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Fractalkine expression and the recruitment of CX_3CR1^+ cells in the prolonged mesangial proliferative glomerulonephritis

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Fractalkine expression and the recruitment of CX_3CR1^+ cells in the prolonged mesangial proliferative glomerulonephritis.

Background. We established the reversible and the prolonged models of mesangial proliferative glomerulonephritis (GN) with anti-Thy 1 antibody 1-22-3. However, the essential factors leading to the prolonged glomerular alterations have not been identified.

Methods. The expressions of several chemokines and cytokines were compared in the reversible and the prolonged models. Expression of fractalkine and the number of the fractalkine receptor CX_3CR1-positive cells in the glomeruli in the prolonged model were significantly higher than those in the reversible model. Then, the localization of fractalkine and the characteristics of CX_3CR1^+ cells were analyzed in glomeruli. To elucidate the significance of the fractalkine expression, we analyzed the expression in the model treated with angiotensin II receptor antagonist, candesartan.

Results. Immunostaining of fractalkine was detected on endothelial cells on the fifth day, and fractalkine staining also was detected in the mesangial area on day 14. Major parts of the CX_3CR1^+ cells in the glomeruli were macrophages, especially $ED3^+$ cells. Candesartan treatment ameliorated the glomerular morphological findings at six weeks after disease induction. Although the treatment did not ameliorate the morphological finding at two weeks, decreased expression of fractalkine and CX_3CR1^+ were already detected at two weeks in rats treated with candesartan.

Conclusions. Fractalkine expression and the recruitment of CX_3CR1^+ cells in glomeruli might play an important role in the development of the prolonged disease. These expressions could be predictors of the prolonged disease of the mesangial proliferative glomerulonephritis.

Key words: candesartan, $ED3^+$ cell, Thy 1.1 glomerulonephritis, inflammation, cell proliferation, chemokine.

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The acute phase of glomerulonephritis (GN) is characterized by glomerular infiltration of inflammatory cells and the proliferation of mesangial cells [1–8]. It has been proposed that the secretary product from macrophage and lymphocytes plays an important role in the development of mesangial proliferative GN in both human and experimental models [1, 2, 9–12]. Thy 1.1 GN is most commonly used as the model of mesangial proliferative GN [13–16]. However, because the Thy 1.1 model is self-limiting, and glomerular architecture returns to normal within six to eight weeks, it is not available to elucidate the mechanism of the role of the inflammatory cells in the development of the prolonged mesangial alterations. We have previously reported that anti-Thy 1.1 mAb 1-22-3 causes severer mesangial alterations than OX-7 and polyclonal anti-thymocyte serum [17]. A single injection of mAb 1-22-3 to uninephrectomized rats caused the prolonged mesangial alterations (prolonged model), although the mesangial alterations induced by an injection of mAb 1-22-3 into rats without uninephrectomy were reversible (reversible model) [18]. It is reported that uninephrectomy increased single nephron glomerular filtration rate (GFR) in the remaining kidney, and also promoted several cytokines production [19]. These alterations caused by uninephrectomy are considered to affect the outcome of the disease, although the precise mechanisms of mesangial alterations in the prolonged model have not been fully elucidated to date.

The purpose of this study was to identify the essential factor that causes the prolonged disease. We analyzed the inflammatory responses in glomeruli in the early stage of the prolonged model and the reversible model. We detected that fractalkine expression and the recruitment of fractalkine receptor CX_3CR1-positive cells in glomeruli were significantly higher in the prolonged model than in the reversible model. Fractalkine is a novel

fourth class of chemokine that possesses three amino acid residues between the first and the second cysteines (C-X_3-C motif) [20]. Unlike other chemokines, fractalkine can exist in two forms, either the membrane anchored or soluble form [21]. The soluble C-X_3-C chemokine is reported to have potent chemoattractant activity for T cells and monocytes, and the membrane-anchored fractalkine, which is mainly expressed by endothelial cells, promotes strong adhesion of those leukocytes. Feng et al recently reported that fractalkine has an essential role in the crescent formation of anti-glomerular basement membrane (GBM) GN in WKY rats [22, 23]. They suggested that fractalkine may be especially important for leukocyte recruitment in tissues with a high blood flow rate, for example, in glomeruli, where the low-affinity binding of other chemokines to proteoglycan may not withstand the high shear environment [22]. These characteristics of fractalkine taken together with our findings, we hypothesized that fractalkine plays an important role in the recruitment and adhesion of inflammatory cells in glomeruli of nephrectomized rats injected with mAb 1-22-3, the glomerular filtration of which was increased.

To elucidate the significance of the fractalkine expression for the development of the disease, we analyzed the expression in the model treated with angiotensin II type I receptor antagonist (AT1RA), candesartan. We observed that the decreased expression of fractalkine and CX_3CR1^+ at two weeks in rats treated with AT1RA, although the treatment did not ameliorate the morphological finding at two weeks. We confirmed that AT1RA treatment ameliorated the glomerular morphological findings at six weeks after disease induction, suggesting that these expressions could be predictors of a prolonged disease in course of mesangial proliferative glomerulonephritis. This study further examined the localization of fractalkine expressing cell and determined the subpopulation of leukocyte CX_3CR1^+ cells. Immunofluorescence (IF) studies suggested that fractalkine was expressed by endothelial cells in the early phase and also by mesangial cells in the late phase. Almost all CX_3CR1^+ cells in glomeruli attracted by fractalkine were not T cells but macrophages. The major parts of CX_3CR1^+ cells in glomeruli were the $ED3^+$ cells that are reported to be activated macrophages. These results suggest that fractalkine and CX_3CR1^+ cells attracted by fractalkine are involved in the development of prolonged mesangial proliferative GN.

METHODS

Animals

Specific pathogen free female Wistar rats (6 weeks old) were purchased from Charles River Japan Inc. (Atsugi, Japan).

Induction of the uninephrectomized prolonged model

Sixty rats were divided into two groups: (*a*) thirty rats were uninephrectomized two weeks before injection of 1.0 mL of saline containing 0.5 mg monoclonal antibody (mAb) 1-22-3; (*b*) thirty rats were sham operated two weeks before the injection of 1.0 mL of saline containing 0.5 mg mAb 1-22-3. Five rats in each group were sacrificed at 30 minutes, 24 hours, and 5, 14, and 56 days after the injection of mAb 1-22-3. Five rats of each group were sacrificed before the injection of mAb 1-22-3 as a control of the baseline (0 hour). As controls, five rats were injected with phosphate-buffered saline (PBS) two weeks after the unilateral nephrectomy, and sacrificed on 56 days after the injection. Unilateral nephrectomy and sham operations were performed under ether anesthesia. A small flank incision was made, the left renal capsule and the adrenal gland were separated from the kidney, the renal artery, vein, and ureter were ligated at renal pedicle and the left kidney was removed. The sham operation involved the same surgical procedure, but the kidney and the renal pedicle were undamaged.

Urinary protein excretion

Urine was collected from the rats in a metabolic cage for 24 hours, and 1, 7, and 14 days after the surgery, and 1, 3, 5, 7, 14, 21, 28, 35, 42, 48, and 56 days after the mAb 1-22-3 or PBS injection. The amount of urinary protein excretion was measured by the Bradford method (Bio-Rad, Oakland, CA, USA).

Morphological and immunohistochemical studies

The right kidney of each rat was removed immediately. For light microscopy, part of the kidney was fixed with Carnoy's solution, embedded in paraffin and a section was stained with periodic acid-Schiff (PAS) and periodic acid-methenamine silver (PAM). Mesangial matrix expansion was analyzed semiquantitatively as grades from 0 to 4 according to the method of Raij, Azar and Keane [24]. The severity of mesangiolysis at 24 hours after the mAb 1-22-3 injection was scored by 0 to 4 according to the method described by Johnson et al [25].

For immunofluorescence microscopy, renal tissue was quickly frozen in n-hexane and cooled to $-70°C$. Four-micrometer thick sections were cut by cryostat. These sections were incubated with anti–α-smooth muscle actin (α-SMA) antibody, (Sigma St. Louis, MO, USA) or anti-rat collagen type I antibody (Chemicon, Temecula, CA, USA), and then stained with FITC-conjugated anti-mouse IgG2a or FITC-conjugated anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL, USA), respectively. The intensity of α-SMA and collagen type I staining was scored as the method described by Floege et al [26]. To identify the subpopulation of inflammatory cells recruited in glomeruli, the cryostat sections were

incubated with anti-leukocyte common antigen (OX-1; Serotec, Oxford, UK), anti-PMN (RP-3; kindly donated by Dr. Sendo, Yamagata University, Japan), anti-pan macrophage (ED1; Chemicon), anti-activated macrophage (ED3; Chemicon), anti-pan T cell (OX-19; European Collection of Animal Cells, Porton Down, Salisbury, UK), and then stained with FITC-conjugated anti-mouse IgG1 (Southern Biotechnology Associates) for OX-1, ED1, and OX-19, or FITC conjugated anti-mouse IgG2a for ED3, or FITC conjugated anti-mouse IgM (Southern Biotechnology Associates) for RP-3. The number of positive cells for these markers was counted in 30 full sized glomeruli. The numbers were expressed as the mean cells per glomeruli.

