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Expression and functional role of formyl peptide receptor in human bone marrow-derived mesenchymal stem cells

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Abstract **We investigated the expression of formyl peptide receptor (FPR) and its functional role in human bone marrow-derived mesenchymal stem cells (MSCs). We analyzed the expression of FPR by using ligand-binding assay with radio-labeled *N*-formyl-met-leu-phe (fMLF), and found that MSCs express FPR. FMLF stimulated intracellular calcium increase, mitogen-activated protein kinases activation, and Akt activation, which were mediated by G_i proteins. MSCs were chemotactically migrated to fMLF. FMLF-induced MSC chemotaxis was also completely inhibited by pertussis toxin, LY294002, and PD98059, indicating the role of G_i proteins, phosphoinositide 3-kinase, and extracellular signal regulated protein kinase. N-terminal fragment of annexin-1, Anx-1(2–26), an endogenous agonist for FPR, also induced chemotactic migration of MSCs. Thus MSCs express functional FPR, suggesting a new (patho)physiological role of FPR and its ligands in regulating MSC trafficking during induction of injured tissue repair.**

Keywords: Mesenchymal stem cells; Formyl peptide receptor; Chemotaxis; fMLF; Annexin-1; Pertussis toxin-sensitive G-protein

1. Introduction

Since mesenchymal stem cells (MSCs) are precursors that can be differentiated into several specialized cell types and tissues, they have been regarded as an important therapeutic tool for clinical application in the field of damaged tissue remodeling and so on [1]. For the proper action of MSCs to differentiate into a certain type of cells in a specific location, MSCs should migrate to a site of injury. Several factors that regulate MSC migration have been reported. They include some chemokines including stromal-derived factor-1 [2,3].

Formyl peptide receptor (FPR), a chemoattractant receptor, is mainly expressed in phagocytic cells and plays an important role in host defense against pathogen infection [4]. Activation of FPR induces diverse cellular responses including chemotactic migration and superoxide generation [4]. Recently FPR also has been reported to be expressed in non-phagocytic cells, such as fibroblasts [5]. Very recently Viswanathan et al. reported that human bone marrow-derived mesenchymal stem cells express functional FPRs [6].

In this study, we investigated the effect of FPR agonists on the chemotactic migration of MSCs and the signaling pathway involved in the process.

2. Materials and methods

2.1. Materials

N-Formyl-met-leu-phe (fMLF) was purchased from Sigma (St. Louis, MO). [^{3}H]-labeled fMLF was obtained from Perkin–Elmer Life Sciences, Inc. (Boston, MA). Cyclosporin H (CsH) was kindly provided by Novartis Pharma (Basel, Switzerland). N-terminal fragment of annexin-1, Anx-1(2–26), was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA). Pertussis toxin (PTX) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) were from Calbiochem (San Diego, CA). Fura-2 pentaacetoxymethylester (fura-2/AM) and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) were purchased from Molecular Probes (Eugene, OR). Enhanced chemiluminescence reagents from Amersham Biosciences (Piscataway, NJ), phospho-extracellular signal regulated protein kinase (ERK)1/2, phospho-p38 and ERK2 antibodies were purchased from New England Biolabs (Beverly, MA). Phospho-Akt antibody, Akt antibody, fibrinogen, and fibronectin were purchased from Sigma (St. Louis, MO). 2′-Amino-3′-methoxyflavone (PD98059) and 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) were obtained from Biomol (Plymouth Meeting, PA) and were dissolved in dimethyl sulfoxide before being added to the cell culture. The final concentrations of dimethyl sulfoxide in culture were 0.1% or less.

2.2. Isolation and culture of MSCs

Human bone marrow stem cells were isolated as described previously [7]. Bone marrow stromal cells were isolated and were plated in T75 flasks for continuous passage in αMEM medium supplemented with 20% fetal bovine serum and 1% antibiotic–antimycotic solution. Medium was changed twice weekly, cells detached by trypsin–EDTA and under passage into fresh culture flasks at a ratio of 1:4 upon

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Abbreviations: MSCs, mesenchymal stem cells; FPR, formyl peptide receptor; fMLF, *N*-formyl-met-leu-phe; CsH, cyclosporine H; PTX, pertussis toxin; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Fura-2/AM, fura-2 pentaacetoxymethylester; BAPTA/AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester; ERK, extracellular signal regulated protein kinase; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole; $[Ca^{2+}]_i$, intracellular calcium concentration; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase

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reaching confluence. Cultures were incubated at 37 °C in a humidified incubator with 5% CO_2. Different phenotypic markers of human mesenchymal stem cells including CD29, CD44 and CD66 were employed to confirm the stem cell-like feature of the isolated stromal cells. All the experiments were done using within passage 6 MSCs. The experimental procedures were approved by the Institutional Review Board of Seoul National University Dental Hospital.

