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Increased Serum Midkine Levels during Hemodialysis Using Heparin in Chronic Renal Failure

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The heparin-binding growth factor midkine (MK) has been implicated in neuron growth, angiogenesis, and inflammation. In this study, to elucidate the involvement of MK in the development of pathologies associated with uremia, we examined the serum MK levels in patients receiving hemodialysis (HD) by a highly sensitive enzyme-linked immunoassay. Although no significant difference was found between control serum and serum before dialysis in HD patients, serum MK levels increased significantly at the early stage of HD sessions using heparin and gradually decreased after dialysis. In normal controls, intravenous administration of heparin induced a similar sudden increase of MK, but the subsequent decrease was also rapid. In an *in vitro* study, MK was released in time- and heparin-dose dependent manner from cultured vessels, but not from peripheral leukocytes. These results indicate that, in HD patients, MK is released mainly from endothelial cells immediately after administration of heparin during HD and disappears gradually from blood due to renal impairment. This phenomenon might affect some complications associated with HD.

Key words: endothelial cells, hemodialysis, heparin, midkine, uremic complications.

Midkine (MK) belongs to a family of recently cloned heparin-binding neurotrophic factors, which are developmentally regulated and may have an important role in angiogenesis and neoplasia (*1-6*). The family includes MK, pleiotrophin, and the avian midkine homologue RI-HB (*7*). MK was discovered as a product of a retinoic acid-responsive gene (*1*). MK protein is a highly basic secreted growth factor with a molecular weight of 13,000, and its structure is highly conserved across species (*8*). In normal adult organs, MK expression is detectable in the kidney, lung, thyroid, and small intestine (*3*).

MK overexpression has been correlated with several pathological conditions. MK expression is increased in various human tumor types, such as gastrointestinal cancer (*3*), neuroblastoma (*9*), breast cancer (*10*), hepatoma (*3, 11*), Wilm's tumor (*3*), and bladder tumor (*12*). The senile plaques in Alzheimer's disease invariably accumulate MK (*13*). MK was also found around the sites of nerve damage in cerebral infarction (*14*). These reports suggest that MK is involved in tumorigenesis and tissue repair. Recently we have demonstrated that the inflammation sites in patients with rheumatoid arthritis and osteoarthritis show significantly strong expression of MK, which induces recruitment of leukocytes (*15*). We have also revealed that MK induces immediate cutaneous response *via* histamine release from mast cells (*16*). These results suggest that MK may also play a role in inflammation processes.

Various abnormalities of the bioregulatory system have been demonstrated in patients on maintenance hemodialysis (HD). Many of the complications, for example, anemia (*17*), secondary hyperparathyroidisms (*18*), and dialysis-related amyloidosis (*19, 20*), can be attributed to the dysregulation of hormones, cytokines, or growth factors. We hypothesized that a novel growth factor, MK, may not be properly regulated in HD patients. In this study, to examine the involvement of MK in the pathogenesis of uremic complications, we determined serum MK levels in HD patients by a highly sensitive enzyme-linked immunoassay. We discovered demonstrate that serum MK levels increased dramatically during HD using heparin and gradually decreased after dialysis. Moreover, we found that, in the presence of heparin, MK is mainly released from endothelial cells, not from leukocytes. Some possible effects *via* MK by heparin utilization are also discussed.

MATERIALS AND METHODS

Patients—Twenty-five patients (13 males and 12 females with a mean age of 61±23 years) with end-stage renal

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Abbreviations: MK, midkine; HD, hemodialysis; NM, nafamostat mesilate; DRA, dialysis-related amyloidosis.

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disease caused by chronic glomerulonephritis were investigated concerning plasma MK levels. All patients had been stable on hemodialysis (HD) thrice weekly (4 h each dialysis) for 9.2 ± 3.5 years and were taking standard medications including calcitriol, vitamin D_3, calcium carbonate, and erythropoietin. HD therapy was characterized by ultrafiltration-control, bicarbonate-base, and the use of different membranes [cellulose (6 patients), cellulose acetate (12 patients), and polysulfone membranes (7 patients)]. For anticoagulation, patients were given an initial dose of 1,500 units and an hourly dose of 500 units of heparin sodium for the first 3 h. Another anticoagulant, nafamostat mesilate, was also used (20 mg for priming and an hourly dose of 40 mg during HD) to investigate the difference between the two anticoagulants. Informed consent was obtained from each patient. As a control population, 25 normal volunteers (14 males and 11 females with a mean age of 56 ± 19 years) were studied.

