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A membrane-tethering pepducin that inhibits formyl peptide receptor 2-induced signaling

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Since formyl peptide receptor 2 (FPR2) plays a key role in the regulation of innate immune response and inflammation, it has been a hot topic to develop molecules which inhibit FPR2-induced cellular responses. In this study, we investigated the effect of an FPR2-derived pepducin in human neutrophils and human umbilical vein endothelial cells (HUVECs). The pepducin (F2pal-12) selectively inhibited FPR2 agonists (MMK-1 and serum amyloid A)-stimulated neutrophil chemotaxis. MMK-1-stimulated superoxide anion production was also inhibited by F2pal-12. HUVECs also express FPR2; FPR2 agonists-stimulated HUVECs migration and tube formation were also selectively inhibited by F2pal-12 but not by scrambled control pepducin. Since FPR2 mediates inflammatory response by inducing chemotactic migration of inflammatory cells, F2pal-12 can be used as a useful material to modulate FPR2-mediated inflammatory responses.

1. Introduction

Activation of G-protein coupled receptors (GPCRs) plays a key role in the regulation of immune response (Kim and Luster 2007; Kolachala et al. 2008). Various kinds of extracellular stimuli that stimulate GPCRs have been reported. They include chemokines and many chemoattractants (Huang et al. 2008; Koelink et al. 2012; Murphy 2002). Formyl peptide receptor 2 (FPR2) is a well-known chemoattactant receptor that regulates trafficking of several important leukocytes, such as monocytes, neutrophils, and dendritic cells (Ye et al. 2009). Moreover, the activation of FPR2 also elicits superoxide anion production from human monocytes and neutrophils (Bae et al. 2003; Lee et al. 2010). The production of several proinflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and CXCL8, and proinflammatory lipid mediators including arachidonic acid, prostaglandin E_2 and leukotriene B_4 are also stimulated by FPR2 agonists (Lee et al. 2008a; Lee et al. 2006; Lee et al. 2009). Keeping in mind the physiological role of FPR2, FPR2 has been regarded as a crucial receptor involved in the regulation of innate immunity and inflammatory responses.

In addition to extracellular ligands that stimulate GPCRs, the membrane-tethering cell permeable peptide, pepducin, can modulate specific GPCRs (Covic et al. 2002). Some pepducins derived from PAR1 or CXCR1 act as agonists or antagonists for their specific receptors (Covic et al. 2002; Kaneider et al. 2005). Previously, we synthesized several FPR2-based pepducins, and characterized one (F2pal-16) as a novel agonist for FPR2. F2pal-16 strongly stimulated FPR2, resulting in the activation of a broad range of cellular signaling (Lee et al. 2010). Functionally, F2pal-16 stimulated not only superoxide anion production but also inflammatory cytokine production (Lee et al. 2010).

In this study, we characterized another FPR2-based pepducin (F2pal-12) and tested the effect of it on the activities of human neutrophils and endothelial cells.

Abbreviations: GPCR, G-protein coupled receptor; FPR2, formyl peptide receptor 2; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; F2pal-12, Pal-KGMIKSSRPLRV; scF2pal-12, Pal-VRLPRSSKUMGK; pep-12, NH_2-KGMIKSSRPLRV-COOH; MMK-1, LESIFRSLLFRVM; LL-37, LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES; SAA, serum amyloid A; HUVECs, human umbilical vein endothelial cells.

2. Investigations and results

2.1. F2pal-12 strongly inhibits neutrophil chemotactic migration induced by FPR2 agonists

FPR2 is well characterized as a classical chemoattractant receptor (Ye et al. 2009). Stimulation of FPR2 by its selective agonist induces chemotactic migration of human neutrophils (Ye et al. 2009; Hartt 2012). In this study, we investigated the effect of F2pal-12 on neutrophil chemotaxis. Addition of various concentrations of F2pal-12 did not induce chemotactic migration of neutrophils (data not shown). However, the well-known FPR2 agonists MMK-1 and SAA strongly induced neutrophil chemotaxis (Fig. 1). Next, we tested the possible inhibitory effect of the pepducin on neutrophil chemotaxis induced by the FPR2 agonists. Preincubation of neutrophils with F2pal-12 (5 μM) prior to applying chemotaxis assay strongly inhibited neutrophil chemotactic migration induced by MMK-1 or SAA (Fig. 1). We also tested the effect of peptide corresponding to F2pal-12 and scrambled pepducin of F2pal-12, neither of which had an effect on chemotactic migration in human neutrophils (Fig. 1, other data not shown). However, a well-known FPR1 agonist, fMLF-stimulated neutrophil chemotaxis was not inhibited by F2pal-12 (Fig. 1). The results strongly indicate that F2pal-12

