Midkine antisense oligodeoxynucleotide inhibits renal damage induced by ischemic reperfusion.

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Background. Midkine, a heparin-binding growth factor, is involved in the migration of inflammatory cells. The inflammatory cell migration to the tubulointerstitium of the kidney after ischemia/reperfusion (I/R) injury is attenuated in midkine gene–deficient mice, resulting in better preservation of the tubulointerstitium compared with wild-type mice. In the present investigation, we planned to evaluate the usefulness of antisense midkine for the therapy of ischemic renal failure.

Methods. Midkine antisense phosphorothioate oligodeoxynucleotide (ODN) at a dose of 1 mg/kg in saline was intravenously administered to mice 1 day before or after I/R. The kidneys were removed for examination 1, 2, 3, and 7 days after I/R.

Results. It was rapidly incorporated into proximal tubular epithelial cells, and inhibited midkine synthesis, leading to reduced migration of inflammatory cells to the injured epithelial layer. Consequently, the midkine antisense ODN-treated animals exhibited less severe renal damage than untreated or midkine sense ODN-treated animals 2 days after I/R as assessed by morphologic criteria and blood urea nitrogen (BUN) and serum creatinine levels. Midkine expression, BUN, and serum creatinine levels were not significantly different between injection of midkine antisense ODN before and after ischemic injury.

Conclusion. These results indicate that intravenous injection of midkine antisense ODN is a candidate for a novel therapeutic strategy against acute tubulointerstitial injury induced by I/R injury.

Migration of inflammatory leukocytes is a key event in the cascade leading to pathologic tissue status such as ischemic and autoimmune injury and atherosclerosis [1–8]. Thus, means to interfere with the leukocyte migration are intensely sought to prevent or treat various diseases.

Key words: gene therapy, infiltration, kidney tubules.

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METHODS

ODNs

Phosphorothioate-modified ODNs were synthesized with an automated solid-phase nucleotide synthesizer (Expedite 8900 Nucleic Acid Synthesis System) (Applied Biosystems, Foster City, CA, USA) and subsequently purified using a Wakopak Handy ODS column (Waters). Midkine antisense ODN, 5′-AGGGCGAGAAGGAAGAG-3′, corresponded to bases 15 to 32 [25] in midkine cDNA [4]. Sense ODN, 5′-CTTCTTCTTCTCGCCCT-3′, and scrambled ODN, 5′-GGAAAAGAAACCGGAGG-3′, were used as a control of the antisense DNA. We also used ODNs labeled with fluorescein isothiocyanate (FITC) at 5′ end, which were synthesized and purified in a manner identical to the unlabeled one [26].

Cell culture and transfection of ODNs

CMT-93 cells (American Type Culture Collection, Rockville, MD, USA) derived from mouse rectal carcinoma were used to evaluate ODNs. Conditions of cell culture and ODN transfection were as described previously [25].

Renal I/R injury

Male 129/SV mice at 8 to 10 weeks old were used and fed normal rodent chow. The experiments described below were carried out according to The Animal Experimentation Guide of Nagoya University School of Medicine. A previously characterized mouse model of renal I/R injury was used [4, 27]. The animals were placed on a heating pad to keep a constant body temperature (37°C). Briefly, 7 days after removing the right kidney, the mice were anesthetized and the left kidney was exposed. The renal pedicles were bluntly dissected, and a nontraumatic vascular clamp was applied across the pedicles for 30 or 90 minutes. After the clamps were released, the flanks were closed in two layers with 5-0 silk sutures. The animals received 100 mL/kg warm saline instilled into the peritoneal cavity during the procedure and were allowed free access to food and water. One, 2, 3, and 7 days after ischemia, the left kidneys were removed for examination. In some experiments, 24 hours before ischemic injury, the mice were treated with ODNs at a dose of 1 mg/kg in saline by intravenous injection. Unless otherwise specified, ODN was added 24 hours after the ischemic injury, and experiments were performed using 12 mice.

