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Original Article

The Macrophage Is a Key Factor in Renal Injuries Caused by Glomerular Hyperfiltration

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Glomerular hyperfiltration is a common pathway leading to glomerulosclerosis in various kinds of kidney diseases. The 5/6 renal ablation is an established experimental animal model for glomerular hyperfiltration. On the other hand, low-grade inflammation is also a common mechanism for the progression of kidney diseases including diabetic nephropathy and atherosclerosis. Here we analyzed the gene expression profile in the remnant kidney tissues of 5/6 nephrectomized mice using a DNA microarray system and compared it with that of sham-operated control mice. The 5/6 nephrectomized mice showed glomerular hypertrophy and an increase in the extracellular matrix in the glomeruli. DNA microarray analysis indicated the up-regulated expression of various kinds of genes related to the inflammatory process in remnant kidneys. We confirmed the up-regulated expression of platelet factor-4, and monocyte chemoattractant protein-1, 2, and 5 in remnant kidneys by RT-PCR. The current results suggest that the inflammatory process is involved in the progression of glomerulosclerosis and is a common pathway of the pathogenesis of kidney disease.

Key words: kidney, inflammation, chemokine

Infiltration of macrophages into the glomeruli and interstitium is one of the characteristic features of chronic renal diseases including diabetic nephropathy [1, 2]. Hemodynamic changes in diabetic glomeruli have also been considered to be an important cause of glomerular injury in diabetic patients. The 5/6 nephrectomy (Nx) is widely used as an animal model for glomerular hyperfiltration followed by glomerulosclerosis.

Recently, low-grade inflammation, "microinflammation" has been considered to be a common mechanism in the pathogenesis of atherosclerosis and of obesity-related insulin resistance. We have reported that low grade inflammation is one of the major pathways for the development of diabetic nephropathy. Intercellular adhesion molecule (ICAM)-1 is one of the major molecules, promoting leukocytes to attach firmly to endothelium followed by transmigration out of the blood vessel lumen [3]. We have previously reported macrophage infiltration and an increased expression of ICAM-1 in the glomeruli of diabetic animals and mice with 5/6 renal ablation [4, 5]. Furthermore, we have found that ICAM-1-deficient mice are resistant to renal injuries after the induction of diabetes [6].

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These data suggest that the inflammatory process may be a common factor in the pathogenesis of various kinds of kidney disease.

The aim of this study was to clarify the change in the gene expression profile of remnant kidney tissues of 5/6 nephrectomized mice and to identify the genes involved in the inflammatory process in the progression of glomerulosclerosis.

Materials and Methods

Animals. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Males at 8 weeks of age were used for the experiments.

Experimental design. Mice were subjected either to a sham operation (sham, n = 10) or subtotal nephrectomy (Nx, n = 10). The Nx operation was performed by surgical resection of the upper and lower thirds of the right kidney followed by a left nephrectomy. All mice had free access to standard chow and tap water. Five mice in each group were sacrificed and kidneys were obtained 4 weeks after surgery for the DNA microarray and at 12 weeks for histological examination. All procedures were performed according to the Guidelines for Animal Protection and Management Law (No. 105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

DNA microarray analysis. DNA microarray analysis was performed using RNA from the kidneys of Nx and sham groups at 4 weeks after surgery (N = 5). Total RNA was extracted from the renal cortex using the RNeasy kit (Qiagen, Valencia, CA, USA). Preparation of cRNA and hybridization of probe arrays (Uniset mouse I) were performed according to the manufacturer's instructions (GE Healthcare, UK). These arrays contain probe sets for > 10,500 transcripts (Uniset mouse I, GE Healthcare, UK). After hybridization, the microarrays were washed, scanned, and analyzed with the software (Codelink System Software version 2.3.2 UK).

