*Full Paper***Effects of Liposome Clodronate on Renal Leukocyte Populations and Renal Fibrosis in Murine Obstructive Nephropathy**

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Abstract. Although liposome-encapsulated clodronate has been used as a means to deplete macrophages from certain tissues, target leukocyte subtypes within the kidney are not clearly known under normal and pathologic conditions. The present study was therefore conducted to examine the effects of liposome clodronate on renal infiltrating cell type following unilateral ureteral obstruction (UUO) and tried to correlate these changes to the mechanisms of early development of renal fibrosis. Renal infiltrating leukocyte subtypes and counts were determined by using multicolor flow cytometric analysis of cell suspensions from obstructed kidneys. UUO for 5 days elicited renal tubular apoptosis and renal fibrosis and showed 4-fold increase in renal leukocytes including monocytes/macrophages, dendritic cells, and T-cells. Repeated administration of liposome clodronate selectively depleted F4/80⁺ monocytes/macrophages and F4/80⁺ dendritic cells but not F4/80⁻ dendritic cells or other cell types in both obstructed and non-obstructed kidneys. Tubular apoptosis and renal fibrosis were also significantly attenuated by liposome clodronate. Increased gene expression of TNF- α and TGF- β observed in obstructed kidneys were markedly attenuated by depletion of renal mononuclear phagocytes. These findings suggest that F4/80⁺ monocytes/macrophages and/or F4/80⁺ dendritic cells play a pivotal role in the development of obstruction-induced tubular apoptosis and renal fibrosis, possibly through TNF- α and TGF- β dependent mechanisms.

Keywords: obstructive nephropathy, ureteral obstruction, macrophage, dendritic cell, liposome clodronate

Introduction

Tubulointerstitial fibrosis is a main determinant that leads to an irreversible loss of renal function in chronic diseases (1). Since infiltration of the monocyte/macrophage lineage often correlates with the degree of renal fibrosis, monocytes/macrophages have been considered to contribute to the development of fibrosis (2).

Liposome-encapsulated clodronate (dichloromethylene-bisphosphonate) is thought to produce a selective depletion of macrophages from tissue (3). When administered intravenously, liposome is delivered to phagocytic cells of the mononuclear phagocyte system and clodronate

is released intracellularly. Clodronate then selectively destroys these phagocytic cells. This macrophage ‘suicide’ technique has been successfully used as a tool to investigate macrophage activities in vivo (4) and also was applied to an animal model of acute kidney injury (5, 6). Liposome clodronate deleted renal ED-1-positive monocytes/macrophages in rats (6, 7) and renal F4/80-positive macrophages (5) in mice and attenuated renal ischemia-reperfusion injury, a direct demonstration of the pathologic role of monocytes/macrophages. In rat unilateral ureteral obstruction (UUO), an animal model of renal fibrosis, however, although single intravenous administration of liposome clodronate one day prior to UUO significantly reduced interstitial collagen deposition at day 5, immunohistochemical analysis failed to detect any decrease in the number of renal cortical ED-1-positive macrophages by liposome clodronate

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either at day 1 or 5 (8). This discrepancy may be due to insufficient dosage and/or timing of liposome clodronate. Alternatively, liposome clodronate may affect different cell types that cannot be detected immunohistochemically by using a single cell surface marker. In fact, Dong et al. have shown that liposome clodronate selectively depleted renal dendritic cells without effects on the number of monocytes in mice by using multicolor flow cytometry (9). The F4/80 marker detects both murine macrophages and dendritic cells that are resident within or infiltrating the kidney (10). These two cell types are main constituents of the mononuclear phagocytic system within normal kidney (11). Renal dendritic cells have been well characterized by their morphology, cell surface markers (e.g., CD11c, major histocompatibility complex class II molecules), maturation responses, phagocytic capacity, and T cell stimulatory capacity (10–13). In order to use liposome clodronate adequately as a pharmacological tool to explore the role of individual infiltrating cell type in the renal disease process, it is essential to characterize the target cell types. The present study was therefore conducted to examine the effects of repeated administration of liposome clodronate on renal infiltrating cell type in obstructed kidneys and tried to correlate these changes to the mechanisms of early development of renal fibrosis. To characterize and quantitatively analyze renal infiltrating cells, renal tissue was subjected to enzymatic digestion and the renal cell suspension was analyzed by multicolor flow cytometry.

