Haptotactic Migration Induced by Midkine

INVolvement of PROTeIN-TyrOsinE Phosphatase ζ, MIToGEN-Activated Protein Kinase, and PhosphatidylinositOL 3-kinase*

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Midkine, a heparin-binding growth factor, plays a critical role in cell migration causing suppression of neoIntima formation in midkine-deficient mice. Here we have determined the molecules essential for midkine-induced migration. Midkine induced haptotaxis of osteoblast-like cells, which was abrogated by the soluble form of midkine or pleiotrophin, a midkine-homologous protein. Chondroitin sulfate B, E, chondroitinase ABC, and orthovanadate, an inhibitor of protein-tyrosine phosphatase, suppressed the migration. Supporting these data, the cells examined expressed PTPζ, a receptor-type protein-tyrosine phosphatase that exhibits high affinity to both midkine and pleiotrophin and harvests chondroitin sulfate chains. Furthermore, strong synergism between midkine and platelet-derived growth factor in migration was detected. The use of specific inhibitors demonstrated that mitogen-activated protein (MAP) kinase and protein-tyrosine phosphatase were involved in midkine-induced migration but not PDGF-induced chemotaxis, whereas phosphatidylinositol 3 (PI3)-kinase and protein kinase C were involved in both functions. Midkine activated both PI3-kinase and MAP kinases, the latter activation was blocked by a PI3-kinase inhibitor. Midkine further recruited PTPζ and PI3-kinase. These results indicate that PTPζ and concerted signaling involving PI3-kinase and MAP kinase are required for midkine-induced migration and demonstrate for the first time the synergism between midkine and platelet-derived growth factor in cell migration.

Cell migration plays a key role in a wide variety of biological phenomena (1, 2). There are three main types of cell migration: chemokinesis, chemotaxis, and haptotaxis. Chemokinesis comprises random, non-directional motility in response to a ligand without any orienting cues. Chemotaxis is the cell movement toward a positive gradient of soluble stimulants such as chemokines and growth factors. Haptotaxis involves cell crawling toward substrate-bound molecules such as various extracellular matrix proteins. Cell migration is the result of a series of complicated, integrated processes and is controlled by many kinds of intracellular molecules (1, 2). These molecules include Rho small G protein family members, PI3-kinases, MAP kinases (Erk1 and Erk2), and protein kinase C.

Midkine (MK) was first identified as the product of a retinoic acid-responsive gene in embryonal carcinoma cells (3, 4). MK and pleiotrophin (PTN, also called HB-GAM for heparin-binding growth-associated molecule) comprise a family of heparin-binding growth/differentiation factors and are not related to other heparin-binding growth factors such as fibroblast growth factor or hepatocyte growth factor (5, 6, 7). MK has been reported to promote neuronal survival and neurite outgrowth (8, 9) and to play roles in carcinogenesis (10, 11) and tissue remodeling (12, 13).

Using MK knock-out mice, it was demonstrated that MK is involved in neoIntima formation in a model of restenosis after angioplasty (14). NeoIntima is the basic lesion in both atherosclerosis and restenosis after angioplasty (15). A variety of stresses to the arterial endothelium can induce the migration of smooth muscle cells from the media into the space between the endothelium and internal elastic lamina to form a neoIntima. One of the most important molecules in this process is PDGF-BB, which is responsible for the migration of smooth muscle cells (16). Macrophages recruited into the arterial wall also play a critical role in this lesion formation (17). NeoIntima formation and macrophage recruitment to the arterial wall were suppressed in MK-deficient mice (14). Because MK induces the migration of both smooth muscle cells and macrophages in vitro (14), it was concluded that the cell migration-inducing activity of MK is crucial for the suppression of neoIntima formation.

These findings also suggest a possible interaction between MK and PDGF in smooth muscle cell migration. In addition, remodeling after bone fractures also supports the interaction of MK and PDGF, because MK expression and PDGF accumulation are induced during this process (13). PTN/HB-GAM promotes the migration of osteoblast-like cells, including UMR106 cells, which provides further evidence (18).