Mice—Female BALB/c mice were obtained from Japan SLC (Hamamatsu) and bred under pathogen-free conditions. Mice were between 8 and 10 weeks of age when they were used in experiments.

Blood Samples—Blood was collected from patients and normal volunteers and allowed to clot by leaving it at room temperature overnight, and the resulting serum was used for the assay. In the case of HD patients, samples were collected immediately before dialysis. To investigate the change in MK levels during and after HD, and the difference in MK levels between the two sides of the dialyzer, 7 of the 25 patients were selected randomly, and their blood was collected both from arterial sides and venous sides at 0, 15, 60, 120, and 240 min after the start of HD session and at 60 and 120 min after dialysis. To determine the differences between dialyzer membranes, blood was drawn from 5 HD patients per membrane type at 0, 15, and 240 min after the start of HD. To compare the effect of heparin administration between normal controls and HD patients, 3 of 25 normal volunteers were given the same dose of heparin as in HD therapy.

In Vitro Culture with Heparin of Human Leukocytes or Murine Vessels—Samples of 10 ml of peripheral blood were collected from healthy volunteers. The blood was mixed with 0.6 ml of 0.1 M EDTA and 1.4 ml of dextran. After sedimentation of erythrocytes at room temperature for 1 h, the upper leukocytes layer was collected and centrifuged at 1,200 rpm for 10 min. Leukocytes were washed twice in Hanks' balanced salt solution (HBSS) and resuspended at a concentration of 5×10^6 cells/ml in RPMI 1640 medium with 5% FCS in 6-well plates.

Femoral arteries were removed aseptically from mice and cut into 3-mm sections. They were allowed to develop in organ culture in RPMI 1640 medium with FCS in 6-well plates (3 sections per well).

Blood and vessel samples were incubated for 10 min at 37°C, then heparin sodium (0, 1, or 50 IU in 1 ml of medium) was added to the culture medium. At 0, 15, 60, 120, and 240 min after addition of heparin, the medium was collected and frozen at -30°C until assay.

Antibodies and Reagents—Chemically synthesized human MK (*21*) was purchased from Peptide Research Institute (Osaka), and recombinant mouse MK produced by L cells was purified as described previously (*22*). Rabbit antibodies against mouse MK or human MK and their biotinylated antibodies were prepared as described previously (*11, 23*). Streptoavidin-β-D-galactosidase and 4-methylumbelliferyl-β-D-galactosidase were purchased from Boehringer Mannheim Biochemica (Germany) and Sigma Chemical (St. Louis, MO), respectively.

Assay for MK in Human Serum and Culture Medium—Enzyme-linked immunoassay of MK was performed as described previously (*11*). Briefly, anti-MK antibodies [1 μg in 50 μl of 50 mM Tris-HCl buffer (pH 8.0)] were added to a well in a microtiter plate. Plates were incubated for 3 h, then washed. After blocking with the washing buffer, 50 μl of a sample solution was added. After incubation, wells were washed, and biotinylated anti-MK antibodies (0.7 ng in 50 μl of washing buffer) were added. Then streptoavidin-β-D-galactosidase conjugate (5 mU in 50 μl of washing buffer) was added and the plate was incubated. After washing, 4-methylumbelliferyl-β-D-galactosidase (5 μg in 50 μl of washing buffer) was added. The enzyme reaction was stopped by adding 0.1 M glycine-NaOH buffer (pH 10.3). The amount of 4-methylumbelliferon released was measured with a microplate fluorescence reader (Cytofluor II, Biosearch), at excitation and emission wavelengths of 360 and 460 nm, respectively.

Statistical Methods—Data were analyzed by unpaired Student's t-test. Differences with p values of less than 0.05 were considered to be statistically significant.

RESULTS

Serum MK Levels Are Significantly High during Hemodialysis Sessions—First, serum MK levels in normal controls and patients on hemodialysis (HD) were observed. As shown in Fig. 1a, MK levels in HD patients were slightly higher than those in normal controls, but there was no significant difference ($p = 0.059$). To determine whether HD therapy affects serum MK levels, we examined these levels before, during and after HD sessions in which heparin sodium (heparin) was used as a routine anticoagulant. Serum MK levels were significantly increased by 15 min after the start of HD, remained at a plateau at 35-fold the level of the baseline until the end of HD, then decreased gradually after HD (Fig. 1b). These results demonstrate that HD therapy itself can induce high levels of serum MK.