Materials and Methods

Animals

Male C57BL/6J mice (6–8-week-old) were purchased from Charles River (Yokohama) and housed in a specific pathogen free facility. All experiments were conducted in accord with “Guiding Principles for the Care and Use of Laboratory Animals” approved by The Japanese Pharmacological Society.

UUO model

After amikacin injection (6 mg/kg, i.m.), mice were anesthetized by pentobarbital sodium (50 mg/kg, i.p.). UUO was performed aseptically by complete ligation of the left ureter at 3 points and cut between the distal ligatures. At 5 days after the ligation, mice were again anesthetized and kidneys were perfused with ice-cold heparinized saline via heart puncture. Kidneys were excised and coronal slices were fixed with methyl-Carnoy’s solution and embedded in paraffin for immunohistochemical examination. A portion of the renal tissue was immediately frozen in liquid nitrogen and stored at

–80°C for further analysis. In some experiments, kidneys were fixed with 4% paraformaldehyde and were used for TUNEL assay.

Administration of liposome-encapsulated clodronate

Clodronate (Sigma-Aldrich, Tokyo) was encapsulated in liposomes as described previously (3). PBS-encapsulated liposomes were used as controls. Mice were injected intravenously with 200 μ l of clodronate or PBS liposomes on 2 consecutive days. At 48 h after the second injection, the third injection of clodronate was made and then mice were subjected to unilateral ureteral ligation. Injection of clodronate was repeated on day 2 and 4 after ureteral obstruction. This regimen of liposome clodronate administration was based on the immunohistochemical observations that 48 h after liposome clodronate, renal macrophages were decreased to one third (5), whereas no obvious reduction was found at 3 days after administration (14).

Immunohistochemistry

Sections (4- μ m-thick) of the paraffin-embedded renal tissue were used for immunohistochemistry. Immunohistochemical examinations were performed for α -smooth muscle actin (α -SMA) (mouse monoclonal antibody, 1A4; Sigma-Aldrich), collagen III (goat polyclonal antibody; SouthernBiotech, Birmingham, AZ, USA), and F4/80-positive mononuclear phagocytes (rat anti-mouse monoclonal antibody, CI:A3-1; Serotec, Oxford, UK) using Histofine SimpleStain Mouse MAX-PO Kits (Nichirei, Tokyo).

TUNEL assay

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end labeling assay using the In situ Apoptosis Detection kit (Takara Bio Inc., Kyoto). TUNEL assay was performed for paraffin sections fixed with 4% paraformaldehyde and were processed according to the manufacturer’s manual. The number of TUNEL-positive apoptotic cells was counted in six tubulointerstitial areas of each sample ($\times 20$).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using ISOGEN (Nippongene, Toyama), according to the manufacturer’s protocols for animal tissue. Genomic DNA was removed by using the TURBO DNA-free Kit (Applied Biosystems, Tokyo). Real-time RT-PCR was performed using the SYBR[®] Premix Ex TaqTM (Takara Bio, Inc.) and the ABI PRISM7700 Sequence Detection System (Applied Biosystems). Target genes were transforming growth

factor- β (TGF- β 1), collagen type III, tumor necrosis factor- α (TNF- α), and monocyte chemoattractant protein-1 (MCP-1). The kinetics of the reaction was determined by using a standard curve. The level of the molecules investigated was corrected for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All values were expressed as fold expression relative to the average of the value in the contralateral kidney from the PBS liposome-treated group.

Preparation of kidney cell suspension for flow cytometry

In order to characterize and quantitatively analyze renal infiltrating leukocytes, renal tissue was enzymatically digested and the suspension was analyzed by multicolor flow cytometry. This methodology has been successfully used to determine renal leukocyte subsets (9, 10, 15, 16). Kidney cell suspension was prepared based on a previously reported method (9). In brief, kidneys were dissected, placed in Hank's Balanced Salt Solution (HBSS) containing 1.6 mg/ml collagenase IA (Sigma Aldrich) and 200 μ g/ml DNase I (Roche, Indianapolis, IN, USA) for 30 min at 37°C with intermittent agitation, and then washed twice in HBSS. Following erythrocyte lysis, cells were resuspended in FACs buffer (PBS / 5% fetal calf serum / 0.5% NaN₂). Kidney cell suspensions to be used for flow cytometry were allowed to settle for 20 min and then the upper two-thirds were collected for use in these assays.

