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Antimicrobial peptide scolopendrasin VII, derived from the centipede *Scolopendra subspinipes mutilans*, stimulates macrophage chemotaxis via formyl peptide receptor 1

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In this study, we report that one of the antimicrobial peptides scolopendrasin VII, derived from *Scolopendra subspinipes mutilans*, stimulates actin polymerization and the subsequent chemotactic migration of macrophages through the activation of ERK and protein kinase B (Akt) activity. The scolopendrasin VII-induced chemotactic migration of macrophages is inhibited by the formyl peptide receptor 1 (FPR1) antagonist cyclosporine H. We also found that scolopendrasin VII stimulate the chemotactic migration of FPR1-transfected RBL-2H3 cells, but not that of vector-transfected cells; moreover, scolopendrasin VII directly binds to FPR1. Our findings therefore suggest that the antimicrobial peptide scolopendrasin VII, derived from *Scolopendra subspinipes mutilans*, stimulates macrophages, resulting in chemotactic migration via FPR1 signaling, and the peptide can be useful in the study of FPR1-related biological responses. [BMB Reports 2015; 48(8): 479-484]

INTRODUCTION

Formyl peptide receptors (FPRs) are important chemoattractant G-protein coupled receptors (GPCRs) that mediate the chemotactic migration of leukocytic cells (1, 2). Several different myeloid leukocytes such as neutrophils, monocytes, macrophages, and dendritic cells express functional FPRs. The activation of FPRs in neutrophils and monocytes elicits superoxide anion production and bactericidal activity (1, 3). FPRs are involved in the negative regulation of dendritic cell maturation induced by lipopolysaccharide (LPS) or tumor necrosis factor (TNF)-α (4). We also reported that natural killer cells express functional FPRs that regulate chemotactic migration, interferon (IFN)-γ production, and cytolytic activity (5).

Unlike other chemokine or chemoattractant receptors, FPRs recognize a diverse variety of ligands such as formyl peptides derived from bacteria, formyl peptides derived from mitochondria, and host-derived agonists (serum amyloid A, annexin A4, LL-37, and humanin) (1, 2). One of the FPR members FPR2 also recognizes the lipid-derived eicosanoid lipoxin A4 (6). Among these FPR agonists, LL-37 is an important antimicrobial peptide (AMP) that is capable of killing bacteria (7). We are particularly interested in the finding that an AMP (LL-37) is capable of achieving chemotactic migration via the stimulation of leukocytic cells. Since chemotactic migration of leukocytes into event (infection or injury) areas is important to regulate immune or inflammatory response, the identification of new molecules that regulate leukocyte chemotaxis has received attention.

Previously we reported on several AMP candidates using *de novo* RNA sequencing from *Scolopendra subspinipes mutilans*, and demonstrated that some of the AMPs showed anticancer activity in leukemia cells (8, 9). In this study, we investigated whether AMPs that have been isolated from the centipede may regulate macrophage activity. We demonstrated that an AMP isolated from the centipede stimulates mouse bone marrow-derived macrophages (BMDMs), resulting in chemotaxis. We also showed that FPR1 is the target receptor for the AMP.

RESULTS

Scolopendrasin VII stimulates BMDM chemotactic migration via pertussis toxin (PTX)-sensitive G-protein

Based on the previous report that an AMP LL-37 stimulates leukocyte chemotaxis via cell surface receptor (7), we also tried to see if we can extend the effect of AMP on leukocyte migration with other AMP. To investigate the putative effect of scolopendrasin VII on macrophage activity, we tested whether scolopendrasin VII regulates chemotactic migration in the cells. The

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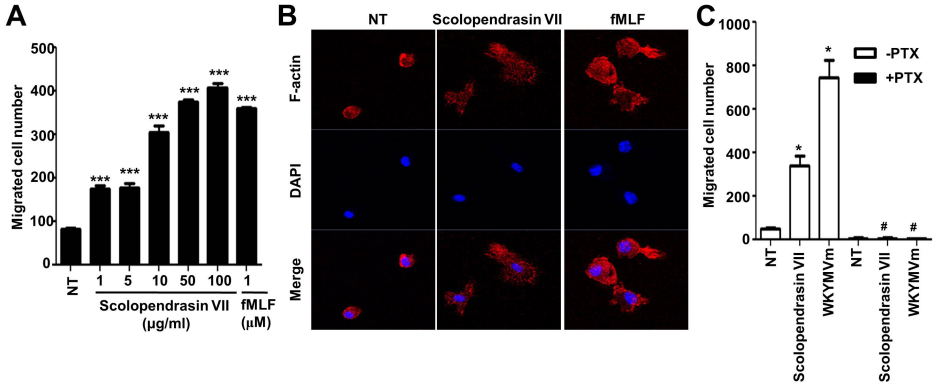

