square-2), where the structure of endothelial cell was obscure. Original magnification: (**a**): $\times$ 3250. (**b**): $\times$ 7875.

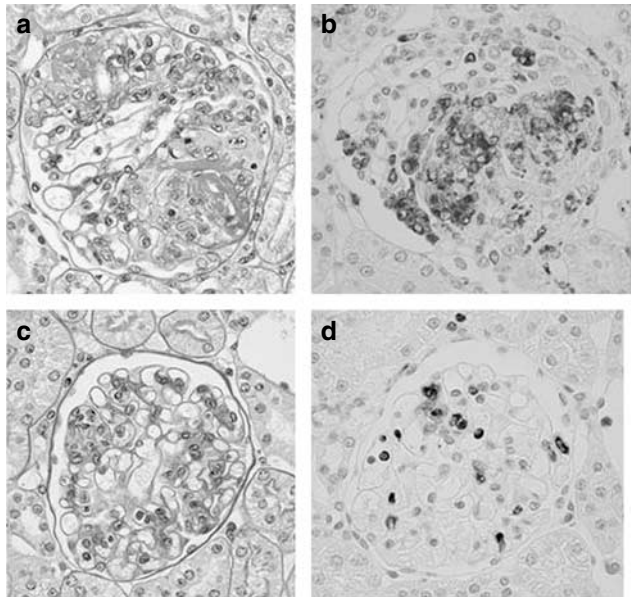

Figure 6 | Renal histology and immunohistochemistry. (**a**, **b**) Periodic acid-Schiff staining. Mononuclear cell infiltration and crescent formation were mostly suppressed by anti-perforin antibody treatment at day 7. (**a**) preimmune serum treatment group, (**b**) anti-perforin antibody treatment group. (**c**, **d**) Immunostaining for ED-1^+ cells. The number of glomerular ED-1^+ Mo/Mϕ was apparently reduced by anti-perforin antibody treatment. (**c**) Preimmune serum treatment group, (**d**) anti-perforin antibody treatment group. (Original magnification: (**a**–**d**): $\times$ 200). The result of immunohistochemistry for ED-1 was confirmed by using isotype-matched control mouse IgG, served as negative control (data not shown).

although perforin was demonstrated in glomerular infiltrating $CD8^+$, the role for $CD8^+$ as effector cells was strongly suggested in this severe crescentic GN model. $CD8^+$ cause target cell apoptosis due to their cytotoxic actions, via perforin-mediated and/or FasL-mediated mechanisms.[11–15] In this study, the *in vivo* blocking of perforin by antibody administration was performed, as the glomerular mRNA expressions of perforin and granzyme B, not of Fas and FasL, corresponded with the number of glomerular $CD8^+$. The results were that the administration greatly reduced the glomerular injury monitored by the amount of urinary protein excretion, the frequency of crescentic glomeruli, and the number of glomerular Mo/Mϕ, although the number of glomerular $CD8^+$ was not reduced by anti-perforin antibody administration. The results strongly suggest that perforin in $CD8^+$ play a role in the course of this GN.

The persistence of $CD8^+$ can be explained that anti-perforin antibody may act by blocking the function of perforin without killing $CD8^+$. And alternatively, even if anti-perforin antibody administration causes the depletion of perforin-positive mature effector $CD8^+$, perforin-negative $CD8^+$ may proliferate and differentiate. Not all of the $CD8^+$ express perforin. $CD8^+$ increase the expression of perforin according to their differentiation and maturation.[20]