2.3. Ligand-binding assay

MSCs were seeded at 5×10^4 cells per well into a 24-well plate and cultured overnight. Several concentrations of [^{3}H]-labeled fMLF were added to the cells in the absence or presence of unlabelled 10 μM fMLF or 10 μM CsH for 3 h at 4 °C with continuous shaking. Specific binding of [^{3}H]-labeled fMLF was counted using a β-ray counter [8].

2.4. Measurement of intracellular calcium concentration

Intracellular calcium concentration $[Ca^{2+}]_i$ was determined using fura-2/AM [9]. Briefly, MSCs were incubated with 3 μM fura-2/AM at 37 °C for 50 min in fresh serum free αMEM medium with continuous stirring. 2×10^6 Cells were aliquoted for each assay into Locke's solution [9]. Fluorescence was measured at 500 nm at excitation wavelengths of 340 nm and 380 nm.

2.5. Western blot analysis

MSCs (2×10^6) were stimulated with the indicated concentrations of fMLF. Cell extracts were separated in 10% SDS–polyacrylamide gel and, the proteins were blotted onto a nitrocellulose membrane. Subsequently, membranes were incubated with specific antibodies. Antigen–antibody complexes were visualized after incubating the membrane with 1:5000 diluted goat anti-rabbit IgG antibody coupled to horseradish peroxidase and detected by enhanced chemiluminescence.

2.6. Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (Neuroprobe Inc. Gaithersburg, MD) [10]. Polycarbonate membrane of 96-well chemotaxis chamber was precoated with fibronectin (20 μg/ml). MSCs were suspended in αMEM at 1×10^6 cells/ml, and 25 μl of this suspension was placed into the upper well of a chamber separated by an 8 μm precoated polyhydrocarbon filter from the peptide containing lower well. After incubation for 12 h at 37 °C, migrated cells were then counted in three randomly chosen high power fields (400×) [10].

2.7. Statistics

The results are expressed as means ± S.E. of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $P < 0.05$.

3. Results

3.1. Expression of FPR in MSCs

To investigate whether MSCs express FPR, we performed a ligand-binding assay using [^{3}H] fMLF in MSCs. The addition of various concentrations of [^{3}H] fMLF demonstrated the concentration-dependent binding of [^{3}H] fMLF to MSCs (Fig. 1A), which was quantified after subtracting non-specific