To find the genes involved in the pathogenesis of glomerulosclerosis, we selected the genes as follows: 1) ratio of the expression level of Nx > 2.0 to that of sham. Functional profiling of the genes was performed based on the Gene Ontology Consortium 〈http://www.geneontology.org〉 (accessed September 2010) terms. The gene annotation procedure was performed using The Database for Annotation, Visualization, and Integrated Discovery 2.1 by the National Institute of Allergy and Infectious Disease 〈http://david.abcc.ncifcrf.gov/〉 (accessed September 2010). Out of the 3 available Gene Ontology Consortium ontologies, the biological annotation process is presented in this article.

Metabolic data. Serum creatinine (S-Cr), blood urea nitrogen (BUN), urinary albumin concentration (UAE), systolic blood pressure (SBP) and body weight were measured before sacrifice at 12 weeks after surgery (n = 5). Urine collection was performed for 24h with each mouse individually housed in a metabolic cage and having free access to food and water. Urinary albumin concentrations were measured by nephelometry. Blood pressure was measured by the tail-cuff method.

Kidney morphology. We performed the morphometric analysis using kidney tissues from Nx and sham groups at 12 weeks after surgery. Kidneys were fixed in 10% formalin for periodic acid-methenamine-silver (PAM). To evaluate the glomerular size and mesangial matrix area, 6 (for glomerular size) and 12 (for mesangial matrix index) glomeruli per animal (n = 5) were examined. The glomerular tuft area was measured by manually tracing the glomerular tuft using Photoshop software version 6 (Adobe systems, San Jose, CA, USA) and Scion Image analysis software (Scion Corp., Frederick, MD, USA). The mesangial matrix area was defined as the periodic acid-methenamine-positive area within the tuft area. The mesangial matrix index represented the ratio of the mesangial matrix area divided by the tuft area, as previously described [6].

Immunoperoxidase staining. Immunoperoxidase staining was performed as previously described [4]. Fresh frozen sections were cut at a 4-μm thickness using a cryostat. The specific rat antibody to mouse macrophage (F4/80, Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used as a primary antibody, followed by a second reaction with biotin-labeled donkey anti-rat IgG antibody (Jackson Immunoresearch Laboratories). Finally, the avidin-biotin coupling reaction was performed on the sections using a Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA). The number of F4/80-positive cells in a glomerulus was counted in 30

glomeruli per one animal in the section (n = 5).

Quantitative real-time RT-PCR. Total RNA was extracted from the renal cortex using an RNeasy Midi kit (Qiagen, Valencia, CA, USA). Single-strand complementary DNA (cDNA) was synthesized from the extracted RNA using a reverse transcription-PCR (RT-PCR) kit (Perkin Elmer, Foster City, CA, USA) according to the instructions provided by the manufacturer. To evaluate mRNA expression of mouse platelet factor-4 (PF-4), monocyte chemotactic protein (MCP)-1, MCP-2, MCP-5 and β-actin in the renal cortex, we performed quantitative real-time RT-PCR using a Light Cycler and Light-Cycler-FastStart SYBR Green 1 (Roche Diagnostics, Tokyo, Japan). After the addition of specific forward and reverse primers (0.3 μM), MgCl2 (3 mM), and template DNA to the master mix, 40 cycles of denaturation (95℃ for 10 s), annealing (62℃ for 10 s), and extension (72℃ for 6 s) were performed. To determine the specificity of each primer set, melting curve analysis was performed. Accumulated levels of fluorescence were analyzed by the fit-point method after melting curve analysis. The mRNA expression levels of PF-4, MCP-1, MCP-2, and MCP-5 were normalized by β-actin in each sample, and the relative expression ratios were calculated. To amplify the cDNA, the following oligonucleotide primers specific for mouse PF-4, mouse MCP-1, mouse MCP-2, MCP-5 and mouse β-actin were used:

