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Recombinant uteroglobin prevents the experimental crescentic glomerulonephritis

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Recombinant uteroglobin prevents the experimental crescentic glomerulonephritis.

Background. Although uteroglobin is known to have an immunomodulatory property and prevents the deposition of immune-complexes on the glomeruli of mice, the therapeutic potential of uteroglobin is uncertain in glomerulonephritis. To test the hypothesis that uteroglobin can prevent glomerulonephritis, we have studied the effects of recombinant uteroglobin on the development of experimental crescentic glomerulonephritis that is induced by anti-glomerular basement membrane (anti-GBM) antibodies.

Methods and Results. Glomerulonephritis was induced by the intravenous injection of rabbit anti-GBM globulin antibodies into mice (C57BL/6), and renal injury was evaluated 7, 14, and 21 days afterward. Recombinant uteroglobin or phosphate-buffered saline (PBS) were given intravenously to mice for 3 days after anti-GBM antibody injection. Proteinuria was significantly reduced in mice treated with recombinant uteroglobin compared with disease-control mice at 7 and 14 days after an anti-GBM antibody injection, although the serum creatinine concentration was similar in both groups. The amount of proteinuria was similar in recombinant uteroglobin-treated and normal control mice. By histologic analysis, mesangial matrix expansion, mesangial proliferation, and cellular crescents representing crescentic glomerulonephritis were markedly attenuated by injection of recombinant uteroglobin. The in vitro proliferative responses of mesangial cells to lipopolysaccharide (LPS) were blunted by the addition of recombinant uteroglobin in a dose-dependent manner. The preventive effects exerted by recombinant uteroglobin treatment were based on the inhibition of antibodies and complement-3 deposition on the glomeruli.

Conclusion. This study demonstrates the preventive effects of recombinant uteroglobin in an experimental model of crescentic glomerulonephritis, and suggests the therapeutic implications of uteroglobin for human chronic glomerulonephritis.

Glomerulonephritis is one of the major causes of end-stage renal disease (ESRD) in Korea [1]. Although the exact nature of the pathogenesis of glomerulonephritis is still unknown, immune complex–mediated inflammatory responses have been suggested as central pathogenetic mechanism [2]. The accumulation of cells and fibrin within Bowman's space of glomeruli produces the histologic appearance of a glomerular crescent. Glomerular crescent formation, the hallmark of severe and rapidly progressive immune renal injury, is characterized by the accumulation of T cells, macrophages, and fibrin in glomeruli [3–5]. Among the various animal models of glomerulonephritis, anti-glomerular basement membrane (anti-GBM) disease (glomerulonephritis) has been widely studied and seems to be the most similar counterpart of the human disease [6–8].

Uteroglobin or Clara cell 10 kD protein is a steroid-dependent, immunomodulatory, and cytokine-like protein [9, 10]. Moreover, it is known to be secreted by the mucosal epithelial cells of all vertebrates studied to date [11, 12]. Uteroglobin appears to function as an anti-inflammatory agent, in both the respiratory and urogenital tracts [12], and micromolar concentrations of uteroglobin may inhibit the chemotaxis of both neutrophils and monocytes and prevent the infiltration of inflammatory cells [13]. Uteroglobin is also a potent inhibitor of phospholipase A_2 activity [14], thereby limits the metabolism of arachidonic acid and the synthesis of prostaglandin and leukotriene mediators [15]. Zheng et al [16] reported that two independent mouse models, both manifesting deficiency of uteroglobin, developed pathologic features of human IgA nephropathy, and suggested that exogenous uteroglobin was sufficient to prevent the glomerular accumulation of exogenous IgA in uteroglobin-null mice [16].

[1]Both authors contributed equally to this work.

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We examined whether exogenous recombinant uteroglobin could prevent the development of experimental glomerulonephritis induced by anti-GBM antibodies in mice, and also evaluated the effects of recombinant uteroglobin on proliferative responses to noxious stimuli on mouse mesangial cells. In addition, we investigated the influence of recombinant uteroglobin on immune complex deposition.

