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Adoptive transfer of macrophages ameliorates renal fibrosis in mice

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Abstract

We performed adoptive transfer of bone marrow-derived (BM) macrophages following pharmacological depletion of leukocytes in a mouse model of unilateral ureteral obstruction (UUO). Treatment with cyclophosphamide (CPM) caused marked decrease in the numbers of F4/80-positive interstitial macrophages as well as in peripheral blood leukocyte counts, and adoptive transfer of BM macrophages to CPM-treated mice resulted in significant increase in the numbers of interstitial macrophages both at day 5 and at day 14 after UUO. At day 5 after UUO, no significant change was observed in the degree of renal interstitial fibrosis either by treatment with CPM or with CPM + macrophage. However, at day 14 after UUO, treatment with CPM caused significant increase in the degree of interstitial fibrosis, and adoptive macrophage transfer to these mice attenuated this enhancement in renal fibrosis. Our result suggests the role of infiltrating macrophages on facilitating tissue repair at late stage of UUO.

Keywords: Renal fibrosis; Renal interstitium; Adoptive macrophage transfer; Bone marrow-derived macrophage; Macrophage depletion; Cyclophosphamide; Unilateral ureteral obstruction; Tissue repair

Many clinical and experimental studies have shown that one of the initial events of progressive renal disease is macrophage infiltration to the kidney [1,2]. Thus, macrophage infiltration has been considered to be a hallmark of all forms of renal injury. However, recent evidence showing that macrophages are involved in all stages of inflammatory process including tissue repair and healing such as in lung [3] and in skin [4] has superseded this classical view of macrophages. Evidence suggesting that macrophages also downregulate renal injury and facilitate repair has recently been reported in a mouse model of glomerulonephritis [5] and renal fibrosis [6]. Renal fibrosis had long been recognized as a prominent feature of diseased kidneys. However, the pathogenic mechanisms during the evolution of fibrotic changes in the kidney, including the role of infiltrating macrophages in each stage of fibrotic process, have not yet been fully elucidated.

Several studies have shown that macrophages transferred into animals can accumulate within the kidney and act as biologically functional cells that may accelerate or attenuate renal injury [7–10]. However, these previous studies have been focused on the pathogenic role of macrophages in glomerular injury. In the present study, we intended to focus on the role of infiltrating macrophages in the evolution of renal interstitial fibrosis. Unilateral ureteral obstruction (UUO) is a well-established experimental model of progressive renal interstitial fibrosis. Macrophage infiltration to the cortical tubulointerstitium is observed as an initial event in this model, and infiltrating macrophages are considered to play a pivotal role in the development of interstitial fibrosis. In this study, we used adoptive transfer of macrophages following pharmacological depletion of leukocytes in mouse UUO model to study the role of infiltrating macrophages in the process of progressive renal fibrosis.

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Methods

Experimental protocol. Experiments were performed on male C57BL/6 mice (10–12 weeks, 20–24 g). Experimental protocol is outlined in Fig. 1. Renal fibrosis was induced to mice by a complete unilateral ureteral obstruction (UUO) as previously described [6] (day 0). Briefly, under sodium pentobarbital anesthesia, the middle portion of the left ureter was ligated and cut between the two ligated points. At 5 and 14 days after surgery, the mice were sacrificed, and the obstructed kidneys were harvested and subjected to the studies described below. In protocol A (Fig. 1A), mice were given intraperitoneal injections of 165 mg/kg cyclophosphamide (CPM) (Shionogi, Osaka, Japan) to deplete circulating leukocytes both on day −2 and on day 1. This caused marked depletion of circulating leukocytes from day 0 (at the time of UUO) to day 5 (at the time of sacrifice). Macrophage transfer was performed repeatedly in CPM-treated mice by intravenous injections of 1×10^7 bone marrow-derived (BM) macrophages in 0.2 ml of serum-free media on day 0 (immediately after a UUO was performed), day 2, and day 4 as previously described [10], and the mice were sacrificed at day 5. In protocol B (Fig. 1B), after UUO (day 0), mice were given intraperitoneal injections of CPM both on day 6 and on day 10. This caused marked depletion of circulating leukocytes from day 8 to day 14 (at the time of sacrifice). Macrophage transfer was performed on day 7, day 9, and day 11, and the mice were sacrificed at day 14. The experimental protocols were performed according to the regulations of the Kyoto Prefectural University of Medicine Animal Care Committee.

