Anti-monocyte chemoattractant protein-1 gene therapy attenuates nephritis in MRL/lpr mice

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Objective. Monocyte chemoattractant protein-1 (MCP-1) is up-regulated and recruits and activates inflammatory cells in human diffuse proliferative lupus nephritis (DPLN) and in nephritis of lupus model MRL/lpr mice. The aim of this study was to examine whether anti-MCP-1 gene therapy inhibits the progression of nephritis in MRL/lpr mice.

Method. An NH₂-terminal deletion mutant of the MCP-1 gene, 7ND, was injected into skeletal muscles of MRL/lpr mice with advanced stage nephritis to blockade MCP-1 and its receptor (CCR2) signalling pathway.

Result. Histological findings of kidneys in treated mice, which received more than four injections of 7ND, showed that protection against renal injury resulted from reduced infiltration of leucocytes. Therefore, this therapy has been shown to prolong the life span of MRL/lpr mice.

Conclusion. Anti-MCP-1 gene therapy is specifically effective in the localized inflammatory region. The data presented here indicate that this anti-MCP-1 gene therapy may be effective adjunct in the management of DPLN.

Key words: Monocyte chemoattractant protein-1 (MCP-1), Systemic lupus erythematosus (SLE), MRL/lpr mice, Diffuse proliferative lupus nephritis (DPLN), Gene therapy.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of pathogenic autoantibodies and tissue deposition of immune complexes, resulting in the rather common but serious complication of lupus nephritis. Diffuse proliferative lupus nephritis (DPLN), which is categorized as World Health Organization (WHO) class IV, is the most common, severe and important form of lupus nephritis. Patients with DPLN usually have an unfavourable prognosis, and a high percentage of these eventually progress to renal failure despite aggressive treatment [1].

MRL/lpr mice are particularly valuable for understanding the pathogenesis of autoimmune diseases, since tissue destruction in this model is spontaneous, predictable, steadily progressive and fatal [2]. Kidney disease consists of glomerular, interstitial and vascular components mediated by infiltrating macrophages and T cells, and MRL/lpr mice are a particularly suitable model for DPLN. We previously reported that in the kidney tissue of human DPLN the majority of the numerous mononuclear cells in the interstitium were positive for CD68 (macrophages) and CD3 (pan-T cells); while there were many CD68+ cells, there were only a few CD3+ cells in the glomeruli [3]. These migrations of leucocytes into the kidneys are dependent on chemokines released by injured kidney tubular epithelial and mesangial cells [4, 5].

In the chemokine family, the monocyte chemoattractant protein-1 (MCP-1) is a potent chemoattractant for monocytes, T cells and natural killer cells [6–8]. It may also be responsible for inflammation in tissues during autoimmune disease [9]. Tissue expression of MCP-1 in human DPLN and rheumatoid arthritis [10–13], and mouse lupus nephritis models (NZB/W mice and MRL/lpr mice) were up-regulated; MCP-1 recruits and activates inflammatory cells [4, 14]. In addition, the fact that MCP-1 deficiency resulted in improvement of glomerulonephritis in MRL/lpr mice [15] and that treatment with a MCP-1 antagonist results in the reduction of arthritis in MRL/lpr mice suggests that MCP-1 may be responsible for inflammation in tissues during the progress of an autoimmune disease [16].

To block MCP-1 and its receptor (CCR2) signalling pathway we used the strategy of anti-MCP-1 gene therapy involving the transfection of a mutant human MCP-1 gene into skeletal muscle [17, 18]. This mutant human MCP-1, designated 7ND, lacks NH₂-terminal amino acids 2–8 and has been shown to work as a dominant-negative inhibitor of human MCP-1 [19, 20]. The amino acid sequence of human MCP-1 and the region of murine MCP-1 that is responsible for the chemoattractant activity share considerable primary sequence homology; 68% sequence identity with conservative amino acid substitutions at most of the non-identical positions [21]. Although murine MCP-1 extends 49 amino acids farther in the C-terminal direction than human MCP-1, which may be a feature common to rodent MCP-1 [22–24], it has a potent chemoattractant activity for human monocytes in vitro [25]. Similarly human 7ND protein blocks the MCP-1/CCR2 signal pathways and suppresses the chemoattractant activities in several different animals [26].

