Anti-Monocyte Chemoattractant Protein-1 Gene Therapy Attenuates Renal Injury Induced by Protein-Overload Proteinuria

HIDEAKI SHIMIZU,* SHOICHI MARUYAMA,* YUKIO YUZAWA,* TOMOMI KATO,* YUSUKE MIKI,* SATOSHI SUZUKI,* WAICHI SATO,* YOSHIKI MORITA,* HIROKI MARUYAMA,† KENSUKE EGASHIRA,‡ and SEIICHI MATSUO*

**Division of Clinical Immunology, Department of Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan; †Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; ‡Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Science, Fukuoka, Japan.*

Abstract. It has been postulated that protein filtered through glomeruli activates tubular epithelial cells, which secrete vasoactive and inflammatory substances including chemokines, leading to tubulointerstitial renal injury. The present study was designed to investigate the role of monocyte chemoattractant protein-1 (MCP-1) in this process and to evaluate the effectiveness of a kidney-targeted gene transfer technique using hydrodynamic pressure. Naked plasmid encoding 7ND (an MCP-1 antagonist) or a control plasmid was introduced into the left kidney of rats. Three days after gene transfer (day 0), intraperitoneal administration of bovine serum albumin (10 mg/g body wt per day) was started and continued for 14 or 21 d. RT-PCR showed that 7ND mRNA was expressed only in the gene-transfected kidney. Immunostaining showed that 7ND protein was localized in the interstitial cells. Macrophage infiltration was significantly reduced in the left kidney of rats treated with 7ND on days 14 and 21. In the right kidney, such effects were not observed. 7ND also attenuated tubular damage and decreased the number of apoptotic cells. Computer-assisted analysis revealed that the areas positively stained for α-smooth muscle actin (αSMA), fibronectin-EDA, type I collagen, and collagen fibrils were significantly reduced in the 7ND-treated kidney on day 21. Furthermore, 7ND gene therapy significantly reduced MCP-1 and TGF-β1 mRNA expression. These results demonstrate that MCP-1 plays an important role in the development of tubulointerstitial inflammation, tubular damage, and fibrosis induced by proteinuria. The fact that 7ND gene therapy had little effect on the contralateral kidney indicates that 7ND acted locally. This strategy may have a potential usefulness as a gene therapy against tubulointerstitial renal injury.

Besides being a hallmark of glomerular disease, proteinuria has been shown to be an independent factor that induces and maintains renal damage. Cellular infiltration and fibrosis in the interstitium are common characteristics for virtually all progressive renal diseases with proteinuria. It has been postulated that protein filtered through glomeruli activates tubular epithelial cells, induces cellular infiltration into the interstitium, and subsequently causes interstitial fibrosis (1). Secretion of vasoactive mediators (*e.g.*, endothelins), and chemokines, (*e.g.*, monocyte chemoattractant protein-1 [MCP-1] and osteopontin) are induced in the tubules by protein-overload proteinuria (2,3). These substances are considered to promote inflammation and fibrosis of the interstitium, resulting in renal scarring (4,5). From among these mediators, we focused on MCP-1, a C-C chemokine with potent monocyte chemotactic and activating properties. It has been shown that MCP-1 is upregulated in various experimental and human renal diseases (6,7). MCP-1 expression is enhanced in tubular cells by bovine serum albumin (BSA) via a NF-κB–dependent pathway (8). MCP-1 can also induce fibrosis through recruitment and activation of macrophages that synthesize TGF-β1, a strong profibrotic factor (9).

The first objective of this study was to examine the role of MCP-1 in the progression of interstitial injury induced by proteinuria. For this purpose, we employed a rat model of protein overload nephropathy. This is a well-established model of non-immunological proteinuria. In this model, interstitial inflammation (mainly by macrophages) develops at one week, followed by the deposition of extracellular matrix proteins at two or three weeks (2,10). Tubular damage is also observed, with tubular cell apoptosis being suggested as a contributing factor (11). Upregulation of MCP-1 has previously been dem-

Received December 9, 2002. Accepted March 5, 2003.
Correspondence to Dr. Shoichi Maruyama, Division of Clinical Immunology, Department of Medicine, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showaku, Nagoya, Japan 466-8550. Phone: 81-52-744-2192; Fax: 81-52-744-2209; E-mail: marus@med.nagoya-u.ac.jp
1046-6673/1406-1496
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000069223.98703.8E

onstrated in this model (2). However, the effect of a MCP-1 blockade has not been studied. To clarify this, we used 7ND, a deletion mutant of human MCP-1 (missing the amino-terminal amino acids 2 to 8) (12). Previous *in vitro* experiments showed that 7ND forms inactive heterodimers with wild-type MCP-1 and inhibits monocyte chemotaxis (13). The effectiveness of 7ND has also been shown *in vivo* by experiments infusing recombinant 7ND protein to MRL/lpr mice (14) or by experiments transferring the 7ND gene to a remote organ (muscle) in animals with atherosclerosis or pulmonary hypertension (15–17).

A second objective of this study was to demonstrate the effectiveness of a recently developed hydrodynamics-based gene transfer technique (18). With this procedure, naked plasmid can be transferred into the interstitial fibroblasts in the kidney, and the gene expression persists for more than 6 mo. This is the first usage of this technique in an animal model.

We report here that the 7ND gene was successfully transferred into the rat kidney and it acted locally. A blockade of MCP-1/CCR2 signaling reduced macrophage infiltration and subsequently inhibited tubular damage and interstitial fibrosis induced by protein-overload proteinuria.

Materials and Methods

Animals

Male Lewis Rats weighing approximately 200 g were purchased from Chubu Kagaku Shizai Co. Ltd. (Nagoya, Japan) and were allowed free access to food and water. The experiments were performed according to the Animal Experimentation Guidelines of Nagoya University School of Medicine (Nagoya, Japan).

Expression Vector

FLAG-tagged (3' C terminus]) mutant MCP-1 (7ND) was constructed and cloned into the *Bam*HI (5') and *Not*I (3') sites of the pCDNA3 expression vector plasmid (Invitrogen Corp., Carlsbad, CA) (17). The plasmid was prepared using a Qiagen EndoFree plasmid Giga kit (Qiagen GmbH, Hilden, Germany). An empty pCDNA3 vector plasmid was used as a control.

Introduction of 7ND Plasmid into Rat Kidney

Naked plasmid encoding 7ND was introduced into the left kidney of rats by means of a recently developed hydrodynamics based gene transfer technique (18). Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt), and the left kidney was exposed by midline incision. After clamping the left renal vein and artery, 700 μl of Ringer solution containing plasmid DNA was injected retrogradely into the left renal vein within 5 s using a 24-gauge SURFLO intravenous catheter (Terumo, Tokyo, Japan). Blood flow was reestablished immediately after the injection.

Experimental Protocols

Bovine serum albumin (BSA) solution was prepared from Sigma preparation (fraction V, A4503), and protein overload nephropathy was induced as described previously (10). In experiment 1, rats were given 100 μg ($n = 6$), 300 μg ($n = 6$), or 1 mg ($n = 3$) of 7ND plasmid into the left kidney. Control rats were treated with 300 μg of empty vector ($n = 6$). Three days after gene transfer (day 0), intraperitoneal administration of BSA (10 mg/g body wt per day) was started and continued for 14 d. In experiment 2, 300 μg of 7ND plasmid ($n = 6$) or a control vector ($n = 6$) was transferred locally to the left kidney (day −3), followed by the daily injection of 10 mg/g body wt of BSA starting on day 0 and continuing for 21 d. Urine samples were collected on days 7, 10, and 13 in rats in the first series of experiments and on days 6, 13, and 20 in rats in the second series of experiments. Rats were sacrificed at the end of the experiments (day 14 or day 21), and kidney samples were collected for the study.