METHODS

Production of recombinant human uteroglobin

Human lung cancer cell line (NCI-H322) (ATCC, CRL5806) was used for cloning the human uteroglobin. To obtain the whole segment of mature uteroglobin, reverse transcription-polymerase chain reaction (RT-PCR) was conducted using CC10-1 (sense primer 5′-CCAGACTCAGAGACGGAA-3′) and CC10-2 (antisense primer 5′-CATATGAAACTCGCTGTCACC-3′). Nested PCR was performed after first round PCR using CC10-3 (sense primer 5′-CTCGAGCTAATT ACACAGTGAGCT-3′) and CC10-4 (antisense primer 5′-GACTCAAAGCATGGCAGC-3′). PCR product was cloned into pET32a plasmid (Novagen, Madison, WI, USA). Recombinant uteroglobin was produced from transformed *Escherichia coli* BL21. His-tagged recombinant uteroglobin was purified by Ni^{++}-affinity chromatography (Novagen). Centricon (Millipore, Bedford, MA, USA) was used to concentrate the recombinant protein after dialysis, and the Bradford assay was used to quantify the protein (Bio-Rad Laboratories, Hercules, CA, USA). Recombinant uteroglobin was confirmed by Western blot where polyclonal rabbit antihuman uteroglobin antibody (urine protein 1) (Dako-Cytomation, Glostrup, Denmark) was used as primary antibody (data not shown).

Induction of anti GBM disease

Anti-GBM antibody was prepared from the serum of a rabbit immunized against homogenized murine renal cortex in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA). Glomerulonephritis was initiated by the intravenous injection of 4.5 mg of rabbit anti-GBM antibody into an 8- to 10-week-old female C57BL/6. Recombinant uteroglobin [500 μg in 300 μL of phosphate-buffered saline (PBS)/mouse for 3 days] was delivered 1 hour after anti-GBM antibody injection via a tail vein ($N = 10$). Disease control mice ($N = 10$) received three doses of 300 μL of PBS after anti-GBM antibody injection. Normal control mice received PBS instead of anti-GBM antibody, and renal injury was studied after 7, 14, and 21 days. Proteinuria and urine creatinine were measured by 24-hour urine collections at 3, 5, 7, 14, and 21 days. Urinary protein concentrations were determined by the Bradford method. Creatinine concentrations were measured using an autoanalyzer. Proteinuria was normalized by urine creatinine (urine protein/urine creatinine) (mg/mg). Serum was collected via retro-orbital bleeding at the same time of evaluation. All the mice were raised in specific pathogen-free animal facility and the treatment protocol was reviewed by Seoul National University Hospital Institute Review Board.

Histologic analysis

To assess the light microscopic appearance, 5 μm paraffin sections were stained with periodic acid-Schiff (PAS) reagent. For grading the induced glomerulonephritis, the numbers of glomeruli forming crescent were counted in a blinded fashion. A minimum of 50 glomeruli per mouse kidney were evaluated, and the mean value was used as representative for the mouse.

For immunohistochemistry study, paraffin-embedded kidney was cut into 5 μm slices. For deparaffinization and hydration, xylene and ethanol were used. Endogenous streptoavidin activity was blocked by 3% hydrogen peroxide (H_2O_2). We used biotinylated goat antirabbit antibody (Vector Laboratory, Burlingame, CA, USA) as primary globulin antibody for rabbit anti-GBM antibody detection and rabbit antihuman complement-3 antibody which is cross-reactive for mouse complement-3 (US Biological, Swampscott, MA, USA) as primary antibody for complement-3 detection. Streptoavidin and diaminobenzidine (DAB) were used for immunohistochemical detection. Concentration of antibodies and exposure time were titrated to get optimal results. Hematoxylin was used for counterstaining.

