Amyloid β_{42} Activates a G-Protein-Coupled Chemoattractant Receptor, FPR-Like-1

Yingying Le,[1] Wanghua Gong,[2] H. Lee Tiffany,[3] Alexei Tumanov,[1] Sergei Nedospasov,[1] Weiping Shen,[1] Nancy M. Dunlop,[1] Ji-Liang Gao,[3] Philip M. Murphy,[3] Joost J. Oppenheim,[1] and Ji Ming Wang[1]

[1]Laboratory of Molecular Immunoregulation, Division of Basic Sciences and [2]Science Applications International Corporation Frederick, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702, and [3]National Institutes of Health, Bethesda, Maryland 20892

Amyloid β (Aβ) is a major contributor to the pathogenesis of Alzheimer's disease (AD). Although Aβ has been reported to be directly neurotoxic, it also causes indirect neuronal damage by activating mononuclear phagocytes (microglia) that accumulate in and around senile plaques. In this study, we show that the 42 amino acid form of β amyloid peptide, Aβ_{42}, is a chemotactic agonist for a seven-transmembrane, G-protein-coupled receptor named FPR-Like-1 (FPRL1), which is expressed on human mononuclear phagocytes. Moreover, FPRL1 is expressed at high levels by inflammatory cells infiltrating senile plaques in brain tissues from AD patients. Thus, FPRL1 may mediate inflammation seen in AD and is a potential target for developing therapeutic agents.

Key words: amyloid β; receptor; FPRL1; monocytes; chemotaxis; Alzheimer's disease

Amyloid β (Aβ) peptides play an important role in the neurodegeneration of Alzheimer's disease (AD). Mutations in the amyloid precursor protein and the presenilin genes are associated with increased production of a 42 amino acid polypeptide (Aβ_{42}) and are linked with exacerbated familial forms of AD (Selkoe, 1999). Although a direct neurotoxic effect of Aβ has been proposed (Du Yan et al., 1997; Lambert et al., 1998), the bulk of evidence favors an "indirect" pathway, based on induction by Aβ of inflammatory responses of microglia, the brain counterpart of the mononuclear phagocytes (Kalaria, 1999; Neuroinflammatory Working Group, 2000). Consistent with this, activated microglia migrate to accumulate in and around the senile plaques in AD and release neurotoxic mediators in response to Aβ *in vitro* (Davis et al., 1992; London et al., 1996; Meda et al., 1996; Klegeris and McGeer, 1997). Clear-cut evidence of infiltration of AD-like plaques by microglia was seen in transgenic mice overexpressing human β amyloid in the brain (Stalder et al., 1999). Moreover, subjects receiving anti-inflammatory drugs showed significantly delayed development of AD dementia (Kalaria, 1999; Neuroinflammatory Working Group, 2000). The importance of Aβ in AD pathogenesis was further substantiated by the fact that vaccination with Aβ_{42} of PDAPP mice, which overexpress human β amyloid in the brain, attenuated the progression of AD-like lesions (Schenk et al., 1999). Searches for a cellular receptor or receptors yielded several molecules that interact with Aβ. The scavenger receptor (SR) and the receptor for advanced glycation end products (RAGE) (El Khoury et al., 1996; Yan et al., 1996) bind Aβ, however, it is controversial whether they mediate a proinflammatory microglial cell response to Aβ. The existence of other functional Aβ receptor or receptors on the cell surface has been suggested (London et al., 1996; Liu et al., 1997; McDonald et al., 1997, 1998; Huang et al., 1999). In this study, we report that a G-protein-coupled seven-transmembrane (STM) receptor, FPR-Like-1 (FPRL1), is used by Aβ_{42} to induce migration and activation of human monocytes. We propose that FPRL1 may serve as a receptor mediating the proinflammatory responses elicited by Aβ_{42}.

MATERIALS AND METHODS

Reagents and cells. Aβ peptide (Aβ_{42}) and the peptide with reversed sequence (Aβ_{42-1}) were purchased from California Peptide Research (Napa, CA). All peptides were examined for endotoxin contamination and were negative at highest concentrations used in the study. Human peripheral blood monocytes were isolated from buffy coats (National Institutes of Health Clinical Center, Bethesda, MD) enriched for mononuclear cells by using iso-osmotic Percoll gradient. The purity of the cell preparations was examined by morphology and was >90%. Rat baso-

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Correspondence should be addressed to Ji Ming Wang, Laboratory of Molecular Immunoregulation, Division of Basic Sciences, National Cancer Institute, Frederick Cancer Research and Development Center, Building 560, Room 31-40, Frederick, MD 21702. E-mail: wangji@mail.ncifcrf.gov.