Histology and Immunohistochemistry

One part of the kidney was fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 2-μm-thick sections. They were stained with periodic acid-Schiff reagent (PAS), periodic acid-methenamine-silver (PAM), or Sirius red (19).

Another part of the kidney was frozen in OCT compound (Miles, Elkhart, IN) and kept frozen at −80°C until use. Sections (2-μm-thick) were cut by a cryostat and fixed in acetone. Immunostaining was performed as described previously (20). Briefly, the sections were incubated with primary antibodies, followed by the fluorescein- or Rhodamine-conjugated secondary antibodies. The primary antibodies employed in this study were monoclonal antibodies against FLAG (M2; Sigma Aldrich, St. Louis, MO), ED1 (anti-rat-macrophage/monocyte, Serotec, Raleigh, NC), rat CD5 (Serotec, Raleigh, NC), α-smooth muscle actin (αSMA, 1A4; Dako, Carpinteria, CA), and cellular fibronectin-EDA domain (IST-9; Harlan Sera-lab, Belton, Loughborough, UK), and rabbit polyclonal antibody against rat collagen type I (Chemicon International, Temecula, CA). The secondary antibodies were fluorescein conjugates of goat anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA) and Rhodamine conjugates of goat anti-mouse IgG and goat anti-rabbit IgG antibodies (Sigma). Negative controls were performed by the replacement of primary antibodies with isotype-matched or species-matched antibodies. For the dual-staining study, anti-FLAG antibody, Rhodamine-conjugated goat anti-mouse IgG and fluorescein labeled anti-ED-1 antibody (Serotec) were used. Normal control rat kidneys ($n = 4$) were also stained to determine the baseline.

TUNEL Staining

Apoptotic cell death was determined on the 6-μm-thick paraffin-embedded kidney section by using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining with *in situ* Apoptosis Detection Kit (Takara, Shiga, Japan) according to the manufacturer's instruction. Apoptotic cells were counted in at least ten randomly chosen non-overlapping cortical fields (×100; 0.58 mm^2) for each kidney.

Morphometric Analysis of Histology and Immunohistology

To assess tubulointerstitial injury, kidney sections were analyzed using a semiquantitative grading (21). Briefly, the extents of tubular cast formation, tubular dilatation, and tubular degeneration (vacuolar change, loss of brush border, detachment of tubular epithelial cells, and condensation of tubular nuclei) were scored according to the following criteria by two observers in a blind manner: 0, normal; 1, less than 25%: 2, 25% to 70%; 3, more than 70% of the pertinent area. For the analysis of each type of immunostaining, 20 randomly selected non-overlapping cortical fields (×200; 0.146 mm^2 each) were examined using a 20× lens with Zeiss microscope (Oberkochen, Germany) as described previously (21). Interstitial macrophage and T cell infiltration were assessed by counting ED-1 and CD5-positive cells in each field. Ratio of areas positively stained for αSMA, fibronectin-EDA, or type I collagen to the total cortical area was

measured by computer-aided planimetry using Mac Scope (Mitani, Fukui, Japan) and expressed as percentages (22). The staining for collagen fibrils was similarly quantified by calculating the ratio of area positively stained with Sirius red using a polarized light.

Analysis of mRNA for 7ND, MCP-1, and TGF-β1

One hundred milligrams of renal cortical tissue was lysed in Trisol (Life Technologies, Frederick, MD), and total RNA was prepared according to the manufacturer's protocol. One μg of total RNA was subjected to reverse transcriptase (RT) -PCR by using One Step RT-PCR kit (Takara). The samples were incubated at 50°C for 30 min and at 94°C for 5 min, followed by denature at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The final extension step was performed at 72°C for 5 min. The primers for 7ND were 5'-GAGACCTTCTGTGCCTGCTGCTCATAGCA-3' (forward) and 5'-GAGAGCTTGTCCAGGTGGTCCATGGAATC-3' (reverse); the primers for MCP-1 were 5'-ATGCAGGTCTCTGTCACG-3' (forward) and 5'-CTAGTTCTCTGTCATACT-3' (reverse); the primers for TGF-β1 were 5'-CCTGCAAGACCATCGACATG-3' (forward) and 5'-CTGGCGAGCCTTAGTTTGGA-3' (reverse); and the primers for β-actin were 5'- TTGTAACCAACTGGGACGATATGG-3' (forward) and 5'- GATCTTGATCTTCATGGTGCTAGG-3' (reverse). Thirty-five cycles of PCR were performed for 7ND. Expression of MCP-1 and TGF-β1 mRNA was assessed semiquantitatively (23). Twenty-two cycles of PCR were performed for MCP-1, 24 cycles for TGF-β1, and 16 cycles for β-actin. PCR products were separated on 2% agarose gel containing ethidium bromide (0.5 μg/ml). The gels were imaged and the fluorescence intensities of the PCR products were analyzed using KODAK 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY). Ratios of the values for MCP-1 or TGF-β1 to β-actin PCR products were determined and compared. To exclude the possibility of plasmid or genomic DNA contamination, experiments were carried out in the absence of RT.

Human MCP-1 ELISA

7ND protein levels in the sera of rats treated with 300 μg of 7ND were studied using a OptiEIA Human MCP-1 Kit (Phamingen, San Diego, CA) according to the manufacturer's instruction.

Serum and Urine Measurements

Blood was taken from the aorta, and serum was collected. Blood urea nitrogen (BUN) and serum creatinine levels were measured using Daiya Auto UN or Daiya Auto Crea Kit (Daiya Shiyaku, Tokyo, Japan). Urinary protein was measured by a pyrogallol red method using Micro TP-Test Wako (Wako, Oasaka, Japan).

Statistical Analyses

All values are provided as mean ± SEM. Statistical analysis was performed by one-way ANOVA. When significant difference was present, statistical analysis was further performed using the Scheffe *F* test between two groups. Significant difference was set when the *P* value was less than 0.05.

Results

7ND Expression

RT-PCR study showed that 7ND mRNA was expressed in the left kidney treated with of 300 μg of 7ND gene on days 14 and 21. The mRNA was not observed in the contralateral kidney or in the control vector treated kidney. The fact that no DNA was amplified when reverse transcriptase was omitted from the reaction indicated that the contamination of plasmid DNA in the total RNA preparations was negligible (Figure 1A). The localization of 7ND protein was studied by dual staining with anti-FLAG mAb and anti-ED-1 Ab. 7ND protein was clearly visible in the interstitial cells in the 7ND gene-treated kidney on days 14. 7ND staining was not localized in the macrophages, nor was it observed in the contralateral kidney or the control vector treated kidney (Figure 1, B and C). On day 21, 7ND protein could still be observed in the interstitial cells, but in a more scattered pattern. 7ND protein was not detectable in the sera of rats treated with 300 μg of 7ND on either day 14 or 21.