MK and PTN/HB-GAM induce the migration of cortical neurons (19, 20). MK also induces the migration of neutrophils...
Fig. 1. MK induces haptotactic migration of UMR-106 cells. A, the migration assay was performed with filters coated with MK on their lower surface at the indicated concentrations. UMR106 cells (~1 × 10⁶ cells in 100 μl of 0.3% bovine serum albumin/Dulbecco’s modified Eagle’s medium) were added to the upper chamber of Chemotaxicell (a modified Boyden chamber), followed by incubation for 4 h. Ten fields at ×400 per filter were counted to obtain the migrated cell number (1 field = 1/160 of entire surface of filter). The value shown as the Migrated Cell Number is the mean ± S.E. (n = 3) per field. A representative of three independent experiments is shown. B, a filter coated on its lower surface with PLL, yeast-produced human MK (γ-hMK), chemically synthesized human MK (c-hMK), or baculovirus-produced mouse MK (mMK), at 20 μg/ml, was used for the assay. MK was added to the lower chamber in a soluble form at 100 ng/ml (sol. MK). C, the effect of soluble MK at the indicated concentrations on the migration of UMR106 cells induced by coated MK on the filters was examined.

(21). However, only a limited body of information concerning the signaling involved in MK-mediated cell migration is available. We conducted the present study to elucidate the molecular components essential for MK-mediated cell migration, and to test our hypothesis that MK and PDGF could cooperate in cell migration.

EXPERIMENTAL PROCEDURES

Cell Line, Reagents, and Antibodies—A rat osteoblast-like cell line, UMR106 (ATCC No. CRL 1661), was purchased from the American Type Culture Collection. Heparin, chondroitin sulfate A, C, D, and E, chondroitinase ABC, AC II, and B, and heparitinase were purchased from Seikagaku, Japan. Dermatan sulfate, phosphatidylinositol, and phosphatidylsulfatidinositol, and phosphatidylcholine 4-monophosphate were obtained from Sigma. The inhibitors for Src (PP1), protein kinase C (Ro318220), and a MAP kinase kinase, MEK (PD98059), were from Alexis Biochemicals, Calbiochem-Novabiochem and Biomol Research Laboratories, respectively. The other inhibitors for protein kinase C (H7 and calphostin C), PI3-kinase (wortmannin), and phospholipase C (U-73122) were products of Calbiochem-Novabiochem and Biomol Research Laboratories, respectively. The peptide inhibitors for protein kinase C (Ro31-8220), and PI3-kinase (wortmannin), and phospholipase C (U-73122) were products of Calbiochem-Novabiochem and Biomol Research Laboratories, respectively. The other inhibitors for protein kinase C (H7 and calphostin C), PI3-kinase (wortmannin), and phospholipase C (U-73122) were products of Calbiochem-Novabiochem and Biomol Research Laboratories, respectively.

The procedure for producing recombinant human MK with yeast has already been described (14), and in this paper MK means human MK produced by yeast unless specified otherwise. Recombinant mouse MK was expressed with baculovirus and purified as described previously (9). Chemically synthesized human MK was purchased from the Peptide Institute, Japan. The procedure for producing recombinant human PTN/HB-GAM with yeast was the same as that for MK described previously (14). The monoclonal anti-PI3-kinase (p85α) antibody and anti-phospho-AKT-(Ser-473) antibody were purchased from Upstate Biotechnology and Cell Signaling Technology, respectively. The rabbit anti-phosphorylated Erk antibody was from New England Biolabs. The monoclonal anti-Erk2 antibody and monoclonal anti-PTPα antibody (PTPα) antibody were purchased from Transduction Laboratories. The rabbit polyclonal anti-PTPα antibody (anti-6B4) was prepared as described previously (22).