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philic leukemia cell line (RBL-2H3) transfected with epitope-tagged FPR (designated ETFR) was a kind gift of Dr. R. Snyderman (Duke University, Durham, NC). cDNA cloning and establishment of FPRL1-transfected human embryonic kidney (HEK) 293 cells (FPRL1/293) were described previously (Gao and Murphy, 1993). All the transfected cells were maintained in culture media as described (Su et al., 1999).

Chemotaxis assays and measurement of calcium mobilization. Chemotaxis assays were performed using 48-well chemotaxis chambers (Deng et al., 1999). The results were expressed as the mean number (± SD) of migrated cells in three high-powered fields in three replicate samples. Chemotaxis index, which represented the fold increase in the number of cells migrated in response to chemoattractants over the cell response to control medium, also was used. Ca^{2+} mobilization was measured by stimulating fura-2 AM-loaded human monocytes or receptor-transfected cells with various agents (Deng et al., 1999; Su et al., 1999) and recording the ratio of fluorescence at 340 and 380 nm in a luminescence spectrometer with FL WinLab program (Perkin-Elmer, Beaconsfield, UK).

In situ *hybridization.* Twenty micrometer serial cryostat sections were prepared from frozen AD or normal brain tissues and mounted on glass slides. The sections were fixed in paraformaldehyde–PBS solution, washed with PBS, then acetylated in 0.25% acetic anhydride. After washing with PBS, slides were prehybridized at room temperature (RT) for 2 hr with hybridization solution (50% formamide, 5× SSC, 5× Denhardt's solution, 250 μg/ml Torula's yeast RNA, and 500 μg/ml herring sperm DNA). Hybridization was performed with digoxigenin-labeled FPRL1 cRNA probe (400 ng/ml). After overnight hybridization at 70°C, slides were washed in 0.2× SSC for 3 hr at 70°C. Anti-digoxigenin antibody conjugated with AP (1:2000 dilution) was applied in buffer B (0.1 M Tris-HCl, pH 7.5, and 0.15 M NaCl) containing 1% heat-inactivated goat serum and incubated overnight at RT. After extensive washing in buffer B, phosphatase reaction was performed for 3 hr in buffer C (0.1 M Tris-HCl, pH 9.5, 0.15 M NaCl, and 50 mM $MgCl_2$) supplemented with 0.34 mg/ml nitro blue tetrazolium, 0.23 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, and 0.24 mg/ml Levamisole.

Immunohistochemistry and Congo Red staining. Serial sections of the brain tissues were fixed and incubated for 30 min with 0.3% H_2O_2, followed by 0.05% Tween 20 for 30 min and blocking serum for 60 min. The sections were reacted for 60 min at room temperature with anti-CD11b (Mac-1) antibody (1:1000) (PharMingen, San Diego, CA). The avidin–biotin–peroxidase method (Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromogen was used to visualize the antibody staining (brown products). Congo Red staining was performed on Mac-1-stained sections according to standard protocols.

Statistical analysis. All experiments were performed at least three times. The significance of the difference between test and control groups was analyzed with Student's *t* test.

RESULTS

Aβ42 activates monocytes

Microglial cells are considered to belong to the monocyte–macrophage lineage (Neuroinflammatory Working Group, 2000). Extensive studies on the biological activity of Aβ have been performed with human monocytes and monocytic cell lines such as THP-1 with similar activation patterns (Davis et al., 1992; London et al., 1996; Klegeris and McGeer, 1997; Klegeris et al., 1997; Lorton, 1997; McDonald et al., 1997, 1998; Combs et al., 1999). To characterize the nature of the putative receptor or receptors used by Aβ, we studied the effect of Aβ_{42} on chemotaxis and activation of human monocytes. Freshly dissolved Aβ_{42} induced a dose-dependent migration of human monocytes starting at a concentration of 20 nM (EC_{50}, 1.5 μM; Fig. 1*A*). In contrast, peptide with the reverse sequence of Aβ_{42} (Aβ_{42-1}), was completely inactive (Fig. 1*A*). Checkerboard analysis indicated that Aβ_{42} functioned chemotactically rather than by increasing random cell migration (data not shown). Because aggregated Aβ is likely to deposit in senile plaques of AD and activates mononuclear phagocytes *in vitro*, we tested the chemotactic activity of Aβ_{42} "aged" at 37°C. Figure 1*A* shows that this form of Aβ_{42} also induced significant monocyte migration, although with lower potency than freshly dissolved peptide. The activation of monocytes