Cellular Infiltration in the Interstitium

After 14 d or 21 d of protein overloading, mononuclear cell infiltration was observed by light microscopy in the cortical interstitial area of the kidneys treated with a control vector. Immunostaining revealed that these were mainly macrophages. Macrophage infiltration was attenuated by 30% in the left kidney of rats treated with 100 μg of 7ND compared to the

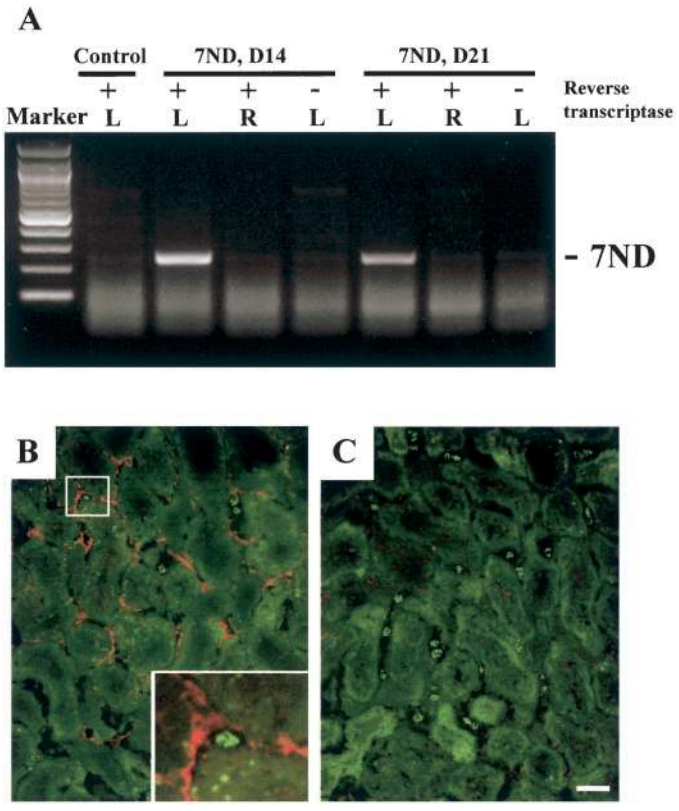

Figure 1. Detection of the transgene expression. (A) RT-PCR showed that 7ND mRNA was expressed in the left kidney (L) treated with of 300 μg of 7ND gene on days 14 and 21. The mRNA was not observed in the right kidney (R) or in the kidney treated with the control vector. No product was observed when reverse transcriptase was omitted from the reaction. Representative micrographs show dual staining for 7ND (red) and the macrophages (green). (B) 7ND protein was localized in the interstitial cells but not in the macrophages in the gene transfected kidney on day 14. (C) No staining was observed in the control rat kidney. Scale bar, 25 μm.

right kidney ($P < 0.05$). In the kidney treated with 300 μg or 1 mg of 7ND plasmid, the reduction of macrophage infiltration was more dramatic (50%, $P < 0.01$) (Figure 2, A through E). The results indicated that the effect of 7ND gene transfer was maximal at a dosage of 300 μg. The following experiments were therefore performed with a dosage of 300 μg. On day 21, an even larger number of macrophages was observed in the interstitium. 7ND decreased the macrophage count by 50% (Figure 2, F through H, $P < 0.01$ *versus* control rats). Right kidney showed no difference from the kidney of the control rats on either day 14 or 21. There was a slight increase in the number of T cells (CD5-positive cells) in the cortex of the control rat kidney on days 14 (4.0 $\pm$ 2.0) and 21 (13.4 $\pm$ 3.2) as compared with the number in the normal rats (2.3 $\pm$ 1.2). However, this increase was not significantly attenuated in the 7ND treated kidney either on day 14 (1.7 $\pm$ 0.9) or day 21 (13.1 $\pm$ 2.0).

Tubular Damage

Histologic examination of the PAS-stained sections showed that mild tubular damage including cast formation, tubular dilatation, and tubular degeneration had developed in the cortex of the control rat kidney by day 14. On day 21, a moderate degree of tubular damage was observed, especially in the area with massive cellular infiltration (Figure 3A). 7ND gene therapy significantly attenuated the tubular damage on day 21 (Figure 3, B and C).

TUNEL-Positive Cells

Apoptosis was evaluated by counting the TUNEL-positive cells in the cortex. The number of TUNEL-positive cells in the tubules increased in the control rat kidney on day 21 (Figure 4, A and C). 7ND reduced the number by 40% ($P < 0.05$) (Figure 4, B and C).

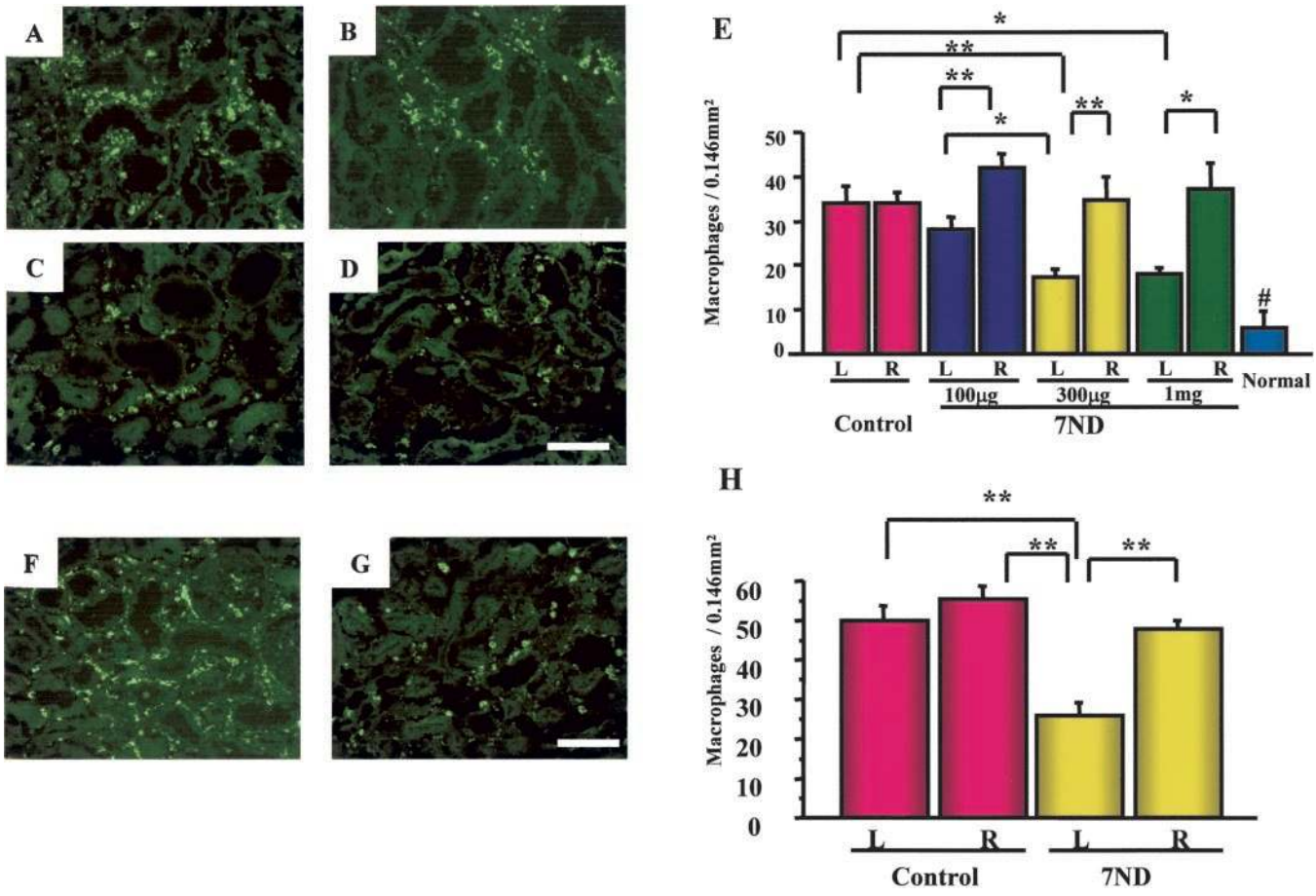

Figure 2. 7ND decreased macrophage infiltration induced by protein-overload proteinuria. Representative micrographs show the immunofluorescence staining for ED-1 on days 14 (A through D) and 21 (E and F). Kidney was treated with 300 μg of control plasmid (A and F), 100 μg of 7ND (B), 300 μg of 7ND (C and G), or 1 mg of 7ND (D). Scale bar, 50 μm. Graphic presentation demonstrates the number of macrophages in the cortical interstitium. (E) On day 14, macrophage infiltration was significantly attenuated in the left kidney (L) of rats treated 7ND plasmid as compared with the right kidney. The effect was maximal at a dosage of 300 μg. (H) On day 21, 7ND decreased the macrophage count by about 50%. Right kidney (R) showed no difference from the kidney of the control rats on either day 14 or 21. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$ as compared with the values in the experimental groups except for those in the left kidney of rats treated with 300 μg or 1 mg of 7ND.