PF-4, forward 5' ATC CAT CTT AAG CAC ATC AC 3',
reverse 5' ACT TTC TTA TAT AGG GGT GC 3'
MCP-1, forward 5' AAG CTG TAG TTT TTG TCA CC 3'
reverse 5' GGG CAG ATG CAG TTT TAA 3'
MCP-2, forward 5' GAA GCT GTG GTT TTC CAG AC 3'
reverse 5' TCA GGT GTG AAG GTT CAA GG 3'
MCP-5, forward 5' ACC AGA TGC GGT GAG CAC 3'
reverse 5' ATG GTC CTG AAG ATC ACA GC 3'
β-actin, forward 5-CCTGTATGCCTCTGGTCGTA-3
reverse 5-CCATCTCCTGCTCGAAGTCT-3
(Nihon Gene Research Labs, Sendai, Japan).

Statistical analysis. The Mann Whitney-U test was used to analyze the real-time RT-PCR data and the Student's *t*-test was used to analyze the other data. Data are expressed as median and interquartile ranges or mean and SE. A p value < 0.05 was considered to denote the presence of a statistically significant difference. A modified Fisher's exact test was used for the gene annotation procedure by The Database for Annotation, Visualization, and Integrated Discovery 2.1 by the National Institute of Allergy and Infectious Disease 〈http://david.abcc.ncifcrf.gov/〉.

Results

Metabolic data at the end of the 12-week observation period. Serum levels of creatinine and urea nitorogen (UN) were significantly higher in the Nx group than in the sham group. There was no difference in body weight, blood pressure and urinary albumin excretion (Table 1).

Light microscopy. Representative findings of glomeruli in PAM-stained sections are shown in Fig. 1. Greater mesangial matrix expansion was observed in the kidney of Nx group compared with the sham group (mesangial matrix index: 14.2 vs. 8.4%, $p < 0.01$) (Fig. 1). The mean value of glomerular size (tuft area) in Nx appeared higher than that in the sham group, although there was no statistically significant difference (glomerular size: $4{,}298 \pm 114$ vs. $4{,}098 \pm 149\,\mu m^2$, n.s.).

Gene expression analysis by DNA microarray. We identified 98 genes with a > 2.0 ratio of expression levels of the Nx group compared with those of the sham group. Functional annotation of these 98 genes revealed that their functions were related to the

Table 1 Body weight, blood pressure, serum creatinine, blood urea nitorogen, urinary albumin excretion in 5/6 nephrectomized mice and sham operated mice at 12 weeks after operation

	Sham operation	5/6 Nephrectomy
Body weight (g)	30.2 ± 1.3	28.4 ± 0.6
SBP (mmHg)	105 ± 1.6	100 ± 3.3
S-Cr (mg/dl)	0.15 ± 0.01	$0.23 \pm 0.03^{\#}$
BUN (mg/dl)	26.9 ± 0.9	$47.3 \pm 2.8^{\#\#}$
UAE (μg/day)	10.5 ± 2.4	13.1 ± 5.3

$^{\#}p < 0.05$ v.s sham operation, $^{\#\#}p < 0.001$ v.s sham operation
N = 6, mean ± SE. Student's *t*-test.