Flow cytometric analysis

Aliquots of cells were pre-incubated with anti-CD16 / CD32 Fc receptor for 10 min to minimize nonspecific antibody binding. Cells were then incubated with various combinations of mAbs for 25 min at 4°C and then washed twice with FACS buffer. Four-color immunofluorescence staining was analyzed using LSR II and FACSDiva software (BD Biosciences, Tokyo). Antibodies used were anti-CD45-PE-Cy7 (Ly-5), anti-CD11b-PE (Mac-1), anti-F4/80-APC (BM8), and anti-CD3e-PE (145-2C11) (all from eBioscience, San Diego, CA, USA) and anti-CD11c-PE, FITC (HL3), anti-CD16 / CD32 (2.4G2), anti-Ly6C FITC (AL-21), and anti-Ly-6G-FITC (1A8) (all from BD Biosciences). To determine the CD45⁺ cell absolute counts, the kidney suspension was mixed thoroughly with a known number of fluorescent counting beads (TruCOUNT Tubes, BD Biosciences) before acquisition by the flow cytometer. The absolute counts of CD45⁺ cells in the sample were determined by comparing cellular events to fluorescent bead events. The leukocyte subset number was multiplied by the CD45 cell numbers and by the percentage of the subset.

Statistical analyses

All data are presented as the mean $\pm$ S.E.M. Data were analyzed using analysis of variance, and individual comparisons were made using Tukey's *post hoc* analysis. As some data showed heteroscedasticity, logarithmic transformation was made before analysis. Statistical significance was defined as $P < 0.05$.

Results

Ureteral obstruction elicited marked accumulation of bone marrow-derived cell population within the kidney and liposome clodronate selectively depleted F4/80⁺ dendritic cells and macrophages

Renal infiltrating leukocyte subsets were identified using antibodies against CD45, CD11b, F4/80, CD11c, Ly6C, Ly6G, and CD3. Figure 1 shows the cell numbers of bone marrow-derived cell populations following UVO and changes by liposome clodronate treatment. UVO for 5 days increased renal accumulation of bone marrow-derived (CD45⁺) cells approximately 4-fold from 3.8×10^5 cells/0.1 g kidney. Cell number of

Fig. 1. Calculated cell numbers of bone marrow-derived cell population following unilateral ureteral obstruction. Suspended kidney cells were stained and then gated for the CD45⁺ leukocyte population. Staining definitions were F4/80⁺ macrophage: CD45⁺ / F4/80⁺ / CD11c⁻, F4/80⁺ dendritic cell: CD45⁺ / F4/80⁺ / CD11c⁺, F4/80⁻ dendritic cell: CD45⁺ / F4/80⁻ / CD11c⁺, neutrophil: CD45⁺ / Ly-6G⁺, T cell: CD45⁺ / CD3⁺. Open and closed columns: cell numbers in non-obstructed (n = 5) and obstructed kidneys (n = 8) receiving liposome PBS, respectively. Light- and dark-gray columns: cell numbers in non-obstructed (n = 3) and obstructed kidneys (n = 9) receiving liposome clodronate, respectively. * $P < 0.05$, compared to non-obstructed kidneys; † $P < 0.05$, compared to those receiving liposome PBS. n = 6–9 for each group.

CD11b⁺ (integrin α M and component of Mac-1) that is highly expressed by monocytes/macrophages, dendritic cells, and granulocytes also increased 5-fold from 2.0×10^5 cells/0.1 g kidney. The number of F4/80⁺ cells that is a marker for both monocytes/macrophages and dendritic cells is comparable to that of CD11b⁺ cells in non-obstructed kidney and increased to a similar extent. CD11c-positive cells (a marker of dendritic cells) increased 3-fold from 1.7×10^5 cells/0.1 g kidney.