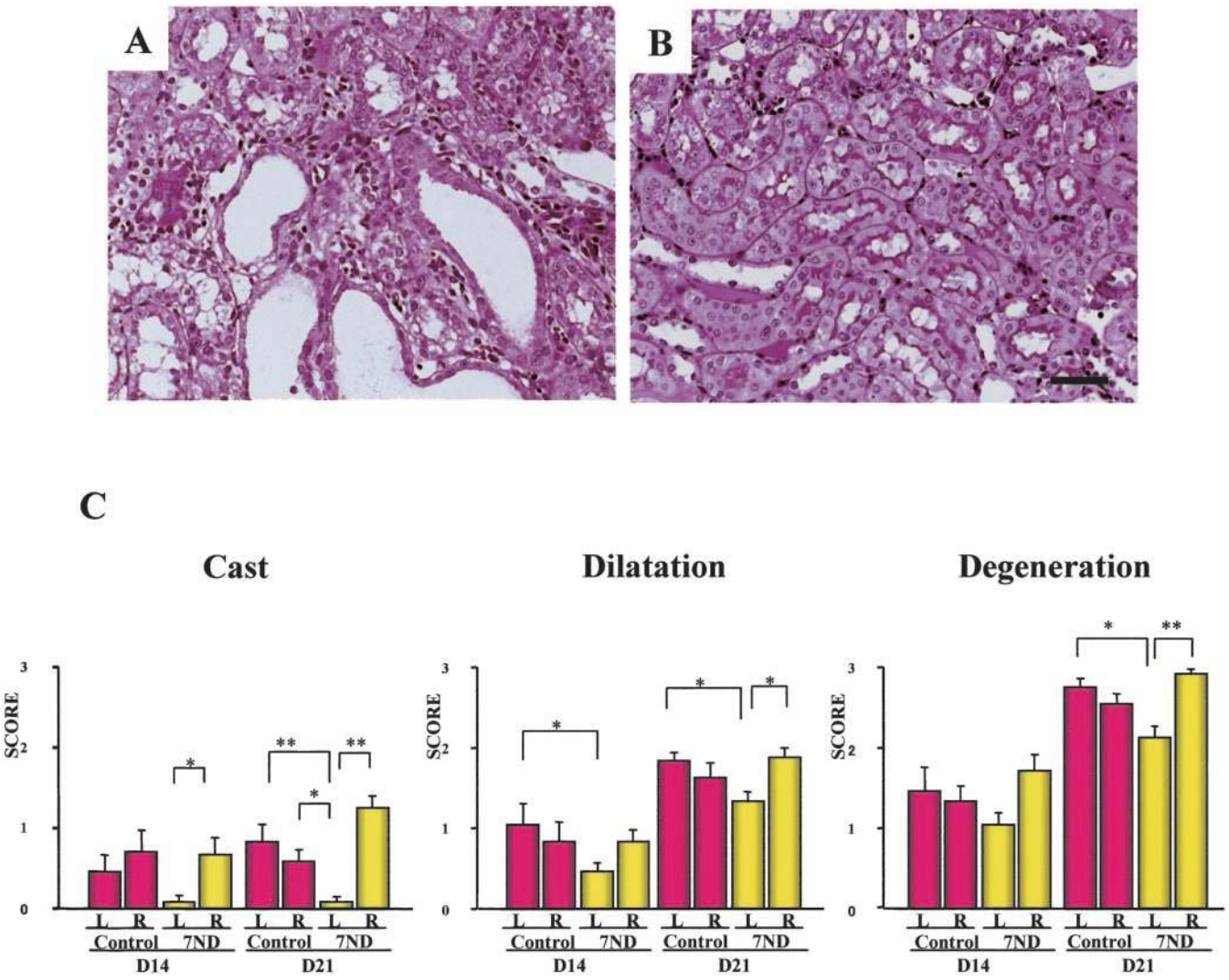

Figure 3. 7ND gene therapy attenuated tubular damage. Representative micrographs show renal histology (PAS) on day 21. (A) Kidney treated with the control vector; (B) kidney treated with 7ND. Scale bar, 50 μm. (C) Graphic presentation demonstrates tubular damage indices in each group on days 14 and 21. On day 21, tubular damage, including cast formation, tubular dilatation, and tubular degeneration, was significantly attenuated in the 7ND treated kidney. $*P < 0.05$, $**P < 0.01$.

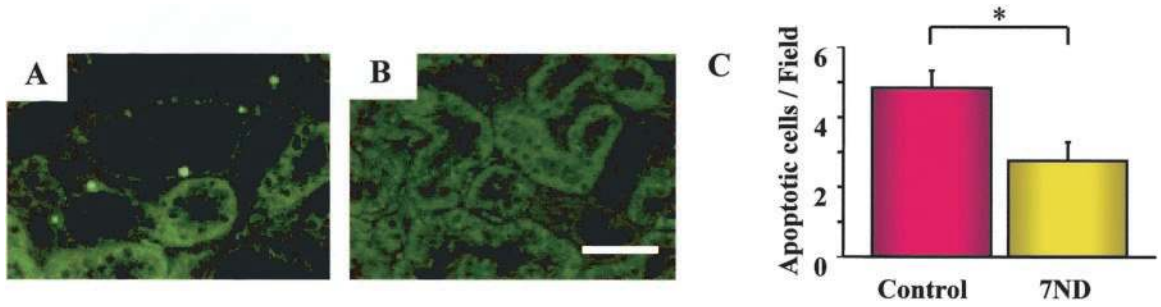

Figure 4. 7ND gene therapy decreased the number of apoptotic cells. Representative micrographs show apoptotic cells detected by TUNEL staining on day 21. (A) Kidney treated with the control vector; (B) kidney treated with 7ND. Scale bar, 50 μm. Graphic presentation demonstrates the number of apoptotic cells per cortical field ($\times$100, 0.58 mm^2). (C) 7ND attenuated apoptosis by 40% on day 21. $*P < 0.05$.