The mechanisms by which perforin causes glomerular injury need to be discussed. From cytosolic T cells, perforin monomers are released and inserted into the target cell membrane, and polymerized to form a pore in a tubular structure, through which granzymes enter the target cell and initiate apoptosis.[21] In the step of apoptosis, the contents of the dying cell are packaged into apoptotic bodies and are scavenged by neighboring phagocytes, so as not to be released into the external milieu.[22] In this model of anti-GBM GN, $CD8^+$ may be activated in the capillary lumen, recognize the glomerular endothelial cells as the target cells, and release perforin and granzymes from their cytoplasmic granules. The increased number of apoptotic cells in the glomerulus supports this hypothesis. In addition, perforin may mediate cell death by uncontrolled influx of small molecules from the extracellular fluid through the pore or by induction of osmotic stress.[21] Otherwise, granzymes may degrade collagenous components of GBM [23] and cause detachment of endothelial cells, accompanied by the release of cytokines and the activation of the leukocyte integrins. All of these events may be involved in perforin/granzyme-induced direct glomerular injury, and the part of Mo/Mϕ infiltrating in glomeruli is considered to be secondary to this direct glomerular injury induced by $CD8^+$.

The decreased number of glomerular Mo/Mϕ by anti-perforin antibody administration can be explained by the reduction of apoptosis and glomerular injury, and the subsequent reduction of cytokines and chemotactic signals. During the course of apoptosis, dying cells display 'eat-me' signals such as phosphatidylserine on the surface, and secrete attraction signals such as the phospholipid lysophosphatidylcholine.[24] Anti-perforin antibody may reduce the migration of professional phagocytes by suppression of these signals from the dying cells.

Based on the results of our previous studies, it has been considered that $CD8^+$ might be involved in glomerular injury indirectly through the action of accumulated Mo/Mϕ. We have also suggested the role for Mo/Mϕ as effector cells by the administration of anti-monocyte chemoattractant protein-1 monoclonal antibody [25] and of liposome-encapsulated dichloromethylene diphosphonate (liposome-MDP).[26] Anti-MCP-1 monoclonal antibody administration reduced the number of glomerular Mo/Mϕ by 34.7% and the amount of proteinuria by 66.2% at day 4, without reducing the number of glomerular $CD8^+$.[25] Therefore, the results of this study do not exclude the role for Mo/Mϕ as effectors cells. Both $CD8^+$ and Mo/Mϕ play the role of effector cells, and they may interact with each other to cause glomerular injury.

Taken together, the pathogenesis of anti-GBM GN in WKY rats may be proposed as follows. After binding of the rabbit IgG to GBM, a small number of Mo/Mϕ first accumulate to the glomerulus.[27] Through the recognition of the rabbit IgG Fc portion by Fcγ receptors, these Mo/Mϕ are stimulated to produce cytokines and chemokines, which accumulate $CD8^+$ into the glomerulus.[10] These $CD8^+$ play essential roles in the initiation and the progression of the disease. $CD8^+$ cause glomerular injury directly by releasing perforin/granzyme B to injure glomerular cells as shown in this study. In addition, $CD8^+$ injure glomerular cells indirectly by accumulating Mo/Mϕ, and the Mo/Mϕ may be activated in the glomerulus to release inflammatory mediators.[28] Massive accumulation of Mo/Mϕ at the late stage of this model is probably mediated by cytokines released by the action of $CD8^+$, which stimulate LFA-1 molecules on Mo/Mϕ to interact with intercellular adhesion molecule-1 molecules on glomerular endothelial cells.[29] In consequence, both glomerular cells and GBM may be injured by perforin/granzyme B released from the $CD8^+$ and by other inflammatory mediators from Mo/Mϕ, resulting in marked destruction of the glomerular structure and excretion of protein in urine.

In summary, this study has, for the first time, provided evidence on the essential roles of perforin in $CD8^+$ in anti-GBM crescentic GN in WKY rats. This suggests that the blocking of perforin might contribute to the aggressive treatment of human severe crescentic GN in future.

MATERIALS AND METHODS

Induction of anti-GBM GN

Inbred male WKY rats aged 12–16 weeks were purchased from Charles River Inc. (Atsugi, Kanagawa, Japan), and maintained in our animal facility. Animal care was in accordance with the guidelines of Niigata University (Niigata, Japan). The rats were anesthetized by diethyl ether inhalation and euthanized by exsanguinations after the removal of kidneys. One group of 16 rats received 25 μl (containing 325 μg of rabbit IgG) of the anti-GBM antibody per 100 g body weight by intravenous injection as described previously.[8] Four rats each were killed at 1 h and at days 1, 3, and 7 to obtain kidneys. As a control group, four normal rats were killed for the renal histological examination and glomerular RNA isolation.