Fig. 2. Calculated cell numbers of F4/80⁺ macrophage subpopulations and immunohistochemistry of F4/80-positive cells in renal cortex. A) F4/80⁺ macrophages (CD45⁺/F4/80⁺/CD11c⁻) were sub-grouped by the presence of Ly-6C. Open and closed columns: cell numbers in non-obstructed (n = 3) and obstructed kidneys (n = 5) receiving liposome PBS, respectively. Light- and dark-gray columns: cell numbers in non-obstructed (n = 3) and obstructed kidneys (n = 6) receiving liposome clodronate, respectively. **P* < 0.05, compared to non-obstructed kidneys; †*P* < 0.05, compared to those receiving liposome PBS. Since the number of replicates for Ly-6C staining was less than that examined in Fig. 1, the absolute counts of F4/80⁺ macrophages were not same as those appearing in Fig. 1. Staining definitions were Ly-6C⁻: CD45⁺/F4/80⁺/CD11c⁻/Ly-6C⁻, Ly-6C⁺: CD45⁺/F4/80⁺/CD11c⁻/Ly-6C⁺. B) Quantitative evaluation of F4/80-positive cells in each group. Ctrl.: contralateral kidney. Obstr.: obstructed kidney. In the liposome PBS-treated group, there was a remarkable increase in F4/80 immunostaining following ureteral obstruction. Liposome clodronate attenuated F4/80 staining in both non-obstructed and obstructed kidney. C) Representative photomicrographs of F4/80 immunostaining.

F4/80⁺ macrophages account for about 30% of total F4/80⁺ cells in non-obstructed kidney. This cell type increased more than ten times following ureteral obstruction. Three fourth of dendritic cells (CD11c⁺) were positive for F4/80, while 70% of F4/80⁺ cells were dendritic cells in non-obstructed kidney. Both types (F4/80⁺ and F4/80⁻) of dendritic cells increased significantly following ureteral obstruction. Neutrophils that account only for 4% of total leukocytes increased 2–3-fold. T cells that explain 15% of total leukocytes in non-obstructed kidneys were increased 4-fold following ureteral obstruction. In obstructed kidney, liposome clodronate significantly decreased cell numbers of total leukocytes (CD45⁺ cells), CD11b⁺ cells, F4/80⁺ cells, and CD11c⁺ dendritic cells. Among those, F4/80⁺ macrophages and F4/80⁺ dendritic cells were eliminated by liposome clodronate administration. However, the numbers of F4/80⁻ dendritic cells, neutrophils or T cells were not affected by clodronate. In the non-obstructed kidneys, liposome clodronate also decreased the cell numbers of total leukocytes, CD11b⁺ cells, F4/80⁺ cells, CD11c⁺ dendritic cells, F4/80⁺ macrophages, and T cells. Within the F4/80⁺ macrophage population, Ly-6C⁻ cells are a principal cell type (Fig. 2A). Following ureteral obstruction, cell numbers of both Ly-6C⁻ and Ly-6C⁺ macrophages increased that were then eliminated by liposome clodronate. Immunohistochemical staining of F4/80⁺ cells was shown and summarized in Fig. 2, B and C. In non-obstructed kidney, F4/80⁺ cells were diminished by liposome clodronate. Following ureteral obstruction, immunohistochemical examination revealed that F4/80⁺ cells were remarkably accumulated in the interstitial space that was almost abolished by clodronate treatment. These changes mirror the results from multicolor flow cytometry (Figs. 1 and 2), a supporting finding of the validity of flow cytometric analysis of the leukocyte population using isolated renal cell suspension obtained by enzymatic digestion.

Liposome clodronate ameliorated early development of renal fibrosis and renal tubular apoptosis

UUO increased interstitial accumulation of extracellular matrix, collagen III, and α -SMA (a marker of myofibroblast and vascular smooth muscle) that represent early development of renal fibrosis (Fig. 3). Liposome clodronate significantly attenuated interstitial accumulation of collagen III and α -SMA. Tubular apoptosis was increased in obstructed kidneys when compared to contralateral non-obstructed kidneys. Liposome clodronate significantly decreased tubular apoptosis (Fig. 3).