Cell Migration Assay, PTPα Extraction, and Chondroitinase Digestion—The migration assay was performed as described previously (14, 19, 20) using Chemotaxicell (Kurabo, Japan; 8-μm pores). The migration assay was performed with medium containing MK (20 μg/ml) or PTN/HB-GAM alone, or together. MK, coated with 20 μg/ml MK; PTN, 10 μg/ml PTN/HB-GAM; P-M10, 10 μg/ml MK and 5 μg/ml PTN/HB-GAM; P-M20, 20 μg/ml MK and 5 μg/ml PTN/HB-GAM. Values are the means ± S.E.; n = 3.

RT-PCR—Five μg of total RNA from 15-day-old rat brain or cultured cells was used for reverse-transcription with TrueScript (Sawady, Japan). The primers used for PCR were: 5'-TCTTCAACATCCTGAA-TCTACTCTCCA-3' and 5'-CTTATGAGTCTGCAACGATGG-AGCCGA-3'. The 474-bp PCR product corresponded to the 1587–2061 fragment of rat PTPα (GenBank™/EBI no. U90357), which was confirmed by DNA sequencing.

Western Blot Analysis—Polystyrene beads (Polysciences) were coated with MK (20 μg/ml) or PLL (5 μg/ml, plus 15 μg/ml bovine serum albumin) at 4 °C overnight or room temperature for 2 h. The beads were washed with phosphate-buffered saline four times before use. UMR106 cells starved for 24 h were stimulated with the coated polystyrene beads. Cells were lysed in a buffer comprising 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, 40 mM leupeptin, and 0.2 mM sodium vanadate. Thirty μg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane, followed by detection with anti-phosphorylated Erk, anti-Erk2, anti-phosphorylated AKT, or anti-AKT antibody.

PI3-kinase Assay—UMR106 cells were treated as described for Western blot analysis of Erk phosphorylation. PI3-kinase activity was meas-
ured by in vitro phosphorylation of phosphatidylinositol, using essentially the same method as described previously (24).

Immunofluorescence Microscopy—UMR106 cells, which had been exposed to MK- or PLL-beads for 15 min, were fixed with 4% paraformaldehyde and then stained with anti-PI3-kinase (p85α) antibody/flourescein isothiocyanate-conjugated anti-rabbit IgG and/or anti-RPTPβ (PTPζ) monoclonal antibody/horseradish peroxidase-conjugated anti-mouse IgG antibody/cyanine 5-labeled tyramide. For PTPζ staining, the TSA system (PerkinElmer Life Sciences) was employed to enhance the signal.

RESULTS

MK Induces Haptotactic Migration of Osteoblast-type Cells—PTN/HB-GAM, another member of the MK family, induces haptotactic migration of several osteoblast cell lines (18). We first investigated whether or not MK had the same activity. When coated on the lower surface of a filter, MK induced the haptotactic migration of UMR106 cells, with the maximum level at the concentration of 20 μg/ml (Fig. 1A). The profile of MK-induced cell migration was similar to that of the colon carcinoma cell migration induced by several extracellular matrix proteins, such as fibronectin, vitronectin, laminin-1, and collagen IV (25). As chemically synthesized human MK and baculovirus-produced mouse MK showed the same activity as that of yeast-produced human MK (Fig. 1B), the migratory effect was not caused by impurities in the MK used. MK contains 30% basic amino acids. However, PLL showed very weak migratory activity (Fig. 1B), suggesting that the effect of MK was not because of its high basicity.

Soluble MK did not induce UMR106 cell migration (Fig. 1B), even if the filter was precoated with collagen I (data not shown). On the contrary, when added to the lower chamber, soluble MK inhibited coated MK-induced migration (Fig. 1C). At the concentration of 10 μg/ml, soluble MK completely abolished coated MK-induced migration. The same concentration of soluble PLL in the lower chamber did not inhibit MK-induced migration (data not shown). These findings indicate that the substratum-bound form of MK is active in cell migration. Thus, MK could induce haptotactic, but not chemotactic, migration of UMR106 cells.