Here, we show that application of 7ND is effective for treatment of kidney disease in MRL/lpr mice at an advanced stage, and that this anti-MCP-1 gene therapy may be a prospective therapy for DPLN.

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Materials and methods

Mice

MRL/lpr mice were purchased from the Jackson Laboratory, and bred and maintained in the Laboratory of Animal Experiments at Kyushu University. The present experiments were reviewed and approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiologic Society.

Plasmid expression vectors

Human 7ND complementary deoxyribonucleic acid (cDNA) was constructed by a recombinant chain reaction using wild-type human MCP-1 cDNA (a generous gift from Dr T. Yoshimura, National Cancer Institute, Bethesda, MD, USA) as a template, and inserted into the BamHI (5′) and NotI (3′) sites of the pcDNA3 expression vector plasmid (Invitrogen, Carlsbad, CA, USA).

Gene transfer

Treatments were initiated in 16-week-old MRL/lpr mice, the kidneys of which showed an advanced stage of glomerulonephritis. Under anaesthesia by intraperitoneal injection of pentobarbital, the 7ND-treatment group mice (7ND-treated mice) were injected with the 7ND gene [50 μg in 50 μl phosphate-buffered saline (PBS)], and empty plasmid treatment group mice (empty plasmid-treated mice) were injected with empty plasmid (50 μg in 50 μl PBS) in the femoral muscle using a 27-gauge needle. The control group mice (PBS-treated mice) were injected with 50 μl PBS. To enhance transgene expression, all mice received electroporation at the injected site. Immediately after the injections into the femoral muscle, a pair of electrode needles (Tokiwa Science Ltd., Fukuoka, Japan) were inserted into the muscle 5 mm apart on either side of the injected site. Six 100 V square wave pulses (150 ms apart) were applied, followed by six pulses of the opposite polarity for 50 ms, using a CUY201 electric pulse generator (Nepagene Co. Ltd, Chiba, Japan). The wound was then closed. No inflammation was observed at the injection site. The three groups received injections at bi-weekly intervals up to 28 weeks of age.

Lymphadenopathy and splenomegaly

After killing, the weights of the total swelling lymph nodes (cervical, brachial, inguinal and mesenteric) and the spleen were measured to evaluate lymphadenopathy and splenomegaly.

Serological analysis

Anti-DNA antibodies in the serum were analysed by immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and the amounts were represented by the value of absorbance at optimal density at 450 nm as described by Burlingame et al. [27]. For serum Ig determination, ELISA was performed using the following antibodies: rat anti-mouse IgG1 (Zymed Laboratories Inc., South San Francisco, CA, USA: 04–6100), rat anti-mouse IgG1-HRP (BioSource International Inc., Camarillo, CA, USA: AMI2311), goat anti-mouse IgG2a (Bethyl Laboratories Inc., Montgomery, TX, USA) and rabbit anti-mouse IgG2a-HRP (Cappel Lab., Cochranville, PA, USA: 50283). Serum mouse MCP-1 concentrations were measured using a commercially available ELISA kit (BioSource International Inc., CA, USA). To evaluate amounts of 7ND released by the transfected skeletal muscle, serum 7ND concentrations were determined using a human MCP-1 ELISA kit (BioSource International Inc., Camarillo, CA, USA). Since this human MCP-1 ELISA kit does not react with mouse MCP-1, it is reasonable to assume that the serum 7ND concentrations can be measured with this kit [27].