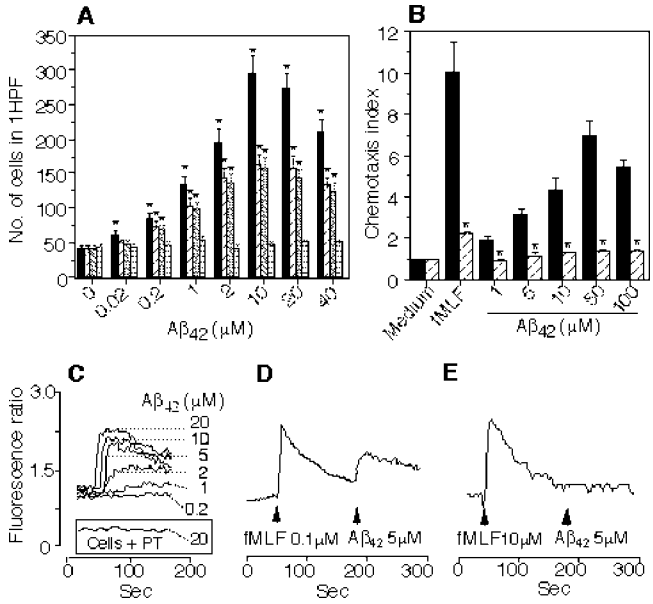

Figure 1. Activation of human monocytes by Aβ_{42}. *A,* Migration of monocytes induced by freshly prepared Aβ_{42} (*black bars*), Aβ_{42} "aged" at 37°C for 3 (*diagonally hatched bars*) or 7 (*shaded bars*) d, and a peptide with reversed sequence of Aβ_{42} (*horizontally hatched bars*, Aβ_{42-1}). $^*p < 0.05$ compared with cell migration in the absence of Aβ_{42}. *B,* Effect of preincubation with medium (*black bars*) or pertussis toxin (PT) (*diagonally hatched bars*) (100 ng/ml, 37°C, 30 min) on monocyte migration to fMLF (100 nM) or Aβ_{42}. $^*p < 0.05$ compared with migration of cells cultured in the absence of PT. *C,* Aβ_{42}-induced Ca^{2+} mobilization in monocytes. *Inset,* Response of cells treated with PT to 20 μM Aβ_{42}. *D, E,* Attenuation of Aβ_{42}-induced Ca^{2+} flux by fMLF.

by Aβ_{42} was further demonstrated by increased Ca^{2+} mobilization (Fig. 1*C*). In both chemotaxis and calcium flux assays, human monocytes responded to a wide range concentrations of Aβ_{42}. These concentrations of Aβ_{42} are comparable with or much lower than those used in other studies. In addition, preincubation of monocytes with pertussis toxin (PT), an inhibitor of G_i-type proteins, completely abolished monocyte migration (Fig. 1*B*) and calcium flux in response to Aβ_{42} (Fig. 1*C, inset*). These results suggest that Aβ_{42} uses G_i-protein-coupled STM receptor or receptors on monocytes.