Serum MK Levels Are High during HD Using Heparin Sodium, but Not Using Nafamostat Mesilate—Two aspects of HD therapy have a potential influence on serum MK levels. One is the blood-membrane interaction, which has been discussed in relation to production of proinflammatory cytokines (*24–26*). The other is the drugs that are used during HD, that is, anticoagulants such as heparin and nafamostat mesilate (NM). To investigate the effects of dialyzer membranes on MK levels, serum from the arterial and venous sides of a dialyzer was examined. As Fig. 2a shows, there was no significant difference between the two sides at either time during HD sessions. Moreover, no significant differences were found in serum MK levels between different dialyzer membranes (Table I). To assess the influence of anticoagulants, we compared serum MK levels during HD using NM with those during HD using heparin. In contrast with HD using heparin, no increase in MK levels was found during HD using NM (Fig. 2b). These results strongly suggest that the increase of serum MK levels may be induced by the presence of extraneous

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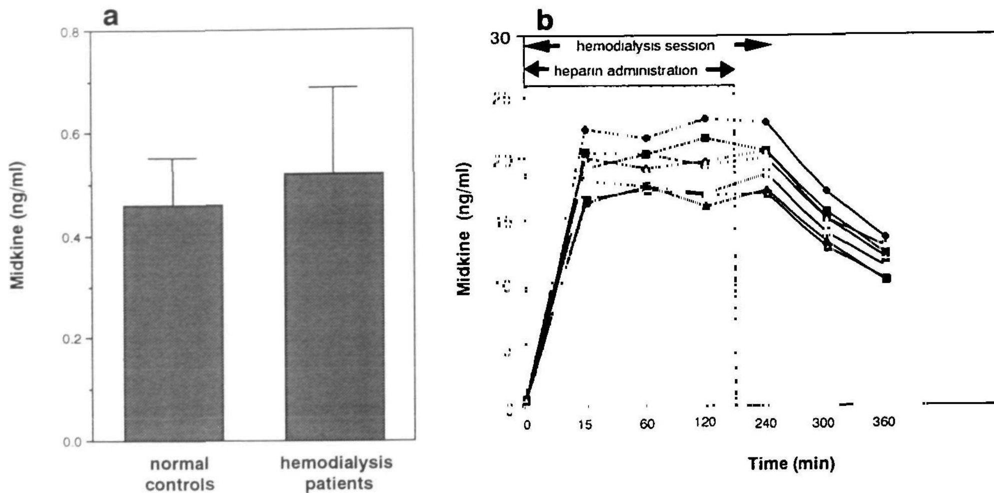

Fig. 1. **(a) Serum MK levels in HD patients and normal controls.** Blood samples were collected from 25 HD patients and 25 normal controls. In HD patients, samples were collected prior to initiation of dialysis. MK was assayed as described in "MATERIALS AND METHODS." No significant difference was found between the two groups ($p=0.059$). **(b) Serum MK levels during and after HD sessions using heparin in 7 HD patients selected randomly.** Serum was diluted 10 times with PBS(−) before assay.