Histopathology and immunohistopathological studies of kidneys

Kidneys were fixed in 10% formalin for 24 h at 4°C. Paraffin sections (4 μm) were stained with periodic acid Schiff (PAS) reagent. For immunohistochemical staining, kidneys were snap-frozen in optimal cutting temperature (OCT) compound (Sakura Japan, Osaka, Japan). To detect immune complex (IC) deposits, cryostat sections (2 μm) were fixed in chilled acetone and stained with a fluorescein (FITC) conjugated goat polyclonal anti-mouse IgG antibody (Organon Teknica, PA, USA). MCP-1 was detected using a goat polyclonal anti-rat MCP-1 antibody (Santa Cruz Biotechnology, CA, USA), followed by biotin-conjugated anti-goat IgG (Nichirei Co. Ltd, Tokyo, Japan). The endogenous peroxidase activity was quenched by incubation in a solution of methanol/0.3% H2O2, and then the sections were incubated with a streptavidin complex (Nichirei Corp., Tokyo, Japan). Staining was visualized by incubation with diaminobenzidine (Nichirei Corp., Tokyo, Japan). For negative controls, sections were treated with normal goat IgG (Santa Cruz Biochemistry, CA, USA).

Immunohistochemical staining of Bouin-fixed and paraffin-embedded sections was performed as follows. After blocking the endogenous peroxidase activity, deparaffinized sections were incubated with the primary antibody, rat monoclonal anti-mouse F4/80 antibody (BMA Biomedicals, Reinstrasse, Switzerland). After incubation with the secondary antibody, biotin-conjugated anti-rat IgG antibody (Vector Laboratories Inc., CA, USA), sections were incubated with a streptavidin complex (Nichirei Corp., Tokyo, Japan). For negative controls, sections were treated with normal rat IgG (Santa Cruz Biochemistry, CA, USA).

The severity of glomerulonephritis was evaluated by histological examination of the sectioned kidneys in a blinded manner; using the crescent formation rate and the number of macrophages in the glomerulus. We evaluated glomerular pathology by assessing 100 glomerular cross sections (gcs) per kidney, and the crescent formation rate was defined as the number of crescents formed. F4/80+ cells were counted in each glomerular cross section and their average among 100 gcs was then calculated. Perivascular cell accumulation was determined semi-quantitatively by scoring the number of cell layers surrounding the majority of vessel walls (score: 0 = none, 1 = <5, 2 = 5–10 and 3 > 10). The scoring was evaluated using coded slides.

Histopathology of the lungs

The lungs were inflated and fixed in 10% formalin, and paraffin sections (4 μm) were stained with haematoxylin and PAS reagents. Peribronchiolar and perivascular infiltrates were assessed semiquantitatively in >20 bronchioli per section and in >20 vessels per section (number of cell layers surrounding the majority of bronchioli or vessels; score 0 = none, 1 = 1–3, 2 = 3–6 and 3 > 6). The scoring was evaluated using coded slides.

Laser capture microdissection (LCM) and RNA isolation

Glomeruli were microdissected from consecutive tissue sections as described by Sgroi et al. [28] using a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). Total ribonucleic acid (RNA) was extracted from the captured cells using a Picopure RNA isolation kit (Arcturus Engineering, Mountain View, CA).
Real-time quantitative polymerase chain reaction (PCR)
We assessed the transcription levels of MCP-1 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the glomeruli. Reverse transcription reactions and TaqMan PCRs were performed according to the manufacturer’s instructions (Applied Biosystems Japan Ltd, Tokyo, Japan). Sequence-specific amplification was detected with an increased fluorescent signal of 5’-[(N′-(3’-diphenylphosphinyl-4’-methoxycarbonyl) phenylcarbonyl) aminoacetamido] fluorescein (FAM) during the amplification cycles using an ABI prism 7700 sequence detection system (Perkin Elmer Japan Co. Ltd, Yokohama, Japan). Oligonucleotide primers and probes were designed using the Primer Express program and synthesized (Applied Biosystems Japan Ltd, Tokyo, Japan). These sequences (5’ to 3’) follow. Forward primer sequence: 5’-CTCTTTCCATTTTTGCATCAAGTTC-3’, Reverse primer sequence: 5’-CCCATCTTTAACCGATCTAGAGTCA-3’ and probe sequence: 5’-CTCCGCCCACTCATCTTCGGTGG-3’.