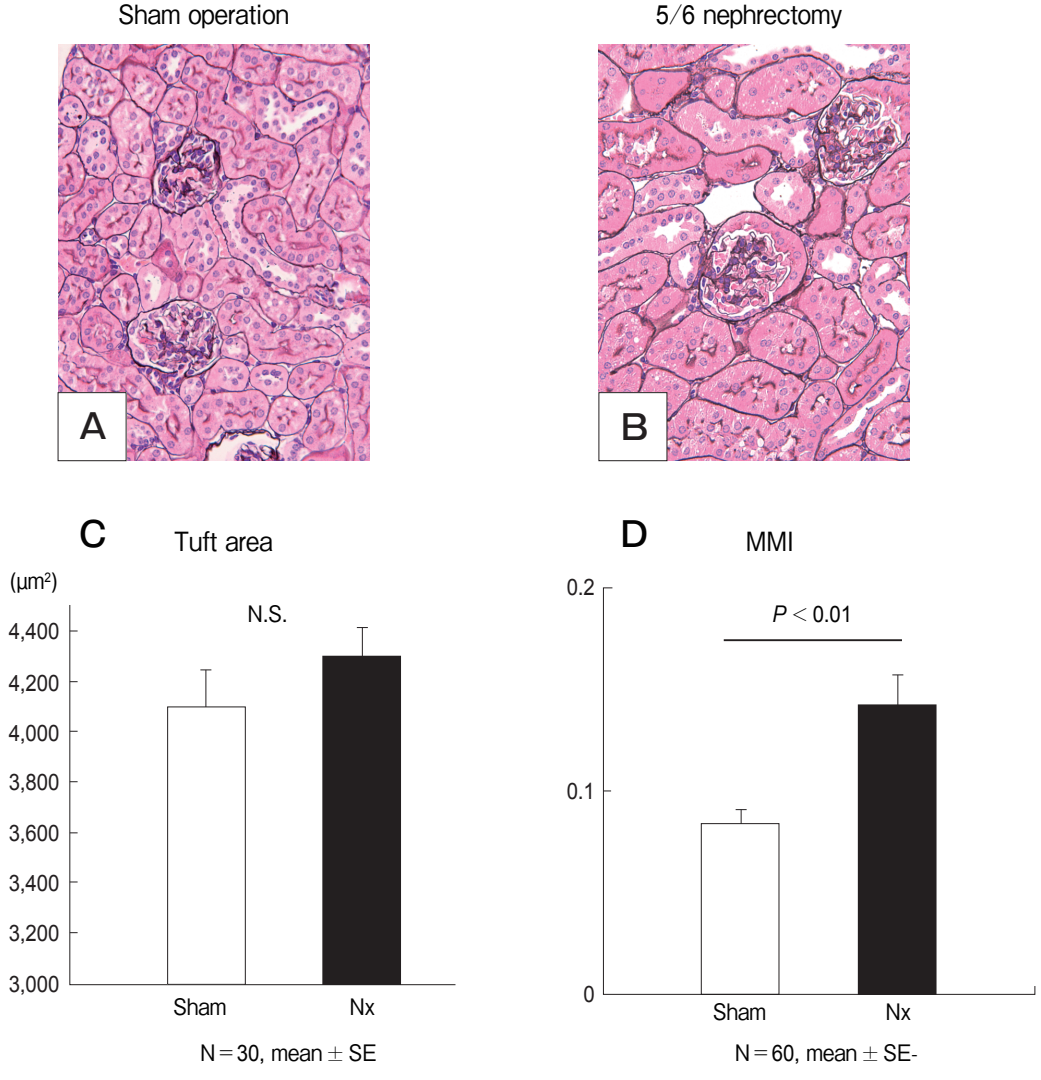

Fig. 1 Periodic acid-methenamine silver (PAM) staining of kidney sections of sham-operated mouse (**A**) and 5/6 nephrectomized mouse (**B**). PAM-staining of mouse glomeruli indicates glomerular hypertrophy and mesangial expansion in the kidneys of 5/6 nephrectomized mice as compared with sham-operated mice. **C**, The mean value of glomerular size (tuft area) in Nx appears higher than that in the sham group, although there is no statistically significant difference (N = 30, n.s. Mean ± SE.) Student's *t*-test; **D**, Mesangial matrix index (MMI) at 12 weeks. MMI, calculated by the PAM-positive area in the tuft area, was increased in 5/6 nephrectomized mouse compared with sham-operated mouse. (N = 60, $p < 0.01$. Mean ± SE.) Student's *t*-test.

immune or inflammatory process: taxis, immune response (Fig. 2). These genes contained several inflammatory-related genes including chemokine (C-X-C motif) ligand 4 (CCL4, platelet factor 4) and CCL12 (MCP-5) (Table 2).