Immunostaining of fractalkine

Rabbit anti-rat fractalkine antibody was purchased from Torrey Pines Biolabs, Inc. (San Diego, CA, USA). To confirm the specificity of the staining of the anti-fractalkine antibody, the antibody was absorbed with the fusion protein of the chemotactic site of the fractalkine of rat. Chemotactic site of rat fractalkine coding 76 amino acid residues and engineered with EcoRI (5′) and SalI (3′) restriction sites so as to maintain the appropriate reading frame were amplified by polymerase chain reaction (PCR), and directly subcloned with pGEX-6P-1 vector (Amersham Pharmacom Biotech, Buckinghamshire, UK). Following bacterial transformation, cells of bacterial culture were pelleted, washed, solubilized, and applied in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fusion protein was extracted from acrylamide gels. As control for absorption test, the fusion protein of pGEX-6P-1 vector without insert was used. The rate of fractalkine positive glomeruli on the 14th day after mAb 1-22-3 injection was determined in randomly selected 20 full sized glomeruli. The glomerulus in which clearly specific staining of fractalkine was detected over 50% area was defined as fractalkine positive glomerulus. To examine the localization of the fractalkine expressing cell, dual labeling IF studies were performed with anti-fractalkine and anti-rat endothelial cell (RECA1; Serotec), or mAb 1-22-3.

Identification of CX_3CR1 expressing cells

To determine which subpopulation of leukocytes the CX_3CR1^+ cells are located, dual-labeling studies were performed. The sections were incubated with anti-CX_3CR1 (Torrey Pines Biolabs, Inc.) and ED1, ED3, or OX-19, and then stained with FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG (Dako, Glostup, Denmark). The CX_3CR1^+ cells, and double positive cells with ED1, ED3, or OX-19 were counted in 20 full sized glomeruli.

Kidney binding and complement fixation

To compare the amount of mAb 1-22-3 bound to the kidney, each of the three nephrectomized or sham operated rats were injected with mAb 1-22-3 labeled with ^{125}I, and kidneys were removed 30 minutes after the mAb 1-22-3 injection. Monoclonal antibody 1-22-3 was labeled with ^{125}I by the chloramine-T method and the level of ^{125}I bound to the kidneys was measured as described previously [27]. To compare the complement fixation in glomeruli, 30 minutes after the injection the kidney sections were incubated with FITC-conjugated anti-rat C3 (Dako). IF intensity of 30 full sized glomeruli from the sections of five rats of each group was measured by an auto-exposure system (Vanox AHBS3; Olympus, Tokyo, Japan) as described previously [17].

Semiquantitative reverse transcriptase (RT)-PCR

Glomeruli were isolated from kidneys pooled from the same group. The total RNA was extracted from isolated glomeruli by TRIZOL (Gibco BRL, Gaithersburg, MD, USA) and stored at −70°C before use. RNA was quantified by absorbance at 260 nm. First strand cDNA was synthesized by total RNA from each group by using a commercial kit (Superscript preamplification system; Gibco BRL). Five micrograms of RNA was incubated at 42°C for 50 minutes in a final volume of 20 μL, which contained 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 1 mmol/L each of dATP, dCTP, dGTP and dTTP, 0.5 mg oligo (dT) and 50 U reverse transcriptase (SuperScript II; Gibco BRL). At the end of the incubation, samples were heated at 90°C for five minutes to eliminate the transcriptase activity. Then, 3 μL of cDNA were amplified in a 50 μL total volume of PCR buffer containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 mmol/L each dATP, dCTP, dGTP and dTTP, 0.4 mmol/L each of the 5′ and 3′ primers and 2.5 U heat-activated Taq DNA polymerase (AmpliTaq Gold; Perkin-Elmer, Foster, CA, USA). The primers of MCP-1, RANTES, lymphotactin and GAPDH were designed according to published sequence [12, 28]. The primers of fractalkine, CX_3CR1 and macrophage derived chemokine (MDC) were designed based on their sequences. The sequences of the primers, the sizes of PCR product expected and annealing temperature were shown in Table 1. PCR products of fractalkine and CX_3CR1 were sequenced and confirmed to be specific products of rat fractalkine and rat CX_3CR1. Amplification was carried out using the PC-800 programmable temperature control system (Astec, Fukuoka, Japan) through 20 to 35 cycles of denaturation at 95°C for 30 seconds, annealing at individual temperatures for 30 seconds, and extension at 72°C for one minute. The optimum number of amplification cycles used for semiquantitative PCR was chosen based on the pre-

Table 1. Primers used in this study

	Sense primer	Antisense primer	Temperature ℃	Size *bp*
MCP-1	5′-CTC TTC CTC CAC TAT GC-3′	5′-CTC TGT CAT ACT GGT CAC TTG-3′	60	457
RANTES	5′-ACC TGC CTC CCC ATA TGG CT-3′	5′-GTA TTC TTG AAC CCA CTT CTT CTT C-3′	60	190
Lymphotactin	5′-CCT GGG AGT CTG CTG CTT CG-3′	5′-TGG CGG ACC TCT GGG CTT GT-3′	60	313
Fractalkine	5′-CTC GCC AAT CCC AGT GAC CTT GCT O-3′	5′-GAT TGG TAG ACA GCA GAA CTC GGC CAA ATG-3′	61	492
TARC	5′-GCG TGC TGC CTG GAT TAC TTC AA-3′	5′-TTC TTC ACA TGT TTG TCT TTG GGG TC-3′	58	154
MDC	5′-TGG CAC TTC AGA CCT CCG ATG-3′	5′-AGG GGA CGG ACA GGA AGT ACA-3′	58	398
CX_3CR1	5′-AGC TGC TCA GGA CCT CAC CAT-3′	5′-GTT GTG GAG GCC CTC ATG GCT GAT-3′	61	319
GAPDH	5′-CTC TAG CCA CGG CAA GTT CAA-3′	5′-GGA TGA CCT TGC CCA CAG C-3′	60	516

liminary trial in the linear phase of amplification. The PCR products were electrophoresed on 1.2% agarose gel containing 0.0001% ethidium bromide in TAE buffer, and band intensities were determined by image analysis using a Macintosh computer and the densitometry program, Densitograph (ATTO, Tokyo, Japan). All results were corrected for the amount of mRNA of GAPDH as an internal standard. The data are shown as ratios relative to control rat expression (0 hr of the sham operated group) and are expressed as mean ± SD of these independent experiments.

Real-time RT-PCR

Real-time PCR was performed basically according to the manufacturer's manual. cDNA was synthesized as described above. cDNA, specific primers and SYBR Green (Takara, Otsu, Japan) were mixed with *Takara Ex Taq* R-PCR Version For Real Time PCR kit (Takara). PCR reactions were run on Smart Cycler System (Takara). The sequences of the primers of fractalkine and GAPDH were described above. The reactions and run for all samples were performed in duplicate.

Preparation and chemotaxis analysis of inflammatory leukocytes from nephritic glomeruli

Inflammatory leukocytes were isolated from glomeruli of rats sacrificed on day 5 after 0.5 mg of mAb 1-22-3 injection following the method of Cook, Smith and Cattell [29]. Glomerular leukocytes were resuspended at 1×10^6/mL in MEM plus 10% heat-inactivated fetal calf serum (FCS). Chemotaxis analysis was performed basically according to the method described by Chen et al [23]. In brief, 200 μL of 10^{-7} mol/L human recombinant fractalkine (Genzyme/Techne, Cambridge, MA, USA) diluted with PBS with 1 mg/mL BSA was placed in the lower well of the blind well chamber, model BW 200S (Neuro Probe, Inc., Cabin John, MD, USA), and separated from 200 μL of cell suspension by an 5-μm pore size polyvinylpyrolidone-free polycarbonate filter (Neuro Probe, Inc). After incubation at 37°C for two hours, sedimented cells on the top surface of the filter were wiped off and migrated cells on the undersurface were fixed in methanol for 30 seconds, and stained with Giemsa's solution (Merck Japan, Tokyo, Japan). The cell numbers were counted with four filters. Results were expressed as mean ± SEM cell number per low power fields (×200). As control, PBS with 1 mg/mL BSA was placed in the lower chamber. For the immunofluorescence (IF) study, the migrated cells on the undersurface of the filters were stained with ED3 and FITC anti-mouse IgG2a, after the sedimented cells on the top surface of the filter were wiped off.