Histology

The kidneys were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 2 μm sections. They were stained with periodic acid-Schiff (PAS) reagent. Using semiquantitative indices, the sections were analyzed to evaluate acute tubulointerstitial damage in each region. Briefly, the extent of tubular cast formation, tubular dilatation, and tubular degeneration (vacuolar change, loss of brush border, detachment of tubular epithelial cells, and condensation of tubular nuclei) in fields of both cortex and medulla was scored according to the following criteria by two observers in a blind manner: 0, normal; 1, <30%; 2, 30% to 70%; and 3, >70% of the pertinent area [28].

Immunohistochemistry

Parts of the kidney tissues were snap-frozen in liquid nitrogen and kept at −80°C until use. Two micrometer thick sections were cut with a cryostat and fixed in acetone. The sections were then stained with rabbit antimouse midkine and FITC-labeled goat antirabbit IgG (Cappel, Durham, NC, USA). Rabbit antimouse midkine was raised by injection of purified midkine produced in bacteria into rabbits, and was purified by affinity chromatographies on protein A and midkine columns [5]. The antibody was specific to midkine and did not react to pleiotrophin/heparin binding growth associated molecule (HB-GAM). After washing with phosphate-buffered saline (PBS), all the sections were covered with 90% glycerol containing p-phenylenediamine and were examined by an epifluorescence microscope (Olympus Optical Co., Tokyo, Japan).

For the double immunofluorescence staining with FITC-labeled midkine antisense ODN and proximal tubule markers, 2 μm thick sections were cut with a cryostat, fixed in acetone, and then incubated with goat antirabbit angiotensin-converting enzyme (ACE) antibody [29], followed by incubation with rhodamine-labeled rabbit antigoat IgG.

Cryosections were stained with a monoclonal rat antimouse monocyte-macrophage marker F4/80 (Serotec, Ltd., Oxford, UK) or a monoclonal rat antimouse neutrophil marker 7/4 [30] (Serotec Ltd.) followed by detection with FITC-rabbit antirat IgG (Zymed Laboratories, Inc, South San Francisco, CA, USA). Leukocytes positive for F4/80 or 7/4 were counted by examining 10 fields of both cortex and medulla under a microscope at ×400 magnification in a blind manner.

Biochemical analyses

The blood was collected into a glass tube, left overnight, and centrifuged at 1500 rpm for 10 minutes. Serum creatinine and blood urea nitrogen (BUN) were measured using a Cre-Kainos Kit (Kainos Co. Ltd., Tokyo, Japan) and Iatrochrom UN kit (Iatron Co. Ltd., Tokyo, Japan), respectively. Proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and midkine protein was detected by Western blotting with antimouse midkine antibody using an enhanced chemiluminescence (ECL) kit (Amersham,
Buckinghamshire, UK). Northern blotting analysis was performed as described previously [31, 32]. For quantitative estimation, blots were scanned with Imaging Densitometer Model GS-700 (Bio-Rad, Tokyo, Japan).

**Statistical analysis**

All values are given as means + SE. Statistical analysis was performed by one-factor analysis of variance (ANOVA). When a significant difference was detected, statistical analysis was further performed using the Scheffe’s F test between two groups. A P value of < 0.05 was taken to indicate a significant difference.

**RESULTS**

**Reperfusion model of 129/SV mice**

We previously demonstrated a striking difference of renal damages induced by reperfusion between wild-type and midkine-deficient mice with 129/SV genetic background [4]. Therefore, in the present study, we used 129/SV mice to establish midkine roles in the reperfusion injury model. Although most works on the reperfusion renal injury model employed 30 minutes ischemia, mouse strains used in these works are C57BL6J, BALB/c, and NIH Swiss rather than 129/SV [27, 33]. We examined several conditions using 129/SV mice. 129/SV mice were relatively resistant to reperfusion-induced renal injury (Fig. 1). Thus, 30-minute ischemia showed little effect on renal function as estimated by BUN and serum creatinine, while 90-minute ischemia resulted in severe renal damage. However, the mortality of 90-minute ischemia was 0% among more than 100 mice examined (data not shown). Therefore, we employed the condition of 90-minute ischemia on 129/SV mice.