Fibrotic Changes in the Interstitium

Histologic examination of the PAM-stained sections showed that interstitial fibrosis observed in the control rat kidney was minimal on day 14 (data not shown) and was moderate on day 21 (Figure 5A). The change was attenuated by the 7ND treatment on days 14 (data not shown) and 21 (Figure 5B). Then,

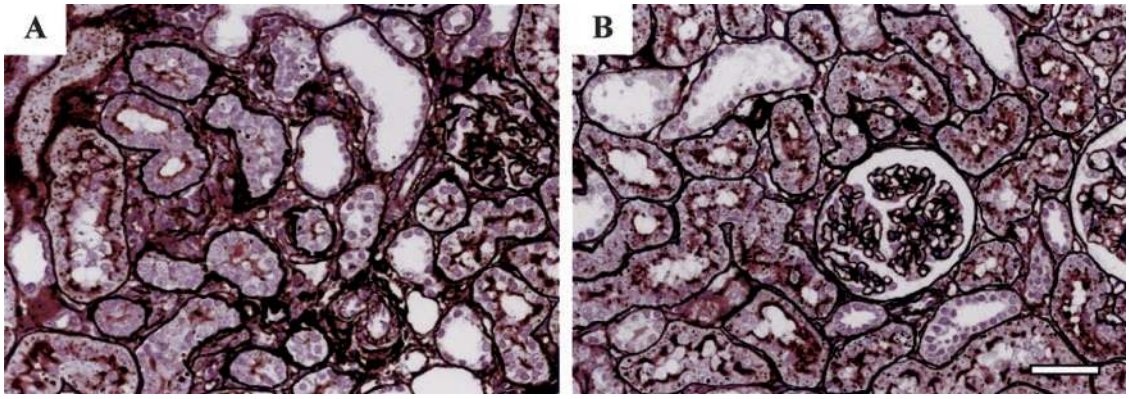

Figure 5. Representative micrographs show the renal histology (PAM) on day 21. (A) Moderate degree of fibrotic change was observed in the cortex of the control rat kidney. (B) The change was mild in the kidney treated with 7ND. Scale bar, 50 μm.

each parameter of fibrotic change was evaluated as follows. In the normal rat kidney, αSMA expression was confined to blood vessel walls. On day 14, αSMA staining was not different from that of a normal rat kidney. On day 21, however, enhanced αSMA staining was observed in the cortex of the control rat kidney mainly in the inflamed interstitium or periglomerular area (Figure 6A). The staining was reduced by the 7ND treatment (Figure 6B). Computer-assisted quantitative analysis showed that 7ND gene transfer reduced the ratio of αSMA-positive area by 40% as compared with the contralateral kidney and the kidney of the control vector–treated rats on day 21 ($P < 0.01$) (Figure 6I). The staining for fibronectin-EDA was observed in the control rat kidney on day 14 mainly in the peritubular and periglomerular area accompanied with massive macrophage infiltration. The staining was more evident on day 21 (Figure 6C). Fibronectin-EDA–positive areas in the 7ND-treated kidney were about 60% and 40% smaller than those in the controls on days 14 and 21, respectively ($P < 0.01$; Figure 6, D and J). The deposition of type I collagen was minimal in the control rat kidney on day 14, but it was enhanced on day 21 (Figure 6E). The type I collagen–positive area was decreased by about 30% by the 7ND gene transfer ($P < 0.01$; Figure 6, F and K). The deposition of collagen fibrils were studied by Sirius red staining. In the control rat kidney on day 14, minimal staining was observed, which was not different from that in the normal rat kidney. However, the staining was significantly enhanced on day 21 (Figure 6G). 7ND gene therapy attenuated the staining by 45% (Figure 6, H and L; $P < 0.01$).

MCP-1 and TGF-β1 mRNA Expression

Semiquantitative analyses of RT-PCR revealed that the levels of MCP-1 and TGF-β1 mRNA expression increased after 21 d of BSA administration compared with those in the normal rat kidneys. These values were significantly suppressed by the 7ND gene transfer ($P < 0.05$; Figure 7).

Serum and Urinary Measurements

BUN levels in the control group (26.50 $\pm$ 0.89 mg/dl on day 14 and 30.17 $\pm$ 2.29 mg/dl on day 21) were not different from the values in the 7ND-treated group (31.8 $\pm$ 3.04 mg/dl on day 14 and 33.7 $\pm$ 1.22 mg/dl on day 21). However, these values were significantly higher than BUN levels in the normal rats (12.67 $\pm$ 0.62 mg/dl). In contrast, no difference was observed in the serum creatinine levels in the control group (0.30 $\pm$ 0.26 mg/dl on day 14 and 0.35 $\pm$ 0.34 mg/dl on day 21), the 7ND-treated group (0.32 $\pm$ 0.17 mg/dl on day 14 and 0.33 $\pm$ 0.21 mg/dl on day 21) or the normal rats (0.37 $\pm$ 0.03 mg/dl). Nor did the levels of urinary protein show any difference at any time point (Table 1).

Discussion

The present study has shown for the first time that a blockade of the MCP-1/CCR2 signaling pathway attenuates interstitial nephritis induced by protein-overload proteinuria. Previous animal studies have shown, using anti-MCP-1 antibody or MCP-1–deficient mice, that MCP-1 is one of the key molecules in glomerular and tubulointerstitial renal injury (24). However, there is a study suggesting that MCP-1 does not play a major role in macrophage recruitment in interstitial nephritis (25). In addition, there are studies suggesting that other chemokines, including MIP-1α (26), MIP-2 (27), and RANTES (28) also play a major role in animal models of renal injury. Although upregulation of MCP-1 has been demonstrated in the kidney after BSA administration (2), whether MCP-1 critically contributes to the inflammation in protein overload nephropathy remained to be determined. In the present study, infiltrating cells were mainly macrophages, and anti-MCP-1 gene therapy significantly reduced macrophage infiltration.

A blockade of MCP-1 signaling also functioned to preserve the normal structure of the tubular epithelial cells. It has been shown that tubular epithelial cells are damaged by BSA, and the contribution of apoptosis to this process was suggested by *in vitro* and *in vivo* experiments (11,29). In our study, anti-MCP-1 therapy significantly suppressed the tubular damage and reduced the number of apoptotic cells induced by proteinuria. It is important to determine the levels of urinary protein in this model, because the degree of tubular injury was shown to be dependent on the amount of urinary protein excretion (10). The fact that the levels of urinary protein excretion were not