Fig. 1. Scolopendrasin VII stimulates BMDM chemotaxis via PTX-sensitive G-protein. (A) Mouse BMDMs were applied to the upper well of the multiwell chamber containing several concentrations (0 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml, 100 μg/ml) of scolopendrasin VII or 1 μM of fMLF for 2 h. (B) Mouse BMDMs were stimulated with 100 μg/ml of scolopendrasin VII or 1 μM of fMLF for 2 min. Polymerized actin were visualized by staining with rhodamine-phalloidin. Nuclei were stained with DAPI. Data are representative of three independent experiments (B). (C) Mouse BMDMs were incubated in the absence or presence of 100 ng/ml PTX for 24 h, and were applied to the upper well of the multiwell chamber containing 100 μg/ml of scolopendrasin VII or 1 μM of WKYMVm for 2 h. The number of migrated cells was determined by counting under a light microscope. Data are presented as means ± S.E. *$P < 0.05$, ***$P < 0.001$ compared to the NT (not treated) control; #$P < 0.05$ compared to the −PTX control.

scolopendrasin VII strongly induced the chemotactic migration of the BMDMs, showing a concentration-dependency (Fig. 1A), and the 50 μg/ml scolopendrasin VII-induced BMDM chemotactic migration is comparable with the migration induced by the fMLF, a well-known potent chemoattractant for BMDM (Fig. 1A). Since actin polymerization is accompanied with the chemotactic migration of leukocyte (10), we tested the effect of scolopendrasin VII on actin polymerization by staining the cells with rhodamine-phalloidin, which specifically binds to polymerized actin. The stimulation of the BMDMs with scolopendrasin VII induced actin polymerization (Fig. 1B). The results suggest that scolopendrasin VII stimulates BMDMs, resulting in chemotactic migration via actin polymerization.

In previous reports, chemokines and chemoattractants stimulated the chemotactic migration of leukocytic cells via PTX-sensitive G-protein(s) (1, 11). In this study we examined the role of the PTX-sensitive G-protein in scolopendrasin VII-induced chemotaxis. Incubation of the BMDMs with PTX prior to the addition of scolopendrasin VII almost completely inhibited the scolopendrasin VII-induced chemotactic migration of the BMDMs (Fig. 1C). PTX also completely inhibited WKYMVm (a well-known synthetic FPR agonist)-induced chemotaxis (Fig. 1C). The results suggest that PTX-sensitive GPCRs may be involved in the process.

Scolopendrasin VII stimulates ERK and protein kinase B (Akt) activity in BMDMs

Because scolopendrasin VII stimulated the chemotactic migration of BMDMs, we examined the signaling pathways involved in the peptide-induced chemotaxis. Many chemokines and chemoattractants that bind to PTX-sensitive GPCRs stimulate

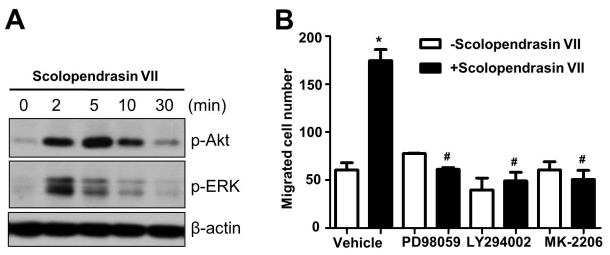

Fig. 2. Scolopendrasin VII stimulates Akt and ERK activity in BMDMs. (A) Mouse BMDMs were stimulated with 100 μg/ml of scolopendrasin VII for several lengths (0 min, 2 min, 5 min, 10 min, 30 min) of time. Total cell lysates were separated in SDS-PAGE, and the levels of p-Akt and p-ERK were measured using a Western blot analysis. Data are representative of three independent experiments (A). (B) Mouse BMDMs were incubated in the absence or presence of PD98059 (50 μM) for 60 min, LY294002 (50 μM) for 15 min, or MK-2206 (2 μM) for 20 min, and were applied to the upper well of the multiwell chamber containing 100 μg/ml of scolopendrasin VII for 2 h. The number of migrated cells was determined by counting under a light microscope. Data are presented as means ± S.E. *$P < 0.05$ compared to the NT (not treated) control; #$P < 0.05$ compared to the scolopendrasin VII alone control.

phospholipase C activity and the subsequent increase of intracellular calcium (1, 12). We checked whether scolopendrasin VII stimulates calcium increase in the BMDMs using fura-2 labeled cells; the stimulation of the fura-2 loaded BMDMs with scolopendrasin VII did not induce an intracellular calcium increase (data not shown).