**Inhibition of midkine synthesis in the kidney with ischemic injury by midkine antisense ODN**

Midkine antisense and scrambled ODNs were evaluated for suppression of midkine expression using CMT-93 cells, a mouse rectal carcinoma cell line. CMT-93 is an adequate cell line for evaluation of the specificity of ODNs because the transfection efficiency of ODN is almost 100% in CMT-93, but is very low in primarily cultured proximal tubule cells (data not shown). Midkine antisense ODN strikingly suppressed midkine expression, while midkine scrambled ODN or saline control did not (Fig. 2A and B). As previously reported, midkine sense ODN showed no effect on midkine expression (data not shown) [25]. Upon I/R injury, midkine expression in the proximal tubular epithelial cells becomes significantly increased [4]. To inhibit the induced expression, we planned to inject midkine antisense phosphorothioate ODN intravenously. We first examined localization of ODN absorbed in the kidney. When midkine antisense ODN labeled with FITC was administered, it colocalized with ACE, a marker of the proximal tubules [29], 8 hours after administration (Fig. 2C to F).

Midkine antisense ODN significantly suppressed the expression of midkine protein 2 days after I/R (i.e., 24 hours after midkine antisense ODN treatment), compared to midkine sense, scrambled ODN, or saline alone (Fig. 3). Midkine protein expression returned to the normal level on day 7 when there was no significant difference between the midkine antisense ODN-treated group and control group (Fig. 3).

Immunohistochemical staining for midkine revealed that midkine expression in the proximal tubules was strongly suppressed by treatment with midkine antisense ODN (Fig. 4A) compared to treatment with sense, scrambled ODN, or saline treatment (Fig. 4B to D).

**Suppression of renal damage by midkine antisense ODN**

Histologic examination revealed that renal damage 2 days after I/R was less severe in the mice treated with
midkine antisense ODN 24 hours after ischemia than in those treated with midkine sense, scrambled ODN, or saline alone (Fig. 5). Semiquantitative estimation of renal damage, based on the degree of tubular cast formation, tubular dilatation, and tubular degeneration, supported the above observation (Fig. 6). Seven days after I/R, the differences in the degree of ischemic injury were not significant between the mice treated with midkine antisense ODN, midkine sense ODN, midkine-scrambled ODN, and saline alone (Fig. 6). Determination of BUN and serum creatinine levels also led to the same conclusion (Fig. 7). Data of Figure 7 also confirmed the overall profile of the renal damage. BUN and serum creatinine levels reach the maximum levels on day 2 in all negative controls, while midkine antisense ODN significantly suppress the levels on day 2 and 3.

DISCUSSION

In the previous study, we showed reduced susceptibility of midkine-deficient mice to reperfusion-induced tubulointerstitial injury, and clarified that midkine plays a key role in the pathological process of renal injury. The present study further confirms the crucial role of midkine in the promotion of renal injury and suggests that targeting midkine could be a potential therapeutic strategy to prevent or attenuate renal injury.
Fig. 4. Effects of midkine antisense oligodeoxyribonucleotide (ODN) in midkine expression on the proximal tubules. One day after ischemic/reperfusion (I/R) injury, either midkine antisense or sense ODN was injected intravenously at 1 mg/kg. The kidney was removed 2 days after the injury, and then examined by immunofluorescence analysis using antimidkine antibody and fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG. (A) Midkine antisense ODN. (B) Midkine sense ODN. (C) Midkine scrambled ODN. (D) Saline control. (E) No ischemia. Bar, 50 μm.