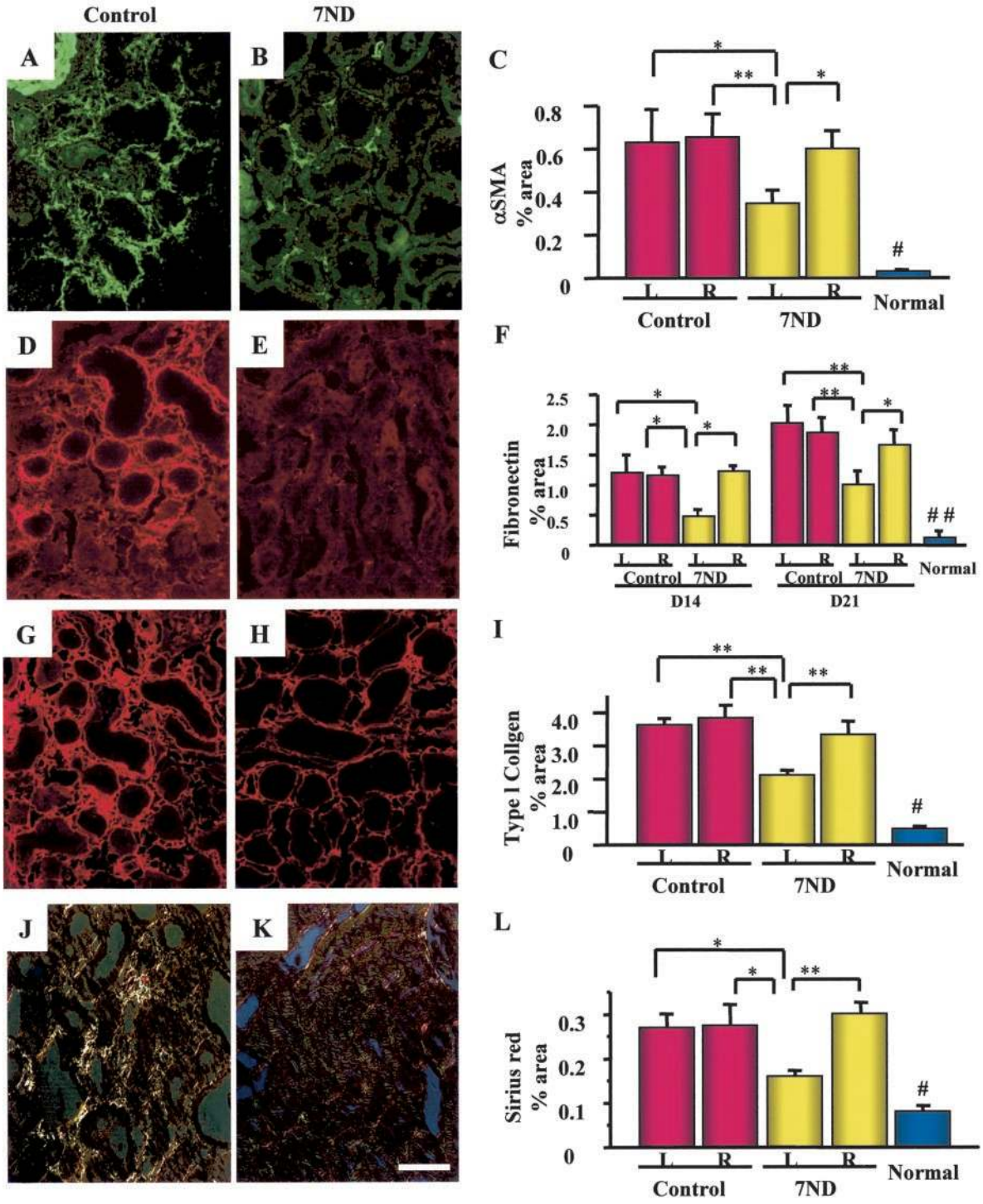

Figure 6. 7ND gene therapy attenuated the fibrotic changes. Representative micrographs show the kidney sections stained for αSMA (A and B), fibronectin-ED (D and E), or type I collagen (G and H), or stained with Sirius red (J and K). (A, D, G, and J) left kidneys of the control rats; (B, E, H, and K) kidneys treated with 7ND. Scale bar, 50 μm. Graphic presentation demonstrates the ratios of the positively stained area to the total cortical field. (C) Quantitative analysis showed that 7ND gene transfer reduced the ratio of αSMA-positive area by 40% as compared with the contralateral kidney and the kidneys of the control rats on day 21. (F) 7ND gene therapy reduced the staining for fibronectin-EDA by about 60% and 40% on days 14 and 21, respectively. (I) The type I collagen-positive area was decreased by 30% by the 7ND gene transfer on day 21. (L) Analysis of the Sirius red staining showed that 7ND gene transfer attenuated the deposition of collagen fibrils by 45% on day 21. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$ as compared with any value in the experimental groups (C, I, and L), $^{\#\#}P < 0.05$ as compared with the others except for the one in the left kidney of the 7ND treated group on day 14 (F).

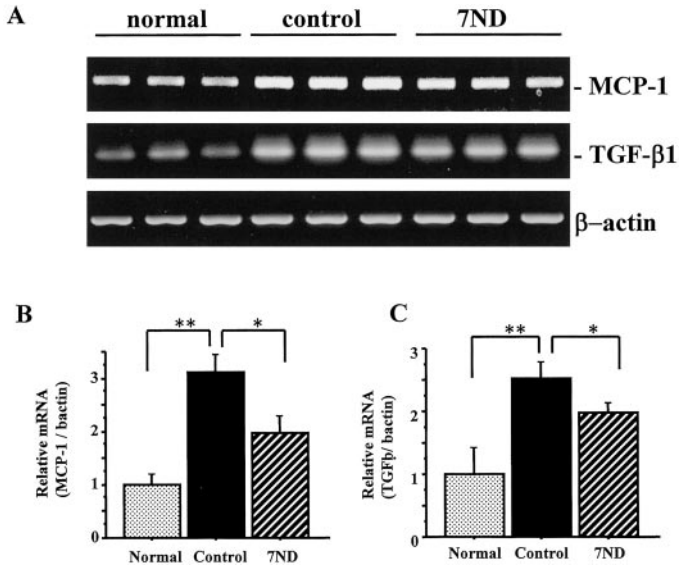

Figure 7. 7ND gene therapy decreased the levels of monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor–β (TGF-β) mRNA expression. (A) Representative photographs show mRNA expression of MCP-1, TGF-β1, and β-actin in the cortex of the normal rat kidney, kidney treated with the control vector, and the kidney treated with 7ND. Semiquantitative analyses revealed that the mRNA expression of MCP-1 (B) and TGF-β1 (C) increased after 21 d of bovine serum albumin (BSA) administration. These values were significantly reduced by the 7ND gene transfer. The ratios of MCP-1/β-actin mRNA and TGF-β1/β-actin mRNA in the normal rat kidney were considered to be the control (1.0). $*P < 0.05$, $**P < 0.01$.

Table 1. Urinary protein excretion[a]

	Urinary Protein (mg/d)	
	7ND (300 μg)	Control
Experiment 1		
day 6	162.0 ± 47.8	205.6 ± 52.6
day 9	174.4 ± 30.4	148.5 ± 23.4
day 12	206.6 ± 32.8	176.9 ± 30.3
Experiment 2		
day 7	176.8 ± 19.7	140.8 ± 28.5
day 14	157.9 ± 29.0	200.4 ± 39.0
day 20	191.2 ± 33.7	160.1 ± 33.7

[a] Results are expressed as mean ± SEM for experimental and control rats at each of these time points.

different between the experimental groups suggests that the amount of BSA that reached the tubular epithelial cells was comparable. It is known that inflammatory macrophages produce a wide array of pro-inflammatory mediators and are capable of inducing apoptosis (30). Our data suggest that, besides the direct effect of BSA (29), infiltrated macrophages may contribute to the tubular damage by inducing apoptosis.

Moreover, the present study clearly showed that MCP-1 plays a significant role in the development of interstitial fibrosis. Computer-aided planimetry analyses revealed that anti-MCP-1 gene therapy significantly suppressed αSMA expression (a marker of activated fibroblasts) and reduced the deposition of ECM proteins and collagen fibrils. Anti-MCP-1 therapy also decreased the expression of mRNA for TGF-β1 (a strong pro-fibrogenic cytokine). Taken together, our results support the hypothesis that MCP-1 (induced by proteinuria) recruits and activates monocytes/macrophages to produce inflammatory and fibrogenic mediators, leading to tubulointerstitial renal fibrosis.

In the present study, as in the past report (10), serum creatinine levels did not show the increase one would expect from the pathologic changes in the kidney. This may have been because the interstitial inflammation and fibrosis were not severe enough to significantly affect renal function. Volume overloading, as was performed in this experiment, could also account for the lower than expected creatinine levels. It was therefore difficult to judge the effects of 7ND by measuring serum creatinine levels. Furthermore, the fact that we did not remove the right kidneys may have masked the effects of anti-MCP-1 gene therapy on the renal function. In contrast, BUN levels in the experimental groups were higher than those in the normal rats, presumably because of the infused albumin catabolism.