Increased expression of chemokines in remnant kidney by real-time RT-PCR. We examined the gene expression levels of PF-4, MCP-5, MCP-1 and MCP-2 by real-time RT-PCR. These genes were up-regulated in the remnant kidneys of 5/6 nephrectomized mice as compared with sham-operated mice (Fig. 3).

Distribution of macrophages in the glomeruli. Immunostaining showed that the number of macrophages (F4/80-positive cell) was increased in the Nx group as compared with the sham group (Fig. 4).

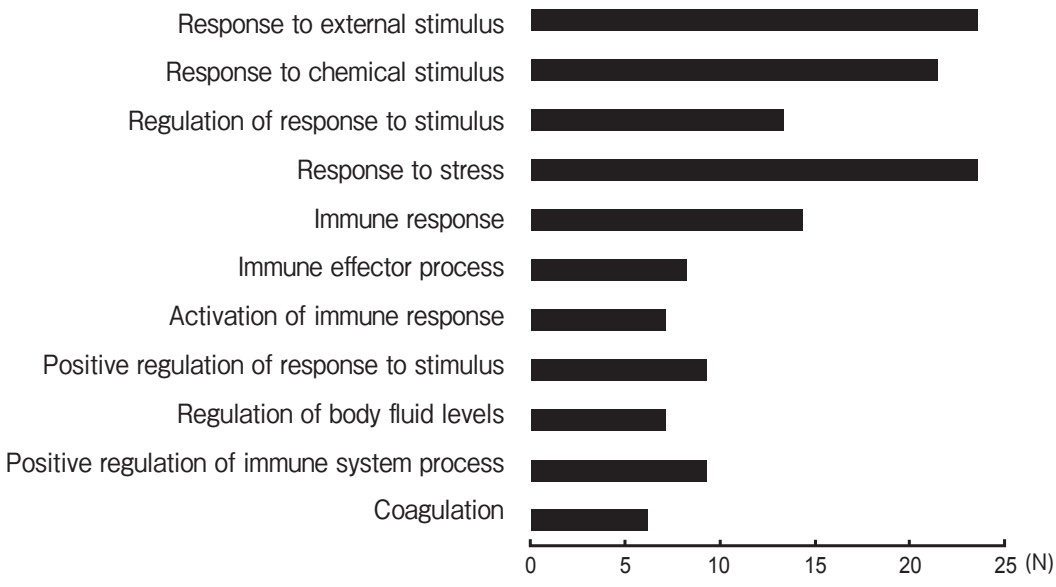

Fig. 2 Functional annotation of the genes up-regulated in the kidneys from 5/6 nephrectomized mice as compared with sham-operated mice in DNA micro array. (ratio > 2.0, $p < 0.0001$.) Modified Fisher's exact test.

Table 2 List of the upregulated genes in positive regulation of immune system process

GENBANK_ACCESSION	(Gene name)	Ratio
NM_019932	platelet factor 4	4.22
NM_010776	mannose-binding lectin (protein C) 2	4.20
NM_011331	chemokine (C-C motif) ligand 12; similar to monocyte chemoattractant protein-5	3.48
NM_011707	vitronectin	3.38
J00544	immunoglobulin joining chain	3.05
NM_020008	C-type lectin domain family 7, member a	3.05
NM_009139	chemokine (C-C motif) ligand 6	2.69
NM_009777	complement component 1, q subcomponent, beta polypeptide	2.31
NM_016982	pre-B lymphocyte gene 1	2.12
NM_010188	Fc receptor, IgG, low affinity III	2.10
NM_010185	Fc receptor, IgE, high affinity I, gamma polypeptide	2.07
NM_007574	complement component 1, q subcomponent, C chain	2.07
NM_007574	complement component 1, q subcomponent, alpha polypeptide	2.07
NM_007686	complement component factor I	2.02

