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Requirement of chondroitin sulfate/dermatan sulfate recognition in midkine-dependent migration of macrophages

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Midkine (MK) is a heparin-binding growth factor that promotes cell migration, cell growth and cell survival. The promotion of migration of inflammatory cells, especially macrophages, by MK is involved in formation of a vascular abnormality, i.e. neointima formation. MK-induced migration of peritoneal exudate macrophages was inhibited by heparin, chondroitin sulfate E and dermatan sulfate, but not by chondroitin sulfate D or chondroitin 6-sulfate. Digestion of macrophages with chondroitinase ABC as well as chondroitinase B decreased the migratory activity. However, heparitinase digestion showed only slight effects. These results indicated that a chondroitin sulfate, i.e. an E-type oversulfated structure with dermatan sulfate domain, is involved in MK-induced migration of macrophages. Although a chondroitin sulfate proteoglycan, receptor-type protein tyrosine phosphatase ζ (PTP ζ), participates in MK-induced migration of neurons and osteoblasts, PTP ζ was not detected in macrophages. The MK-induced migration was inhibited by PP1, wortomanin, PD 98059 and vanadate, indicating that the downstream signaling system, which includes Src, PI3 kinase and ERK as important components, is shared with other MK signaling systems in which PTP ζ is involved.

Keywords: **chondroitin sulfate, macrophage migration, midkine, protein tyrosine phosphatase ζ**

Abbreviations: **ERK, extracellular signal-regulated kinase; MEK, extracellular signal-regulated kinases kinase; MK, midkine; PBS, Dulbeco's phosphate buffered saline; PEM, peritoneal exudates macrophage; PI3-kinase, phosphatidyl-inositol 3-kinase; PTN/HB-GAM, pleiotropin/heparin-binding growth-associated molecule; PTP ζ, protein tyrosine phosphatase ζ; RT-PCR, reverse-transcription polymerase chain reaction.**

Introduction

The results of intensive investigations have indicated the importance of inflammatory cell migration in the complex processes leading to atherosclerosis and post-angioplasty restenosis [1,2]. A common feature of these vascular pathological processes is intimal lesion formation; upon vascular injury, smooth muscle cells migrate from the media to the intima where they proliferate to form intimal lesions. Factors that promote this process are partly derived from inflammatory cells, which migrate to the injured tissue earlier than smooth muscle cells.

Recently, a critical role of midkine (MK), a heparin-binding growth factor, in inflammatory cell migration was demonstrated [3]. MK was first identified as the product of a retinoic acid-responsive gene in embryonal carcinoma cells [4,5]. MK has about 50% sequence identity with pleiotrophin/heparin-binding growth-associated molecule (PTN/HB-GAM) [6,7] and is distinct from fibroblast growth factors. MK promotes survival [8] and neurite outgrowth [9,10] of embryonic neurons, growth of fibroblasts [10], migration of embryonic neurons [11], neutrophils [12], macrophages [3] and osteoblasts [13], and plays roles in carcinogenesis and tumor invasion [14,15].

MK deficient-mice show severely impaired migration of macrophages and neutrophils to injured blood vessels [3]. As a result of the suppression of inflammatory cell migration, neointima formation is markedly inhibited in the injured blood vessels in these knockout mice [3]. In terms of MK-induced migration of neurons and osteoblasts, we found the importance of a chondroitin sulfate proteoglycan, receptor-type protein tyrosine phosphatase ζ (PTP ζ) [11,13]. However, nothing is known about the mechanism of MK-induced migration of macrophages, although the physiological significance of the

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process has been established using MK-deficient mice [3]. The present study was performed principally to explore the involvement of chondroitin sulfate proteoglycans in macrophage migration, which is important in in atherosclerosis and post-angioplasty restenosis.

Materials and methods

Reagents

Heparin, chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitin sulfate D and E, dermatan sulfate, chondroitinase ACI, ACII, B and ABC and heparitinase were purchased from Seikagaku Kogyo Co (Tokyo, Japan). PP1 and PD 98059 were from Alexis Biochemicals (San Diego, CA, U.S.A.), and Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.), respectively. Wortmannin and U-73122 were products of Sigma-Aldrich Inc (St. Louis, MO, U.S.A.). Genistatin, Herbimicin A and sodium vanadate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human MK produced in yeast [16] was a kind gift from Dr. S. Sakuma, Meiji Milk Co. Ltd. (Odawara, Japan).