Anti-GBM GN in $CD8^+$-depleted rats

The other group of 16 rats received monoclonal antibody against rat CD8 (MRC-OX8, IgG1, PHLS Centre for Applied Microbiology & Research, Wiltshire, UK) at a dose of 2.8 mg of γ-globulin fraction/100 g body weight intraperitoneally and 0.6/100 g body weight intravenously 2 days before the administration of anti-GBM antibody to deplete $CD8^+$ in their circulation.[8] Four rats each were killed at the same time points as the group mentioned above.

Histology and immunohistochemistry

Each kidney specimen for histology was divided; one part was fixed in methyl-Carnoy's solution and embedded in paraffin for histology and immunohistochemistry, and the other part was quick-frozen in N-hexane at -70°C for cryostat sectioning. The paraffin-embedded tissues were sectioned at a 3 μm-thickness and stained with periodic acid-Schiff reagent for the light microscopic examination. For immunohistochemistry, the paraffin-embedded sections were dewaxed and incubated sequentially with normal goat serum (1:20 dilution) for blocking, a mouse monoclonal antibody against rat Mo/Mϕ (ED-1; Dainippon Seiyaku Co., Tokyo, Japan, 1:500 dilution), or $CD8^+$ (MRC-OX8; 1:200 dilution) for 1 h, and horseradish peroxidase-conjugated goat anti-mouse Igs (EnVision, DAKO JAPAN Co., Kyoto, Japan) for 1 h. The peroxidase reaction

product was visualized with 0.5 mg/ml of 3′-diaminobenzidine tetrahydrochloride-0.01% hydrogen peroxide. The sections were counterstained with hematoxylin. Cell stained with these antibodies were counted in more than 50 gcs in each kidney under a light microscope. The results of immunohistochemistry for ED-1 and MRC-OX8 have been confirmed by using isotype-matched control IgG (mouse IgG), served as negative controls. Cryostat sections were stained with fluorescein isothiocyanate goat anti-rat IgG (Seikagaku Kogyo Co., Tokyo, Japan) to evaluate the humoral immune response against the rabbit IgG. The sections were also stained with fluorescein isothiocyanate-goat anti-rabbit IgG (Seikagaku Kogyo Co.) to evaluate the intensity of the rabbit anti-GBM antibody localized along the GBM during the course of the experiment.

Quantitation of glomerular RNA for perforin, granzyme A, B, Fas, and FasL

Isolation of glomerular RNA. Glomeruli were isolated from the renal cortex by standard sieving method, and RNA was isolated by acid guanidinium-phenol-chloroform method.

Preparation of cDNA templates. Rat cDNA for perforin was isolated from the cDNA library of a rat large granular lymphocyte[30] by hybridization with a cDNA probe for mouse perforin (kindly provided from Dr Ishikawa, Department of Immunology, Juntendo University, Tokyo, Japan), and a 314 bp fragment was subcloned into the *Hin*dIII and *Pst*I site of pGEM-3Z (Promega Corp., Madison, WI, USA).