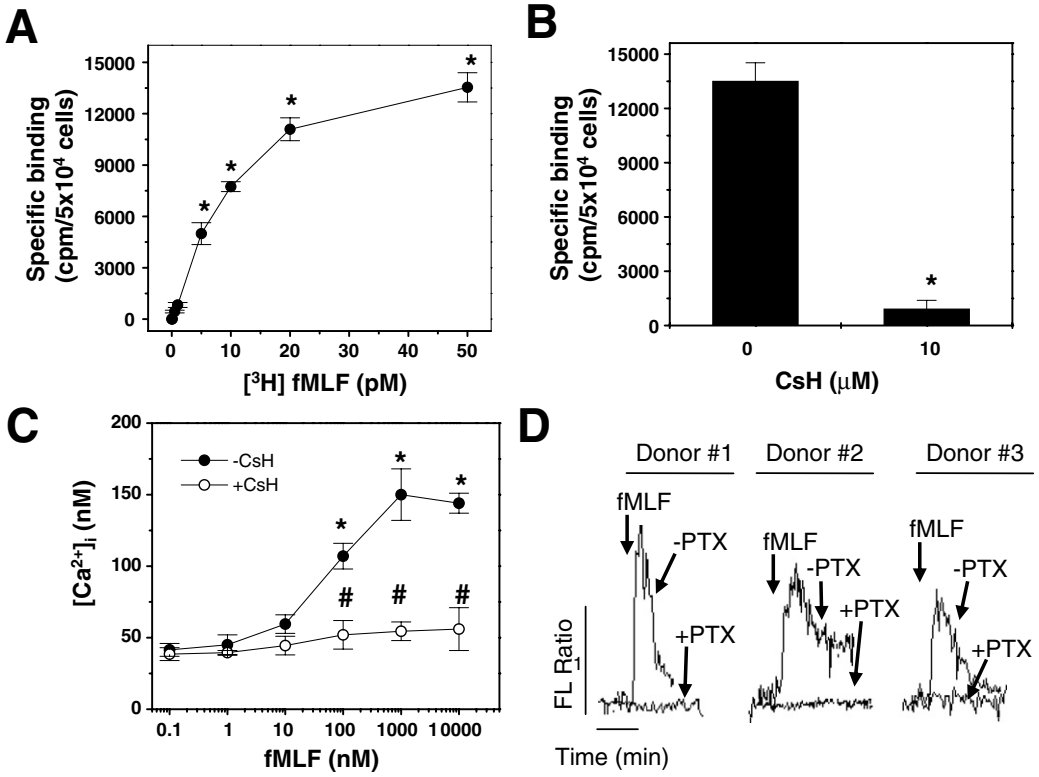

Fig. 1. Functional expression of FPR in MSCs. Several concentrations of [^{3}H]-labeled fMLF was incubated with MSCs for 3 h at 4°C in the absence or presence of 10 μM of unlabeled fMLF (A). 50 pM [^{3}H]-labeled fMLF was incubated with MSCs for 3 h at 4 °C in the absence or presence of 10 μM of CsH (B). The quantity of bound [^{3}H]-labeled fMLF was determined using a β-ray counter. The results are presented as means ± S.E. of three independent experiments (A, B). MSCs were then loaded with fura-2/AM and $[Ca^{2+}]_i$ was determined fluorometrically after stimulation with several concentrations of fMLF in the absence or presence of CsH (10 μM) (C). The results are presented as means ± S.E. of three independent experiments (C). Three differently prepared MSCs were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h. Cells were stimulated with 1 μM of fMLF (D). Relative intracellular Ca^{2+} concentrations are expressed as fluorescence ratios (340:380 nm). Data are representative of four independent experiments (D). * indicates results significantly different at the $P < 0.05$ probability levels as compared to the values obtained from the control (-fMLF). # indicates results significantly different at the $P < 0.05$ probability levels as compared to the values obtained from the control (DMSO treated).

binding. The specific binding of fMLF in MSCs was proven by adding 0.5 pM of [^{3}H] fMLF, which achieved saturation at ca. 20 pM of [^{3}H] fMLF for 5×10^4 cells (Fig. 1A). The specific binding of 50 pM of [^{3}H] fMLF was almost completely inhibited by 10 μM of CsH (Fig. 1B). These results indicate that MSCs express FPR.

3.2. FMLF stimulates intracellular calcium increase via PTX-sensitive G-protein in MSCs

Previously it has been demonstrated that the activation of FPR by fMLF causes $[Ca^{2+}]_i$ increases [11]. To confirm that FPR on MSCs is functional, we examined the effect of fMLF upon $[Ca^{2+}]_i$ in MSCs. Stimulation of MSCs with several concentrations of fMLF caused $[Ca^{2+}]_i$ increase in a concentration-dependent manner, showing maximal activity at 1 μM fMLF (Fig. 1C). We also examined the effect of PTX, a specific inhibitor of $G_{i/o}$ type G-proteins, on fMLF-induced $[Ca^{2+}]_i$ increase. When MSCs were preincubated with 100 ng/ml of PTX prior to being stimulated with 1 μM fMLF, fMLF-induced $[Ca^{2+}]_i$ increase was completely inhibited (Fig. 1D). FMLF-induced $[Ca^{2+}]_i$ increase was commonly observed from three differently prepared MSCs (Fig. 1D). These results indicate that fMLF stimulates $[Ca^{2+}]_i$ increase via PTX-sensitive pathway.

3.3. FMLF stimulates mitogen-activated protein kinases in MSCs

We examined whether fMLF stimulates mitogen-activated protein kinases (MAPKs) by Western blotting with anti-phospho-specific antibodies to each enzyme. When MSCs were stimulated with 1 μM fMLF for different times, the phosphorylation levels of ERK and p38 MAPK transiently increased, showing maximal activity after 5–10 min of stimulation (Fig. 2A). We also found that stimulation of MSCs with various concentrations of fMLF-induced MAPK phosphorylation. FMLF-induced ERK and p38 kinase phosphorylations in a concentration-dependent manner, showing dramatic activity at 10–1000 nM (Fig. 2B). We investigated the role of PTX-sensitive G-protein on fMLF-induced MAPK activation. MSCs were preincubated with 100 ng/ml of PTX prior to being stimulated with 1 μM fMLF. Pretreatment with PTX dramatically blocked ERK phosphorylation by fMLF (Fig. 2C), showing that fMLF induces ERK activation in a PTX-sensitive manner.