FIG. 3. Effect of glycosaminoglycans on MK-induced cell migration. A, UMR106 cells were preincubated at 37 °C for 30 min with glycosaminoglycans at the concentration of 20 μg/ml or that indicated. The migration assay was carried out with the same glycosaminoglycan concentration in the lower chamber as in the upper chamber. CS means chondroitin sulfate. B and C, UMR106 cells were preincubated at 37 °C with heparitinase for 90 min (B), or chondroitinase ABC for 30 min (C). D, at 20 milliunits/ml, chondroitinase ABC and B, but not AC II, inhibited the migration. Values are the means ± S.E.; n = 3.

FIG. 4. Possible role of PTPζ in MK-induced cell migration. A, 5 μg of total RNA were used for RT-PCR. L cell, fibroblast cell line; G401 cell, Wilms' tumor cell line. Upper panel, PTPζ; lower panel, GAPDH. B, Western blot analysis with anti-RPTPβ (PTPζ) antibody before and after chondroitinase digestion. Arrowheads and an arrow indicate the positive bands after chondroitinase ABC digestion. The bracket indicates the smear detected for UMR106 cells. Note the nonspecific bands for all lysates of UMR106 and L cells. C, sodium vanadate (NaVa) inhibited MK-induced migration of UMR106 cells. D, preincubation with anti-PTPζ (anti-6B4) antibodies, but not control rabbit IgG, enhanced MK-induced migration. Values are the means ± S.E.; n = 3.
Soluble MK and PTN Abrogate Haptotaxis Mediated by MK and PTN/HB-GAM—We next addressed the question of whether or not MK and PTN/HB-GAM share the same cell surface binding site(s). Soluble MK abrogated PTN/HB-GAM-induced haptotaxis and vice versa (Fig. 2A). Coating with a combination of MK and PTN/HB-GAM did not enhance the migration as compared with MK or PTN/HB-GAM alone (Fig. 2B). These data suggest that if the total molar concentration of MK and PTN/HB-GAM reaches a critical level, the migration activity will be saturated, and MK and PTN/HB-GAM may function through common molecule(s) on the cell surface.

Proteoglycan Is Involved in MK-induced Migration—Heparan sulfate proteoglycans, such as syndecan-1, -3, and -4, and a chondroitin sulfate proteoglycan, PTP\(\zeta\), are supposed to act as a receptor or co-receptor for MK (20, 26, 27, 28). We investigated the effects of different glycosaminoglycans on MK-mediated migration. In addition to heparin, dermatan sulfate (chondroitin sulfate B) and chondroitin sulfate E showed comparable effects. They abolished the migration at the concentration of 20 \mu g/ml (Fig. 3A). At 20 \mu g/ml, chondroitin sulfate A, C, and D showed only minor effects (Fig. 3A). Treatment with heparitinase to remove the heparan sulfate on the cell surface did not affect MK-mediated migration (Fig. 3B). On the contrary, chondroitinase ABC digestion and chondroitinase B digestion down-regulated MK-mediated migration (Fig. 3, C and D), suggesting the involvement of cell surface chondroitin sulfate.