Mesangial cell culture and proliferation assay

Mesangial cells were isolated from the glomeruli of C57/BL6 mice using the differential sieving technique. The glomeruli were enriched by more than 95% by centrifugation. MTS/PMS analysis [17, 18] was used to assess the proliferation of mesangial cells. In brief, 5×10^3 mesangial cells/well were plated in a 96-well plate. After 24 hours, the culture media was changed to Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fetal bovine serum (FBS). Cells were washed the cells with PBS two times after 48 hours, and the culture media was changed for one containing 1% FBS, 1% FBS + lipopolysaccharide (LPS) (100 ng/mL, 1 μg/mL, and 100 μg/mL, respectively), and 1% FBS + LPS (1 μg/mL) + uterglobin (50, 100, and 150 μg/mL, respectively). After 24 hours, 20 μL of MTS (3-[4,5-dimethylthiazol-2yl-5]-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium) (MTS/PMS) solution (Promega, Madison, WI, USA) was added with light shield. Light absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Spectra Max 250; Molecular

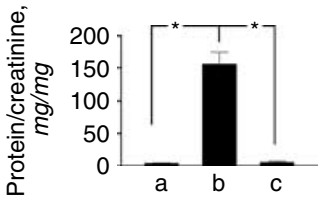

Fig. 1. Marked attenuation of proteinuria by uretoglobin treatment at day 7. Normal control mice (4 ± 0.7 protein/creatinine) (mg/mg) (lane a), disease control mice received three doses of 300 μL phosphate-buffered saline (PBS) after anti-glomerular basement membrane (GBM) antibody injection (156 ± 18.2) (lane b), and recombinant uteroglobin–treated mice received three doses of 0.5 mg of uteroglobin in 300 μL PBS daily 1 hour after anti-GBM antibody injection (6 ± 1.8) (lane c). Values are mean ± SD ($N = 10$/group, $P = 0.0059$, t test).

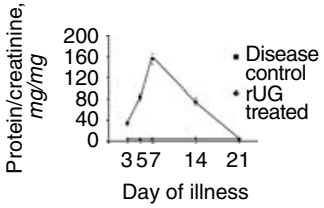

Fig. 2. Persistent anti-proteinuric effect of recombinant uteroglobin (rUG) after the induction of anti-glomerular basement membrane (anti-GBM) glomerulonephritis. Antiproteinuric effect of recombinant uteroglobin against glomerulonephritis was persistent for 2 weeks. Proteinuria occurred from the third day of illness and peaked at the seventh day of illness in anti-GBM glomerulonephritis mice. Proteinuria was resolved after 3 weeks of disease. Recombinant uteroglobin-treated mice did not show any significant proteinuria throughout observation period. At 2 weeks of disease, disease control mice showed significant amount of proteinuria (80.4 ± 11.19) compared to recombinant uteroglobin-treated mice (5.7 ± 2.82) ($P = 0.0079$, t test) ($N = 5$/group at 2 weeks and 3 weeks).

Devices, Sunnyvale, CA, USA) at 490 nm. Experiments were repeated three times.

Statistics

Data were presented as the mean ± standard deviation (SD). Comparisons between variables were made and P values of less than 0.05 were considered statistically significant.

Table 1. Serum creatinine levels (mg/dL) in mice with anti-glomerular basement membrane (GBM) antibody-induced glomerulonephritis

Day	Normal control mice	Disease control mice	Recominant uteroglobin treated mice
7	0.6 ± 0.05	0.5 ± 0.05	0.6 ± 0.12
14	0.5 ± 0.05	0.5 ± 0.12	0.6 ± 0.15

RESULTS

Recombinant uteroglobin treatment prevented the development of proteinuria and crescentic glomerulonephritis