Effect of AT1RA on the expression of fractalkine and CX_3CR1

To analyze the long-term consequence of AT1RA, candesartan treatment, the prolonged mesangial GN was induced by a single injection of 0.5 mg mAb 1-22-3 into uninephrectomized rats. Six rats of each group were injected daily from day 0 (1 hour after disease induction) through experimental period intraperitoneally with 0.5 mg/kg body wt/day of candesartan or PBS basically according to the method described previously [30] and then sacrificed at six weeks after disease induction. Mesangial matrix expansion was scored as described previously. To analyze the effect of AT1RA candesartan on the expressions of fractalkine and CX_3CR1, the prolonged mesangial GN was induced in 12 rats as described above. Six rats of each group were injected daily with candesartan or PBS as described above, and then sacrificed on day 14 after disease induction. The expression of fractalkine and the recruitment of CX_3CR1^+ cells were counted in 30 full sized glomeruli. For further analyses of mRNA expressions of chemokines and renal function, the prolonged mesangial GN was induced in 10 rats. Five rats each were injected with candesartan, or PBS and then sacrificed on day 14 after disease induction. Glomerular RNA was prepared from pooled kidney of each group. mRNA expressions for fractalkine, CX_3CR1, MCP-1 and MDC were analyzed by semiquantitative RT-PCR. Glo-

Table 2. Kidney bound mAb1-22-3, complement fixation, and mesangiolysis score

Group	Kidney bound mAb1-22-3 *μg/kidney* ($N = 3$)	C3 fluorescence intensity *seconds* ($N = 5$)	Mesangiolysis score ($N = 5$)
Uninephrectomized	11.29 ± 1.63	15.46 ± 3.51	2.75 ± 0.96
Sham operated	8.55 ± 1.87	12.4 ± 3.40	2 ± 0

The C3 fluorescence intensity in glomeruli was expressed as the autoexposure time that was considered to reciprocally reflect the staining intensity.

merular mRNA expression of fractalkine was also analyzed by the real-time RT-PCR method. Systolic blood pressure (SBP) was measured in conscious, restrained rats using a tail-cuff sphyngmanometer on day 11 after disease induction. Serum levels of creatinine were measured on day 14 according to the standard method. Creatinine clearance (C_{Cr}) was calculated from a urine sample taken 24 hours before the animal was sacrificed.

Expression of fractalkine and CX_3CR1 in the prolonged model induced by two consecutive injections of 1-22-3

To investigate the expression in another prolonged model, the prolonged mesangial alterations were induced by two consecutive injections of 1.0 mL of saline containing 0.5 mg of mAb 1-22-3 twice an interval of two weeks as already reported [31]. As control, the reversible mesangial alteration was induced by injecting once with 0.5 mg of the mAb 1-22-3. The rats of each group were sacrificed 30 minutes, 24 hours, 5, and 14 days after the last injection. Glomerular mRNA expressions of fractalkine and CX_3CR1 were examined by RT-PCR.

Statistical analysis

Statistical significance was evaluated using the unpaired *t* test or the Mann-Whitney U test. All values were expressed as the mean ± SD. Differences at $P < 0.05$ were considered significant. Data were analyzed using StatView for Macintosh (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Initiation events, urinary protein excretion and the mesangial alterations of the uninephrectomized prolonged model

The amount of kidney binding mAb 1-22-3, C3 fluorescent intensity and the mesangiolysis score of uninephrectomized and sham operated groups were summarized in Table 2. There was no statistically significant difference in the intensity of the C3 deposition and the mesangiolysis score in both groups. The kinetics of urinary protein excretion was shown in Figure 1A. The amount of urinary protein excretion peaked on day 3 after injection in both groups. No difference of the amount of proteinuria on days 5 and 7 was detected between both groups. Although urinary protein excretion of the sham operated group decreased gradually and normalized by four weeks after the injection, abnormal proteinuria persisted for two months in the uninephrectomized group. No abnormal proteinuria was detected

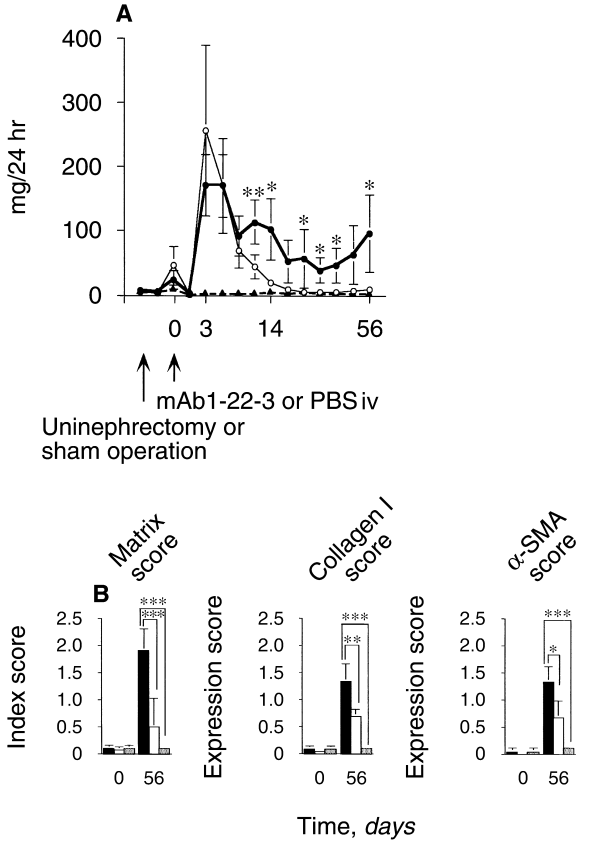

Fig. 1. (*A*) Time course of urinary protein excretion after monoclonal antibody (mAb) 1-22-3 injection. The amount of 24-hour proteinuria was measured 1, 7 and 14 days after the surgery and 1, 3, 5, 7, 14, 21, 28, 35, 42, 48, and 56 days after the mAb 1-22-3 or PBS injection. Symbols are: (●) uninephrectomy + 1-22-3; (○) sham operation + 1-22-3; (▲) uninephrectomy + PBS. Abnormal proteinuria persisted for 2 months in the uninephrectomized group, whereas urinary protein excretion in the sham operated group was normalized by the 28th day after mAb 1-22-3 injection. No abnormal proteinuria was detected in uninephrectomized rats without injection of mAb 1-22-3. (*B*) Matrix score, collagen type I score, and α-SMA score in the uninephrectomized and sham operated groups. Symbols are: (■) uninephrectomy + 1-22-3; (□) sham operation + 1-22-3; (▩) uninephrectomy + PBS; $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. The mesangial matrix expansion and the extension of αSMA and collagen type I staining on the 56th day was higher in the uninephrectomized group than in the sham operated group.

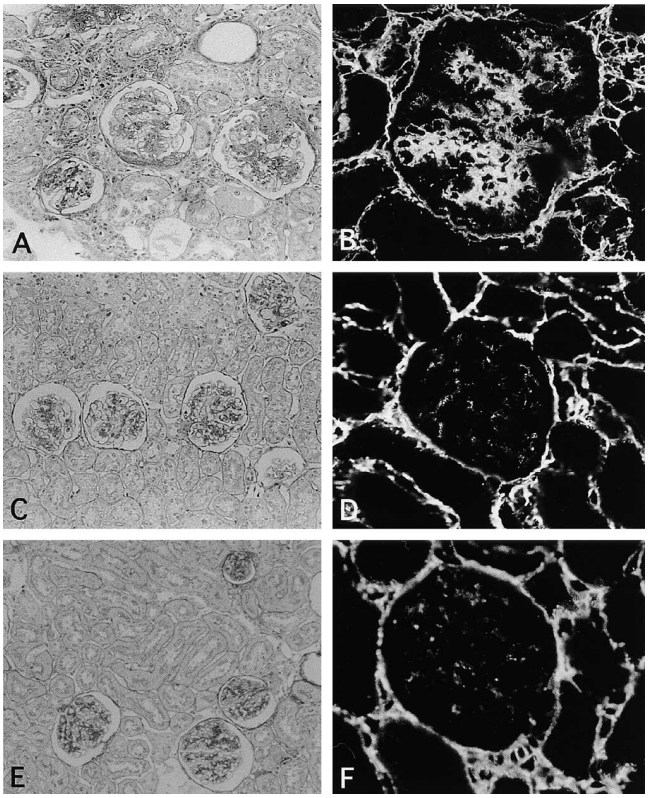

Fig. 2. Light micrographs (*A*, *C*, *E*) and immunofluorescence micrographs of anti-type I collagen (*B*, *D*, *F*) on the 56th day after mAb 1-22-3. Mesangial cell proliferation and severe matrix expansion are still detected on the 56th day after mAb 1-22-3 injection in uninephrectomized rats (A), whereas LM findings of sham operated rats were almost normalized (C). Intense staining of collagen type I was detected in the mesangial area in uninephrectomized rats injected with mAb 1-22-3 (B). The staining of collagen type I in sham operated rats was weak and focal (D). (E) and (F) show the control findings of uninephrectomized rats without injection of mAb 1-22-3 (A, C, E ×200, B, D, F ×400).