Involvement of PTP\(\zeta\) in MK-induced Migration—As PTP\(\zeta\) exhibits high affinity to both MK and PTN/HB-GAM (20, 29), the results shown in Figs. 1, 2, and 3 suggest that PTP\(\zeta\) is involved in MK-induced haptotaxis. Using RT-PCR, a band corresponding to PTP\(\zeta\) was detected for UMR106 cells and rat brain (Fig. 4A). The PCR product was confirmed by DNA sequencing (data not shown). No positive band was detected for L cells or Wilms’ tumor cells (Fig. 4A). This is consistent with the observation that coated MK did not induce the migration of these cells with a 4-h incubation (data not shown). On Western blotting with monoclonal anti-RPTP\(\beta\) (PTP\(\zeta\)) antibody, which recognizes the intracellular domain of PTP\(\zeta\), a smear was detected for the lysate of UMR106 cells (Fig. 4B, bracket, lane 3). This changed to one band corresponding to about 240 kDa after chondroitinase ABC digestion (Fig. 4B, lane 4, arrow). For rat brain, smears were also detected (Fig. 4B, lane 1), which shifted to 380 and 220 kDa upon digestion with chondroitinase ABC (Fig. 4B, lane 2, arrowheads), which represent long- and short-type receptors, respectively (23). The size difference around 220-240 kDa between the brain and UMR106 cells is probably caused by differential glycosylation, which is dependent on the cell type (30). Chondroitinase AC II and B appeared to digest the glycosaminoglycan chains of PTP\(\zeta\) only partially, because the band around 240 kDa was broader than the band digested with chondroitinase ABC (Fig. 4B, lanes 8, 9, 10). This is reasonable because chondroitinase AC II and B recognize different structures (31, 32). Nevertheless, only chondroitinase ABC and B, but not AC II, suppressed MK-induced migration (Fig. 3D), suggesting that structures susceptible to chondroitinase ABC and B are important. As chondroitinase B can digest both dermatan sulfate and E-type dermatan sulfate (32), and chondroitinase ABC can digest all chondroitin and dermatan sulfates (33), this is consistent with the finding that MK-induced cell migration was inhibited by dermatan sulfate (chondroitin sulfate B) and chondroitin sulfate E (Fig. 3A).

PTP\(\zeta\) is also known to be a receptor-type protein-tyrosine

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**Fig. 5. Effect of different inhibitors on MK-induced cell migration.** UMR106 cells 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 concentration of the same inhibitor. A, concentrations of inhibitors: tyrosine kinase-herbimycin A (herb), 1 \mu g/ml; tyrosine kinase-genistein (geni), 50 \mu g/ml; Src-PPI, 10 nm; protein kinase C-Ro318220 (Ro), 2 \mu M; PI 3 kinase-wortmannin (wort), 100 nm; MEK-PD98059 (PD), 20 \mu M; phospholipase C-U73122 (U-7), 10 \mu M. B and C, dose dependence of protein kinase C inhibitors (B) and MEK, PI3 kinase, and phospholipase C inhibitors (C). The units are: PD98059, \mu M; wortmannin, nm; U73122, \mu M. Values are the means ± S.E.; \(n = 3\).

**Fig. 6. Erk activation after MK stimulation.** UMR106 cells stimulated with polystyrene beads coated with PLL (5 \mu g/ml) or MK (20 \mu g/ml) for the indicated times were analyzed by Western blotting with anti-phosphorylated Erk (P-Erk1/2) and anti-Erk2 (Erk2) antibodies. The densitometric data obtained on Western blotting are shown in the graph. In B, inhibitors were used as described in the legend to Fig. 5.
phosphatase. The effect of orthovanadate, an inhibitor of protein-tyrosine phosphatase, was examined. Sodium vanadate decreased the migration in a dose-dependent manner (Fig. 4C).

Sodium vanadate had no effect on PLL or PDGF-BB-induced migration of UMR106 cells (data not shown). If the cells were preincubated with anti-PTPζ (anti-6B4) antibodies, which recognize the ectodomain of the full-length PTPζ (23), MK-mediated cell migration was enhanced (Fig. 4D). This suggests that ligation of cell surface PTPζ with its ligands or antibodies may transduce signals essential for the migration of UMR106 cells.

MK Induces MAP Kinase and PI3-kinase Activity — To identify the intracellular molecules that participate in MK-mediated cell migration, various specific inhibitors were screened. Tyrosine kinase inhibitors (genistein and herbimycin A), a Src inhibitor (PP1), protein kinase C inhibitors (H7, Ro318220, and calphostin C), a PI3-kinase inhibitor (wortmannin), a MAP kinase kinase (MEK) inhibitor (PD98059), and a phospholipase C inhibitor (U-73122) effectively inhibited MK-mediated cell migration (Fig. 5). This probably indicates that a dynamically concerted signaling interaction is essential for the cell migration induced by MK.