Fig. 5. Tubulointerstitial damage in mice after ischemic/reperfusion (I/R) injury. The mice were injected with midkine antisense oligodeoxyribonucleotide (ODN) (A), midkine sense ODN (B), midkine scrambled ODN (C), or saline (D) 1 day after the injury and, 2 days after, the kidneys were removed and stained with periodic acid-Schiff (PAS). No ischemia control is also shown (E). Midkine antisense ODN-treated mice showed less tubulointerstitial injury compared to the midkine sense or scrambled ODN- or saline-treated mice by all three criteria examined [i.e., tubular cast formation (black arrow), tubular dilatation (arrowhead), and tubular degeneration (white arrow)]. Bar, 50 μm.

role in pathogenesis of this model [4]. Based on these results, we intended to cure the renal injury by knocking down midkine in the present study. This study provided two important findings, namely, the therapeutic potential of the midkine antisense reagent to counteract the tubulointerstitial injury and the molecular hierarchy and interaction of midkine and other cytokines in the injury. Our result that the antisense ODN administered 24 hours following injury ameliorated the renal damage is important and clinically relevant, since most previous studies required pretreatment, prior to the initiation of injury [34, 35]. Importantly, administration of midkine antisense ODN after the initiation of the injury exerted similar effects as the preventive administration of ODN before the injury (Fig. 10). Tubulointerstitial injuries by renal ischemia are often observed in human kidneys after renal transplantation or in kidneys with hypovolemic shock. Our data clearly indicate that the effects of midkine antisense ODN on mouse I/R injuries resulted mainly from the interference of de novo synthesis of midkine in the diseased tubules. Although midkine antisense ODN failed to completely block I/R injuries, our results showing that treatment began after the onset of the disease had beneficial effects support its therapeutic value. Further studies are necessary to determine the therapeutic effects of midkine antisense ODN in a chronic model of tubular injury.

As to the molecular hierarchy and interaction, we should point to two issues before discussing the present results. First, midkine has a direct chemotactic activity to neutrophils and macrophages, as discussed in the previous report [4]. Second, the previous report demonstrated that midkine deficiency causes decreased MIP-2 expression and diminished neutrophil infiltration during early phase [4]. In addition, early phase infiltration of macrophages is also suppressed in midkine-deficient mice, but MCP-1 expression is not affected, and is closely associated with late-phase macrophage infiltration [4]. The present study demonstrated that the midkine antisense ODN suppressed not only midkine expression but also MIP-2 expression (but not MCP-1 or RANTES expression). Therefore, it is likely that decreased MIP-2 expression observed in midkine-deficient mice is due to a direct effect of loss of midkine rather than a chronic, indirect effect. Suppression of both midkine and MIP-2 expression led to diminished infiltration of neutrophils and macrophages during the early phase, and consequently ameliorates the renal damage. Supporting our data, anti-MIP-2 antibody suppresses neutrophil infiltration and ameliorates renal damage in a glomerulonephritis model, in which MIP-2 is produced in the proximal renal tubules [36]. Taken together, the present study strongly suggests that MIP-2 is downstream of midkine, and both are essential for early phase neutrophil infiltration in the reperfusion model.

In addition, the previous [4] and present studies collectively elucidated that midkine is also involved in early phase infiltration of macrophages, while MCP-1 expression is independent of midkine, and is closely associated
with late-phase macrophage infiltration. The gradual induction of MCP-1, reaching the maximum level 7 days after ischemia, in the present study is in contrast to previous studies, which report early responses, being the maximum 6 hours to 3 days after ischemia in a rat ischemia model with 30-minute ischemia [37]. We speculate that the discrepancy was caused by differences of species and ischemic time. Considering that suppression of MCP-1 by a dominant negative form of MCP-1 (an amino terminal deletion mutant of MCP-1) diminishes macrophage infiltration and suppresses renal damage in a reperfusion model [38], it is possible that incompleteness to ameliorate the I/R injury by the midkine antisense ODN is, in part, due to the persistent expression of MCP-1 and macrophage infiltration. In addition, RANTES is important for infiltration of T cells and macrophages [39]. The previous [39] and present study showed that the expression profile of RANTES is the same as that of MCP-1 in reperfusion models. Therefore, attention should be also paid to RANTES in the studies of pathogenesis of the reperfusion-induced tubulointerstitial injury.
The results of the present investigation reinforce the idea that midkine plays a central role in inflammatory diseases. Midkine-targeted therapy should be generally considered for the treatment of such diseases. Midkine antisense ODN, which proved to be effective in inhibiting renal damage, may be also useful for treatment of other inflammatory diseases. However, it should be borne in mind that while delivery of the reagent to the kidney presented no problems, this may not be the case for other organs. ODN, administered into the systemic circulation, has been demonstrated to be selectively taken up by the renal tubular epithelial cells in mice and rats [40–42]. The intact ODNs are present in the cells even 4 days after administration; reabsorption of ODNs filtered into the Bowman’s space is considered to occur [40]. Proximal tubular epithelial cells are thought to take up ODN efficiently, without any detectable degradation, through the activity of ODN binding proteins on the brush border membrane [43]. Indeed, we demonstrated here that midkine antisense ODN was efficiently taken up in the proximal tubules. Since such a mechanism is not known in other organs, the choice of delivery method should be examined more closely for the treatment of other organs.