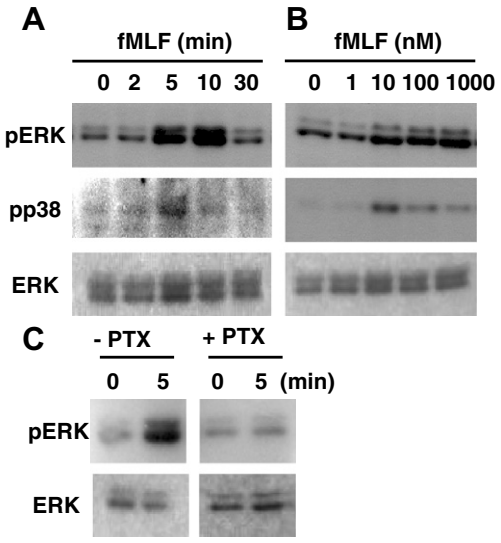

Fig. 2. Activation of MAPKs by fMLF in MSCs. MSCs were stimulated with 1 μM of fMLF for various times (A). The cells were stimulated with various concentrations of fMLF for 5 min (B). MSCs were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h. Cells were stimulated with 1 μM of fMLF for 5 min (C). Western blot analysis was performed using anti-phospho-ERK antibody or anti-phospho-p38 kinase antibody. The results shown are representative of at least three independent experiments (A–C).

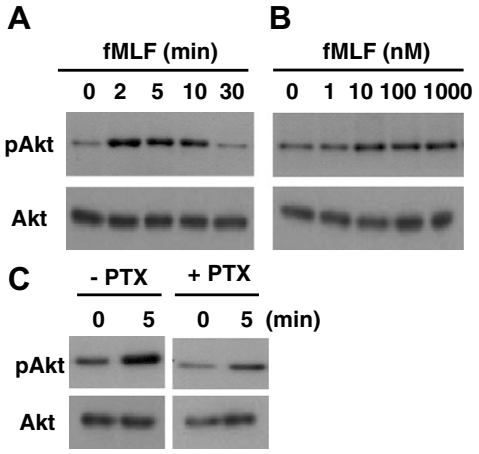

Fig. 3. Activation of Akt by fMLF in MSCs. MSCs were stimulated with 1 μM of fMLF for various times (A). The cells were stimulated with various concentrations of fMLF for 5 min (B). MSCs were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h. Cells were stimulated with 1 μM of fMLF for 5 min (C). Western blot analysis was performed using anti-phospho-Akt antibody. The results shown are representative of at least three independent experiments (A–C).

3.4. FMLF stimulates Akt in MSCs

Akt has been reported to play important roles in the regulation of several cellular responses, such as, cell migration and cell survival [12]. When MSCs were stimulated with 1 μM fMLF for different times, Akt phosphorylation was transiently increased, showing maximal activity after 2–10 min of stimulation and return to baseline 30 min after stimulation (Fig. 3A). In addition, when MSCs were stimulated with different concentrations of fMLF, Akt was activated in a concentration-dependent manner. At 10–1000 nM fMLF caused dramatic Akt (Fig. 3B). FMLF-induced Akt phosphorylation was found to be almost completely inhibited by PTX (Fig. 3C), indicating that fMLF stimulates Akt activation via a PTX-sensitive pathway.

3.5. FMLF induces MSC chemotaxis via PTX-sensitive G-proteins

Since FPR is a chemoattractant receptor and it is involved in the chemotactic migration of phagocytes [4]. We investigated the effect of fMLF on MSCs chemotaxis. It was found that fMLF induced the chemotactic migration of MSCs. Fig. 4A shows the concentration–responsive curve of fMLF-induced MSCs migration, and shows maximal activity at 0.1–1 μM. When MSCs were preincubated with 100 ng/ml of PTX prior to chemotaxis assays, the numbers of cell migrating toward fMLF was dramatically reduced (Fig. 4A), which strongly suggested the involvement of PTX-sensitive G-proteins. We also