Statistical analysis
Data are expressed as the mean ± S.E. Statistical differences were compared by Mann–Whitney U-tests. A level of P < 0.05 was considered statistically significant.

Results
Effect on glomerulonephritis
The kidneys of the PBS-treated (Fig. 1A) and empty plasmid-treated (Fig. 1B) 24-week-old mice showed typical glomerulonephritis lesions, which included an enlarged glomerulus, proliferation of glomerular cells, infiltrating inflammatory cells, increased mesangial matrix and crescent formation. In contrast, those of the 7ND-treated 24-week-old mice showed a lesser degree of glomerulonephritis changes, varying degrees of focal glomerular cell proliferation, occasional inflammatory cells and slight increases in the mesangial matrix (Fig. 1C). It was apparent that there was a great reduction in the number of F4/80+ cells (macrophages) in the interstitium (Fig. 2C), periglomeruli and intraglomeruli (Fig. 3C) in the kidneys of the 7ND-treated mice compared with those of the PBS-treated and empty plasmid-treated mice (Figs. 2A, 3A and 2B, 3B). The significant difference in the severity of the kidney lesions among the three groups is represented by the statistically different crescent formation rate (PBS: 0.220 ± 0.180; empty plasmid: 0.251 ± 0.150; 7ND: 0.109 ± 0.082) (Fig. 4A) and the number of macrophages that accumulated in the glomeruli (PBS: 6.01 ± 1.89; empty plasmid: 6.29 ± 1.64; 7ND: 2.91 ± 1.58) (Fig. 4B). In contrast to the results for interstitium, periglomeruli and intraglomeruli, no meaningful reduction in the number of infiltrating cells was seen in the perivascular region in 7ND-treated mice. The vasculitis scores were 2.70 ± 0.48 in the PBS-treated mice (Fig. 5A), 2.55 ± 0.50 in the empty plasmid-treated mice (Fig. 5B) and 2.60 ± 0.52 in the 7ND-treated mice (Fig. 5C), and there was no significant difference among them.

Fig. 1. Histopathological findings of kidneys from MRL/lpr mice at 2 months after the initial treatment. Representative PAS-stained sections in 400× magnification features of PAS-stained kidney sections of (A) PBS-treated mice, (B) empty plasmid-treated mice and (C) 7ND-treated mice. PBS-treated and empty plasmid-treated mice developed proliferative glomerulonephritis with glomerular crescents. Proliferative changes and crescent formation were attenuated in 7ND-treated mice.

Fig. 2. Immunohistochemical staining for F4/80+ cells of the kidney sections at 100× magnification: (A) PBS-treated mice, (B) empty plasmid-treated mice and (C) 7ND-treated mice.

Fig. 3. 400× magnification features of immunohistochemical staining kidney sections. F4/80+ cells are stained dark brown with pale green nuclei, while F4/80– cells are identified by nuclear staining only. Note the presence of numerous F4/80+ macrophages in the interstitium and glomeruli in the kidney of PBS-treated and empty plasmid-treated mice. However, kidneys of 7ND-treated mice show a reduced number of infiltrating F4/80+ macrophages.
The protection from proteinuria

Bi-weekly, urine protein levels for 15 mice in each group were assessed semiquantitatively by dip-stick analysis. There was no significant difference concerning proteinuria among the three groups (data not shown).