Of interest is that anti-MCP-1 gene therapy decreased the levels of MCP-1 mRNA expression in our study. This result suggests that, directly or indirectly, MCP-1 enhances MCP-1 production in the kidney. MCP-1 is known to be expressed by infiltrating macrophages as well as intrinsic renal cells (*e.g.*, tubular cells and mesangial cells) (6,24). Macrophages have a MCP-1 receptor (CCR2) (31), and they produce MCP-1 upon activation (32). An amplification loop of inflammatory cytokine production in monocytes (33) and in mesangial cells (34) is suggested. Thus, an amplification cascade may also have a function in the process of interstitial nephritis induced by proteinuria. Hypothetically, MCP-1 secreted from the tubular epithelial cells stimulates monocytes/macrophages to produce and secrete inflammatory cytokines (including MCP-1 itself), and the secreted cytokines enhances MCP-1 expression leading to further recruitment and activation of monocytes/macrophages. Although direct proof of this hypothesis is beyond the present study, 7ND may have attenuated macrophage infiltration by blocking this amplification cascade.

Another goal of this study was to demonstrate for the first time the effectiveness of a recently established kidney-targeted naked plasmid transfer technique (18) in an animal model of renal injury. Various method have been tried to transfer the therapeutic genes into the kidney (35,36). Plasmid DNA mixed with the HVJ liposome was introduced into the mesangial cells when infused via the renal artery or into the interstitial fibroblasts when infused via the ureter (37). Similar results were obtained when naked plasmid was injected into the kidney followed by electroporation (37,38). When an adenoviral vector was used, gene expression was observed in the tubules or glomerulus (39). In the present study, naked plasmid was used and the transgene was expressed in the interstitial cells. A previous study using an electron microscope suggested that these are interstitial fibroblasts and not endothelial cells. The

fact that the gene transfer site differs in each method allows us to choose the proper one depending on the kind of renal injury. We assume that the technique employed in the present study will be useful to treat interstitial renal injury, but it may not be effective in treating the glomerular injury.

Various methods of gene transfer have been tried for the treatment of renal injury in animals (35,36). However to date, none of them have been applied to humans. The technique employed in the present study has several advantages over the previous methods. First of all, it is a safe technique, which does not require possibly toxic agents such as liposomes (37) or viral vectors (39). No damage was observed in the kidney from this procedure. Second, it is rather simple because it does not require special techniques such as electroporation (40). It is therefore theoretically possible to apply this method to the human kidney by using proper catheters. Third, it was demonstrated that the gene expression persists for a relatively long period with this technique. In this study, expression of 7ND mRNA and protein was detectable, and the therapeutic effects of 7ND were observed even at 24 d after gene transfer. Of note is the fact that the effects of the 7ND were confined to the gene-transfected kidney and that 7ND protein was not detectable in circulation by ELISA. These data indicate that 7ND expressed and acted locally in the targeted kidney. In the present study, we did not directly compare the effects of kidney-targeted gene transfer with those of systemic gene therapy such as intramuscular gene transfer using electroporation. Previous studies suggested that the serum levels of the transgene product (erythropoietin) obtained by these two gene transfer methods are comparable (18,41). We can therefore assume that a higher local concentration is likely to be achieved by transferring the gene directly into the target organs. Moreover, possible adverse effects may be minimized by transferring the gene locally. Because MCP-1 plays an important role in wound healing and host defense (42,43), systemic suppression of MCP-1 might be harmful. In this context, the kidney-targeted gene transfer technique is considered to be superior to systemic delivery.

In a previous study, rat erythropoietin was successfully expressed in pCAGGS vector by means of the same technique as was used in this study (18). However in that study, transgene expression was not detected when pCI was used. Although pCDNA3 and pCI share the same CMV promoter, factors other than the promoter may affect the transgene expression. In the present study, 7ND was expressed using the pCDNA3 vector. The efficiency of 7ND expression in the different types of expression vectors (*e.g.*, pCAGGS, pCDNA3, and pCI) bears further study.

In summary, we were able to successfully introduce 7ND, a MCP-1 antagonist, using a hydrodynamics-based kidney targeted gene transfer technique. 7ND was expressed and acted locally in the target kidney. The results demonstrated that MCP-1/CCR2 signaling plays an important role in the development of tubulointerstitial inflammation, tubular damage, and interstitial fibrosis induced by proteinuria. Our results showed that the present strategy has potential therapeutic application for tubulointerstitial renal injury in the clinical setting.

Acknowledgments

We thank Ms. Asano N, Katahara T, Fujitani Y, and Mr. Suzuki N for excellent technical assistance. This work was supported by grants from Aichi Kidney Foundation 2001.

References

1. Remuzzi G, Bertani T: Pathophysiology of progressive nephropathies. *N Engl J Med* 339: 1448–1456, 1998
2. Eddy AA, Giachelli CM: Renal expression of genes that promote interstitial inflammation and fibrosis in rats with protein-overload proteinuria. *Kidney Int* 47: 1546–1557, 1995
3. Zoja C, Benigni A, Remuzzi G: Protein overload activates proximal tubular cells to release vasoactive and inflammatory mediators. *Exp Nephrol* 7: 420–428, 1999
4. Eddy A: Role of cellular infiltrates in response to proteinuria. *Am J Kidney Dis* 37: S25–S29, 2001
5. Johnson RJ: Cytokines, growth factors and renal injury: Where do we go now? *Kidney Int Suppl* 63:S2–S6, 1997
6. Mezzano SA, Barria M, Droguett MA, Burgos ME, Ardiles LG, Flores C, Egido J: Tubular NF-kappaB and AP-1 activation in human proteinuric renal disease. *Kidney Int* 60: 1366–1377, 2001
7. Wada T, Yokoyama H, Matsushima K, Kobayashi K: Chemokines in renal diseases. *Int Immunopharmacol* 1: 637–645, 2001
8. Wang Y, Chen J, Chen L, Tay YC, Rangan GK, Harris DC: Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 8: 1537–1545, 1997
9. Schneider A, Panzer U, Zahner G, Wenzel U, Wolf G, Thaiss F, Helmchen U, Stahl RA: Monocyte chemoattractant protein-1 mediates collagen deposition in experimental glomerulonephritis by transforming growth factor-beta. *Kidney Int* 56: 135–144, 1999
10. Eddy AA: Interstitial nephritis induced by protein-overload proteinuria. *Am J Pathol* 135: 719–733, 1989
11. Thomas ME, Brunskill NJ, Harris KP, Bailey E, Pringle JH, Furness PN, Walls J: Proteinuria induces tubular cell turnover: A potential mechanism for tubular atrophy. *Kidney Int* 55: 890–898, 1999
12. Zhang Y, Rollins BJ: A dominant negative inhibitor indicates that monocyte chemoattractant protein 1 functions as a dimer. *Mol Cell Biol* 15: 4851–4855, 1995
13. Zhang Y, Ernst CA, Rollins BJ: MCP-1: Structure/Activity Analysis. *Methods* 10: 93–103, 1996
14. Gong JH, Ratkay LG, Waterfield JD, Clark-Lewis I: An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. *J Exp Med* 186: 131–137, 1997
15. Ikeda Y, Yonemitsu Y, Kataoka C, Kitamoto S, Yamaoka T, Nishida K, Takeshita A, Egashira K, Sueishi K: Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary hypertension in rats. *Am J Physiol Heart Circ Physiol* 283: H2021–H2028, 2002
16. Ni W, Egashira K, Kitamoto S, Kataoka C, Koyanagi M, Inoue S, Imaizumi K, Akiyama C, Nishida KI, Takeshita A: New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 103: 2096–2101, 2001
17. Egashira K, Koyanagi M, Kitamoto S, Ni W, Kataoka C, Morishita R, Kaneda Y, Akiyama C, Nishida KI, Sueishi K, Takeshita A: Anti-monocyte chemoattractant protein-1 gene