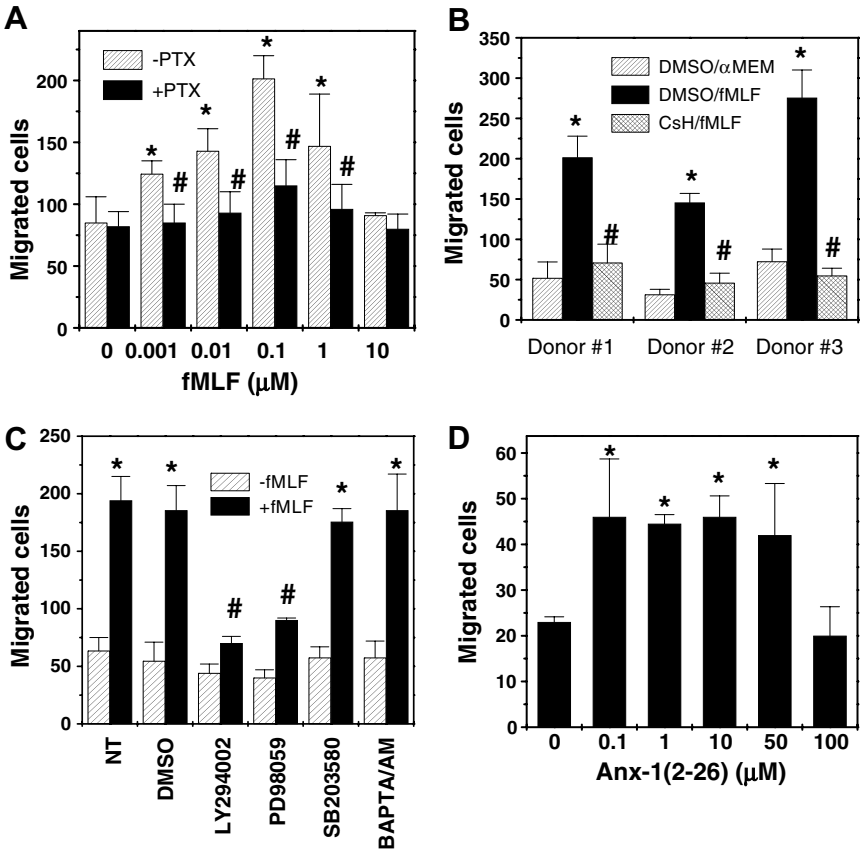

Fig. 4. MSC chemotaxis by fMLF and Anx-1(2–26). MSCs were preincubated in the absence or presence of 100 ng/ml of PTX for 24 h (A). Three differently prepared MSCs were preincubated in the absence or presence of 10 μM CsH for 30 min (B). MSCs were preincubated in the absence or presence of DMSO, LY294002 (50 μM), PD98059 (50 μM), SB203580 (20 μM), or BAPTA/AM (10 μM) for 15 min (60 min for PD98059 and BAPTA/AM) (C). The cells (1×10^6 cells/ml in serum free αMEM) were used for chemotaxis assay in the presence of several concentrations of fMLF (1 μM for B) for 12 h at 37°C (A–C). Various concentrations of Anx-1(2–26) were used for the chemotactic migration (D). Migrated cell numbers were determined by counting in three high power fields (400×). Data are presented as means ± S.E. of three independent experiments performed in duplicate (A–D). * indicates results significantly different at the $P < 0.05$ probability levels as compared to the values obtained from the control (-fMLF). # indicates results significantly different at the $P < 0.05$ probability levels as compared to the values obtained from the control (DMSO treated).

observed that fMLF-induced chemotactic migration was observed in three differently prepared MSCs (Fig. 4B). Furthermore, fMLF-induced MSC chemotaxis was almost completely inhibited by CsH, indicating that fMLF induces MSC chemotaxis via FPR (Fig. 4B). Several chemoattractants stimulate phosphoinositide 3-kinase (PI3K)-mediated Akt activity, and that the PI3K pathway is involved in the chemotactic migration of cells [13,14]. Preincubation of cells with LY294002 (50 μM), a well-known PI3K inhibitor, for 15 min at 37 °C prior to stimulation with fMLF was found to inhibit cellular chemotaxis (Fig. 4C), indicating that MSC activates the PI3K pathway and that this signaling is required for the fMLF-induced chemotaxis of MSCs. We also examined the roles of ERK and p38 kinase on fMLF-induced MSC chemotaxis. When MSCs were preincubated with PD98059 (50 μM) or SB203580 (20 μM) prior to chemotaxis assay, fMLF-induced MSC chemotaxis was found to be dramatically blocked by PD98059, but not by SB203580 (Fig. 4C). The results indicate that ERK-mediated signaling is involved in fMLF-induced MSC chemotaxis. Because fMLF stimulated $[Ca^{2+}]_i$ increases in MSCs, we examined the role of calcium signaling on fMLF-induced MSC chemotaxis using a calcium chelator, BAPTA/AM. At first we observed that preincubation of MSCs with 10 μM BAPTA/AM for 60 min prior to stimulation with fMLF completely inhibited fMLF-induced intracellular calcium increase (data not shown). As shown in Fig. 4C, BAPTA/AM did not affect on MSC chemotaxis induced by fMLF, indicating that calcium signaling is not required for fMLF-induced chemotaxis.