When UMR106 cells were stimulated with MK-coated polystyrene beads, a transient increase in phosphorylation of MAP kinases (Erk1 and Erk2) was detected, the maximum level being observed ~20 min after stimulation (Fig. 6A). At 60 min, it was still higher than the basal level (Fig. 6A). PLL also enhanced Erk phosphorylation, but the increase was less and the duration was shorter than in the case of MK (Fig. 6A).

Interestingly, the PI3-kinase inhibitor wortmannin partly blocked MK-induced Erk phosphorylation, whereas the protein kinase C inhibitor Ro318220 did not (Fig. 6B). MK also increased PI3-kinase activity, the profile being similar to that in the case of PDGF-BB (Fig. 7A). Neither Ro318220 nor MEK inhibitor PD98059 affected MK-induced PI3-kinase activation (Fig. 7B).

MK Recruits PTPζ and PI3-kinase — To further confirm MK-induced PI3-kinase activation, phosphorylation of AKT was examined. AKT (protein kinase B) plays important roles in many biological phenomena, such as cell survival (34, 35). AKT contains a pleckstrin homology (PH) domain, and is phosphorylated (activated) at Thr-308 and Ser-473 residues by PDK1, which also carries a PH domain. The PH domain recognizes a phosphoinositide headgroup, and phosphorylation at the 3 position of inositol ring of phosphatidylinositol in the cell membrane is critical for AKT- and PDK1-binding and recruitment to the cell membrane (35). As this phosphorylation is mediated by PI3-kinase, AKT is an important PI3-kinase effector, and its activation is often used as a marker of PI3-kinase activation (34, 35).

MK enhanced AKT phosphorylation (Fig. 8A, left), which is consistent with the data for MK-induced PI3-kinase activation shown in Fig. 7A. When the cells were preincubated with anti-PTPζ (anti-6B4) antibodies, MK-induced AKT activation was stronger than that of the cells treated with control IgG (Fig. 8A, right). This was consistent with that anti-PTPζ (anti-6B4) antibodies enhanced MK-induced migration of UMR106 cells (Fig. 4D). It is of interest that, in addition to the PI3-kinase inhibitor wortmannin, the protein-tyrosine phosphatase inhibitor orthovanadate and Src inhibitor PP1 blocked MK-induced AKT activation (Fig. 8A, right), but neither protein kinase C inhibitor Ro318220 nor MEK inhibitor PD98059 affected it (data not shown). As the effect of orthovanadate on MK-induced AKT phosphorylation suggested the possibility of a close connection between PTPζ and PI3-kinase, we localized...
PTPζ and PI 3-kinase after MK beads stimulation on UMR106 cells. MK beads induced PI3-kinase recruitment to the sites of the beads (Fig. 8B, left two panels). Furthermore, MK-beads induced colocalization of PI3-kinase and PTPζ (Fig. 8B, lower two panels).

**FIG. 8.** Involvement of PI3-kinase and PTPζ in MK signaling. A, left; UMR106 cells were exposed to MK- or PLL-beads at 37 °C for 15 min. Cell extracts were then analyzed for phosphorylated AKT (Ser-473) (p-Akt) or AKT expression by Western blotting. The densitometric ratio (p-Akt versus Akt) was calculated for each condition, and the relative density of p-Akt is shown at the bottom. A, right; UMR106 cells were incubated with either anti-PTPζ (anti-6B4) antibodies or control rabbit IgG together with the indicated inhibitor at 4 °C for 2 h. The cells were then exposed to MK-beads in the presence of indicated inhibitor at 37 °C for 15 min. B, UMR106 cells were exposed to MK- or PLL-beads at 37 °C for 15 min. The localization of PI3-kinase and PTPζ was examined by immunofluorescence microscopy (PI3-kinase, FITC; PTPζ, cyanine 5) using a confocal microscope (MRC-1024, Bio-Rad). The lower two panels show the same cell that was double-stained with anti-PI3-kinase and anti-RPTPβ (PTPζ) antibodies. Arrows and asterisks indicate cells examined and beads, respectively.