therapy inhibits vascular remodeling in rats: blockade of MCP-1 activity after intramuscular transfer of a mutant gene inhibits vascular remodeling induced by chronic blockade of NO synthesis. *FASEB J* 14: 1974, 1978., 2000
18. Maruyama H, Higuchi N, Nishikawa Y, Hirahara H, Iino N, Kameda S, Kawachi H, Yaoita E, Gejyo F, Miyazaki J: Kidney-targeted naked DNA transfer by retrograde renal vein injection in rats. *Hum Gene Ther* 13: 455–468, 2002
19. De Heer E, Sijpkens YW, Verkade M, den Dulk M, Langers A, Schutrups J, Bruijn JA, van Es LA: Morphometry of interstitial fibrosis. *Nephrol Dial Transplant* 15[Suppl 6]: 72–73, 2000
20. Ikeguchi H, Maruyama S, Morita Y, Fujita Y, Kato T, Natori Y, Akatsu H, Campbell W, Okada N, Okada H, Yuzawa Y, Matsuo S: Effects of human soluble thrombomodulin on experimental glomerulonephritis. *Kidney Int* 61: 490–501, 2002
21. Nomura A, Nishikawa K, Yuzawa Y, Okada H, Okada N, Morgan BP, Piddlesden SJ, Nadai M, Hasegawa T, Matsuo S: Tubulointerstitial injury induced in rats by a monoclonal antibody that inhibits function of a membrane inhibitor of complement. *J Clin Invest* 96: 2348–2356, 1995
22. Kasuga H, Ito Y, Sakamoto S, Kawachi H, Shimizu F, Yuzawa Y, Matsuo S: Effects of anti-TGF-beta type II receptor antibody on experimental glomerulonephritis. *Kidney Int* 60: 1745–1755, 2001
23. Ito Y, Goldschmeding R, Bende R, Claessen N, Chand M, Kleij L, Rabelink T, Weening J, Aten J: Kinetics of connective tissue growth factor expression during experimental proliferative glomerulonephritis. *J Am Soc Nephrol* 12: 472–484, 2001
24. Segerer S, Nelson PJ, Schlondorff D: Chemokines, chemokine receptors, and renal disease: From basic science to pathophysiologic and therapeutic studies. *J Am Soc Nephrol* 11: 152–176, 2000
25. Eddy AA, Warren JS: Expression and function of monocyte chemoattractant protein-1 in experimental nephrotic syndrome. *Clin Immunol Immunopathol* 78: 140–151, 1996
26. Wu X, Dolecki GJ, Sherry B, Zagorski J, Lefkowith JB: Chemokines are expressed in a myeloid cell-dependent fashion and mediate distinct functions in immune complex glomerulonephritis in rat. *J Immunol* 158: 3917–3924, 1997
27. Feng L, Xia Y, Yoshimura T, Wilson CB: Modulation of neutrophil influx in glomerulonephritis in the rat with anti-macrophage inflammatory protein-2 (MIP-2) antibody. *J Clin Invest* 95: 1009–1017, 1995
28. Grone HJ, Weber C, Weber KS, Grone EF, Rabelink T, Klier CM, Wells TN, Proudfood AE, Schlondorff D, Nelson PJ: Met-RANTES reduces vascular and tubular damage during acute renal transplant rejection: blocking monocyte arrest and recruitment. *FASEB J* 13: 1371–1383, 1999
29. Erkan E, De Leon M, Devarajan P: Albumin overload induces apoptosis in LLC-PK(1) cells. *Am J Physiol Renal Physiol* 280: F1107–F1114, 2001
30. Aliprantis AO, Diez-Roux G, Mulder LC, Zychlinsky A, Lang RA: Do macrophages kill through apoptosis? *Immunol Today* 17: 573–576, 1996
31. Kurihara T, Bravo R: Cloning and functional expression of mCCR2, a murine receptor for the C-C chemokines JE and FIC. *J Biol Chem* 271: 11603–11607, 1996
32. Martin CA, Dorf ME: Differential regulation of interleukin-6, macrophage inflammatory protein-1, and JE/MCP-1 cytokine expression in macrophage cell lines. *Cell Immunol* 135: 245–258, 1991
33. Colotta F, Borre A, Wang JM, Tattanelli M, Maddalena F, Polentarutti N, Peri G, Mantovani A: Expression of a monocyte chemotactic cytokine by human mononuclear phagocytes. *J Immunol* 148: 760–765, 1992
34. Luo Y, Lloyd C, Gutierrez-Ramos JC, Dorf ME: Chemokine amplification in mesangial cells. *J Immunol* 163: 3985–3992, 1999
35. Imai E: Gene therapy approach in renal disease in the 21st century. *Nephrol Dial Transplant* 16[Suppl 5]: 26–34, 2001
36. Imai E, Isaka Y: Targeting growth factors to the kidney: Myth or reality? *Curr Opin Nephrol Hypertens* 11: 49–57, 2002
37. Imai E, Akagi Y, Isaka Y: Towards gene therapy for renal diseases. *Nephrologie* 19: 397–402, 1998
38. Nakamura H, Isaka Y, Tsujie M, Rupprecht HD, Akagi Y, Ueda N, Imai E, Hori M: Introduction of DNA enzyme for Egr-1 into tubulointerstitial fibroblasts by electroporation reduced interstitial alpha-smooth muscle actin expression and fibrosis in unilateral ureteral obstruction (UUO) rats. *Gene Ther* 9: 495–502, 2002
39. Yin M, Wheeler MD, Connor HD, Zhong Z, Bunzendahl H, Dikalova A, Samulski RJ, Schoonhoven R, Mason RP, Swenberg JA, Thurman RG: Cu/Zn-superoxide dismutase gene attenuates ischemia-reperfusion injury in the rat kidney. *J Am Soc Nephrol* 12: 2691–2700, 2001
40. Nakamura H, Isaka Y, Tsujie M, Akagi Y, Sudo T, Ohno N, Imai E, Hori M: Electroporation-mediated PDGF receptor-IgG chimera gene transfer ameliorates experimental glomerulonephritis. *Kidney Int* 59: 2134–2145, 2001
41. Maruyama H, Ataka K, Gejyo F, Higuchi N, Ito Y, Hirahara H, Imazeki I, Hirata M, Ichikawa F, Neichi T, Kikuchi H, Sugawa M, Miyazaki J: Long-term production of erythropoietin after electroporation-mediated transfer of plasmid DNA into the muscles of normal and uremic rats. *Gene Ther* 8: 461–468, 2001
42. Low QE, Drugea IA, Duffner LA, Quinn DG, Cook DN, Rollins BJ, Kovacs EJ, DiPietro LA: Wound healing in MIP-1alpha(-/-) and MCP-1(-/-) mice. *Am J Pathol* 159: 457–463, 2001
43. Kaufmann A, Salentin R, Meyer RG, Bussfeld D, Pauligk C, Fesq H, Hofmann P, Nain M, Gemsa D, Sprenger H: Defense against influenza A virus infection: Essential role of the chemokine system. *Immunobiology* 204: 603–613, 2001