For rat granzyme A, B, and rat Fas, total RNAs were isolated from lipopolysaccharide-stimulated rat spleen and rat heart, respectively. Then they were subjected to reverse transcription to cDNAs and used for PCR amplifications. Primers of rat Granzyme A, B were designed from the conserved regions of the cDNAs of human and mouse. The sequences of sense and antisense primers for rat granzyme A were (5′-GGGAATTCTNCTGCTNATTCCTGAA-3′), and (5′-GGGGATCCTTTTTAGGTAGNTGAAGGAT-3′), respectively, and for rat granzyme B were (5′-ACTGAATTCGTGCTGACN GCTGCTCACTG-3′) and (5′-ACTGGATCCAAGNGGGCCTCCAG A-3′), respectively. The sequences of sense and antisense primers for rat Fas were (5′-ACTGGATCCTGCACAGAAGGGGAGGAGTA-3′) and (5′-ACTGAATTCGGTGTTGCTGGTTCGTGTGC-3′), respectively. The PCR cycle conditions were denaturing at 94°C for 60 s, annealing at 55°C for 90 s, and elongating at 72°C for 120 s through 30 cycles. The PCR products were subcloned into pGEM-4z (Promega) for rat granzyme A and B, or pBluescriptII-SK$^+$ (Stratagene Co., La Jolla, CA, USA) for rat Fas, and their DNA sequences were confirmed by an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems, Tokyo, Japan). The nucleotide sequence for the PCR-cloned rat granzyme A cDNA (360 bp) was approximately 87% homologous with mouse cDNA for granzyme A,[31] and that for rat granzyme B cDNA (451 bp) was approximately 83% homologous with mouse cDNA for granzyme B.[32] The nucleotide sequence for the PCR-cloned rat Fas cDNA was 240 bp.

Rat full length of cDNA for FasL in a plasmid pBL-KA15 [33] was kindly provided from Dr Nagata (Department of Genetics, Osaka University Graduate School of Medicine, Osaka, Japan) and a 261 bp fragment of FasL cDNA was subcloned into the *Kpn*I and *Xba*I sites of pBluescriptII-SK$^+$.

Rat cDNA for housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 114 bp) was prepared as described previously.[34]

Generation of ^{32}P-labeled antisense RNA probes

The recombinant plasmids were linearized with *Hin*dIII (perforin), *Eco*RI (granzyme A, B, and Fas), *Kpn*I (FasL), and *Bam*HI (glyceraldehyde-3-phosphate dehydrogenase), respectively. Antisense cRNA probes were prepared by *in vitro* transcription with RNA polymerase T7, with the incorporation of [α-^{32}P] UTP.

Ribonuclease protection assay. One glomerular RNA sample was randomly selected from each group and three different sets of the RNAs were examined by ribonuclease protection assay.[35] Ten micrograms of glomerular total RNA were hybridized with 1×10^5 of each ^{32}P-labeled antisense RNA probe overnight at 50°C. Unhybridized RNA probes were digested with ribonuclease T1 (GIBCO/BRL, Gainsberg, MD, USA) and ribonuclease A (Boehringer Mannheim, Tokyo, Japan) for 1 h at 30°C. The ribonucleases were then digested with proteinase K (Promega) for 30 min at 37°C. After phenol/chloroform extraction and sodium acetate/ethanol precipitation, the hybridized RNA probes were denatured at 85°C for 5 min and electrophoresed on a 6% polyacrylamide gel. The dried gels were exposed to X-ray film (Fuji Photo Film Co. Ltd, Tokyo, Japan). The blots were scanned by a scanner (ScanJet 3C, Hewlett Packard, Sagamihara, Japan) and the density of each protected band was quantified by a computerized image analysis system (NIH Image, NIH, Bethesda, MD). After calibration of the images and plotting lanes to generate the profile plots, the areas of the peaks were measured. The data were represented as a ratio of specific mRNA/glyceraldehyde-3-phosphate dehydrogenase mRNA to ensure a constant quantity of mRNA in each sample.