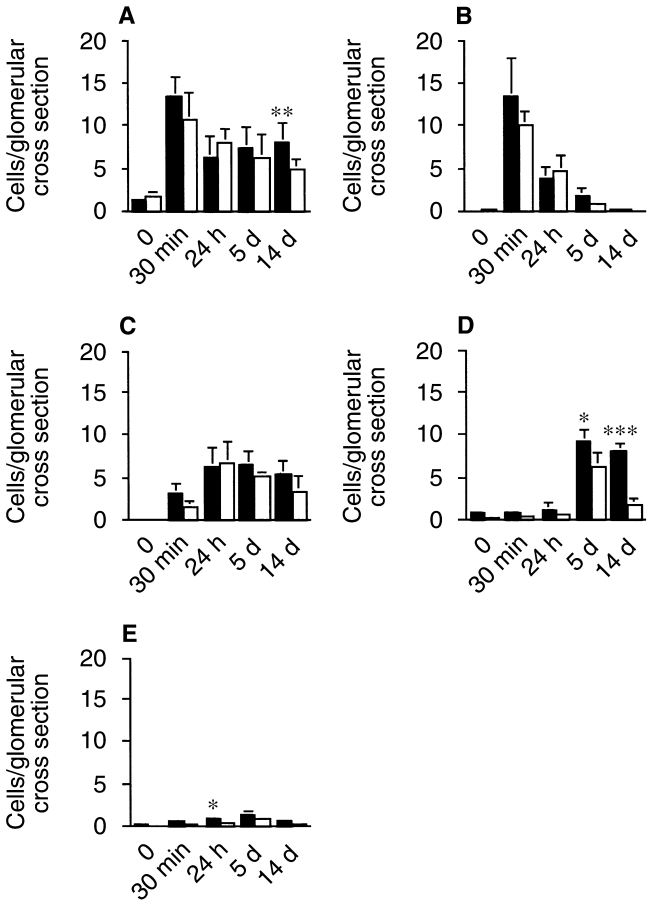

Fig. 3. Time course of the number of infiltrating inflammatory cells in glomeruli after injection of mAb 1-22-3 in the uninephrectomized group and the sham operated group. Symbols are: (■) uninephrectomized prolonged model; (□) sham-operated reversible model; $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ vs. corresponding time point of sham operated model. OX-1^+ cells in glomeruli increased in both groups, peaked at 30 minutes and decreased gradually. (*A*) A larger number of OX-1^+ cells were detected on the 14th day in the uninephrectomized group than that of the sham operated group. (*B*) RP-3^+ cells infiltration into glomeruli peaked within 30 minutes. No significant difference of the infiltration of RP-3^+ cells was detected in both groups. (*C*) In the uninephrectomized group, ED1^+ cells infiltrated into glomeruli earlier than in the sham operated group. (*D*) Increased number of ED3^+ cells in glomeruli was detected on the 5th day and the 14th day in both groups. The number of ED3^+ cells in the uninephrectomized group was larger than that of the sham-operated group. (*E*) Few of OX-19^+ cells were detected in both groups.

in the uninephrectomized rats without an injection of mAb 1-22-3 during the experimental period. No difference of mesangial alteration on day 5 was observed between both groups. In the sham operated group, although mesangial morphological alterations were still detected on the 14th day, mesangial alterations were gradually decreased by the 56th day. Severe mesangial alterations were still detected on the 56th day in the uninephrectomized group. The mesangial matrix score and the IF intensity scores of α-SMA, and collagen type I on the 56th day were shown in Figure 1B. Mesangial matrix expansion in the uninephrectomized group was 3.85 times higher than that of the sham operated group. The IF intensity score of α-SMA and the collagen type I of the uninephrectomized group was 2.01, and 1.93 times higher than those of the sham group. No abnormal morphological alterations were observed in the uninephrectomized rats without injection of mAb 1-22-3. The representative findings of the light microscopy and collagen type I IF staining on the 56th day after injection are shown in Figure 2. These results support the previous report that the mesangial alteration induced by mAb 1-22-3 injection into uninephrectomized rats are prolonged.

Infiltration of inflammatory cells in glomeruli

The kinetics of the recruitment of OX-1^+, RP-3^+, ED1^+, ED3^+, and OX-19^+ cells were shown in Figure 3. OX-1^+ cells in glomeruli increased in both groups, peaked at 30 minutes after mAb 1-22-3 injection and

decreased gradually. A larger number of OX-1^+ cells were detected on the 14th day in the uninephrectomized group than in the sham operated group (Fig. 3A). RP-3^+ cells accumulated rapidly, peaked at 30 minutes after the injection of mAb 1-22-3, and had returned to normal levels by day 5. No significant difference of the infiltration of RP-3^+ cells was observed in both groups (Fig. 3B). In the uninephrectomized group, ED1^+ cells infiltrated into glomeruli earlier than in the sham operated group. However, no difference of the peak number of ED1^+ cells was observed in both groups (Fig. 3C). Few of ED3^+ cells were detected in normal glomeruli and at the early stage of the 1-22-3 induced glomerulonephritis, but the number of ED3^+ cells increased at the later stage (days 5 and 14) in both groups. The number of ED3^+ cells recruited in glomeruli in the uninephrectomized group was already significantly higher on day 5 than that of the sham operated rats ($P < 0.05$). The number of ED3^+ cells on the 14th day was 4.5 times higher in the uninephrectomized group than in the sham operated group (Fig. 3D). Few of OX-19^+ cells were detected in both groups (Fig. 3E).

mRNA expression for chemokines in glomeruli

mRNA expression of MCP-1 in the uninephrectomized group was already increased at 30 minutes (2.70 times to the normal expression) and decreased to 1.33 times at 24 hours, whereas MCP-1 mRNA expression in the sham operated group at 30 minutes is 1.99 times to the normal expression and peaked at 24 hours (2.62 times). Increased mRNA expression of the lymphotactin was detected on the fifth day in both groups. There was no significant difference of lymphotactin expression on day 5 between the uninephrectomized group (2.26 times to normal expression) and the sham operated group (2.32 times). Increased mRNA expression of the TARC was detected on the 14th day in both groups, and there was no significant difference between the uninephrectomized group (1.85 times to normal expression) and the sham operated group (1.45 times). No increased expression of RANTES was detected in both groups at any time points examined. In the uninephrectomized group, mRNA expression of the fractalkine dramatically increased on the 14th day (6.5 times to normal expression). The fractalkine expression of the uninephrectomized group was significantly higher than that in the sham operated group (2.5 times to sham operated group). The kinetics of the expression of fractalkine and a representative RT-PCR finding on the 14th day is shown in Figure 4.