Discussion

Glomerular hyperfiltration is considered to be a common mechanism leading to the development of glomerulosclerosis in immunological and nonimmunological human kidney diseases. Subtotal renal ablation (5/6 nephrectomy) has been used as an animal model to explore the mechanism of glomerular hyperfiltrations [7]. Partial ablation of renal mass initiates the cycle of progressive renal injury in the remnant kidney associated with glomerular hypertrophy and systemic hypertension [8, 9]. Histopathological studies of remnant kidney tissue have revealed a complex response consisting primarily of 3 steps: the hypertrophic phase, the quiescence phase with minimal histological alterations, and the development of segmental glomerular sclerosis and tubulointerstitial fibrosis [10, 11]. There have been many reports that various molecules are up-regulated during different periods in nephrectomized kidneys including hormones [12], cytokines, and growth factors [13–16], growth factor receptors [14, 17], transcription factors [18],

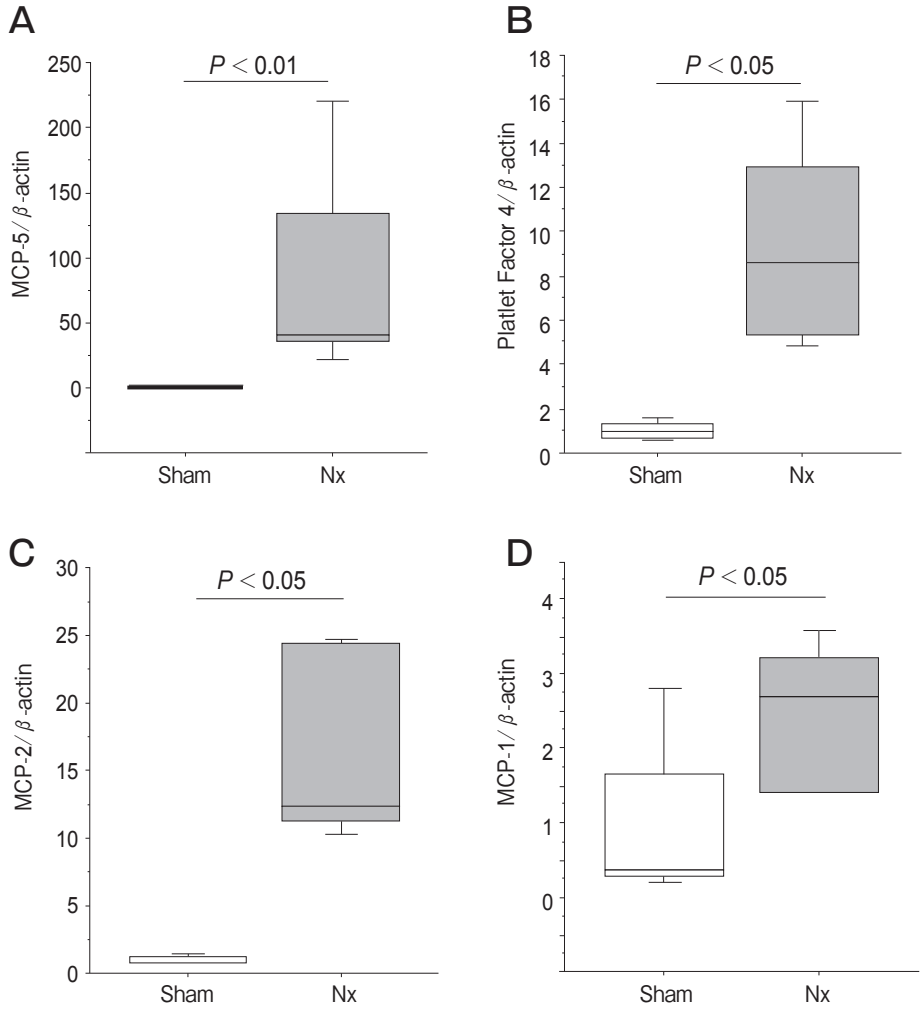

Fig. 3 Quantitative Realtime RT-PCR for MCP-5 (**A**), Platelet factor-4 (**B**), MCP-2 (**C**) and MCP-1 (**D**) (N = 5). The horizontal lines in the box denote the 25th, 50th and 75th percentile values. The error bars denote the 5th and 95th percentile values. Mann Whitney-U test. Data are expressed as median and interquartile ranges.