Culture of macrophages and migration assay

Peritoneal exudate macrophages (PEMs) were collected from the ICR mouse peritoneum as described [17]. Briefly, PEMs were collected by peritoneal lavage with Dulbeco's phosphate buffered saline (PBS) from mice 4 days after intraperitoneal injection of 1.5 ml of thioglycollate broth. The PEMs were collected by centrifugation and resuspended in RPMI1640 medium supplemented with 0.3% bovine serum albumin. After 2 h, the cells were washed twice with PBS. The migration of macrophages was observed using Chemotaxicell (Kurabo, Japan) with pores 5.0 μm in diameter as described previously [3]. Aliquots of 1.0×10^6 cells/ml in RPMI1640 medium containing 0.3% bovine serum albumin (100 μl) were placed in the upper chamber. Then, 600 μl of RPMI1640 medium containing 0.3% bovine serum albumin was placed in the lower chamber. Chondroitinase and heparitinase digestion of PEMs were performed as described previously [13].

RT-PCR

Aliquots of 1 μg of total RNA from 15-day-old rat brain, PEMs or L cells were used for reverse-transcription with Super-Script II, Gibco BRL (Rockville, MD, U.S.A.). The primers used for PCR were those for PTP ζ described previously [13]: 5′-GTTCTCAACACATCCCTGAATCCTACTTCCCA-3′ and 5′-CTTTAGTGATTCTTCTGAACCTGATGGAGCCGA-3′.

Analysis of radioactively labeled products released by chondroitinase ABC digestion from cultured macrophages

PEMs from 5 mice (5×10^6 cells) were cultured in a Falcon 3002 dish containing 3 ml of RPMI1640 medium with 10% fetal calf serum and 0.37MBq of D-1-^{14}C-glucosamine (2.07 GBq/mmol). After 20 h, cells were washed with PBS twice and were incubated with 50 mU of chondroitinase ABC in RPMI1640 medium for 1 h. The released supernatant was again digested with chondroitinase ABC, and the released disaccharides were analyzed as described by Zou et al. [18]

Results

Chondroitin sulfate is involved in MK-dependent migration of macrophages

MK coating of the lower surface of the Boyden chamber induced migration of PEMs [3]. This enhanced migration was inhibited by soluble MK (100 ng/ml) added to the lower chamber (Figure 1A). Coating with poly-L-lysine at a concentration of 20 μg/ml showed a smaller migration-promoting effect (Figure 1A). Heparin at a concentration of 20 μg/ml inhibited the haptotactic migration caused by MK (Figure 1B). We found that chondroitin sulfate E and dermatan sulfate inhibited the migration at 10 μg/ml; chondroitin 4-sulfate showed an inhibitory effect at the higher concentration of 100 μg/ml (Figure 1C). Chondroitin 6-sulfate showed no inhibitory effect at 100 μg/ml. Digestion of PEMs with chondroitinase ABC or B strongly suppressed the migration (Figure 1D). Chondroitinase ACI (50 mU/ml) showed similar suppressive effects, while ACII (250 mU/ml) was slightly less effective (data not shown). Heparitinase digestion showed less effects; only a slight, statistically non-significant reduction of the migratory activity was observed after heparitinase treatment. Digestion with the mixture of chondroitinase ABC and heparitinase reduced the migration activity to the control level, i.e. the migration on poly-L-lysine-coated chambers (Figure 1D). To confirm that chondroitinase digestion indeed released oligosaccharides from chondroitin sulfates, macrophages were metabolically labeled with ^{14}C-glucosamine and disaccharide composition of the released material was analyzed. We detected the peak of the unsaturated disaccharide derived from chondroitin 4-sulfate; other components were too small to be identified (data not shown). These results indicated that chondroitin sulfate is involved in MK-dependent haptotactic migration of macrophages. Furthermore, the results suggested that the structure of chondroitin sulfate involved is the E-type structure with a dermatan sulfate domain.

PTP ζ does not participate in MK-induced migration of macrophages

A chondroitin sulfate proteoglycan PTP ζ serves as a receptor of MK in MK-induced migration of embryonic neurons [11]