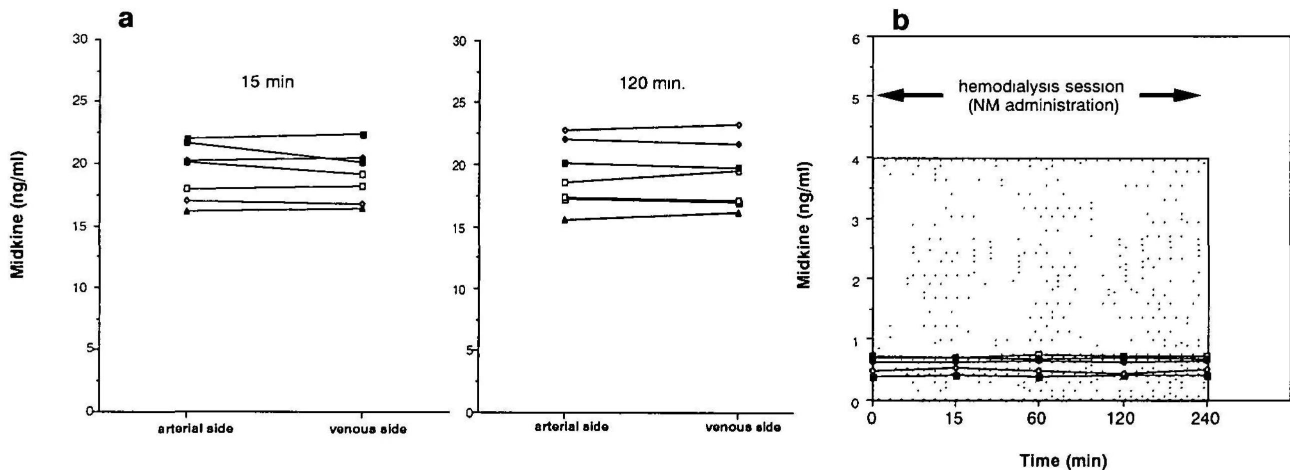

Fig. 2. **(a) A comparison of serum MK levels between arterial sides and venous sides during HD.** Blood was collected from arterial sides and venous sides of dialyzers at 15 (left) and 120 (right) min after the start of HD. There was no significant difference between them each time. Similar results were given from blood at 60 and 240 min. **(b) Serum MK levels during HD sessions using nafamostat mesilate.**

TABLE I. **Serum MK levels during hemodialysis using heparin with different dialyzers.**

Time (min)	Midkine (ng/ml)		
	Cellulose	Cellulose acetate	Polysulfone
0	0.5±0.1	0.5±0.1	0.5±0.1
15	19.9±2.3	20.1±2.8	19.9±2.0
240	20.4±2.7	20.4±2.5	20.4±2.1

Blood in five HD patients per group was drawn at 0, 15, and 240 min after the start of HD. No significant differences in serum MK levels were found between cellulose, cellulose acetate, and polysulfone membranes.

heparin in the circulation.

Serum MK Levels in Normal Human Subjects Are Also Increased by Intravenous Administration of Heparin, but Decrease Rapidly—To investigate whether the MK increase is specific to HD patients, normal volunteers were given the same dose of heparin as in HD therapy, and serum MK levels were examined during and after administration. As shown in Fig. 3, MK levels increased dramatically in the same way as in HD patients, but decreased rapidly at 120

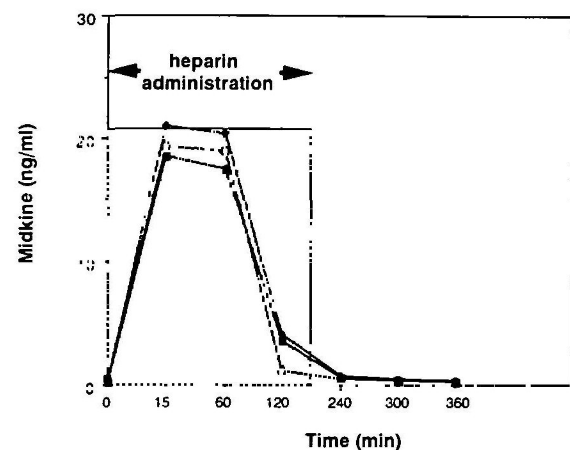

Fig. 3. **Serum MK levels during and after administration of heparin in three normal human subjects.** Serum was diluted 10 times with PBS(−) before assay.

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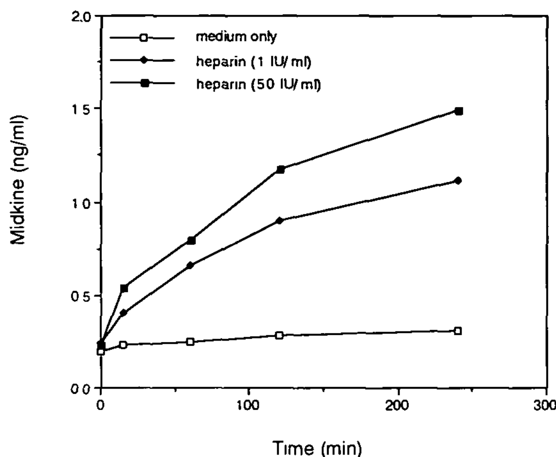

Fig. 4. **MK concentrations in medium in *in vitro* culture of vessels with heparin.** Murine artery was isolated as described in "MATERIALS AND METHODS" and cultured with the indicated concentrations of heparin sodium.

min after the start of infusion, in contrast to HD patients. These results indicate that the MK increase following heparin administration is observed both in HD patients and normal controls, and that the rate of disappearance of MK differs between these patients and the controls.