**FIG. 9.** Synergistic effect of MK and PDGF-BB on cell migration. A, PDGF-BB-induced migration was inhibited by protein kinase C and PI3-kinase inhibitors. PDGF-BB (20 ng/ml) was added to the lower chamber. Concentrations of reagents: PD98059, 20 μM; Ro318220, 2 μM; wortmannin, 100 nM; heparin, 20 μg/ml. B, MK and PDGF-BB showed synergistic effect to induce UMR106 cell migration. The filter of Chemotaxicell was coated with collagen I on both its upper and lower surfaces or with MK on only its lower surface. Migration was then monitored in the presence or absence of PDGF-BB in the lower chamber. C, MK/PDGF-BB-mediated migration was not affected by MEK inhibitor. PD98059, Ro318220, and wortmannin were used as described in A.

**DISCUSSION**

MK has been reported to be induced in areas of a variety of types of tissue injury, such as cerebral and heart infarction, bone fractures, skin burns, and arterial endothelial injury (12, 13, 14, 36, 37). In the case of arterial endothelial injury, we found not only the induction of MK expression in wild-type mice, but also dramatically suppressed neointima formation in MK-deficient mice (14). The administration of the protein kinase C inhibitor to MK-deficient mice caused resumption of neointima formation. Thus, MK seems to be vital for tissue remodeling. The present study revealed another important aspect of the mode of MK action, namely, that it acts synergistically with PDGF in cell migration. PDGF was first purified from platelet α granules and has several important activities, such as mitogenesis.
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and chemotaxis. Because tissue injury is usually accompanied by bleeding and/or cessation of blood flow, an abundance of PDGF can be found in these areas. PDGF expression is also induced during the process of wound healing (38). Furthermore, PDGF induces the migration of smooth muscle cells (16, 39) and osteoblasts (40), and the migration of both is also induced by MK (Ref. 14 and the present study). Taken together, the synergism between MK and PDGF appears to be pivotal in many in vivo situations.

Several possibilities should be considered for the mechanism underlying the synergism between MK and PDGF. The present study revealed a difference in the signaling mechanism between MK and PDGF. Heparin inhibited MK-induced cell migration but did not enhance PDGF-induced cell migration (Figs. 3A and 9A). MAP kinases were essential for MK-mediated cell migration but not for PDGF-induced cell migration (Figs. 5 and 9). Orthovanadate inhibited MK-induced cell migration but not PDGF-induced cell migration (Fig. 4C and data not shown). Because distinct signaling molecules are used for cell migration, depending on the ligand (41, 42), the synergism between MK and PDGF might be attributed to the integration of distinct signaling pathways. Alternatively, induction of MK receptor(s) by PDGF or vice versa should also be considered, as in the case of the induction of the Interleukin-1 receptor or PDGF receptor by the neuropeptide substance P in their synergism in bone marrow fibroblast proliferation (43). In addition, a third cell surface molecule, such as integrin β1, might be involved in the synergism, like in the case of the synergism between lysophosphatidic acid and epidermal growth factor or PDGF in cell migration (44).

MAP kinases (Erk1 and Erk2) can activate myosin light chain kinase and induce changes in the cytoskeletal structure, leading to cell migration (45). In this context, it is noteworthy that MK enhances collagen gel contraction by dermal fibroblasts (46). MK-induced MAP kinase activation appeared to be at least partly regulated by PI3-kinase (Fig. 6B). PI3-kinase functions as an early intermediate in Gβγ-mediated MAP kinase activation (47). Several papers have reported that PI3-kinase acts upstream of MAP kinase, e.g. in insulin signaling in 3T3-L1 adipocytes and PDGF signaling in Swiss 3T3 cells (48, 49).