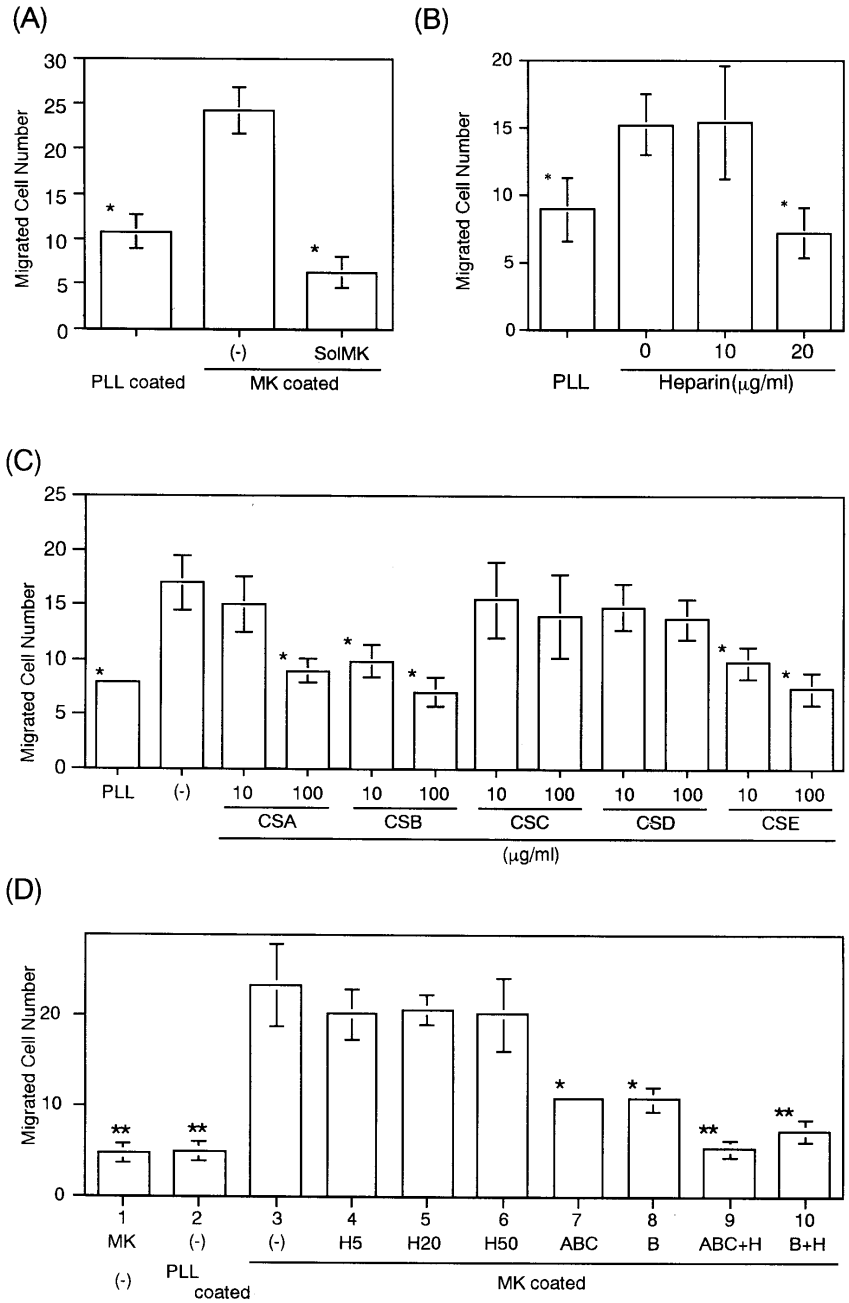

Figure 1. Involvement of glycosaminoglycans in MK-induced haptotactic migration of PEMs. The migration assay was performed with filters coated with MK or poly-L-lysine (PLL) on their lower surface at 20 μg/ml. PEMs (approximately 1×10^5 cells in 100 μl 0.3% BSA/RPMI1640) were added to the upper chamber of the Chemotaxicell (a modified Boyden chamber), followed by incubation for 6 h. Ten fields at ×400 magnification per filter were counted to determine the migrated cell number (1 field corresponds to 1/160 of entire surface of the filter). The value shown as the "Migrated Cell Number" is the mean ± S.E. ($n = 3$) per field. *, $P < 0.05$; **, $P < 0.001$. A: Effects of soluble MK. The results shown are representative of three independent experiments. SolMK, MK was added to the lower chamber in a soluble form at 100 ng/ml; (-), MK was not added as a soluble form. B: Effects of heparin. The lower surface of the chamber was coated with MK except for the case of PLL. PEMs were incubated with the indicated concentration of heparin at 37°C for 30 min, and the same concentration of heparin was added to both chambers. C: Effects of chondroitin sulfates. PEMs were pre-incubated at 37°C for 30 min with chondroitin sulfates at concentrations of 10–100 μg/ml. The migration assay was carried out with the same concentration of chondroitin sulfates added to both lower and upper chambers. The lower chamber was coated with MK, except for PLL in which PLL was used for coating. CSA, chondroitin 4-sulfate; CSB, dermatan sulfate; CSC, chondroitin 6-sulfate; CSD, chondroitin sulfate D; CSE, chondroitin sulfate E. D: Effects of chondroitinase and heparitinase. Filters were coated with MK (lane 3–10), PLL (lane 2) or nothing (lane 1). PEMs were preincubated at 37°C for 30 min with heparitinase (H; 5–50 mU/ml)(lane 3–6, 9, 10), for 60 min with chondroitinase ABC (ABC; 50 mU/ml)(lane 7, 9) or B (B; 50 mU/ml)(lane 8, 10). Numbers in the columns of H indicated mU of heparitinase added. In columns 9 and 10, two enzymes were added.