Detection of apoptosis by TUNEL method

Cells undergoing apoptosis were identified by the TUNEL technique, using an *in situ* apoptosis detection kit (ApopTag, Intergen, Purchase, NY, USA). Paraffin-embedded tissues fixed in methyl-Carnoy's solution were sectioned at a 3 μm-thickness, deparaffinized, rehydrated, and digested with proteinase K 4 μg/ml for 15 min at room temperature. Endogenous peroxidase was quenched by 3% hydrogen peroxidase for 10 min. After equilibration, the sections were incubated in a humidified chamber with terminal deoxynucleotidyl transferase enzyme for 1 h at 37°C. Nucleotides labeled with digoxigenin are enzymatically added to 3′-hydroxyl DNA ends by terminal deoxynucleotidyl transferase. Afterwards, sections were soaked in stop-wash buffer for 30 min followed by incubation with an alkaline phosphatase-conjugated anti-DIG antibody (1:500 dilution, Roche Diagnostics, Penzberg, Germany) at 37°C for 30 min. The signal was detected using a nitro blue tetrazolium chloride 5-bromo-4-cloro-3-indolyl phosphate toluidine salt (NBT/BCIP) substrate solution. The positive result was confirmed by omitting terminal deoxynucleotidyl transferase enzyme served as negative control.

Localization of perforin in glomerulus

In situ *hybridization.* *In situ* hybridization was performed as described previously[36] with some modifications.[37] Briefly, kidneys were perfused and prefixed with 4% paraformaldehyde. The tissues were embedded in OCT (Miles Laboratories, Elkhart, IN, USA) and were then frozen on dry ice. ^{35}S-labeled antisense and sense riboprobes were transcribed from both directions using rat perforin cDNA cloned in pGEM-3Z. Frozen sections (4–6 μm-thickness) were fixed in 4% paraformaldehyde for 10 min on ice and deproteinated in proteinase K solution (1 μg/ml) for 10 min at room temperature. After washing in 0.5 × standard sodium citrate for 10 min, prehybridization was performed at 42°C for 2 h in 50 μl hybridiza-

tion buffer per section. The buffer contained 10 mM dithiothreitol, 0.3 N NaCl, 20 mM Tris (pH 8.0), 5 mM ethylenediaminetetraacetic acid, 1 × Denhardt's, 10% dextran sulfate, and 50% formamide. Ten microliter of hybridization buffer with radiolabeled probe (3×10^5 c.p.m. per section) was added directly into the bubble of prehybridization solution covering the sections. Hybridization was performed at 55°C overnight. After washing in 2 × standard sodium citrate, slides were immersed in RNase digestion buffer (20 μg/ml RNase A) at room temperature for 30 min. The slides were then washed twice in 0.5 × standard sodium citrate without ethylenediaminetetraacetic acid and β-ME for 10 min. Following dehydration, the slides were dried. After autoradiography for 3 days, the slides were dipped in Kodak NTB2 nuclear emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with water at 42°C. Exposure was performed at 4°C in the dark for 4 weeks before development.

Dual-staining immunohistochemistry for CD8 and perforin. The paraffin-embedded sections were dewaxed and used. At first, sequential serial sections were prepared for the purpose of comparing the location of glomerular $CD8^+$ with that of perforin-positive cells. The results were confirmed by using isotype-matched control IgG (mouse or rabbit IgG, respectively), served as negative controls. For dual-staining immunohistochemistry, endogenous peroxidase was quenched by 3% hydrogen peroxidase for 10 min. Slides were incubated sequentially with normal goat serum for blocking, a mouse monoclonal antibody against rat $CD8^+$ for 1 h, biotinylated anti-mouse IgG (Vector labs, Burlingame, CA, USA) for 30 min, followed by alkaline phosphatase streptavidin (Vector labs) for 30 min, and the sections were visualized with a nitro blue tetrazolium chloride/5-bromo-4-cloro-3-indolyl phosphate toluidine salt substrate solution. The second primary antibody, a rabbit polyclonal antibody against rat perforin (Dainippon Seiyaku Co., 1:20 dilution), was applied next after heating for 10 min by microwave in 0.01 M sodium citrate buffer (pH 6.0). Slides were then incubated overnight at 4°C. After washing in phosphate-buffered saline, horseradish peroxidase-conjugated goat anti-rabbit Igs (EnVision) was applied for 30 min. The peroxidase reaction product was visualized with 0.5 mg/ml of 3′-diaminobenzidine tetrahydrochloride-0.01% hydrogen peroxide.