Mitogen-activated protein kinases (MAPKs) such as ERK, p38 MAPK, and JNK are activated by chemokine or chemoattractant

receptors (1, 13). To see whether scolopendrasin VII stimulates MAPKs in BMDMs, we performed a Western blot analysis. Stimulation of the BMDMs with scolopendrasin VII also induced a transient ERK phosphorylation that was apparent in the region of 2 min to 10 min after stimulation (Fig. 2A); however, scolopendrasin VII did not stimulate p38 MAPK and JNK phosphorylation in the BMDMs (data not shown). Akt is an important enzyme that mediates a diverse range of cellular responses including proliferation, survival, and migration (14). We also examined the effect of scolopendrasin VII on Akt activity by monitoring the phosphorylated level of Akt after the scolopendrasin VII stimulation. Scolopendrasin VII strongly increased the Akt phosphorylation level in a time-dependent manner, showing marked activity in the region of 2 min to 10 min after stimulation (Fig. 2A).

Since scolopendrasin VII stimulated ERK and Akt activity in the BMDMs, we examined the roles of ERK and Akt in scolopendrasin VII-induced BMDM chemotaxis using a specific inhibitor for each enzyme. Incubation of the BMDMs with the ERK inhibitor PD98059 prior to the addition of scolopendrasin VII almost completely inhibited the peptide-induced BMDM chemotaxis (Fig. 2B), moreover the Akt inhibitor MK-2206 completely blocked scolopendrasin VII-induced chemotaxis. Akt activation is mediated by upstream signaling molecules, and PI3K activity in particular is essential for the activation of Akt (14). We also found that the incubation of the BMDMs with the PI3K inhibitor LY294002 completely inhibited scolopendrasin VII-induced BMDM chemotaxis (Fig. 2B). The results suggest that scolopendrasin VII-induced BMDM chemotaxis is mediated by ERK and PI3K-dependent Akt activity.

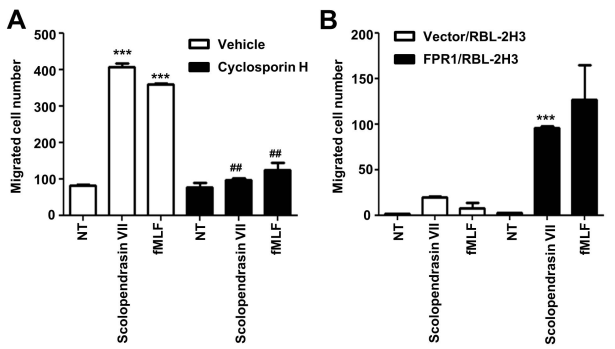

Fig. 3. Scolopendrasin VII-induced BMDM chemotaxis is mediated by FPR1. (A) Mouse BMDMs were incubated in the absence or presence of cyclosporin H (1 μM) for 30 min, and were applied to the upper well of the multiwell chamber containing 100 μg/ml of scolopendrasin VII or 1 μM of fMLF for 2 h. (B) Vector- or FPR1-expressing RBL-2H3 cells were applied to the upper well of a multiwell chamber containing 100 μg/ml of scolopendrasin VII or 1 μM of fMLF. The number of migrated cells was determined by counting under a light microscope. Data are presented as means ± S.E. ***P < 0.001 compared to the NT (not treated) control; ##P < 0.01 compared to the scolopendrasin VII alone control.

Scolopendrasin VII-induced BMDM chemotactic migration is mediated by FPR1

FPR1 is one of the important chemoattractant GPCRs expressed in BMDMs (15). To test the possible role of FPR1 in scolopendrasin VII-induced chemotaxis, we used the FPR1-selective antagonist cyclosporine H (16). Scolopendrasin VII-induced BMDM chemotaxis was almost completely inhibited by cyclosporine H (Fig. 3A), which also completely blocked fMLF-induced BMDM chemotaxis (Fig. 3A). To confirm the role of FPR1 in peptide-induced chemotactic migration, we also tested the effect of scolopendrasin VII on the chemotactic migration of vector- or FPR1-expressing RBL-2H3 cells. Scolopendrasin VII markedly induced the chemotactic migration of the FPR1-expressing RBL-2H3 cells but not that of the vector-expressing cells (Fig. 3B).