N-terminal fragment of annexin-1, Anx-1(2–26), is an endogenous host-derived agonist for FPR [15]. In this study we also tested the effect of annexin-1 on MSC chemotaxis. As shown in Fig. 4D, stimulation of MSCs with several concentrations of Anx-1(2–26) elicited chemotactic migration of MSCs.

4. Discussion

MSC migration is crucially associated with damaged tissue remodeling [1]. In response to tissue damage, MSCs migrate into damaged sites along chemotactic gradient [1]. Until now various molecules that modulate chemotactic migration of MSCs have been reported [2,3]. FPR is also important

chemoattractant receptor, and expression of FPR has been identified mainly in phagocytic cells, including monocytes, neutrophils, and dendritic cells [4,16]. Very recently some other non-leukocytic cells have been demonstrated to express FPR [5,17]. Some epithelial cells such as lung epithelial cells and hepatocyte express FPR [18,19]. Furthermore, activation of FPR by its selective agonist (fMLF and annexin-1) has been shown to stimulate adhesion, motility, and chemotactic migration of these epithelial cells [18,19]. However, the role of FPR agonists in MSC chemotaxis has not been studied. In the present study, we found that MSCs express functional FPR and its specific agonists (fMLF and Anx-1(2–26)) stimulate the chemotactic migration of the cells. This finding suggests that FPR has a potential role in tissue remodeling, wound healing, and various functional aspects related to MSC migration.

Previously annexin-1, a family of phospholipid-binding proteins, has been reported to be found in many tissues such as lung, bone marrow, and intestine [20]. Annexin-1 is also known as a 2–4% of the total cytosolic protein in neutrophils [21]. Annexin-1 has been reported to regulate several cellular responses [22,23]. Annexin-1 is involved in membrane trafficking and epithelial cell migration and invasion [17]. In Fig. 4D, we demonstrated that Anx-1(2–26), an endogenous FPR agonist, induced chemotactic migration of MSCs. Since in this study we demonstrated that Anx-1(2–26) induced chemotactic migration of MSCs, we suggest a new aspect of annexin-1 as a regulator of MSC migration which is need for tissue repairing.

In our study we also investigated the effect of PTX, which specifically blocks the coupling of G-protein-coupled receptors to G_i, on fMLF-induced signaling. When MSCs were treated with 100 ng/ml of PTX for 24 h prior to fMLF stimulation, fMLF-induced intracellular calcium elevation, ERK phosphorylation, and Akt phosphorylation were almost completely inhibited (Figs. 1D, 2C and 3C). Furthermore, fMLF-induced chemotactic migration was also completely inhibited by PTX treatment, as shown in Fig. 4A. These results were correlated with previous reports that demonstrate fMLF utilizes PTX-sensitive G-protein-coupled receptor, FPR [4]. We also found that fMLF-induced MSC chemotaxis via PI3K, and ERK pathways (Fig. 4C). However, phospholipase C-mediated calcium signaling pathway and p38 kinase pathway were not found to be involved in fMLF-induced chemotaxis (Fig. 4C). In view of the fact that calcium signaling and p38 kinase regulate several kinds of cellular physiologies and that fMLF stimulates phospholipase C-mediated intracellular calcium increase and p38 kinase, it would be interesting to know the other functional roles of fMLF in MSCs related to calcium signaling or p38 kinase-dependent processes.

In conclusion, the present study shows that MSCs express FPR and two of FPR agonists (fMLF and Anx-1(2–26)) induce chemotactic migration of MSCs. Since this study describes the functional expression of FPR and role of its agonists in MSC chemotaxis, further studies on the pathologic and physiologic roles of FPR and its specific agonists are required.

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