In addition to the mode of delivery, the side effects are issues of primary concern in antisense therapeutics. The adverse effects common to phosphorothioate ODN and independent of the molecular target are lymphoid proliferation, mononuclear cell infiltration to multiple tissues, and degeneration and necrosis of the liver and renal proximal tubules [44–47]. However, the side effects of
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Fig. 9. Macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1), or regulated on activation, normal T-cell expressed and secreted (RANTES) expression 2 and 7 days after ischemic/reperfusion (IR) injury. RNA was extracted from the kidney and subjected to Northern blot analysis for MIP-2 and MCP-1. The mice were administered with oligodeoxyribonucleotide (ODN) or saline 1 day after the injury. (A) Northern blot analysis for MIP-2 mRNA, MCP-1 mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Lanes 1 and 4, midkine antisense ODN; lanes 2 and 5, midkine sense ODN; lanes 3 and 6, saline control. C is no ischemia. Intensity of bands relative to that of no ischemia, mRNA represents MIP-2 (B), MCP-1 (C), and RANTES (D) mRNA normalized to GAPDH mRNA. Midkine antisense ODN (■). Midkine sense ODN (□). Saline control (★). No ischemia (▲). *P < 0.01 (N = 12).

Fig. 10. Midkine (MK) expression (A), blood urea nitrogen (BUN) (B) and serum creatinine (sCr) (C) levels of mice administrated with oligodeoxyribonucleotide (ODN) or saline 1 day before or after ischemic injury. The kidney and blood was removed 2 days after the initiation of the injury, and then examined by Western blot analysis and biochemical analysis. C is control, no ischemia. Midkine antisense ODN (■). Midkine sense ODN (□). Saline control (★). No ischemia (▲). *P < 0.01; ***P < 0.05 (N = 12).
phosphorothioate ODNs are generally observed at doses above 50 mg/kg. Indeed, in phase I clinical trials phosphorothioate ODNs are generally well tolerated at a dose of 10 mg/kg [21–23]. The dose of midkine antisense ODN used in the present experiment was 1 mg/kg, and we observed no apparent side effects caused by phosphorothioate ODNs.

Phosphorothioate ODN containing cytosine phosphodiester-guanine (CpG) motifs is recognized by macrophages, dendritic cells, and B cells as a danger signal indicating bacterial infection [48, 49], and induces murine B cells to proliferate and secrete immunoglobulins in vitro and in vivo [50]. Since midkine antisense ODN and control ODNs used in the present study had no CpG immune stimulatory sequence, the above-mentioned nonspecific stimulation of immune cells was left out of consideration.

CONCLUSION

Midkine antisense ODN administered after I/R inhibited midkine synthesis, leading to reduced migration of inflammatory cells to the injured epithelial layer and reduced renal damage. These results indicate that intravenous injection of midkine antisense ODN is a candidate for a novel therapeutic strategy against acute tubulointerstitial injury induced by I/R injury.

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