Immunostaining of fractalkine

In the uninephrectomized group, clearly positive staining of fractalkine in glomeruli was already detected at 24 hours, and its intensity peaked on the 14th day (Fig. 5A). No positive staining of fractalkine was detected on

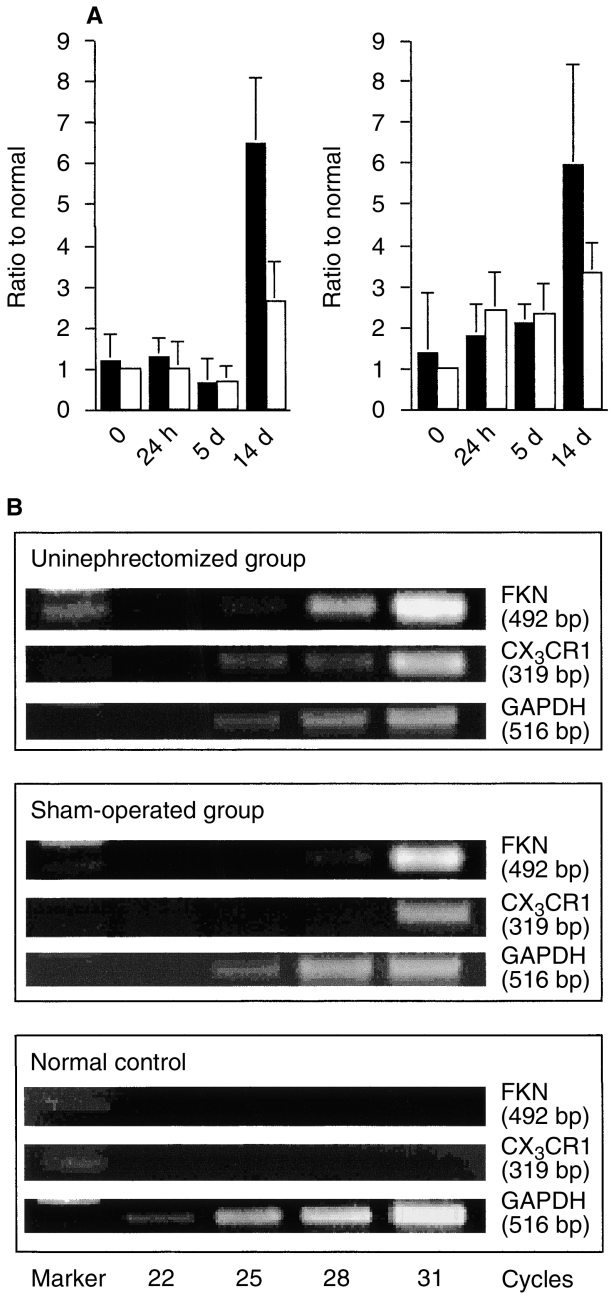

Fig. 4. Kinetics of fractalkine (FKN) and CX_3CR1 mRNA expression on the 14th day after mAb 1-22-3 injection. (*A*) mRNA expression was semiquantitated by RT-PCR using cDNA corresponding to 750 ng RNA. Symbols are: (■) nephrectomized; (□) sham-operated. The optimal cycle numbers were determined in a preliminary trial to be in the linear phase of amplification. Ratios of the densitometric signals of FKN (left panel) or CX_3CR1 (right panel) and the internal control (GAPDH) were analyzed. The data are shown as the ratio relative to normal rat findings and are expressed as mean ± SD of three independent experiments. FKN mRNA expression of uninephrectomized rats on day 14 was 2.52 times higher than that in the normal rat findings. (*B*) Representative agarose gel electrophoretic patterns of PCR products of FKN, CX_3CR1, and GAPDH on day 14 in 3 cycle intervals.

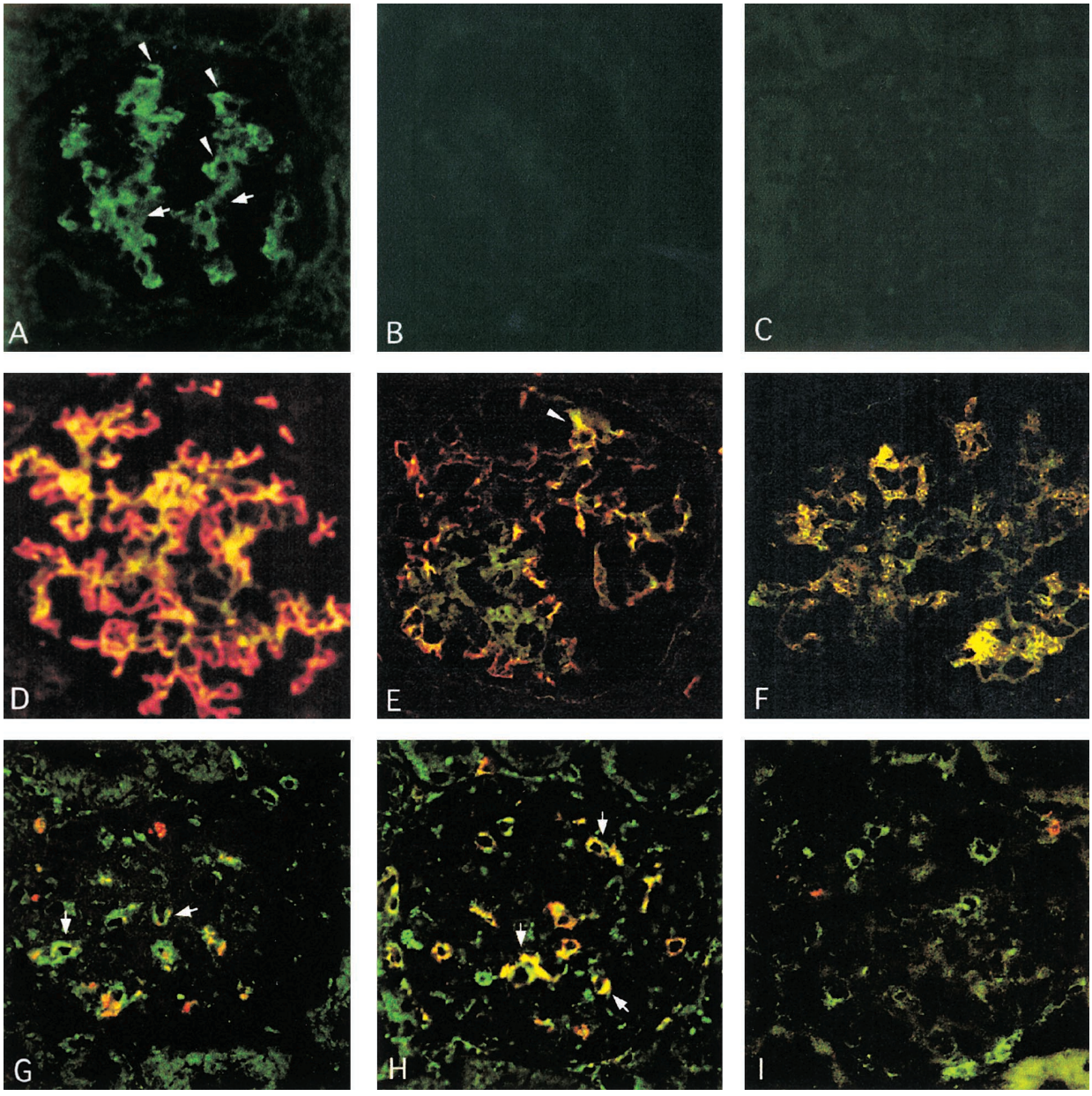

Fig. 5. Immunofluorescence findings of fractalkine (FKN) and CX_3CR1 in glomeruli after mAb 1-22-3 injection in the uninephrectomized rat. (*A*) Immunofluorescence micrograph of FKN on the 14th day after mAb 1-22-3 injection into a uninephrectomized rat. Intense staining of FKN is detected at the site facing the capillary lumen (arrowheads), and the staining is also detected at the mesangial area (arrows). (*B*) No staining is detected with anti-FKN antibody (Ab) pre-incubated with fusion protein of the extracellular site of FKN in the same rat material as used in panel A. (*C*) FKN staining is not detected in normal rat glomeruli. (*D*) Dual labeling staining with anti-FKN Ab (green) and anti-RECA1 Ab (red) on the 5th day. A part of RECA1 positive cells (endothelial cells) is positive for FKN (yellow). (*E*) Dual labeling staining with anti-FKN Ab (green) and anti-RECA1 Ab (red) on the 14th day. Positive staining of FKN is detected in endothelial cell (yellow, arrow head) and in the mesangial area (green). (*F*) Dual labeling staining with anti-FKN Ab (green) and mAb 1-22-3 (red) on the 14th day. Monoclonal antibody (mAb) 1-22-3 was used as a marker for mesangial cell. Positive staining of FKN is detected in the mesangial area (yellow). (*G*) Dual labeling staining with anti-CX_3CR1 (green) and with ED1 (red) on the 5th day. A part of the $ED1^+$ cells are also stained with anti-CX_3CR1 (yellow, arrows). (*H*) Dual labeling staining with anti-CX_3CR1 (green) and with ED3 (red) on the 5th day. Major parts of $ED3^+$ cells are also stained with anti-CX_3CR1 (yellow, arrows). (*I*) Dual labeling staining with anti-CX_3CR1 (green) and with OX-19 (red) on the 5th day. No double positive cells were detected.

