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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 radiolabeled *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

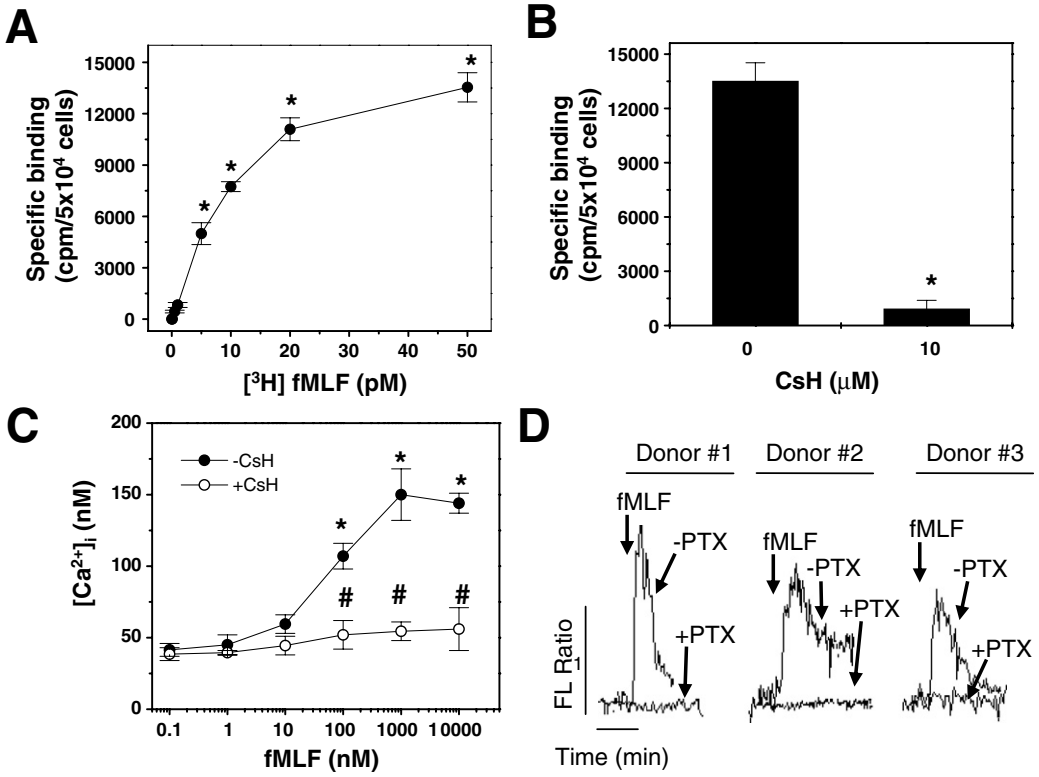

Fig. 1. Functional expression of FPR in MSCs. Several concentrations of [^{3}H]-labeled fMLF was incubated with MSCs for 3 h at 4°C in the absence or presence of 10 μM of unlabeled fMLF (A). 50 pM [^{3}H]-labeled fMLF was incubated with MSCs for 3 h at 4 °C in the absence or presence of 10 μM of CsH (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).