To assess the effects of recombinant uteroglobin on the development of anti-GBM glomerulonephritis, we measured the amount of proteinuria in normal controls, a disease control (anti-GBM antibody + PBS), and in recombinant uteroglobin-treated (anti-GBM antibody + recombinant uteroglobin) mice. The amount of proteinuria was normalized by urine creatinine. The proteinuria/creatinine (mg/mg) was similar between normal control and recombinant uteroglobin-treated mice at the seventh day of glomerulonephritis (4 ± 0.7, 6 ± 1.8, respectively) ($P = 0.3059$), but recombinant uteroglobin-treated mice showed the significantly reduced amount of proteinuria compared to that of disease control mice (6 ± 1.8 vs. 156 ± 18.2) ($P = 0.0059$) (Fig. 1). In disease control mice, significant proteinuria was detectable from the third day of illness and peaked at seventh day of illness. But in recombinant uteroglobin-treated mice did not have significant proteinuria throughout the observation period. Antiproteinuric effect of recombinant uteroglobin was maintained until 2 weeks of glomerulonephritis (recombinant uteroglobin-treated mice 6 ± 0.7, disease control mice 75 ± 11.4) ($P = 0.0079$). After 3 weeks, the amount of proteinuria was normalized in disease control mice to the levels shown by the normal controls (disease control vs. recombinant uteroglobin-treated vs. normal control 4 ± 1.1 vs. 3 ± 1.1 vs. 4 ± 0.6) ($P = 0.567$) (Fig. 2). Serum creatinine levels were unchanged during the period of massive proteinuria (Table 1).

To determine the correlation between the histologic and the biochemical changes, we examined the glomerular lesions in mice. Cellular proliferation was evident in mesangial areas (Fig. 3B) and cellular crescents developed in more than 50% of glomeruli in the disease controls (Fig. 3C). The cellular crescents appeared to be of similar age and cellularity. Also, glomerular sclerosis was found frequently by 2 weeks of disease. Tubules and interstitium revealed minimal atrophy and fibrosis. Cellular crescents reduced to 2% to 3% of glomeruli after 2 weeks in the disease control mice. Whereas after 1 week of induction of glomerulonephritis, the glomeruli from recombinant uteroglobin-treated mice showed no or little crescents (Fig. 3D), and sclerotic glomerulus was not evident by 2 weeks of disease. Overall, mice that were treated with recombinant uteroglobin after anti-GBM antibody injection showed similar histologic findings as normal controls (Fig. 3A).

Uteroglobin reduced the proliferative responses of mesangial cells to LPS stimulation and prevented deposition of immune complexes

To investigate the renal protective characteristics of recombinant uteroglobin, we evaluated the effect of recombinant uteroglobin using a mouse mesangial cell culture system. Mouse mesangial cells showed proliferative

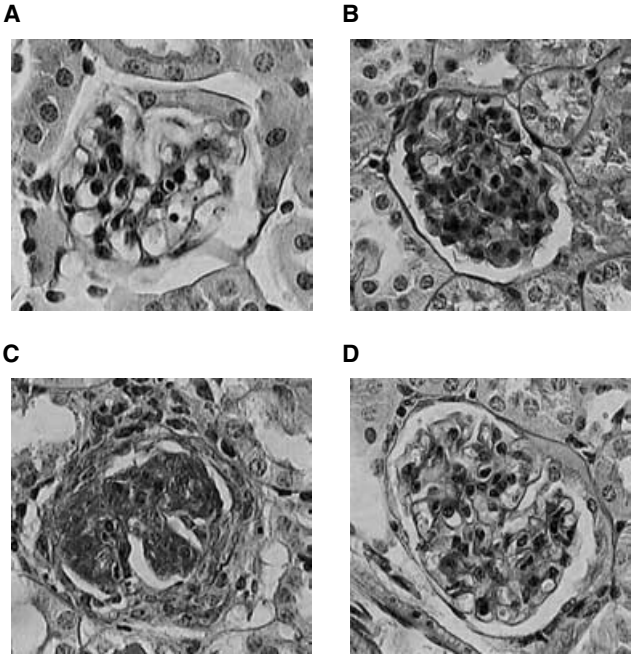

Fig. 3. Recombinant uteroglobin treatment prevented the development of anti-glomerular basement membrane (anti-GBM) glomerulonephritis in C57/BL6 mice. Recombinant uteroglobin treatment after anti-GBM antibody prevented crescent formation and mesangial proliferation. (*A*) Normal control mouse. (*B*) Mesangial proliferation in disease control mice. (*C*) Cellular and fibrocellular crescents in disease control mice. More than 50% of glomeruli showed crescents. (*D*) Recombinant uteroglobin-treated mice showed crescents in 2% to 3% of glomeruli [periodic acid-Schiff (PAS) staining, original magnification ×400]. Pictures were taken at 1 week of disease.