Immunoelectron microscopy. Immunoelectron microscopic observations of kidneys were performed as reported previously.[38,39] In brief, 1 mm^3 kidney blocks were placed in the paraformaldehyde-lysine-periodate fixative for 4 h at 4°C, hydrated, and then embedded in hydrophilic methacrylate resin. The ultrathin sections collected on nickel grids were stained with the immunogold technique.

In vivo blocking of perforin in anti-GBM GN

Preparation of anti-perforin antibody for blocking. A neutralizing rabbit antibody against rat perforin was generated using a recombinant perforin peptide expressed by *Escherichia coli*. The antibody recognized perforin expressed in transformed African green monkey kidney fibroblast cell line (COS-7) by immunohistochemical staining and could block perforin-mediated cytotoxicity of cytotoxic T cells *in vitro*, which was tested by ^{51}Cr release assay.[40,41] Briefly, ^{51}Cr-labeled target cells (P815/S) were incubated with effector cells (6C2, clones of CTL line) for 4 h, and supernatants were removed and measured radioactivity of released ^{51}Cr. The radioactivity was counted by a γ-counter. The percent-specific lysis was calculated using the following formula: $100 \times (a-b/c-b)$, where a is the radioactivity in the supernatant of target cells mixed with effector cells (= *experimental release*), b is the radioactivity in the supernatant of target cells incubated without effector cells (= *spontaneous release*), and c is the radioactivity in the supernatant after complete lysis of target cells with 2% Triton X-100 (= *maximum release*). To demonstrate perforin-dependent cytotoxicity, anti-perforin antibody (5, 10, or 20 μl), or control rabbit IgG (20 μl) were added to mixtures of effector cells and labeled target cells in the assay. And as a controlled experiment, 6C2 clones were pretreated with concanamycin A (CMA; Wako Pure Chemical Industries, Osaka, Japan) for 2 h and mixed with target cells (A20). Concanamycin A was reported to inhibit perforin-dependent cytotoxicity mostly due to accelerated degradation of perforin by an increase in the pH of lytic granules.[42] The result was depicted as Table 1. The percent specific lysis by effector cells was shown to be inhibited by addition of anti-perforin antibody in a dose-dependent manner.

Table 1 | *In vitro* assay for neutralizing ability of anti-perforin antibody

Effector cells	Target cells	Treatment	Effector/target ratio	% specific lysis
GC2	P815/S	Nontreatment	10	90
			2	76
			0.4	25
		Anti-perforin Ab		
		5 μl/well	10	62
		10 μl/well	10	50
		20 μl/well	10	35
		Rabbit IgG		
		20 μl/well	10	84
	A20	Nontreatment	10	78
		CMA	10	31

CMA, concamamycin A.
The percentage of target cell lysis increased as the ratio of effector cells/target cells increased. In the case of anti-perforin antibody treatment, the target cell lysis by effector cells was suppressed in a dose-dependent manner, which verified the perforin blocking effect of this antibody. As a control, rabbit IgG was shown to have no effects on suppressing the lysis via perforin blocking. CMA was also shown to suppress lysis mostly due to accelerated degradation of perforin.

Administration of anti-perforin antibody. One group of five rats was given anti-perforin antibody (1 ml/rat) for 5 successive days from 1 day after anti-GBM antibody administration. As a control, the other group of five rats was given preimmune serum (1 ml/rat) instead of anti-perforin antibody. All rats were killed at day 7 for histological analysis.

Urine samples were collected for 24 h before the induction of the GN and at days 3, 5, and 7. The amounts of protein excreted in the urine were determined by using a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan).

Each kidney specimen for histology was divided for paraffin or cryostat sectioning, and examined for light microscopy and immunohistochemistry as described above.

Statistical analysis

The data were expressed as the mean ± 1SD. Statistical significance was analyzed by Mann–Whitney's *U*-test. Differences with $P < 0.05$ were considered statistically significant.

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