the 56th day in the uninephrectomized group. Fractalkine staining in the sham operated group was weak and focal at the any of the time points examined. Fractalkine expression was not detected in the normal kidneys (Fig. 5C). On the fifth day, a part of the endothelial cells was positive for fractalkine (Fig. 5D), and fractalkine expression also was detected in the mesangial area on the 14th day (Fig. 5 E, F). No positive staining was detected with

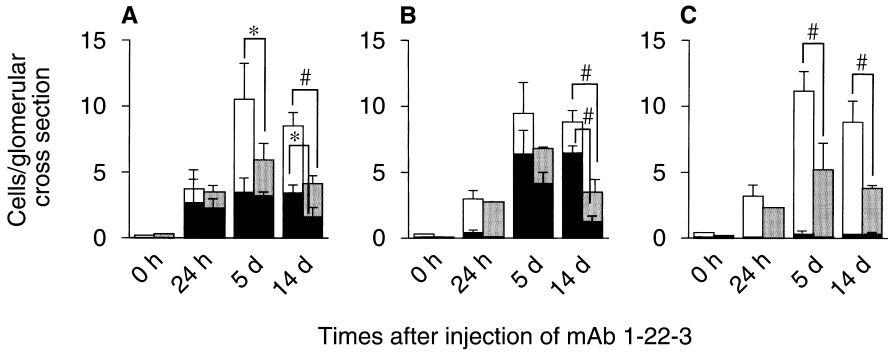

Fig. 6. Time course of number of CX_3CR1 positive cells and double positive cells co-stained with (*A*) ED1, (*B*) ED3, or (*C*) OX-19. Symbols are the numbers of: CX_3CR1-positive cells in the (□) uninephrectomized group and (▩) sham operated group; (■) double positive cells with CX_3CR1 and each macrophage and T cell marker. $*P < 0.05$, $\#P < 0.005$. Infiltration of CX_3CR1^+ cells in glomeruli peaked on the 5th day after injection of mAb 1-22-3. The number of CX_3CR1^+ cells was larger in the uninephrectomized group than in the sham operated group. The majority of the CX_3CR1^+ cells were positive for macrophage marker. Almost all CX_3CR1^+ cells were negative for T cell marker.

anti-fractalkine antibody that was pre-absorbed with fusion protein of chemotactic site of fractalkine (Fig. 5B), although positive staining was detected with anti-fractalkine antibody that was preincubated with fusion protein of pGEX-6P-1 without insert. The rate of fractalkine positive glomeruli was significantly higher in the uninephrectomized group than in the sham operated group (60 ± 17 vs. $18 \pm 13\%$, $P < 0.01$).

mRNA expression of CX_3CR1 and identification of CX_3CR1 expressing cells

Increased mRNA expression of CX_3CR1 was already detected at 24 hours in the uninephrectomized group. The CX_3CR1 mRNA expression in the uninephrectomized group on the 14th day was significantly higher than that in the sham operated group. The kinetics of the expression of CX_3CR1 and a representative RT-PCR finding on the 14th day is shown in Figure 4. The expression of CX_3CR1 mRNA was decreased to almost normal level on 56th day. The dual labeling of the immunofluorescence findings of the anti-CX_3CR1 with ED1, ED3, or OX-19 is shown in Figure 5 (G-I), and the kinetics of the double positive cells of CX_3CR1 and ED1, ED3, or OX-19 is in Figure 6. The number of CX_3CR1^+ cells in glomeruli peaked on the fifth day and were significantly increased in the uninephrectomized group (1.42 times more to the number of sham operated groups on day 5). The majority of the CX_3CR1^+ cell was positive for macrophage marker: 66.7% of the CX_3CR1^+ cells were $ED3^+$ cells on the fifth day and 73.9% on the 14th day in the prolonged model. On the other hand, 69.5% of $ED3^+$ macrophages in glomeruli on the fifth day in the prolonged model were positive for CX_3CR1, and 80.7% on the 14th day.

Chemotaxis analysis of inflammatory leukocytes from nephritic glomeruli

The mean numbers of leukocytes on the undersurface of the filter attracted by fractalkine or PBS/BSA were shown in Figure 7A. The representative finding of Giemsa staining specimens of the cells attracted by fractalkine and the finding of negative control with PBS/BSA were shown in Figure 7 B and C, respectively. Figure 7D shows that a part of leukocytes attracted by fractalkine was $ED3^+$ cells.

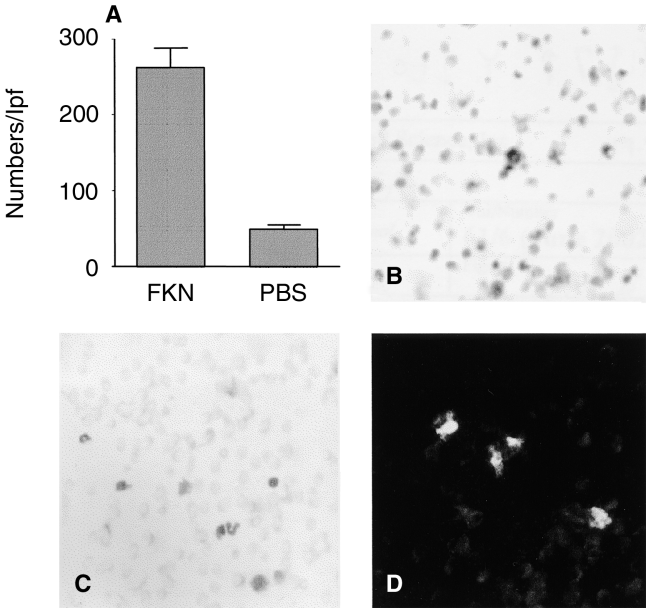

Fig. 7. Chemotaxis of glomerular leukocytes to fractalkine (FKN). Chemotaxis of inflammatory leukocytes isolated from glomeruli of rats injected with mAb 1-22-3 to FKN was assayed. The mean numbers of migratory cells per low power field (×200) on the undersurface of the filter were shown (*A*). The representative Giemsa staining specimens of the inflammatory leukocytes migrated to undersurface of the filter of the chamber with FKN (*B*), and of the chamber with PBS/BSA only (*C*). (*D*) Immunofluorescence finding of the filter with ED3 shows that $ED3^+$ cells were attracted by FKN (B, C ×200; D ×400).

Effect of candesartan on the expression of fractalkine and the recruitment of CX_3CR1^+ cells

We confirmed that the candesartan treatment ameliorated the matrix score at six weeks after disease induction

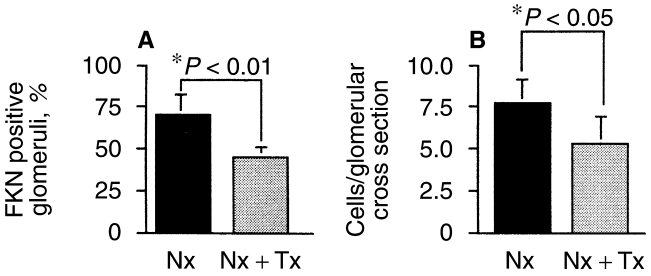

Fig. 8. Comparison of fractalkine (FKN) expression and the recruitment of CX_3CR1^+ cells in the glomeruli between candesartan-treated (Nx + Tx) and non-treated rats (Nx). The rate of FKN positive glomeruli (*A*) and the number of CX_3CR1^+ cells in glomeruli (*B*) on the 14th day after mAb 1-22-3 injection into uninephrectomized rats was determined. FKN expression and the number of CX_3CR1^+ cells in glomeruli were significantly decreased by treatment with candesartan.