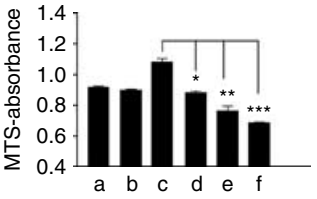

Fig. 4. Recombinant uteroglobin reduced the proliferative responses of mesangial cells to lipopolysaccharide (LPS) stimulation in a dose-dependent manner. LPS stimulation induced mesangial cellular proliferation, but it was inhibited by the addition of recombinant uteroglobin. Mesangial cells (5000 cells/well) were cultivated in media containing 1% fetal bovine serum (FBS) (lane a), 1% FBS + recombinant uteroglobin (150 μg/mL) (lane b), 1% FBS + LPS (1μg/mL) (lane c), 1% FBS + LPS (1 μg/mL) + recombinant uteroglobin (50 μg/mL) (lane d), 1% FBS + LPS (1 μg/mL) + recombinant uteroglobin (100 μg/mL) (lane e), and 1% FBS + LPS (1 μg/mL) + recombinant uteroglobin (150 μg/mL) (lane f). Cellular proliferation was measured by MTS absorbance. $^{*}P = 0.0295$; $^{**}P = 0.0027$; $^{***}P = 0.0035$ (*t* test). Each condition was triplicated and this represents one of three independent experiments.

responses to LPS stimulation in a dose-dependent manner (MTS absorbance control media, 0.9 ± 0.02; media containing LPS, 1 μg/mL, 1.1 ± 0.04; media containing LPS, 100 μg/mL, 1.2 ± 0.08). However, with the addition of recombinant uteroglobin, the proliferation of mesangial cells was attenuated in the presence of LPS. Inhibitory capacity against cellular proliferation was more evident with the increment of recombinant uteroglobin concentration (Fig. 4).

To evaluate the notion that in vivo administration of recombinant uteroglobin would prevent the development of crescentic glomerulonephritis, we evaluated immunologic cascades after injection of anti-GBM antibodies. As we expected, linear deposition of rabbit anti-GBM antibodies along GBM was pathognomonic in disease control group after 1 week of induction, and it was persistent by 2 weeks of disease (Fig. 5A). But with administration of recombinant uteroglobin after anti-GBM antibody injection, the antibody deposition was lessened dramatically in 1 week and 2 weeks of disease induction (Fig. 5A). Also, consistent with findings in antibody deposition, coadministration of recombinant uteroglobin prevented the deposition of complement-3 on glomeruli, while complement-3 depositions were evident on disease control group (Fig. 5B).

DISCUSSION

In present study, we demonstrate the renal protective characteristics of uteroglobin. Upon injecting recombinant uteroglobin, anti-GBM glomerulonephritis was prevented in terms of the amount of proteinuria and histologic changes. Also, the cellular proliferative responses to exogenous stimuli were blunted in the presence of recombinant uteroglobin in a dose-dependent manner. We suggested that the inhibition of pathogenetic globulin binding to glomeruli and hence the inactivation of immunologic cascade were the main mechanisms of the protective role of recombinant uteroglobin in experimental crescentic glomerulonephritis.

The causes of glomerulonephritis are still obscure even though several systemic diseases or antigens have been associated with the development of glomerulonephritis [19]. Genetic predisposition and environmental factors also have been suggested to be associated with glomerulonephritis [20, 21]. Anti-GBM glomerulonephritis is a representative example of glomerulonephritis, which was initiated by the deposition of antibodies against GBM. Following antibody deposition, immune systems such as the complements and inflammatory cells were activated as mediators of glomerular injuries. Cellular proliferation, mesangial matrix expansion, and crescent formation are responses to glomerular injuries, which, in turn, may progress to the irreversible glomerulosclerosis.