Macrophage culture. Culture of bone marrow-derived (BM) macrophages was performed as previously described [10]. Briefly, bone marrow cells were harvested from the femurs and tibias of male C57BL/6 mice by flushing with RPMI-1640 media (Gibco-BRL, Gaithersburg, MD, USA). Cells were washed and resuspended in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Gibco-BRL) supplemented with 10% fetal calf serum (Microbiological Associates-Bioproducts, Walkersville, MD, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. After a 2 h culture in petri dishes to remove adherent stromal cell population, the non-adherent fraction was cultured for 5 days in the presence of 10% L-cell conditioned medium as a source of macrophage colony-stimulating factor at 37 °C in incubators under 95% air and 5% CO_2. At the end of the culture period, more than 90% of the cells were F4/80-positive macrophages as judged by flowcytometry. Cells were also incubated with 50 μg/ml bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO, USA) during the last 24 h of culture, washed, and then transferred to the mice to identify transferred cells in the kidney.

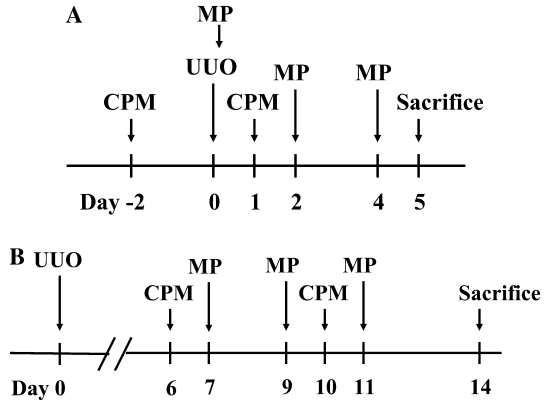

Fig. 1. Experimental protocol for adoptive transfer of bone marrow-derived macrophages. (A) Renal fibrosis was induced to mice by a complete unilateral ureteral obstruction (UUO) (day 0). In protocol A, mice were given intraperitoneal injections of 165 mg/kg cyclophosphamide (CPM) to deplete circulating leukocytes both on day −2 and on day 1. This caused marked depletion of circulating leukocytes from day 0 to day 5. Macrophage transfer (MP) was performed repeatedly in CPM-treated mice by intravenous injections of 1×10^7 bone marrow-derived macrophages on day 0, day 2, and day 4, and the mice were sacrificed at day 5. (B) In protocol B, mice were given intraperitoneal injections of CPM both on day 6 and on day 10. Macrophage transfer was performed on day 7, day 9, and day 11, and the mice were sacrificed at day 14.

Hematologic analysis. At sacrifice, blood samples were collected by retro-orbital venous plexus puncture for determination of peripheral blood leukocyte count. Total leukocyte count was determined using a SE9000 automated counter (Sysmex, Kobe, Japan).

Histological study. For histological examinations, kidneys were fixed with 4% buffered paraformaldehyde for 6 h, embedded in paraffin, and sectioned transversely with a thickness of 4 μm. A standard point-counting method was used to quantitate the collagen fractional volume in the renal cortical interstitium on Masson trichrome-stained sections (magnification, 400×), as previously described [6]. The index of interstitial collagen fractional volume was defined as the number of trichrome-positive points in every 1000 points evaluated. To detect infiltrating macrophages, sections were incubated with monoclonal rat anti-mouse F4/80 (Selotec, Oxford, United Kingdom) for 1 h at room temperature, followed by standard ABC immunostaining using ABC-alkaline phosphatase kit (Vector, Burlingame, CA, USA). Macrophage infiltration was determined by enumerating F4/80-positive cells within the cortical interstitium in 10 randomly selected cortical fields under magnification (400×), and the numbers were averaged for each field [6]. BrdU-positive cells were also immunohistochemically identified with monoclonal anti-BrdU antibody (Oncogene Research Products, San Diego, CA, USA) following the manufacturer's protocol. Sections were also treated with DNase 1 (0.33 U/μl) (Roche Applied Science, Mannheim, Germany) at 37 ° for 1 h following digestion with trypsin.

Collagen content. The amount of hydroxyproline in the renal cortex was measured as an index of collagen content as previously described [11]. Briefly, pieces of renal cortex for the hydroxyproline assay were weighed and snap-frozen in liquid nitrogen at sacrifice. After hydrolyzed in 1 ml of 6 N hydrochloric acid and neutralized with sodium hydroxide, the concentrations of hydroxyproline of the samples were determined by high-performance liquid chromatography (HPLC) using HPLC system 800 series (JASCO, Tokyo, Japan).