Scolopendrasin VII directly binds to FPR1

Since scolopendrasin VII stimulated the chemotactic migration of the BMDMs via PTX-sensitive G-proteins, and FPR1 plays a role in peptide-induced chemotaxis (Fig. 3), we determined whether or not scolopendrasin VII directly binds to FPR1. For this purpose, we synthesized fluorescein isothiocyanate (FITC)-conjugated scolopendrasin VII and incubated the labeled peptide with vector- or FPR1-expressing RBL-2H3 cells. FITC-conjugated scolopendrasin VII did not bind to the vector-express-

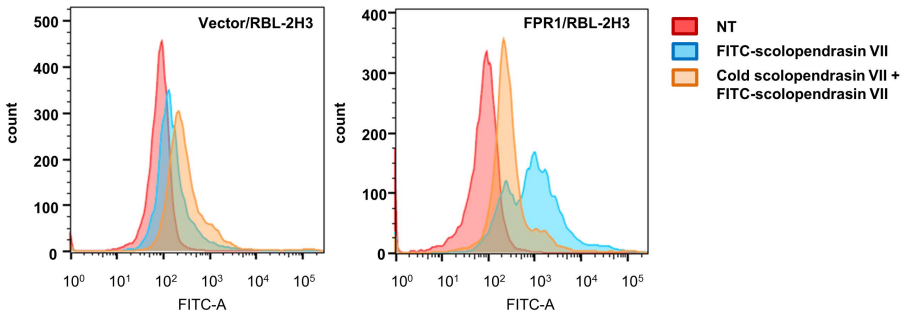

Fig. 4. Scolopendrasin VII directly binds to FPR1. FITC-labeled scolopendrasin VII (1 μg/ml) were incubated for 3 h at 4°C with vector- or FPR1-expressing RBL-2H3 cells in the absence or presence of excess unlabeled scolopendrasin VII (200 μg/ml). Binding of the FITC-labeled scolopendrasin VII was determined using a FACS analysis. Data are representative of two independent experiments.

ing RBL-2H3 cells, but the labeled peptide bound to the FPR1-expressing RBL-2H3 cells (Fig. 4). Moreover, the capability of the FITC-conjugated scolopendrasin VII to bind to the FPR1-expressing RBL-2H3 cells was blocked by the addition of the unlabeled scolopendrasin VII (Fig. 4). The results indicate that scolopendrasin VII directly binds to FPR1, resulting in chemotactic migration.

DISCUSSION

Although FPR2 can recognize AMPs such as LL-37 and CRAMP (7, 17), it has not been reported whether FPR1 can detect an AMP. In this study, we demonstrated that FPR1 detects one of the AMPs derived from *Scolopendra subspinipes mutilans*, scolopendrasin VII. The binding of scolopendrasin VII to FPR1 elicits actin polymerization, resulting in the chemotactic migration of the macrophages (Fig. 1). In terms of the chemotactic activity in macrophages, several other AMPs derived from *Scolopendra subspinipes mutilans* also markedly induced macrophage chemotaxis (data not shown). The results suggest that AMPs from *Scolopendra subspinipes mutilans* may carry immune modulating activity in macrophages.

On the signaling pathway downstream of FPR1, we found that scolopendrasin VII stimulates both ERK and Akt activity, and that these activities are required for the chemotactic migration of the cells (Fig. 2). Previous reports demonstrated that the activation of FPR1 by fMLF and other agonists induces a diverse range of signaling pathways that includes intracellular calcium increase and several kinase activations (1, 2). Intracellular calcium increase is associated with an increased production of reactive oxygen species and heightened bactericidal activity (18). In this study, however, we observed that scolopendrasin VII did not result in an intracellular calcium increase in the macrophages, nor did it evoke a generation of reactive oxygen species from the macrophages (data not shown). We already showed that FPR1 can be differentially regulated by different peptide ligands in a ligand-selective manner (19). Taken together, our results and previous findings show that scolopendrasin VII may selectively regulate FPR1-induced signaling in a ligand-selective manner.