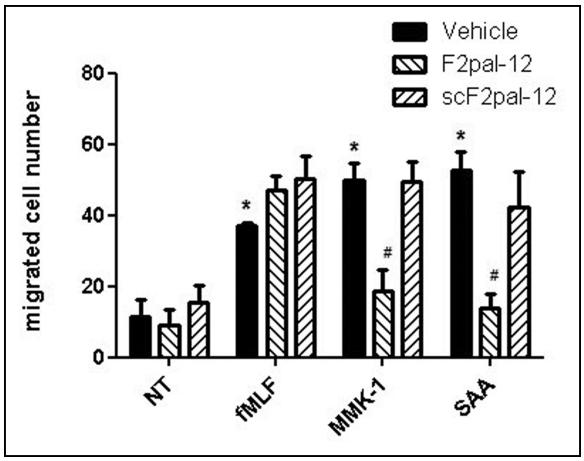

Fig. 1: Inhibition of FPR2 agonists-stimulated neutrophil chemotaxis by F2pal-12. Assays were performed using a modified Boyden chamber assay. Isolated human neutrophils (1×10^6 cells/ml of serum-free RPMI) were added to the upper wells of a 96-well chemotaxis chamber, and migration across the polycarbonate membrane with 3-μm pore size was assessed after 1.5 h of incubation at 37°C. Human neutrophils were incubated with or without 5 μM of F2pal-12 or scF2pal-12 for 15 min. Then, the cells were used for chemotaxis assay using fMLF (1 μM), MMK-1 (1 μM), or SAA (2 μM). The numbers of migrated cells were determined by counting them under light microscope. The data are presented as mean ± S.E. of two independent experiments, each performed in duplicate. *, $P < 0.05$, compared with the value obtained from the NT control; #, $P < 0.05$, significantly different from agonist alone control.

specifically inhibits FPR2-mediated chemotactic migration in human neutrophils.

2.2. F2pal-12 inhibits FPR2 agonists-stimulated superoxide anion production in human neutrophils

One of important roles of human neutrophils in innate immune response is the production of reactive oxygen species such as superoxide anion (Hurst 2012). We checked the effect of F2pal-12 on the production of superoxide anion by using cytochrome c reduction assay. Stimulation of human neutrophils with 5 μM of F2pal-12 failed to enhance superoxide anion production (Fig. 2). However a well-known FPR1 agonist, fMLF strongly stimulated superoxide anion production from human neutrophils (Fig. 2). MMK-1, a specific FPR2 agonist also strongly enhanced superoxide anion production from human neutrophils (Fig. 2). Moreover, preincubation of human neutrophils with F2pal-12 (5 μM) but not with scF2pla-12 (5 μM) prior to addition of MMK-1 almost completely inhibited superoxide anion production induced by the FPR2 agonist, MMK-1. However, fMLF-stimulated superoxide anion production was not inhibited by F2pal-12 (Fig. 2).

2.3. F2pal-12 strongly inhibits chemotactic migration of HUVECs induced by FPR2 agonists

HUVECs also express FPR2, and FPR2 is involved in the chemotactic migration of HUVECs (Koczulla et al. 2003). Like in human neutrophils, F2pal-12 alone failed to stimulate chemotactic migration of HUVECs (Fig. 3A). However, F2pal-12 strongly inhibited HUVECs chemotactic migration induced by LL-37 or MMK-1 in a concentration-dependent manner (Fig. 3B). Another important chemoattractant for HUVECs, sphingosine 1-phosphate (S1P), which acts on its specific receptor $S1P_1$ (Lee et al. 2000), also strongly induced chemotactic migration of HUVECs, which was not affected by F2pal-12 (Fig. 3B).