Statistical analysis. Data are presented as means ± SEM. Statistical analysis was performed by ANOVA, and significance was defined as $P < 0.05$.

Results

Effect of cyclophosphamide pretreatment on peripheral blood leukocyte count

Pretreatment with CPM caused marked decrease in peripheral blood leukocyte counts both at day 5 and at day 14 after UUO compared with those of control mice that were treated with saline only (day 5: 6800 ± 660/μl vs. 1420 ± 153/μl, $P < 0.0001$; day 14: 8157 ± 353/μl vs. 1267 ± 201/μl, $P < 0.0001$, $N = 6$–8 in each group).

Effect of cyclophosphamide pretreatment and adoptive transfer of macrophages on interstitial macrophage infiltration in the UUO kidneys

Pretreatment with CPM caused marked decrease in the numbers of F4/80-positive interstitial macrophages

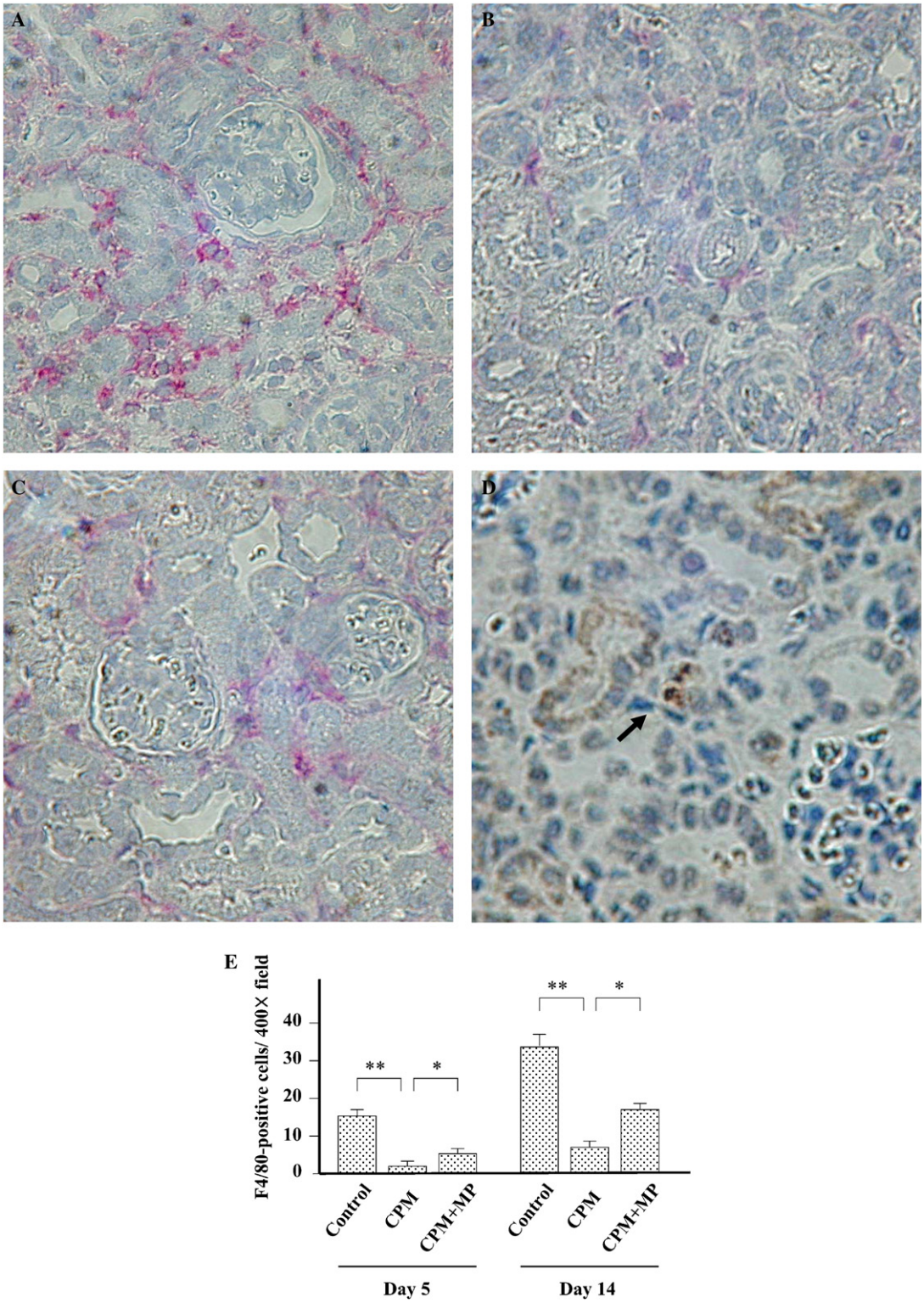

Fig. 2. Effect of cyclophosphamide pretreatment and adoptive transfer of macrophages on interstitial macrophage infiltration in the UUO kidneys. (A–C) Immunohistochemical demonstration of macrophages with anti-F4/80 antibody in mice with no treatment (A), in mice treated with CPM (B), and in mice treated with CPM + macrophage (C) at day 14 after UUO. (D) Detection of BrdU-positive cells in the kidneys of mice treated with CPM + macrophage at day 14 after UUO (arrow). Bone marrow-derived cells were incubated with BrdU during the last 24 h of culture and then transferred to the mice. BrdU-positive cells were occasionally observed in the interstitium of UUO kidney both at day 5 and at day 14 after UUO. Original magnification, 400×. (E) Macrophages (F4/80-positive cells) infiltrating to the interstitium in mice with no treatment, in mice treated with CPM, and in mice treated with CPM + macrophage at day 5 (left) and day 14 (right) after UUO. Data are expressed as means ± SEM. $*P < 0.01$. $**P < 0.0001$.