cell-cycle regulators [19], proto-oncogenes [20], vasoactive peptides [21–24], adhesion molecules [5], extracellular matrix (ECM) glycoproteins [25], ECM-degrading proteases, and their inhibitors [26]. The mRNA expression of ECM glycoproteins, is up-regulated, and the expansion of mesangial and interstitial matrix and the accumulation of ECM glycoproteins has been observed by immunohistochemistry [27]. In the hypertrophic period associated with hyperfiltration, numerous mediators are up-regulated in glomeruli and tubules and are believed to be involved in the progression of the subsequent glomerulosclerosis and the interstitial fibrosis. Transforming growth factor-β (TGF-β) is one of the major mediators implicated in regulating the production and degradation of matrix glycoproteins, and the enhanced glomerular and interstitial expression is noted especially in the initial stage [13]. Other growth factors and their related receptors are also reported to be up-regulated in the kidney, for example, insulin-like growth factor-I (IGF-I) [14, 15], EGF [14, 17], platelet-derived growth factors, platelet-derived growth factor (PDGF) receptors [16], and basic fibroblast growth factor (bFGF) [14]. Furthermore, vasoactive peptides [22–24], proto-oncogenes (c-*fos* and c-*jun*) [20], and cyclin E [19] are also increased. Our

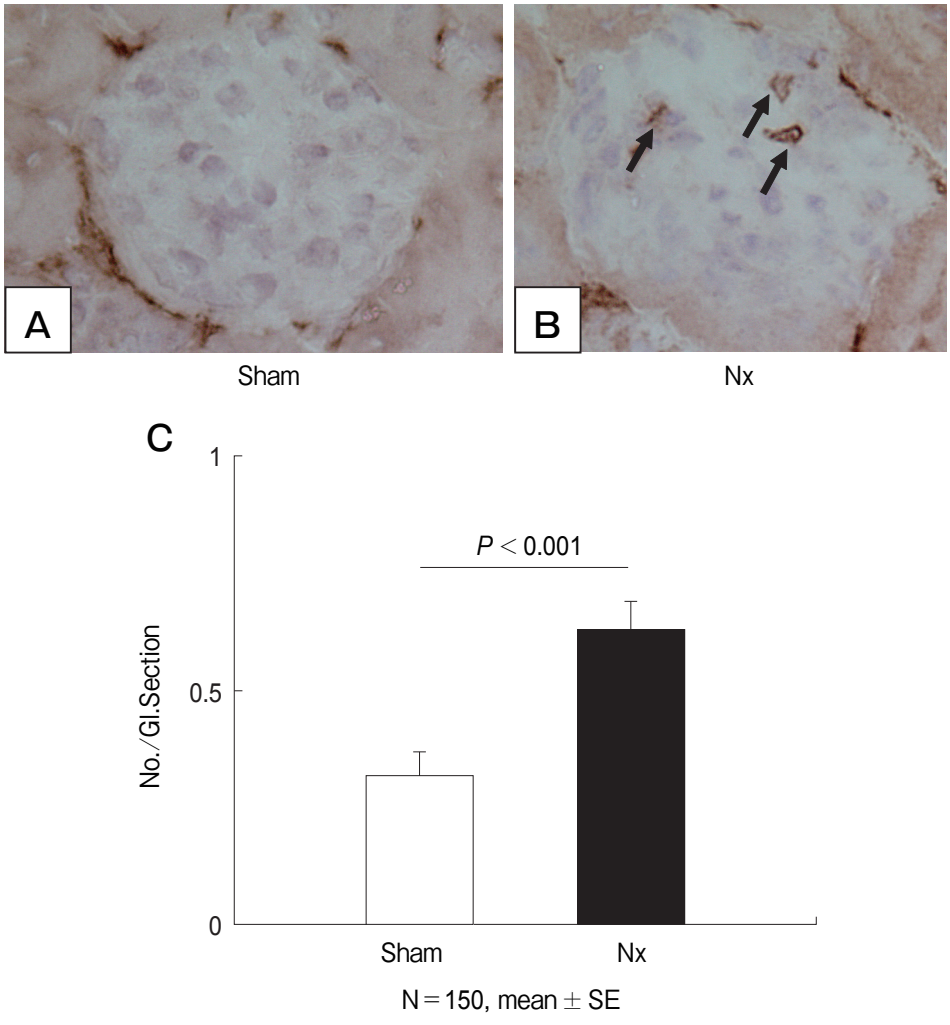

Fig. 4 Immunostaining for mouse macrophage (F4/80) in the glomerulus of sham-operated mouse (**A**) and 5/6 nephrectomized mouse (**B**). Student's *t*-test.

group previously conducted a representational difference analysis of cDNA (cDNA-RDA) based on the PCR-based subtraction method using 5/6 nephrectomized mouse [28].

In the present study, we found that the known genes included kidney androgen-regulated protein, major urinary protein, lysozyme M, metalloproteinase-3 tissue inhibitor, chaperonin 10, cytochrome oxidase I, ε-sarcoglycan, ribosomal protein S3a, the G-protein$^{\gamma}{}_{10}$ subunit, and splicing factor 9G8. Not all of the isolated known genes have been reported to be up-regulated in the nephrectomized mouse kidney, which suggests the possible role of androgen action, mitochondrial functions, matrix metabolism, cell-matrix interactions, and intracellular signaling events in the initiation of progressive renal injury of the remnant kidney.