Fig. 3. Quantitative evaluations of collagen III and α -SMA immunostaining and TUNEL assay. The percentages of positive area of collagen III and α -SMA (α -smooth muscle actin) ($n = 7$ in each group) and nuclei of apoptotic cells stained with TUNEL assay ($n = 4$ in each group) were calculated and summarized in the upper panels. All data were shown as mean $\pm$ S.E.M. * $P < 0.05$ compared to non-obstructed kidneys, $^{\dagger}P < 0.05$ compared to those receiving liposome PBS. Representative photomicrographs of obstructed kidneys were shown in the lower panels. Intense staining of collagen III and α -SMA (α -smooth muscle actin) observed in obstructed kidney (liposome PBS-treated) was markedly attenuated by liposome clodronate. Apoptotic tubular cells seen in obstructed kidney were also decreased by the treatment with liposome clodronate.

Liposome clodronate reduced enhanced mRNA expression of various cytokines and extracellular matrix

Figure 4 summarizes the relative mRNA expression of MCP-1, TNF- α , TGF- β , and collagen III. Gene expressions of MCP-1, TNF- α , and TGF- β were increased 7–30-fold in obstructed kidneys. Liposome clodronate markedly attenuated these increases. Renal mRNA expression of collagen III increased more than 10-fold following UUO that was significantly attenuated by clodronate.

Discussion

UUO for 5 days elicited early manifestation of renal fibrosis and tubular apoptosis concomitantly with accumulation of leukocytes mainly due to the increase in monocytes/macrophages and dendritic cells. Repeated administration of liposome clodronate specifically depleted renal F4/80 $^{+}$ monocytes/macrophages and F4/80 $^{+}$ dendritic cells without affecting the numbers of F4/80 $^{-}$ dendritic cells, neutrophils, or T cells. Liposome clodronate significantly attenuated renal fibrosis and tubular apoptosis. These results provide direct evidence that either F4/80 $^{+}$ monocytes/macrophages, F4/80 $^{+}$ dendritic cells, or both cell types contribute, at least in

part, to the early development of renal fibrosis and tubular apoptosis.

One of the aims of the present study is to characterize which cell types are depleted by liposome clodronate within the kidney. Our results indicate that F4/80 $^{+}$ monocytes/macrophages and F4/80 $^{+}$ dendritic cells are specifically eliminated from either obstructed or non-obstructed kidneys. Thus, when liposome clodronate is used to study renal pathophysiology, role of both cell types should be considered. Since liposomes are not able to cross the vascular barrier formed by capillary walls (3), the site of intravenous liposome clodronate-induced depletion of phagocytes is generally considered to be within the systemic circulation. Systemic depletion of monocytes then would diminish renal influx and result in reduced accumulation of mononuclear phagocytes within the kidney. This notion together with our present findings suggests that increased renal mononuclear phagocytes were mainly recruited from their pool in peripheral blood and liposome clodronate depleted the systemic pool of these cells or their precursors. The turnover of resident macrophages and dendritic cells within the kidney may be relatively as short as within a week since both flow cytometry (Fig. 1) and immunohistochemistry (Fig. 2) confirmed that clodronate also

Fig. 4. Effects of liposome clodronate on mRNA expression of cytokines and collagen III. Average mRNA expression in non-obstructed kidney (liposome PBS-treated) was assigned to unity. MCP-1; monocyte chemoattractant protein-1, TNF- α ; tumor necrosis factor- α , TGF- β ; transforming growth factor- β . All data were shown as mean $\pm$ S.E.M. $n = 4 - 9$ in each group. * $P < 0.05$ compared to non-obstructed kidneys, † $P < 0.05$ compared to those receiving liposome PBS.

depleted F4/80⁺ mononuclear phagocytes from non-obstructed kidneys by liposome clodronate treatment over a period of a week.

The spectrum of renal leukocyte subsets eliminated by clodronate in our study is somewhat different from that reported by others who observed selective depletion of CD11c⁺ dendritic cells (9, 15). They have shown that liposome clodronate depleted renal dendritic cells but not Ly-6C⁺ monocytes/macrophages either following renal ischemia/reperfusion or ureteral obstruction in mice. In contrast, both Ly-6C⁺ and Ly-6C⁻ monocytes/macrophages as well as dendritic cells were effectively depleted from the kidney in our study. Such differences presumably arise from the differences in the schedules of clodronate dosing and the timing of renal cell analysis. We administered liposome clodronate 5 times in a week and renal leukocytes were analyzed 24 h after the last

dose, whereas they examined kidney cells at 72 or 96 h after the second dose. Although clodronate reversibly reduces circulating number of monocytes, monocyte repopulation from the bone marrow occurs within 48 h (17). Thus, it is conceivable that such a monocyte repopulation presumably occurred and was accompanied by a substantial renal infiltration to a comparable level without liposome clodronate in their study. Those authors also interpreted this in a similar way.