both at day 5 and at day 14 after UUO compared with those of control (day 5: 16.1 ± 1.0 vs. 1.9 ± 0.5/400× field, $P < 0.0001$; day 14: 32.9 ± 2.2 vs. 8.2 ± 0.6, $P < 0.0001$, $N = 6$–8 in each group) (Figs. 2A, B, and E). Adoptive transfer of BM macrophages to CPM-treated mice resulted in significant increase in the numbers of interstitial macrophages both at day 5 and at day 14 after UUO compared with those of mice that were treated with CPM and injected with media without macrophages (day 5: 1.9 ± 0.5 vs. 4.7 ± 0.4, $P < 0.01$; day 14: 8.2 ± 0.6 vs. 16.6 ± 1.4, $P < 0.01$, $N = 6$ in each group) (Figs. 2B, C, and E). In mice that had received adoptive transfer of macrophages, BrdU-positive cells were occasionally observed in the interstitium of UUO kidney both at day 5 and at day 14 after UUO (Fig. 2D). However, BrdU-positive cells were not seen in contralateral non-obstructed kidneys either at day 5 or at day 14.

Effect of cyclophosphamide pretreatment and adoptive transfer of macrophages on renal fibrosis in the UUO kidneys

At day 14 after UUO, pretreatment with CPM caused a significant increase in interstitial collagen index (99 ± 7 vs. 158 ± 17/1000 points, $P < 0.005$) (Figs. 3A, B, and D). Furthermore, adoptive transfer of BM macrophages to CPM-treated mice resulted in a significant decrease in interstitial collagen index at day 14 after UUO compared with those of mice that were treated with CPM only (158 ± 17 vs. 118 ± 7/1000 points, $P < 0.05$) (Figs. 3B–D). However, at day 5 after UUO,