Uteroglobin/Clara cell 10 kD (UG/CC10) was first discovered in the rabbit uterus as a steroid-inducible secreted protein [22, 23]. Thereafter, it was found that uteroglobin is also secreted by many types of mucosal epithelial cells, including respiratory and urogenital

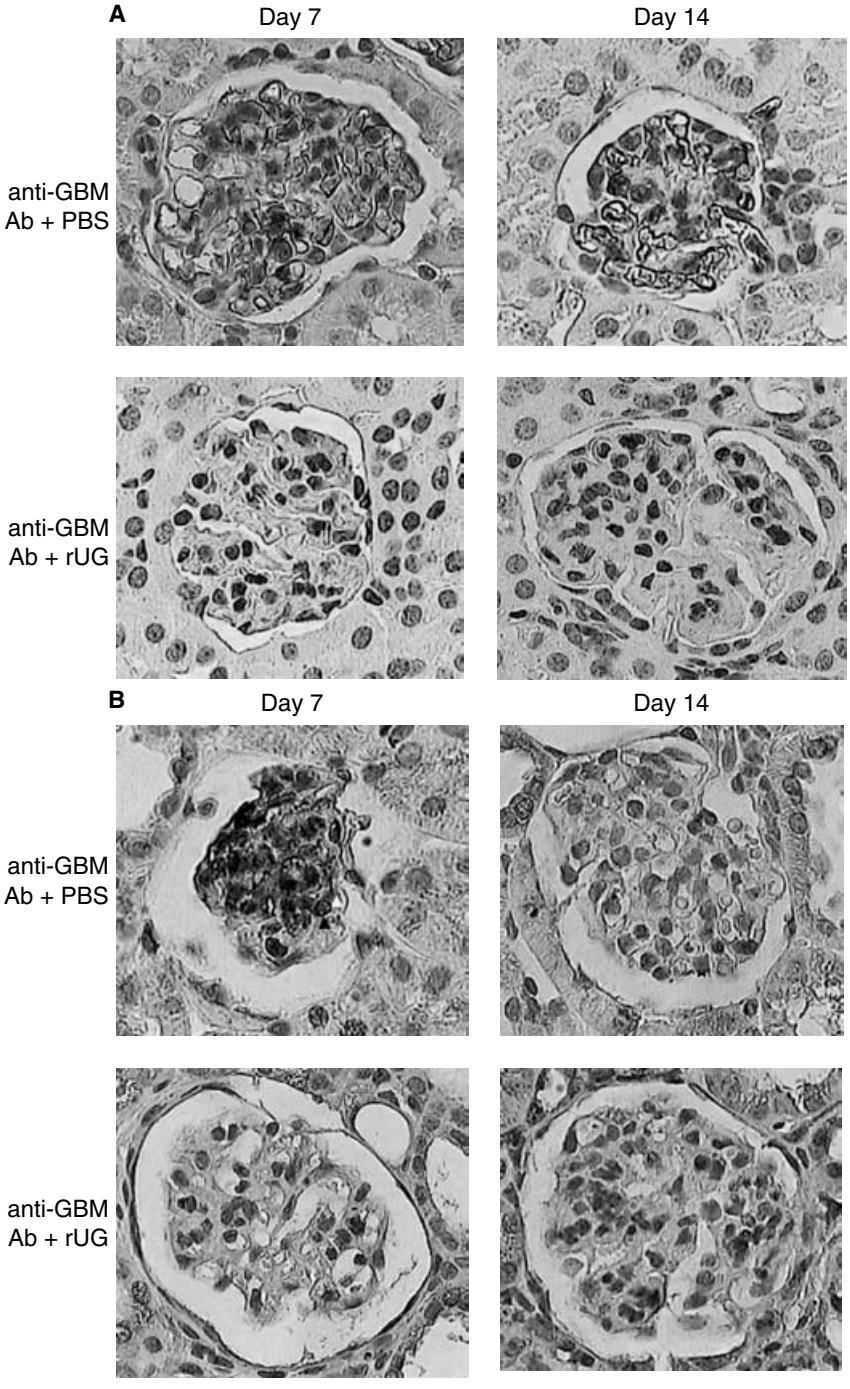

Fig. 5. Antibody and complement deposition was inhibited by the injection of recombinant uteroglobin. Linear deposition of anti-glomerular basement membrane antibodies (anti-GBM Ab) (*A*) and complement-3 (B) along GBM was evident in disease control mice but was markedly inhibited in recombinant uteroglobin-treated mice. Biotinylated secondary antibodies, streptoavidin, and diaminobenzidine (DAB) were used for immunohistochemical detection. Counterstained by hematoxylin (original magnification $\times 400$).