Lung histopathology

It was apparent that there was a great reduction in the number of infiltrating cells in the peribronchiolar region of the lung of 7ND-treated mice (Fig. 6C) compared with those of PBS-treated (Fig. 6A) and empty plasmid-treated mice (Fig. 6B). The infiltration scores were 1.52 ± 0.13 in the PBS-treated mice, 1.64 ± 0.10 in the empty plasmid-treated mice and 0.323 ± 0.12 in the 7ND-treated mice, respectively. There were significant differences between the score of 7ND-treated mice and those of other two mice groups (Fig. 7A), but there is no significant difference in the perivascular region among three groups (Fig. 7B).
The 7ND-treated mice had a prolonged lifespan compared with those of the PBS-treated and empty plasmid-treated mice. After the fourth gene injection (at 24 weeks of age), the differences in survival rates among the three groups were distinct (Fig. 8A). The fact that apparent improvements in the pathological changes in the kidneys of the treated mice were seen at 24 weeks of age may reflect the significantly prolonged survival rate which had the same timing.

Lymphadenopathy and splenomegaly
To determine the effects of 7ND treatment in MRL/lpr mice on lymphadenopathy and splenomegaly, total body weight, total weight of the cervical, axillary, inguinal and mesenteric lymph nodes and spleen weight were determined in the three groups at 24 weeks. No significant differences were observed among the three groups (Table 1).

Transgene expression and serum concentrations of MCP-1 and 7ND
We measured MCP-1 and 7ND after 7ND transfection. Serum MCP-1 concentrations did not change during the course of the experiments, whereas 7ND was detected in serum at 3, 5 and 7 days after transfection with the intramuscular injection of 7ND plasmid DNA (50 µg) (Fig. 8B).

Mouse MCP-1 production in the kidney
To investigate whether 7ND injection had an effect on local tissue MCP-1 production we examined mouse MCP-1 expression in kidney, serum concentrations of MCP-1 and mRNA production of MCP-1 in kidney in each of the three groups. The immunohistochemical staining with anti-MCP-1 of renal tissues failed to show a difference among three groups. RNAs extracted from micro-dissected glomeruli by LCM were analysed by real-time quantitative PCR. There were no significant differences among three groups in the serum concentration (Fig. 9A) or the values of mRNA (Fig. 9B).

Glomerular IgG deposits
Although statistically significant differences in the crescent formation rate and the number of macrophages accumulated in the glomeruli were observed, serum IgG concentrations and IgG deposits in the kidneys were not reduced after treatment (data not shown). These results show that 7ND could not prevent immune complex deposition in kidneys but could protect against accumulation of inflammatory cells, which infiltrate into kidneys to clean up IC deposits.

Discussion
We have demonstrated that intramuscular transfer of a mutant MCP-1 gene inhibits the MCP-1/CCR2 signalling pathway...
and significantly attenuates glomerulonephritis in MRL/lpr mice. Histological findings showed that protection against renal injury resulted from a reduced infiltration of leucocytes (macrophages and T cells) into kidney interstitium and glomeruli. Therefore, this mode of treatment improved the survival rate of MRL/lpr mice, even though it was initiated at an advanced stage of the disease. However, we could not see any significant effect on protection from proteinuria, which was documented in the study of MCP-1 deficient MRL/lpr mice [15]. There was considerable interindividual variation in levels of proteinuria, and the number of the mice we treated might have been too small for the confirmation of any existing difference.