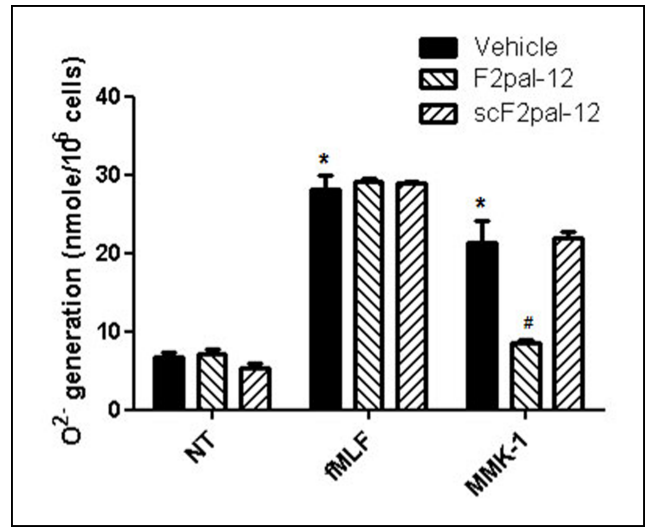

Fig. 2: Inhibition of FPR2 agonist-stimulated superoxide anion production by F2pal-12. Human neutrophils were incubated with or without 5 μM of F2pal-12 or scF2pal-12 for 15 min prior to stimulation with MMK-1 (1 μM) or fMLF (1 μM). The data are presented as mean ± S.E. of two independent experiments, each performed in duplicate. *, $P < 0.05$, compared with the value obtained from the NT control; #, $P < 0.05$, significantly different from agonist alone control.

2.4. F2pal-12 inhibits FPR2 agonists-induced tube formation in HUVECs

In this study, we checked whether F2pal-12 affect on tube formation in HUVECs. We could not detect any stimulatory effect of F2pal-12 on tube formation (Fig. 4A). However, a well-known FPR2 agonist, LL-37, which has been reported to induce physiologic and pathologic angiogenesis *in vivo* (Koczulla et al. 2003), induced tube formation in HUVECs (Fig. 4). Next, we tried to see whether F2pal-12 alters tube formation in the LL-37- or MMK-1-treated HUVECs. The addition of several concentrations of F2pal-12, coupled with LL-37 or MMK-1, markedly decreased LL-37- or MMK-1-induced tube formation. Unlike LL-37- or MMK-1-induced ones, S1P-induced tube formation was not affected by F2pal-12 (Fig. 4).

3. Discussion

Although FPR2 has been regarded as an important leukocyte chemoattractant receptor, which mediates innate immune response and defense mechanism against infectious disorders (Ye et al. 2009; Le et al. 2002), it is also involved in inflammatory response (Ye et al. 2009). Keeping in mind this crucial role of FPR2 in immune response, it has been an important issue to develop a specific antagonist capable of selectively inhibiting FPR2-induced signaling. Until now, a limited number of FPR2 antagonists has been reported. Previously, we identified a specific FPR2 antagonist, WRW^4 (Bae et al. 2004). WRW^4 was identified by screening of peptide libraries with monitoring of competition of ^{125}I-labelled WKYMVm in FPR2-expressng RBL-2H3 cells (Bae et al. 2004). WRW^4 acts on the extracellular milieu of FPR2, competing with the binding of WKYMVm to FPR2. Concerning the mode of action of F2pal-12, pepducin has been reported to act in the intracellular microenvironment. Since the amino acid sequence corresponding intracellular loop3 of FPR2 has been conjugated with palmitic acid, the lipidated peptide may act inside of the cells after flip-flop. A previous report already demonstrated that palmitic acid-conjugated peptide localizes inside the milieu of cell membrane, where pepducin mediates the activation of heterotrimeric G-protein or inhibits the binding of heterotrimeric G-protein to a specific