and osteoblasts [13]. Therefore, we explored the possibility that PTP ζ also participates in the migration of macrophages. First, we examined the effects of sodium vanadate, an inhibitor of protein phosphatase. In agreement with the hypothesis that PTP ζ is involved, sodium vanadate inhibited the action of MK (Figure 2A). However, using cDNA from PEMs as the template, we could not detect expression of PTP ζ in PEMs by RT-PCR (Figure 2B), although PTP ζ expression was detected in the rat brain but not in control L cells.

Effects of various inhibitors

To gain insight concerning the intracellular molecules that participate in MK-mediated PEM migration, the effects of various specific inhibitors of signal transduction were examined. A tyrosine kinase inhibitor (herbimycin A), a Src inhibitor (PP1), a PI3-kinase inhibitor (wortmannin), a MAP kinase kinase (MEK) inhibitor (PD 98059), and a phospholipase C inhibitor (U-73122) significantly inhibited MK-mediated cell migration (Figure 3). This probably indicates that a dynamically concerted signaling interaction is essential for the cell migration induced by MK. Among the inhibitory substances, PP1, wortmannin and PD 98059 also inhibited MK-dependent survival of embryonic neurons [8, Sakaguchi et al., submitted] and MK-induced migration of osteoblasts [13]. Thus, Src, ERK and PI3-kinase appear to play central roles in much of the signaling systems of MK, including those involved in macrophage migration.

Discussion

We have verified that chondroitin sulfate proteoglycan is involved in reception of MK signal for promotion of macrophage migration. So far MK is known to promote migration of four kinds of cells, neurons [11], osteoblasts [13], macrophages [3] and neutrophils [12]. The importance of chondroitin sulfate proteoglycan has also been demonstrated in MK-dependent migration of neurons [11] and osteoblasts [13]. However, one important difference is that in the case of neurons [11] and osteoblasts [13], PTP ζ, a chondroitin sulfate proteoglycan, has been identified as a signaling receptor [11, 19]. In the case of macrophages, however, PTP ζ was not detected. It is likely that another receptor-type protein tyrosine phosphatase similar in nature to chondroitin sulfate proteoglycan is involved in the reaction. However, this point remains to be clarified. The usage of inhibitors of signal transduction yielded results suggesting that the downstream signaling

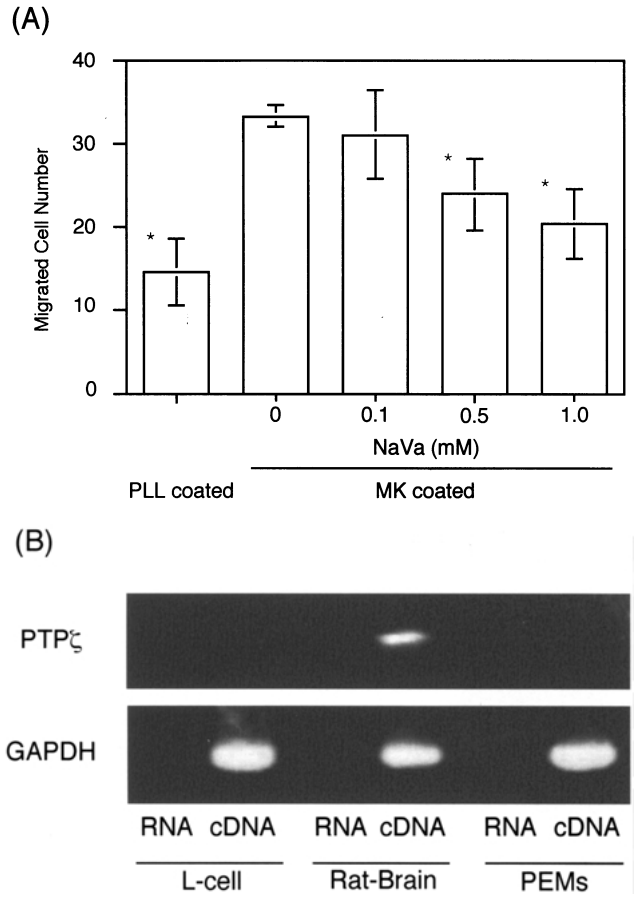

Figure 2. Examination of possible involvement of PTP ζ in MK induced migration of PEMs. A: Sodium vanadate (NaVa) inhibited MK-induced cell migration of PEMs. Experiments were performed as in Figure 1 with either PLL- or MK-coating. Values represent means ± S.E. (n = 3). *, $P < 0.05$. B: RT-PCR analysis for the presence of PTP ζ. One μg of total RNA obtained from L cells (negative control) from, the rat brain at 14 days old (positive control) or PEMs was used for analysis. Upper panel, PTP ζ; lower panel, GAPDH.