(0.38 ± 0.096 vs. 1.40 ± 0.16 candesartan treatment vs. PBS treatment, $P < 0.01$). However, the candesartan treatment did not ameliorate the matrix score on day 14 (1.22 ± 0.133 vs. 1.23 ± 0.068, candesartan treatment vs. PBS treatment). There were no significant differences of C_{Cr} or serum creatinine on the 14th day between candesartan and PBS treated groups (candesartan treatment vs. PBS treatment, 4.20 ± 0.59 vs. 4.38 ± 0.35 mL/min/kg body wt; serum creatinine: 0.46 ± 0.03 vs. 0.50 ± 0.04 mg/dL; serum creatinine of normal rat findings: 0.29 ± 0.05 mg/dL). Candesartan treatment did not decrease SBP on day 11 (112.64 ± 49.1 vs. 128.94 ± 24.21 mm Hg candesartan treatment vs. PBS treatment; SBP of normal rat, 129.22 ± 12.2 mm Hg). By contrast, decreased immunostaining of fractalkine was observed in the candesartan treated group on the 14th day (Fig. 8A). The number of CX_3CR1^+ cells in the glomeruli of the candesartan treated group on the 14th day was significantly less than in the PBS treated group (Fig. 8B). Decreased mRNA expressions of fractalkine and CX_3CR1 were observed in the candesartan treated group on the 14th day (Fig. 9). Real-time RT-PCR analysis also showed that decreased expression of glomerular mRNA for fractalkine in rats treated with candesartan. Although candesartan treatment decreased mRNA expression of MDS, the decrease of MDC was not so remarkable if compared with the decrease of fractalkine (Fig. 9). Candesartan treatment increased mRNA expression of MCP-1 (Fig. 9).

mRNA expression of fractalkine and CX_3CR1 in mAb1-22-3 two consecutive injections model

In the two consecutive injections model, mRNA expression of fractalkine and CX_3CR1 in the glomeruli after the second injection was higher than those after the first injection. The RT-PCR data on the 14th day after the first and second injections are shown in Figure 10.

DISCUSSION

We previously reported that a single injection of anti-Thy 1 mAb 1-22-3 [32, 33] into the uninephrectomized rats was capable of inducing the persistent proteinuria and the prolonged mesangial alterations (prolonged model) [18, 30], although the mesangial alterations induced by a single injection of mAb 1-22-3 to normal rats without uninephrectomy were reversible (reversible model) [32]. While the hemodynamic change caused by a uninephrectomy is considered to affect the outcome of the disease, the precise mechanism of the prolonged model has not been clarified yet. To compare the initiation events just after mAb 1-22-3 injection and the consequent inflammatory responses in the glomeruli between the sham operated reversible model and the uninephrectomized prolonged model is a successful way to identify the essential factor in bringing about the prolonged alterations.

First, the initiation events just after the mAb 1-22-3 injection were compared in these models. The amount of the kidney binding mAb 1-22-3 was higher in the nephrectomized group than in the sham operated group (11.3 vs. 8.55 μg/kidney; Table 1). However, our previous quantitative study with various injection doses of mAb 1-22-3 showed that there was no significant difference in the infiltrating cells and the severity of mesangial alterations between 0.5 mg injected rats (kidney binding, 7.2 μg/kidney) and 10 mg injected rats (kidney binding, 32.7 μg/ kidney) [27]. We experienced that the prolonged mesangial alterations could be induced by a half dose of mAb 1-22-3 injection into uninephrectomized rats (data not shown). We did not detect the significant differences of the complement deposition and the severity of the mesangiolysis between the nephrectomized and the sham operated models (Table 2). These observations indicate the difference of the kidney binding antibody between the sham operated and the uninephrectomized groups had little effect on the different outcomes of the disease.

Next, we compared the expression of chemokine and the recruitment of inflammatory cells in glomeruli after mAb 1-22-3 injection in the sham operated reversible model and the uninephrectomized prolonged model. Fractalkine expression of the uninephrectomized group was significantly higher than that in the sham operated group, although no difference in the expressions for MCP-1, TARC, lymphotactin, or RANTES was detected. The mRNA expression of the fractalkine on the 14th day of the uninephrectomized prolonged model was 2.5 times higher than that of the sham-operated reversible model (Fig. 4). In addition, clear positive immunostaining of fractalkine in glomeruli was detected in the uninephrectomized prolonged model at 24 hours, whereas fractalkine staining in the sham-operated reversible model was weak and focal at any time points examined.

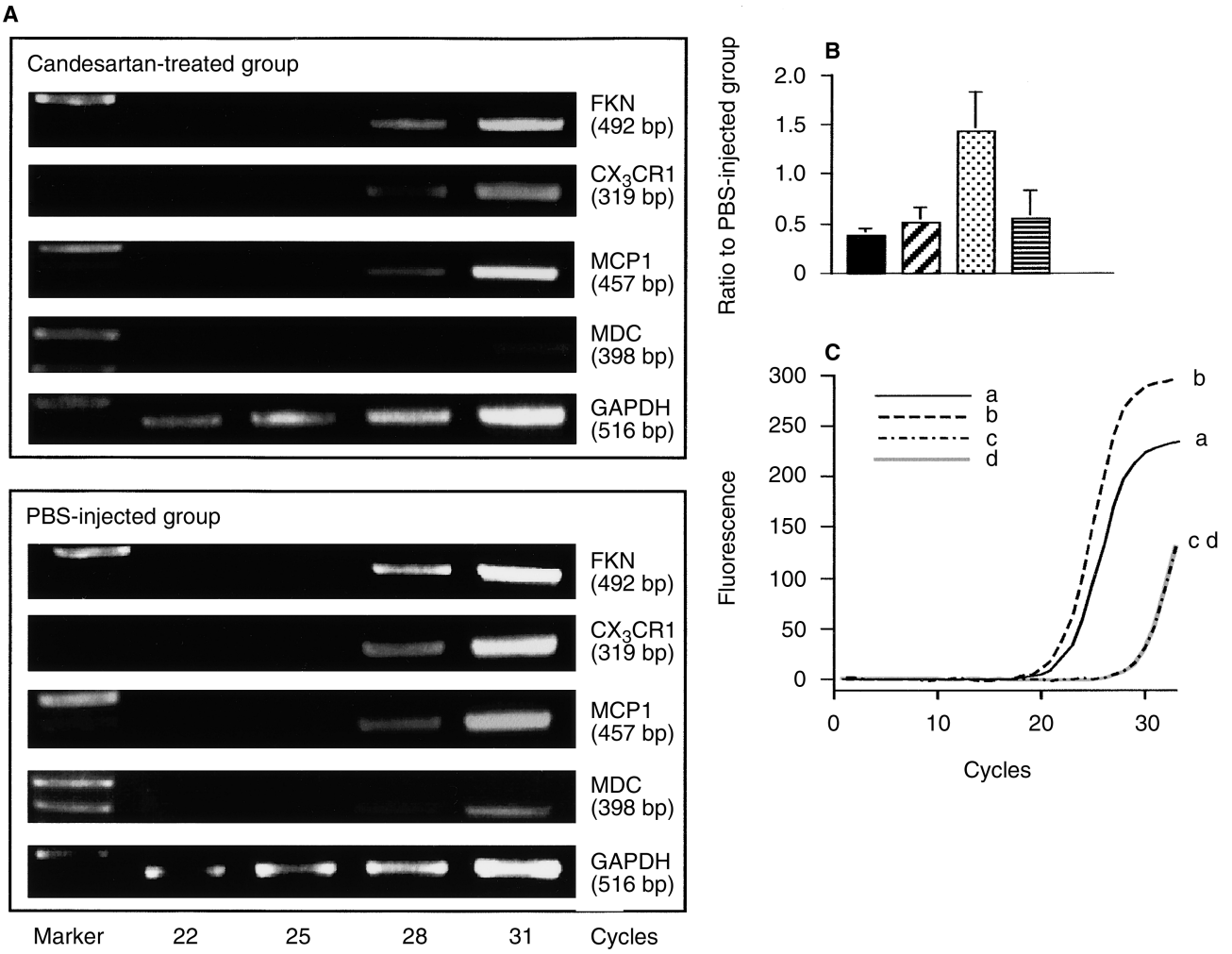

Fig. 9. Chemokines and CX_3CR1 mRNA expression on the 14th day after mAb 1-22-3 injection with or without treatment of candesartan. mRNA expression was semiquantitated by RT-PCR using cDNA corresponding to 750 ng RNA. The optimal cycle numbers were determined in a preliminary trial to be in the linear phase of amplification. (*A*) The findings of candesartan treated group are shown in the upper panel, and those of the nephritic control without treatment of candesartan (PBS injected group) are in the bottom panel. Representative agarose gel electrophoretic patterns of PCR products of FKN, CX_3CR1, MCP-1, MDC, and GAPDH in 3 cycle intervals are shown. (*B*) Ratios of the densitometric signals of (■) FKN, (▨) CX_3CR1, (▩) MCP-1 or (▤) MDC and the internal control (GAPDH) were analyzed. The data are shown as ratio relative to PBS injected group and are expressed as mean ± SD of three independent experiments. Candesatran treatment reduced the expression of FKN and CX_3CR1. By contrast, candesartan treatment increased MCP-1 mRNA expression. (*C*) Real-time RT-PCR analysis of fractalkine in rats treated with candesartan. The x-axis shows the number of PCR cycles, and the y-axis is the difference between measured fluorescence and the baseline. Decreased expression of fractalkine in rats treated with candesartan was confirmed. Lines are: (a) GAPDH, PBS injection; (b) GAPDH candesartan treated; (c) FKN, PBS injection; (d) FKN, candesartan-treated.