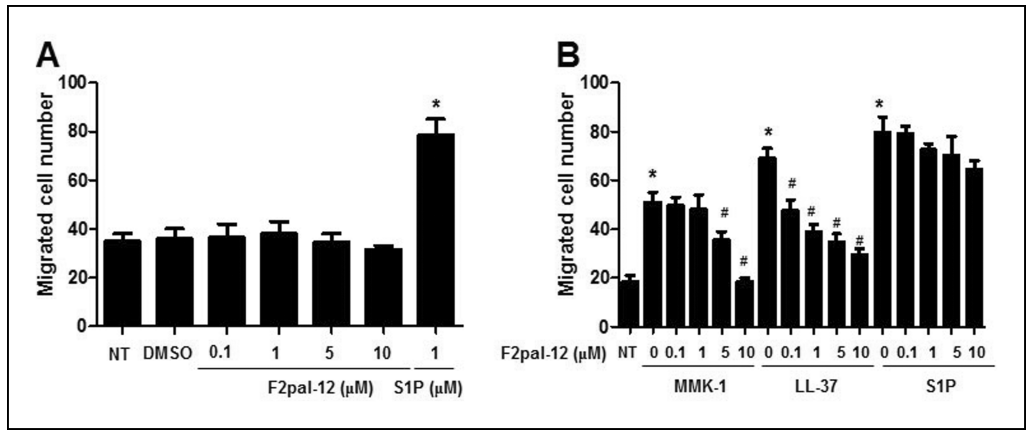

Fig. 3: Inhibition of FPR2 agonists-stimulated HUVEC chemotaxis by F2pal-12. Polycarbonate membrane of 96-well chemotaxis chamber was precoated with fibronectin (20 μg/ml) in 0.25% acetic acid solution overnight at room temperature. Cultured HUVECs (1×10^6 cells/ml in serum free RPMI) were added to the upper well of a 96-well chemotaxis chamber and migration across a polycarbonate membrane of 8 μm pore size was assessed in the presence of several concentrations of F2pal-12 or S1P (1 μM) for 4 h at 37 °C (A). HUVECs preincubated in the presence of several concentrations of F2pal-12 (0, 0.1, 1, 5, 10 μM) for 30 min, were subjected to chemotaxis assays at MMK-1 (1 μM), LL-37 (1 μM), or S1P (1 μM) (B). Migrated cell numbers were determined by counting under light microscope. The Data are presented as the means ± S.E. of three independent experiments performed in duplicate (A,B). *, $P < 0.05$, compared with the value obtained from the NT control; #, $P < 0.05$, significantly different from agonist alone control.

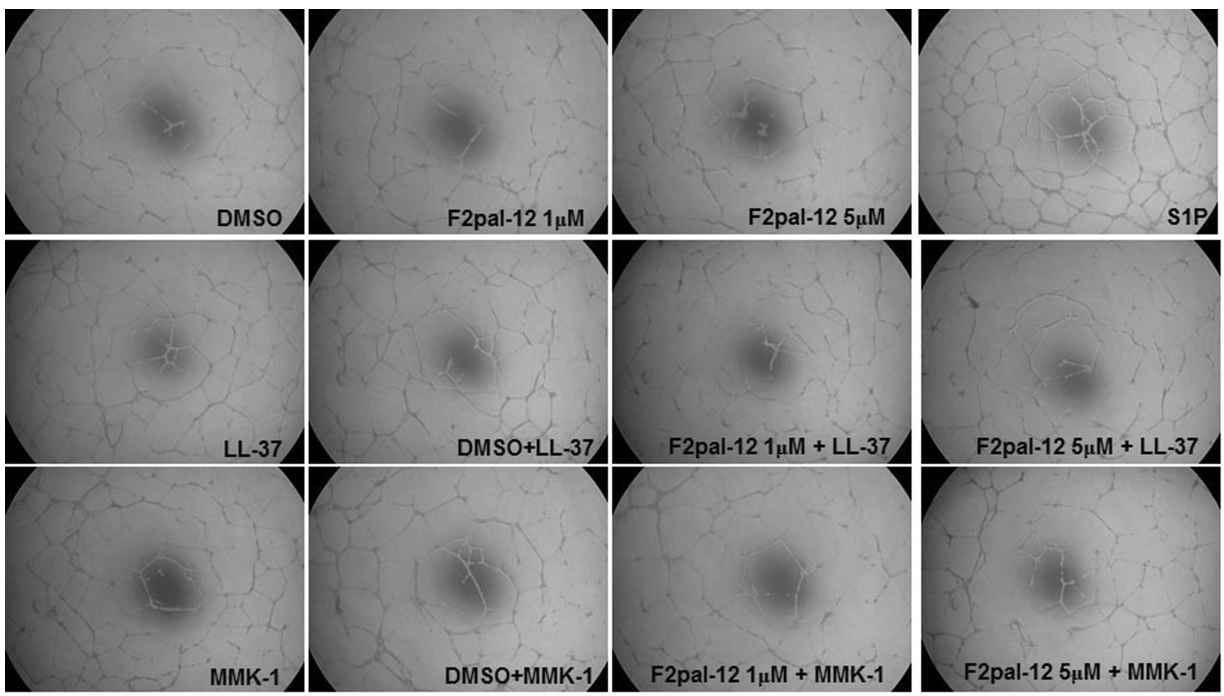

Fig. 4: Inhibition of FPR2 agonists-stimulated tube formation by F2pal-12. HUVECs were seeded onto 48-well plates pre-coated with Matrigel, and incubated in the presence of vehicle, F2pal-12 (1 or 5 μM), S1P (1 μM), MMK-1 (1 μM), LL-37 (5 μM), F2pal-12 plus MMK-1, F2pal-12 plus LL-37, or F2pal-12 plus S1P. After 18 h of incubation, tube formation was observed and photographed using an inverted phase contrast microscope (50 ×). The results shown are representative of three independent experiments.