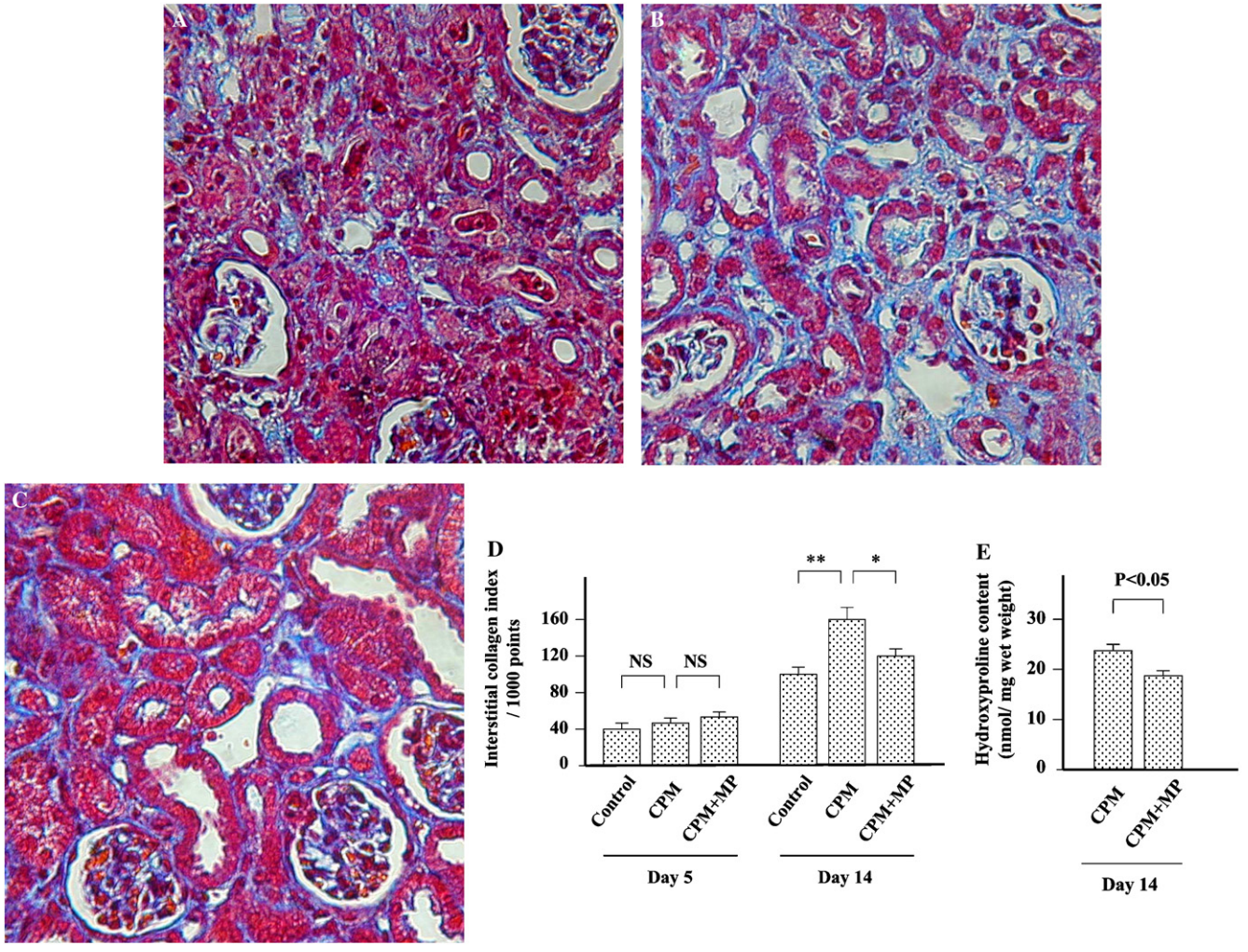

Fig. 3. Effect of cyclophosphamide pretreatment and adoptive transfer of macrophages on renal fibrosis in the UUO kidneys. (A–C) Masson trichrome staining of the renal cortex in mice with no treatment (A), in mice treated with CPM (B), and in mice treated with CPM + macrophage (C) at day 14 after UUO. Original magnification, 400×. (D) Interstitial collagen index assessed by point-counting method on Masson trichrome-stained sections in mice with no treatment, in mice treated with CPM- and in mice treated with CPM + macrophage at day 5 (left) and day 14 (right) after UUO. (E) Hydroxyproline content in renal tissue assessed by HPLC in mice treated with CPM (left) and in mice treated with CPM + macrophage (right) at day 14 after UUO. Data are expressed as means ± SEM. *$P < 0.05$. **$P < 0.005$. NS, not significant.

no significant difference was observed in interstitial collagen index among these three groups (41 ± 3 in no treatment, 45 ± 4 in CPM only, 50 ± 4 in CPM + macrophage/1000 points, NS between groups) (Fig. 3D). At day 14 after UUO, the collagen content in renal tissue assessed by HPLC was also significantly lower in mice treated with CPM and macrophage compared to those in mice treated with CPM only (hydroxyproline: 23.4 ± 1.5 nmol/mg wet weight in CPM only vs. 18.5 ± 1.3 in CPM + macrophage, $P < 0.05$) (Fig. 3E).