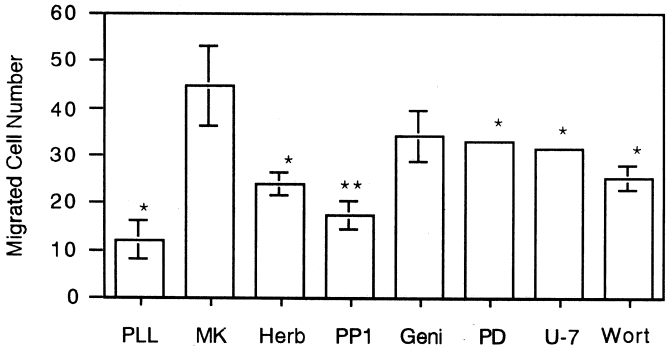

Figure 3. Effects of different inhibitors on MK-induced migration of PEMs. The assay was performed as described in Figure 1. PEMs were incubated with the indicated inhibitors for 30 min. The migration assay was then performed with both the upper and lower chambers containing the same concentrations of the respective inhibitors. The lower surface was coated with MK, except for PLL in which it was coated with PLL. Concentrations of inhibitors are as follows: harbimycin A (Herb), 1 μg/ml; PP1, 10 nM; genistein (Geni), 50 μg/ml; U-73122 (U-7), 10 μM; wortmanin (Wort), 100 nM; PD 98059 (PD), 20 μM. Values represent means ± S.E. (n = 3). *, $P < 0.05$; **, $P < 0.001$.

system includes Src family kinase, PI3 kinase and ERK. Thus, despite the absence of PTP ζ, the downstream signaling system appears to be shared between neurons [8], osteoblasts [13] and macrophages.

The chondroitin sulfate structure recognized by MK in macrophages was also revealed to a certain extent. As chondroitin sulfate E and dermatan sulfate have inhibitory effects, and chondroitinase B digestion abolished the MK action, the likely structure recognized by MK is an over-sulfated E type structure with a dermatan sulfate domain. The strong binding of MK to chondroitin sulfate E has been demonstrated [20]. Furthermore, fine structure of E type structure with a dermatan sulfate domain has been established [21]. The E type structure, the GlcA-GalNAc (4,6-sulfate) unit, was originally found in squid chondroitin sulfate [22]. Although it is present in certain mammalian tissues, this distribution is limited. Nevertheless, the presence of the E unit in macrophages has been demonstrated by previous investigations [23,24]. In addition, the E unit in macrophage is carried by chondroitin sulfate/dermatan sulfate hybrid molecule [24] in agreement with the result of the present investigation. The E unit on macrophages might be involved in interactions with migration-promoting factors including MK.

Heparitinase digestion of macrophages appeared to reduce the MK action slightly, and the combined digestion with heparitinase and chondroitinase appeared to exhibit stronger effects than chondroitinase alone. MK is known to bind to syndecans, transmembrane heparan sulfate proteoglycans, with strong affinity [25,26]. Participation of heparan sulfate proteoglycans in MK-dependent neurite extension and enhancement of fibrinolytic activity has been demonstrated [9,27]. Furthermore, syndecan-3 has been shown to be the receptor of heparin-binding growth-associated molecule (HB-GAM)/pleiotropin in HB-GAM-dependent neurite outgrowth [28]. HB-GAM and MK are closely related in structure and show about 50% amino acid identity [29]. Our current view is that both chondroitin sulfate proteoglycans and syndecans serve as components of MK signal receptor and which type of proteoglycan plays a more significant role is dependent on the cell type. Macrophages belong to the cell type in which chondroitin sulfate proteoglycans play a major role. It is intriguing that Src has been identified as the downstream signaling molecule upon HB-GAM-induced neurite outgrowth [30]. The inhibitor of Src family kinases abolished MK action in this system, osteoblast migration [13,30] and neurite outgrowth by oligodendrocyte precursors [31]. It is likely that Src family kinases generally play important roles in signaling from proteoglycan-type receptors of the MK family.

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