Regardless of the information described above, data from the expression profiles of genes related to the inflammatory process have been insufficient to explore the therapeutic targets of non-immune mediated glomerular diseases including diabetic nephropathy. We therefore, applied a DNA microarray to screen the up-regulated genes related to the inflammatory process in remnant kidney. The 5/6 nephrectomized mice showed glomerular hypertrophy and an increase in size of extracellular matrix in the glomeruli in our present study. This finding is similar to

the pathological features of early-stage diabetic nephropathy. Immunostaining showed increased infiltration of macrophages in the glomeruli of remnant kidneys from 5/6 nephrectomized mice. These findings are consistent with those in our previous study and suggest that the inflammatory process is involved in the progression of hyperfiltration induced glomerulosclerosis [4, 5].

DNA microarray demonstrated an increased expression of many kinds of genes related to the inflammatory process including CC chemokines; PF-4 and MCP-5. Realtime RT-PCR confirmed an up-regulated expression of these 2 genes. Realtime RT-PCR also demonstrated increased expression of MCP-1 and MCP-2 in the kidney tissues of 5/6 nephrectomized mice. CC chemokines are well known to play a major role in the infiltration of monocytes and lymphocytes into inflammatory lesions. MCP-1 is reported to promote macrophage migration into the renal tissue of experimental animal models of diabetic nephropathy and glomerulonephritis [29]. These results suggest that chemokines including PF-4, and MCP-1, 2, and 5, contribute to the inflammatory process in the development of kidney diseases and may be a common therapeutic target of kidney diseases.

In conclusion, our current study indicates that many kinds of proinflammatory genes are up-regulated in remnant kidneys of 5/6 nephrectomized mice, suggesting the that inflammatory process is involved in the mechanism of glomrulosclerosis. Chemokines including PF-4, and MCP-1, 2, and 5 are suggested to be involved in the inflammatory process during glomerulosclerosis and might be potential therapeutic targets of chronic kidney diseases.

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