MK Was Released from Endothelial Cells but Not Leukocytes—The above findings prompted us to assess what cells were stimulated to release MK in the presence of heparin. Since MK was released immediately after stimulation by heparin during HD, we inferred that circulating blood cells or vascular endothelial cells, which could be in direct contact with heparin, could be the source. To investigate this possibility, peripheral leukocytes and vessels were incubated with heparin. Leukocyte-containing medium did not show detectable MK levels even after prolonged culture with a high dose of heparin (data not shown), whereas MK levels in vessel-containing medium increased in a time- and heparin-dose-dependent manner (Fig. 4). It is notable that the vessel-containing medium incubated with heparin showed increased MK levels after only 15 min, the same as for human serum during HD using heparin. These results indicated that the release of MK was induced immediately upon stimulation by heparin from vascular endothelial cells, but not circulating leukocytes.

DISCUSSION

In this report, we have demonstrated that serum MK levels are dramatically increased in association with utilization of heparin, similar to the case for HD therapy, and that this growth factor may originate from endothelium. However, we have not elucidated the mechanism of MK release by heparin. If MK has the same ability to bind glycosaminoglycans on the surface of endothelial cells as do other heparin-releasable proteins, such as lipoprotein lipase (*27*) and superoxide dismutase (*28*), then extraneous heparin, which is an acidic proteoglycan, may compete for glycosaminoglycan-binding sites and induce detachment of MK from the endothelial surface. Alternatively, MK in intracellular vesicles might be released through exocytosis triggered by heparin-mediated signals.

Whichever mechanism is responsible for the release of MK from cells, it is reasonable to assume that MK might have some function in vascular endothelial cells. However, the key physiological roles of MK remain to be elucidated. A role of MK in angiogenesis is suggested by the report that it is a mitogen for endothelial cells and that it stimulates angiogenesis in a number of *in vitro* and *in vivo* assays (*5*). Recently, MK has been shown to induce plasminogen activators in aortic endothelial cells (*29*). Activation of plasminogen activators within a tumor is known to facilitate tumor growth and metastasis (*30-32*). Malignant tumors, which occur in HD patients with a high incidence (*33, 34*), might be exacerbated by these biological activities of MK.

Patients on long-term hemodialysis often develop dialysis-related amyloidosis (DRA), a complication which causes chronic arthralgia, carpal tunnel syndrome, subchondral bone erosions, and cysts (*35*). Histologically, amyloid deposits are surrounded by inflammatory cells in the affected tissues, suggesting the potential involvement of these cells in the pathogenesis of DRA (*36, 37*). Since MK directly triggers recruitment of leukocytes *in vitro* (*15*), the inflammation stage of DRA may be induced by higher levels of MK.

Furthermore, it is commonly known that most of patients undergoing periodic hemodialysis often suffer pruritus. It has been reported that plasma histamine concentrations are significantly elevated in HD patients compared to normal controls, and even more elevated in HD patients with pruritus (*38-40*). Recently we found that MK could induce histamine release from rat mast cells (*16*) and also from human basophils (unpublished results). These findings together suggest that MK may contribute to high levels of plasma histamine and subsequent pruritus in HD patients.

The difference in kinetics of serum MK levels during heparin administration between HD patients and normal controls is another issue to be addressed. MK disappeared more rapidly from blood in normal controls than in HD patients. If MK is metabolized mainly in the kidney, decreased metabolism due to renal failure may result in prolonged high levels of MK during and after HD. Alternatively, factors associated with dialysis, including extracorporeal blood circulation and dialysates, might inhibit the return of released MK to the endothelium; yet even at 2 h after the end of HD the serum MK levels were 20-fold higher than baseline (Fig. 1b). Therefore, our results suggest that the low rate of MK disappearance in HD patients was caused by failure of MK to be metabolized in the kidney.

This study has demonstrated increased serum MK levels during HD in patients with end-stage renal disease due to the release of MK from endothelial cells triggered by intravenous heparin. Synthesis and release of other inflammatory cytokines such as IL-1β and TNF-α are known to be induced in patients during HD. These are particularly intense in patients dialyzed with bioincompatible membranes (*41, 42*). It is important to remember that factors other than the dialysis membranes, such as anticoagulants and dialysates, may have a role in the pathogenesis associated with uremia. Further investigations of heparin-releasable proteins including MK will contribute to our understanding of endothelial functions and may yield new insights into some complications in HD patients.

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