Rollins and co-workers [19, 20] clearly demonstrated under in vitro conditions that mutant MCP-1 forms inactive heterodimers with wild-type MCP-1 and exerts its inhibitory activity in a dominant-negative manner. Actually NH₂-terminal-truncated mouse MCP-1 secreted by a non-metastatic fibroblastoid cell line subcutaneously injected into MRL/lpr mice ameliorates the initiation and progression of nephritis [29]. Similarly Egashira et al. [30] reported that mutant human MCP-1 7ND protein secreted from the transfected skeletal muscle cells into the circulating blood blocks the MCP-1/CCR2 signal pathway in remote target organs or tissue, and suppresses monocyte recruitment. The same effect of anti-MCP-1 gene therapy as in our study was shown in renal ischaemia-reperfusion injury mice by Furuichi et al. [31]. It is surprising that MCP-1/CCR2 signalling should be effectively blocked by a dominant-negative protein produced in a distant muscle tissue. Mir et al. [32] showed the advantages of in vivo electroporation in muscle tissues as follows. In vivo electroporation increases the efficiency of gene transfer not only by cell permeabilization but also by promoting DNA migration and cellular uptake, resulting in a 2- to 4-log enhancement of gene expression. There is no fast extracellular degradation of plasmid DNA in muscle tissue, and there is less interindividual variability. The expression level can be regulated by modulating the amount of DNA injected, electric-pulse parameters or the volume of tissue exposed to the electric pulses. Thus, intramuscular transfer of the 7ND gene into MRL/lpr mice could achieve an effective and sufficient blockade of MCP-1 activity in the kidneys.

Usui et al. [18] demonstrated that 7ND gene therapy inhibited restenotic changes in the common carotid artery after balloon injury in rats and monkeys. They reported that 7ND was detected in the plasma until 14 days after transfection, and that the histological appearance of matrigel plug sections showed significant suppression of MCP-1-induced angiogenesis at 7 and 14 days, but not at 28 days after the 7ND gene injection [18]. In our study, mice that received 7ND injections at 14-day intervals could significantly attenuate the glomerulonephritis, while those at 28-day intervals could not, supports the conclusion that this strategy was effective against glomerulonephritis in a dose-dependent manner.

In the kidneys of 24-week-old MRL/lpr mice injected with PBS or empty plasmid, the majority of macrophages and T cells were localized in the interstitium adjacent to tubules and glomeruli, whereas fewer were identified within glomeruli. The numbers of macrophages and T cells in the interstitium and macrophages in the glomeruli were significantly reduced in 7ND-treated
MRL/lpr mice (Fig. 1). In contrast to this improvement in the interstitium and glomeruli, a decreased accumulation of macrophages or T cells surrounding vessels was not detected in 7ND-treated MRL/lpr mice (Fig. 5). The effects on lungs were also evaluated. Comparing numbers of infiltrated cells in the lung of PBS-treated or empty plasmid-treated mice, 7ND-treated mice showed dramatically reduced numbers of infiltrated cells surrounding bronchioles (Figs. 6C, 7C), but not surrounding vessels (Fig. 7D). Tesch et al. [15] reported that the reduction in the number of perivascular infiltrating cells was not seen and demonstrated a substantial increase in locally proliferating cells in the perivascular region, which showed weak MCP-1 expression, as compared with surrounding glomeruli and tubules in MRL/lpr MCP-1-deficient mice. Although Hasegawa et al. [29] showed NH2-terminal-truncated mouse MCP-1 attenuated renal vasculitis in MRL/lpr mice, the effectiveness on granulomatous lesion in perivascular lesions was insufficient. These findings support that regional proliferation of the infiltrating cells rather than recruitment of them into the lesion plays a central role in the pathogenesis of vasculitis, and this mechanism may be a cause for ineffectiveness of the blockade of the MCP-1 signalling pathway on the attenuation of the vasculitis.

In this study, RT-PCR and immunohistochemical examination of kidney tissues showed that 7ND transfection did not influence intrinsic MCP-1 transcription, or production. Furthermore, 7ND did not alter the amount of anti-dsDNA antibody, the isotype of immunoglobulin or the IgG deposit pattern. These results indicate that 7ND has little effect on helper T-cell polarization, but acts directly on leukocytes to inhibit their migration and activation in the kidney of MRL/lpr mice. The administration of 7ND may not induce alteration of the systemic immunological state. Thus anti-MCP-1 gene therapy may be a useful practical strategy for the patients with SLE.

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References