epithelia [9, 24]. A variety of biological activities of uteroglobin were claimed and it is now considered that uteroglobin is one of the most potent endogenous immunomodulatory and anti-inflammatory proteins [10]. Since uteroglobin was suggested as a protective factor for glomerulonephritis in mouse models [16, 25], we and others tried to examine the role of uteroglobin in human glomerulonephritis [20, 26] as well as in mouse glomerulonephritis model. In our anti-GBM glomerulonephritis mouse model system, recombinant uteroglobin-treated group secreted similar amounts of urine protein as the normal control group (6 ± 1.8 vs. 4 ± 0.7) ($P = 0.3059$), but considerably less than the disease control groups (6 ± 1.8 vs. 157 ± 18.2) ($P = 0.0059$). Reduction of proteinuria by recombinant uteroglobin was most evident on seventh day of illness. Thereafter the amount of urine protein decreased continually, and by the end of third week, even the disease control group showed the same amount

of proteinuria as the normal control group (Fig. 2). In a murine model of glomerulonephritis using rabbit antimouse GBM antibody, the disease process is usually reported to be irreversible [3]. The reversibility of the disease in our model might be due to the difference in the amount of anti-GBM globulin antibody injected or the different protocols used. Most of the models described to date include two consecutive antibody injections or prior sensitization with normal rabbit IgG. In a preliminary study involving dual antibody injection protocols, we were able to obtain a consistent induction of glomerulonephritis, but the low survival rate of mice during the experiment period prevented us from continuing these dual injection protocols. Recombinant uteroglobin coadministration dramatically improved the histologic changes which were consistent with the reduced proteinuria. Cellular crescent formation and mesangial cell changes were hardly observed in the recombinant uteroglobin-treated group. In the disease control group, on day 7, we observed an expansion of mesangial cells and crescent formation in more than 50% of the glomeruli, whereas in recombinant uteroglobin-treated groups, these were found in 2% to 3% of the glomeruli (Fig. 3). However, serum creatinine level did not change before and after the induction of glomerulonephritis, suggesting that the anti-GBM antibody we used was sufficient to induce disease but insufficient to reduce renal function. Another interpretation could be that our observation period was too short to evaluate renal function properly.

We used mesangial cell culture system to evaluate the direct effect of recombinant uteroglobin on cellular components of kidney because mesangial cells are responsible for the development of various glomerulonephritis. LPS-induced mesangial cell activation and proliferation has been reported [13, 27]. Moreover, the severity of inflammation in glomeruli paralleled the LPS concentration [28]. In our primary mouse mesangial culture system, mesangial cells responded proportionately to increased concentrations of LPS. On using a constant amount of LPS (1 μg/mL), uteroglobin prevented mesangial cell proliferation in a dose-dependent manner. This in vitro effect of mesangial cell modulation would have an impact on reduced mesangial cell expansion and crescent formation. As it was proposed that uteroglobin prevented IgA deposition on glomeruli [16], we investigated the influence of recombinant uteroglobin on immune complex deposition. As shown in Figure 5, recombinant uteroglobin showed profound prevention of pathogenic globulin antibody deposition, and blocked the immunologic downstream cascade. Inhibition of cellular proliferation to noxious stimuli (LPS in our system) and decreased immune complex activation may be the central protective mechanisms exerted by recombinant uteroglobin in the murine glomerulonephritis model.

To our knowledge, this is the first demonstration that recombinant uteroglobin leads to the prevention of experimental glomerulonephritis. We believe that an understanding the way in which this protein work could have therapeutic implications for the treatment of human glomerulonephritis.

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