In the present experiments, gene expression of MCP-1, TNF- α , and TGF- β was markedly up-regulated in the obstructed kidney and liposome clodronate attenuated this increase. By in situ hybridization, Vielhauser et al. showed a prominent expression of MCP-1 mRNA in the interstitial mononuclear cell infiltrates and also cortical tubular epithelial cells of mouse obstructed kidney (18). Tubular cells are primary sources of TNF- α following 3 days of ureteral obstruction in rats (19), whereas Dong et al. demonstrated that dendritic cells are the main source in mouse obstructed kidney (15). Interstitial infiltrates express TGF- β mRNA in mice (20) while tubular epithelial cells, particularly those of the proximal tubules, are the main contributors to increased TGF- β mRNA expression in rat obstructed kidney (21). Thus, one of the possible explanations of the decreased gene expression of these cytokines by liposome clodronate is that clodronate eliminated infiltrating leukocytes that expressed these cytokines. Alternatively, increased mononuclear phagocytes may have stimulated tubular production of these cytokines directly or via release of pro-inflammatory substances. As liposome clodronate depleted these infiltrating cells, subsequent stimulation may have ceased. In this context, it is interesting that monocytes directly interact with proximal tubular epithelial cells via ICAM-1-dependent binding and stimulate TGF- β transcription (22).

Our results showed the depletion of renal mononuclear phagocytes attenuated renal tubular apoptosis concomitantly with attenuation of gene expression of TNF- α and TGF- β . Neutralization of TNF- α by soluble TNF receptor attenuated obstruction-induced tubular apoptosis (23). Antibody to TGF- β ameliorates tubular apoptosis following UUO (24). TGF- β potentiates renal tubular cell apoptosis initiated by other death cues in vitro (25). Thus, it is reasonable to speculate that infiltrating mononuclear phagocytes in obstructed kidney play a pivotal role in eliciting tubular apoptosis by stimulating expression of TNF- α and/or TGF- β . TNF- α neutralization also ameliorates obstruction-induced renal fibrosis (26). TGF- β is highly correlated to the fibrogenic process, directly stimulates production of extracellular matrix and myofibroblast transdifferentiation, and plays a central role in renal fibrosis (27). Neutraliza-

tion of this cytokine also attenuates obstruction-induced renal fibrosis (24). Therefore, our findings together with these reports suggest that mononuclear phagocytes elicit early development of renal fibrosis through stimulation of TNF- α and TGF- β expression.

Since liposome clodronate depleted both F4/80⁺ macrophages and F4/80⁺ dendritic cells, we are not able to attribute liposome clodronate-induced renal alterations to either of one cell type or both. Accumulating evidences suggest the importance of macrophages in the pathogenesis of renal fibrosis (28). However, it should be pointed out that detection and/or differentiation of dendritic cells from macrophages is difficult by conventional immunohistochemistry. Furthermore, now we know that functions and phenotypes of these two cell types are considerably more overlapped than considered previously (29). In order to explore the role of macrophages and dendritic cells separately, transgenic conditional cell ablation models using the restricted expression of the human diphtheria toxin receptor (DTR) have been developed and have proven useful, including CD11b-DTR mouse (30) and CD11c-DTR mouse (16). Use of these animal models may solve the problems. A recent study suggested the possible role of dendritic cells in inducing interleukin 17-secreting CD4⁺ memory cells during the early inflammatory response to urinary obstruction (24–72 h) in mice (15). Although this study did not correlate such responses to renal fibrosis, it provides an important insight into understanding the role of renal dendritic cells in the pathogenesis of tubulointerstitial diseases.

In summary, we showed in the present study that liposome clodronate selectively depleted F4/80-positive macrophages and dendritic cells from obstructed kidneys. It is suggested that these cells are involved in the early development of renal fibrosis and tubular apoptosis presumably through TNF- α and TGF- β .

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