Discussion

In this study, pretreatment with CPM caused marked decrease in the numbers of F4/80-positive interstitial macrophages as well as in peripheral blood leukocyte count both at day 5 and at day 14 after UUO. To deplete circulating leukocytes, we have treated with CPM twice for each protocol, i.e., day −2 and day 1 for protocol A, and day 6 and day 10 for protocol B. Because treatment with CPM by these protocols caused marked decrease in circulating leukocytes during a certain period until sacrifice in mice, it is suggested that the depletion in circulating leukocytes resulted in decrease in the numbers of F4/80-positive interstitial macrophages both at day 5 and at day 14 after UUO.

In protocol A, no significant change was observed in renal interstitial collagen index by pretreatment with CPM at day 5 after UUO. However, in protocol B, pretreatment with CPM caused a significant increase in the degree of renal interstitial fibrosis at day 14 after UUO. A possible explanation for this altered renal fibrotic change by treatment with CPM is the direct profibrotic action of CPM. Nevertheless, the effect of CPM to cause renal interstitial fibrosis has not been reported previously, and furthermore, the difference in profibrotic action of CPM between the early and the late stage of UUO makes this assumption more complex. Another view for this effect on fibrosis is the action of CPM through modulating the effect of infiltrating macrophages to renal interstitium, because the number of macrophages infiltrating to renal interstitium was profoundly affected by treatment with CPM both in protocol A and in protocol B in our study. This view makes sense in notion of a close relationship between the progression of renal fibrosis and the role of macrophages infiltrating to the kidney, that is supported by many previous studies [1,2,5,6].

To further investigate the action of macrophages infiltrating to renal interstitium on renal fibrosis, we attempted an adoptive macrophage transfer study. We have performed adoptive transfer of BM macrophages three times on every other day during the period of leukocyte depletion caused by CPM pretreatment. This resulted in significant increase in the numbers of macrophages infiltrating to renal interstitium both at day 5 (protocol A) and at day 14 (protocol B) after UUO compared with those of mice treated with CPM only. The mechanism for how the numbers of infiltrating macrophages are increased by macrophage adoptive transfer is uncertain. However, our BrdU-labeling study showed that BrdU-positive bone marrow-derived cells exist in renal interstitium both at day 5 and at day 14 after UUO. This suggests that the source of infiltrating macrophages in mice with adoptive macrophage transfer is, at least in part, transferred macrophages themselves which may have contributed to increased numbers of infiltrating macrophages.

In protocol A, although an increase in interstitial infiltrating macrophages was observed by adoptive macrophage transfer to leukocyte depleted mice, no significant change was observed in renal interstitial collagen index by adoptive macrophage transfer at day 5 after UUO. Thus, at day 5 after UUO, no significant change was observed in the degree of renal fibrosis either by a decrease or an increase in the number of interstitial infiltrating macrophages. However, in protocol B, a significant decrease in the degree of renal interstitial collagen index was observed at day 14 after UUO along with the increased number of interstitial infiltrating macrophages by adoptive macrophage transfer. This ameliorated renal fibrosis by adoptive macrophage transfer was confirmed independently by a biochemical determination of collagen content. Thus, at day 14 after UUO, a decrease in the number of interstitial macrophages by CPM treatment was associated with an enhanced renal interstitial fibrosis, and adoptive macrophage transfer to these mice attenuated this enhancement in renal fibrosis, which was associated with an increased number of interstitial macrophage infiltration.

Macrophage infiltration is a feature of inflammatory tissue injury. Recent more global view of inflammation as a response to injury designed to restore normal function with the minimal tissue damage highlighted a complex role of macrophages in maintaining tissue integrity [12]. This includes responding to tissue injury and initiating inflammatory process often destructively to the damage, and then, promoting resolution of acute inflammation and facilitating tissue repair to restore normal function. This view led us to face the importance of understanding the role of macrophages at different time points in the evolution of renal fibrosis. Thus, we have studied the effect of macrophage depletion and adoptive macrophage transfer on renal fibrosis at two different time points, i.e., early stage (day 5) and late stage (day 14) of UUO. From this perspective, our result indicating that the degree of renal fibrosis was altered along with the alteration in the number of infiltrating macrophages at late stage, but not at early stage of UUO, suggests the role of infiltrating macrophages limited on late stage in the process of inflammatory

response to an insult to the kidney. Furthermore, our result indicating that the degree of renal fibrosis correlates inversely with the number of infiltrating macrophages at late stage of UUO also suggests the role of infiltrating macrophages on facilitating tissue repair at this stage of UUO. Further studies for the pathogenic mechanisms during the evolution of renal fibrosis are necessary. However, our data presented here suggest a new insight which is of importance in understanding the role of macrophages in renal fibrosis.

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