AMPs carry well-conserved common characteristics including an amphipathic structure and a positive charge (20). In addition to bactericidal activity, AMPs may show several important biological activities including the regulation of innate immune responses (21). It has been reported that LL-37 reduced LPS-induced production of TNF-α, but augmented the expression of CXCR4, CCR2, and IL8RB from macrophages (21); LL-37 also enhanced IL-8 production in epithelial cells (21). LL-37 may contribute to a regulation of an immune response by recruiting immune cells into an infection site (21). In this study, we demonstrated that one of the AMPs from *Scolopendra subspinipes mutilans* recruits macrophages via FPR1, a well-known chemotactic receptor, and our results suggest that AMP can recruit immune cells into an event area by using FPR1.

MATERIALS AND METHODS

Materials

Scolopendrasin VII (sequence: FCTCNVKGFNAKNKRGIIYP-NH_2), FITC-conjugated scolopendrasin VII, and WKYMVm were synthesized from Anygen (Gwangju, Korea) with a purity > 99.6%. fMLF and cyclosporine H were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Rhodamine-phalloidin was obtained from Life Technologies (Carlsbad, CA, USA). Boyden chambers were purchased from Neuroprobe Inc. (Gaithersburg, MD, USA). Anti-phospho-Akt, anti-phospho-ERK, and anti-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Generation of BMDMs

The bone marrow cells were isolated by flushing the femurs and tibias of wild-type C57BL/6 mice, 5 weeks to 7 weeks of age, with ice-cold PBS. Bone marrow progenitor cells were cultured in 10% FBS containing α-MEM with 30 ng/ml M-CSF (Peprotech, Rocky Hill, NJ, USA) under standard incubator conditions for 1 day. The non-adherent cells were removed, and the 10% FBS containing α-MEM with 30 ng/ml M-CSF was added, and the cells were subsequently maintained for 3 days.

Chemotaxis assay

Chemotaxis assays were performed in accordance with a previous report using a multiwell Boyden chamber (22, 23). The mouse BMDMs and the vector- or FPR1-expressing RBL-2H3 cells were applied to different-sized polycarbonate filters (5 μm pore size for the BMDMs, 8 μm pore size for the RBL-2H3 cells) for 2 h (4 h for the RBL-2H3 cells) at 37°C. The migrated cells were stained with hematoxylin (Sigma, St. Louis, MO, USA) and then counted under a light microscope as previously described (22, 23).

Measurement of actin polymerization

Actin polymerization was measured in accordance with a previous report (24). The mouse BMDMs (4×10^4 cells/300 μl) were briefly stimulated with scolopendrasin VII (100 μg/ml) or 1 μM fMLF for 2 min at 37°C. The cells were then fixed for 10 min at room temperature in 4% paraformaldehyde, followed by the addition of PBS, and rhodamine-phalloidin (2 unit/ml) was then added for 30 min. The cells were visualized using a Zeiss LSM 500.

Western blot analysis

The mouse BMDMs were stimulated with 100 μg/ml of scolopendrasin VII for 0 min, 2 min, 5 min, 10 min, and 30 min. The extracted proteins were then separated using 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The membranes were incubated with specific antibodies (anti-phospho-Akt, anti-phospho-ERK, or anti-actin), and antigen- antibody complexes were visualized after the membrane was incubated with 1:2,500 diluted goat anti-rabbit IgG antibodies

that were combined with horseradish peroxidase, whereby detection was achieved using enhanced chemiluminescence as previously described (25, 26).

Ligand binding assay

The scolopendrasin VII ligand binding assay was performed as previously described (27). The vector-expressing or FPR1-expressing RBL-2H3 cells were briefly seeded at 2×10^5 cells per well in a 12-well plate and cultured overnight. After blocking the cells with blocking buffer (33 mM HEPES, pH 7.5, 0.1% BSA in RPMI) for 2 h, 1 μg/ml of FITC-labeled scolopendrasin VII was added to the cells in a binding buffer (PBS containing 0.1% BSA) in the absence or presence of the unlabeled scolopendrasin VII (200 μg/ml). After incubating the samples for 4 h at 4°C with continuous agitation, the samples were then washed 5 times with ice-cold binding buffer. After adding 300 μl of fixation buffer (PBS containing 5% FBS, 0.1% sodium azide, 0.1% paraformaldehyde) to each well, samples were acquired on a FACScantoII flow cytometer, and Flow Jo 7.6.5 was used for the data analysis.

Data analysis

Results are expressed as mean ± S.E. The Student's *t*-test was used to compare individual treatments with their respective control values. Statistical significance was set at $P < 0.05$.

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