The present study demonstrated that MK recruited PTPζ and PI3-kinase (Fig. 6B). Furthermore, MK-induced PI3-kinase activation was inhibited by the Src inhibitor PP1 and protein-tyrosine phosphatase inhibitor orthovanadate (Fig. 8A). Src activation is sometimes needed for PI3-kinase activation (50) and requires dephosphorylation at its C-terminal phosphotyrosine, which can be mediated by PTPs (51). Taken together, the present results suggest a possible MK signaling cascade that is, MK binds and activates PTPζ, which then activates Src and PI3-kinase and further activates MAP kinases. However, details of the precise mechanism underlying the interaction between these molecules remain to be elucidated, and other unidentified important molecules may be involved in MK signaling.

With regard to the sugar structure essential for the binding, the MK and heparin interaction needs all the three sulfate groups in the heparin disaccharide unit (2-O-β-, N-, and O-sulfation) (52). Dextran sulfate, which has 1.5 sulfate residues per sugar residue, strongly inhibits MK-sulfate binding (53). In the case of MK binding to PG-M/versican, a matrix chondroitin sulfate proteoglycan, disulfated disaccharides were identified (54). Furthermore, chondroitin sulfate E specifically inhibits MK-dependent neuronal cell adhesion (55). PTPζ harbors chondroitin sulfate chain, which is important in MK-induced migration of neurons (20). The present study revealed that the chondroitin sulfate chain in PTPζ is also important in migration of osteoblast-like cells. Differential susceptibility of the migratory activity to chondroitininas with different specificities gave further insights into the nature of chondroitin sulfate chain in PTPζ. Digestion with chondroitinase B abolished MK-dependent migratory activity, whereas chondroitinase AC II did not. The former enzyme acts on chondroitin sulfate with the iduronic acid residue, namely dermatan sulfate, whereas the latter does not. Thus, it is concluded that chondroitin sulfate, which is important in MK-signaling in PTPζ, has a dermatan sulfate domain. The finding that the MK-induced migration is inhibited by dermatan sulfate is consistent with the view. The MK activity was also inhibited by chondroitin sulfate E, which is an oversulfated chondroitin sulfate with 4,6-disulfo-N-acetylgalactosamine residue. Taken together, these observations strongly suggest the presence of dermatan sulfate domain. Indeed, E-type structure with a dermatan sulfate domain was found in PG-M/versican, which was isolated from mouse embryos and has MK binding activity (54).

The characteristics of MK-induced migration of UMR106 cells are very similar to those of MK- and PTN/HB-GAM-induced neuronal migration in that PTPζ is involved in haptotactic migration (19, 20). In this context, the effect of anti-PTPζ antibodies on MK-mediated cell migration was unexpected. These antibodies effectively inhibited PTN/HB-GAM-mediated neuronal migration in the previous study, probably because of competitive inhibition for the PTN/HB-GAM-binding sites of cell surface PTPζ by the antibodies (20). In the present study, the antibodies rather enhanced MK-mediated osteoblast-like cell migration. One possible interpretation of this is that PTPζ on UMR106 cells may physically associate with another unidentified component necessary for signal transduction and thus can be readily activated by the oligomerization or conformational change induced by a specific antibody. On nerve cells, PTPζ might need MK to associate with such a component. Supporting our data, Revest et al. (56) recently reported that cross-linking of PTPζ with antibodies enhances the protein-tyrosine phosphatase activity of C6 astrocytoma cells.

In this study, we confirmed the involvement of Erk1 and 2 and PI3-kinase in MK-induced cell migration by detecting their activities or activity induced by MK, in addition to by demonstrating the effects of inhibitors of them. Consistent with our data, Soutou et al. reported that Erk1 and 2 and PI3-kinase are involved in PTN/HB-GAM-mediated cell proliferation (57). In addition, Src, JAK1, and 2 and β-catenin have been reported to be involved in PTN/HB-GAM signaling (58, 59, 60). A study on the possible involvement of these molecules in MK-induced cell migration is underway in our laboratory.

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