We also observed that mRNA expression of CX_3CR1 (Fig. 4) and the number of the recruited CX_3CR1^+ cells in glomeruli were much higher in the uninephrectomized model than in the sham operated model. We confirmed that fractalkine was concerned with the recruitment of leukocytes into glomeruli in mAb 1-22-3-induced nephropathy by the chemotaxis experiment using the leukocytes prepared from glomeruli of rats injected with mAb 1-22-3 (Fig. 7). To analyze whether the expression of fractalkine and CX_3CR1 is the unique finding in the uninephrectomized prolonged model, or if they commonly participate in the development of the prolonged mesangial alterations, the fractalkine expression was investigated in another prolonged model induced by two consecutive injections of mAb 1-22-3 into rats without pre-uninephrectomy. The expressions of fractalkine and CX_3CR1 after the second mAb 1-22-3 injection were much higher than those after the first injection (Fig. 10). Together, these findings suggested that fractalkine expression and the recruitment of CX_3CR1^+ cells play an important role on the development of the prolonged glomerular alterations.

The reason that fractalkine expression is higher in these prolonged models than in the reversible model

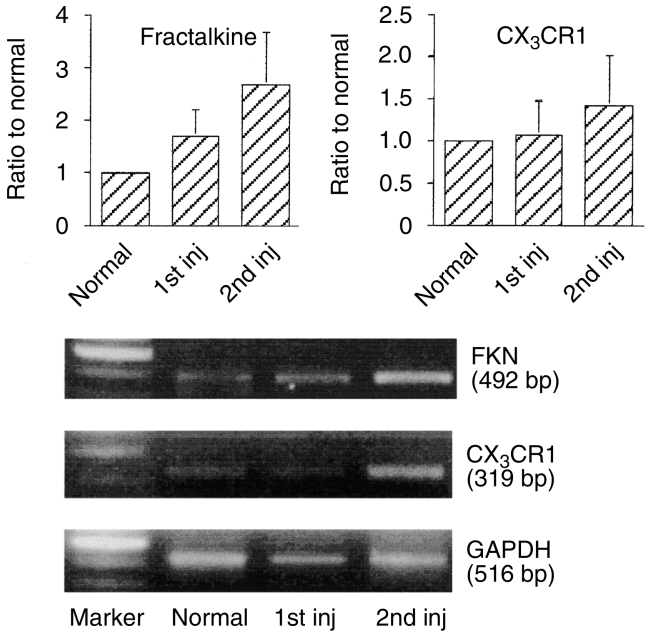

Fig. 10. Fractalkine (FKN) and CX_3CR1 expression on the 14th day after the first and the second mAb 1-22-3 injections. mRNA expression was semiquantitated by RT-PCR using cDNA corresponding to 750 ng RNA. The optimal cycle numbers were determined in a preliminary trial to be in the linear phase of amplification. Ratios of the densitometric signals of FKN or CX_3CR1 and the internal control (GAPDH) were analyzed. The data are shown as ratio relative to normal rat findings and are expressed as mean ± SD of three independent experiments. FKN and CX_3CR1 mRNA expressions after the second mAb injection are higher than those after the first injection.

remains unclear. However, the findings obtained in this study give us a possible clue. Fractalkine is considered to be important for leukocytes recruitment especially in tissue with a high blood flow rate [34, 35], because fractalkine functions as both a chemoattractant and an adhesion molecule for leukocytes [20, 21]. Feng et al reported that fractalkine might play an important role for the recruitment of leukocytes in glomeruli, because glomeruli have a high blood flow rate [22]. Because uninephrectomy causes glomerular hypertension in the remaining kidney, it is conceivable that fractalkine might play a more important role in the remaining kidney of our nephrectomized model. Fractalkine was expressed more actively under the condition with glomerular hypertension for the development of inflammatory responses. In the prolonged model caused by the consecutive injections of mAb 1-22-3, the locally increased blood pressure in the glomeruli—the structure of which was damaged by the first injection of mAb 1-22-3—might be involved in the highly expression of fractalkine. Of course, another possibility cannot be ruled out. Two recent reports have suggested that glomerular hypertension is not essential for the development of prolonged glomerular alterations [36, 37]. Another possibility is that some cytokines act on mesangial cells to produce fractalkine. Whatever the mechanism, we believe that the increased expression of fractalkine in the injured glomeruli contributes to causing the prolonged glomerular alterations.

This study shows that AT1RA treatment ameliorated the glomerular morphological findings at six weeks in the nephrectomized prolonged model, although the treatment did not ameliorate the glomerular morphological findings at two weeks. We clearly demonstrated that the immunostaining and mRNA expression of fractalkine and the number of CX_3CR1^+ cells recruited in glomeruli were already decreased at two weeks after disease induction in rats treated with AT1RA (Figs. 8 and 9). These findings indicate that the expression of fractalkine/CX_3CR1 could be a predictor of the prolonged disease, and suggest that fractalkine/CX_3CR1 contributes to the development of the glomerular alterations. Although the precise therapeutic mechanism of AT1RA on proteinuria and mesangial proliferation remains unclear [38–42], it has been reported that AT1RA ameliorates the glomerular hypertension by relaxing the efferent microartery [42]. AT1RA did not influence the glomerular alterations detected at two weeks after disease induction. The early stage of Thy 1 nephritis is characterized by the mesangiolysis caused by the binding of anti-Thy 1 antibody and the consequent mesangial cell proliferation and the matrix expansion detected at two weeks. It is accepted that these alterations are self-limiting. These glomerular alterations of the early stage were observed similarly in both prolonged and the reversible models. We consider that glomerular hypertension contributes to the development of the prolonged glomerular alterations. It is conceivable that AT1RA prevents the development of the prolonged alterations by reducing the glomerular hypertension. Interestingly, we observed the increased MCP-1 expression in rats treated with AT1RA. Stahl et al reported that MCP-1 is produced by mesangial cell in Thy 1 nephritis [43]. Our current study shows that fractalkine also is produced by mesangial cells. The reason why AT1RA treatment increased the expression of MCP-1 is not clear. However, this might be explained by the following mechanism: In the high shear environment, mesangial cells produce fractalkine more actively than MCP-1. The AT1RA treatment might prevent this alternative response of the mesangial cell, which then would increase the MCP-1 expression.

Using dual-labeled IF studies, the localization of fractalkine was analyzed to determine the exact subpopulations of the leukocyte containing the CX_3CR1^+ cells. Fractalkine was expressed by endothelial cells in the early phase of the uninephrectomized model (Fig. 5D). Interestingly, fractalkine staining was detected not only at the endothelial cells but also in the mesangial area (Fig. 5 E, F) in the later phase of the prolonged model. Dual labeling studies with markers of inflammatory cells

showed that the major parts of the CX_3CR1^+ cells in glomeruli are not T-lymphocytes but macrophages (Fig. 5 G-I and Fig. 6). Our study used two phenotypic markers of macrophages, ED1 and ED3. ED1 is widely used as a pan-monocyte/macrophage marker, and ED3 was originally reported as confined to lymphoid organs [44] but $ED3^+$ cells have been found at sites of immunologically-mediated tissue inflammation in several experimental models [45–48]. Although the precise characteristics of the $ED3^+$ cells has not been clarified, $ED3^+$ cells are reported to be activated macrophages. Our findings showed that 66.7% of CX_3CR1^+ cells in glomeruli were $ED3^+$ cells, and 69.5% of the total $ED3^+$ cells in glomeruli were CX_3CR1^+ cells on the fifth day of the prolonged model. In vitro chemotaxis analysis showed that $ED3^+$ cells in glomeruli in mAb 1-22-3-induced GN could be attracted by fractalkine (Fig. 7D). All of these findings suggest that the $ED3^+/CX_3CR1^+$ cells attracted by fractalkine play important roles for the development of the prolonged glomerular alterations.

In conclusion, immunohistological study and chemotaxis analysis shows that fractalkine is highly concerned with the recruitment of leukocytes into glomeruli in mAb 1-22-3–induced nephritis. Our study demonstrates that the expression of fractalkine and the recruitment of CX_3CR1^+ cells in glomeruli are dominant in the prolonged models of mesangial proliferative GN. Taken together, these findings suggest that the fractalkine/CX_3CR1 might be involved in the development of the prolonged mesangial alterations.

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