GPCR, resulting in the activation or inhibition of GPCR signaling, respectively (Covic et al. 2002). Previously, we also demonstrated that a palmitic acid-conjugated FPR2 agonistic pepducin F2pal-16 stimulated human monocytes, which was not inhibited by an FPR2 antagonist WRW[4] (Bae et al. 2004), suggesting that F2pal-16 may act on the intracellular milieu but not on the extracellular milieu. We assume that F2pal-12 also may act on the intracellular milieu. Keeping in mind this novel mode of action of F2pal-12, F2pal-12 may be useful for the study of FPR2-mediated signaling and cellular response.

FPR2 has been regarded as an important chemoattractant receptor for human phagocytes such as monocytes and neutrophils. Since neutrophil migration is important in the initiation of inflammatory response, it has been important to develop certain molecules that inhibit neutrophil recruitment. Here, F2pal-12 strongly blocked FPR2 agonists-induced neutrophil chemotactic migration. The results indicate that F2pal-12 may be useful to control FPR2-mediated neutrophil migration into event area. Taken together, the data support the view that the inhibitory effect of F2pal-12 on neutrophil chemotaxis in human neutrophils, F2pal-12 may be very useful to control inflammatory response.

In addition to phagocytic cells such as neutrophils and monocytes, FPR2 is also expressed in endothelial cells. Stimulation of HUVECs with FPR2 agonist LL-37 caused chemotactic migration and tube formation in HUVECs, which were strongly inhibited by F2pal-12 (Fig. 4). Keeping in mind that endothelial cell migration is closely associated with inflammation, and tube formation is associated with angiogenesis, F2pal-12 may be effective in controlling FPR2-mediated endothelial inflammation and angiogenesis.

4. Experimental

4.1. Materials

F2pal-12 (Pal-KGMIKSSRPLRV), scrambled pepducin for F2pal-12 (scF2pal-12; Pal-VRLPRSSKUMGK), pep-12 (NH_2-KGMIKSSRPLRV-COOH), MMK-1 (LESIFRSLLFRVM), and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) were synthesized by Anygen (Gwangju, Korea). Recombinant human serum amyloid A (SAA; endotoxin level < 0.1 ng/μg) was purchased from Peprotech (Rocky Hill, NJ). Formyl-Met-Leu-Phe (fMLF), peripheral blood mononuclear cell separation medium (Histopaque-1077), and cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO).

4.2. Isolation of human neutrophils

Peripheral blood leukocytes were isolated from healthy donors. Human neutrophils were isolated according to standard procedures for dextran sedimentation, hypotonic lysis of erythrocytes, and lymphocyte separation medium gradient as described previously (Kim et al. 2011).

4.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) were cultured on 0.02% gelatin-coated (Sigma-Aldrich) 10 cm-diameter dishes in endothelial basal medium (EBM) enriched with 10% fetal bovine serum and supplements (Lonza) as described previously (Lee et al. 2008b). HUVECs were used for experiments between passages 3 to 10.

4.4. Chemotaxis assay

Neurophil chemotaxis assays were performed using multiwell chambers (Neuroprobe, Gaithersburg, MD) as described previously (Bae et al. 2004). Chemotaxis assay with HUVECs were performed using polycarbonate filters (8 μm pore size) as described previously (Lee et al. 2008b).

4.5. Measurement of superoxide anion production

To measure superoxide anion generation from human neutrophils, we used cytochrome c reduction assay as described previously (Bae et al. 2003).

4.6. Tube formation assay

The formation of vascular-like structures for HUVECs on growth factor-reduced was performed as previously described (Lee et al. 2008b).

4.7. Data analysis

The results are expressed as mean ± S.E. of the number of determinations indicated. Statistical significance of differences was determined by Student's *t*-test and statistical significance was considered significant at $P < 0.05$.

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