Desensitization of Aβ_{42} signaling

To identify the monocyte receptor or receptors for Aβ_{42}, we examined the capacity of Aβ_{42} to cross-desensitize cell signaling with chemoattractants known to elicit Ca^{2+} mobilization. This approach can distinguish between unique and/or shared STM receptors for different chemoattractants (Deng et al., 1999). Aβ_{42} signaling in monocytes was not affected by previous stimulation of the cells with a number of chemokines (data not shown), suggesting that Aβ_{42} did not use a chemokine receptor. However, a classical chemoattractant, the bacterial chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLF), clearly inhibited the subsequent Ca^{2+} flux response to Aβ_{42} (Fig. 1*D,E*). Because high concentrations of fMLF were required, we postulated that Aβ_{42} might share a low-affinity fMLF receptor. Such a receptor was cloned 10 years ago and has been designated FPRL1 or LXA4R based on its homology to the high-affinity fMLF receptor FPR (Murphy, 1994; Prossnitz and Ye, 1997) and its reported function as a lipoxin A4 receptor (Fiore et al., 1994). Moreover, FPRL1 in our previous study has been identified as a functional receptor for serum amyloid A (SAA), which is chemotactic for

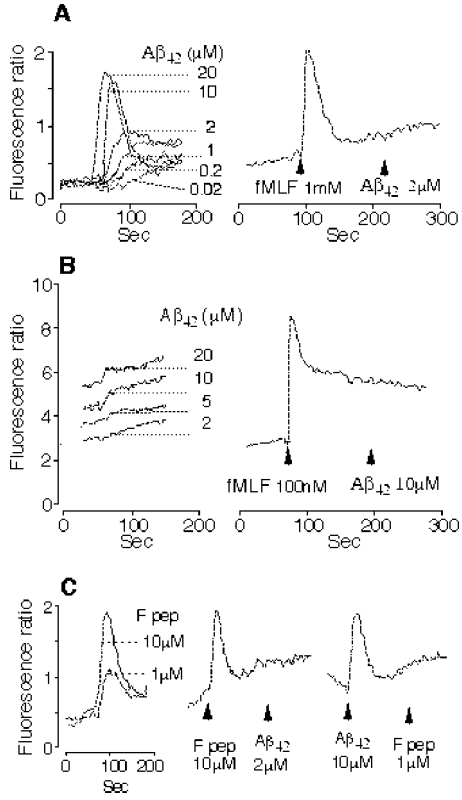

Figure 2. Activation of cells expressing FPRL1 and FPR by $A\beta_{42}$. *A,* Ca^{2+} mobilization in FPRL1/293 cells induced by $A\beta_{42}$ and attenuation by fMLF. *B,* Signaling of $A\beta_{42}$ in FPR expressing ETFR cells and attenuation by fMLF. *C,* Signaling of F peptide (*F pep*) in FPRL1/293 cells and cross-desensitization with $A\beta_{42}$.

human leukocytes (Su et al., 1999) and is one of the major amyloidogenic proteins involved in chronic inflammation in various organs and tissues (Malle and De Beer, 1996) but has not been implicated in AD.

Activation of FPRL1 by $A\beta_{42}$

We then tested the capacity of $A\beta_{42}$ to activate cells transfected to express solely FPRL1 or FPR. $A\beta_{42}$ dose-dependently induced Ca^{2+} mobilization in FPRL1-transfected HEK 293 cells (FPRL1/293 cells) (Fig. 2*A*). $A\beta_{42}$ also induced Ca^{2+} mobilization in a rat basophilic leukemia cell line transfected with FPR (ETFR cells), yet with much lower potency and efficacy than fMLF (Fig. 2*B*). $A\beta_{42}$ signaling was dependent on FPRL1 and FPR, because untransfected parental cells or cells transfected with other chemoattractant receptors did not respond to $A\beta_{42}$ (data not shown). Consistent with the effects on monocytes, $A\beta_{42}$ signaling in both FPRL1/293 and ETFR cells was desensitized by previous stimulation of the cells with high concentrations of fMLF (Fig. 2*A,B*), which were not toxic to the cells and did not inhibit the cell response to other Ca^{2+} flux inducers (Y. Le, unpublished observations). In addition, a synthetic HIV-1 envelope protein domain F peptide, which specifically activates FPRL1 (Deng et al., 1999), also desensitized $A\beta_{42}$-induced Ca^{2+} flux in FPRL1/293 cells and vice versa (Fig. 2*C*). Furthermore, FPRL1/293 cells exhibited a significant chemotactic response to $A\beta_{42}$ (EC_{50}, 200 nM), whereas ETFR cells migrated only weakly, albeit significantly, in response to high concentrations (>10 μM) of $A\beta_{42}$ (Fig. 3*A,B*). The $A\beta_{42}$ concentrations required to activate FPRL1 is similar to those for monocytes, indicating a major role

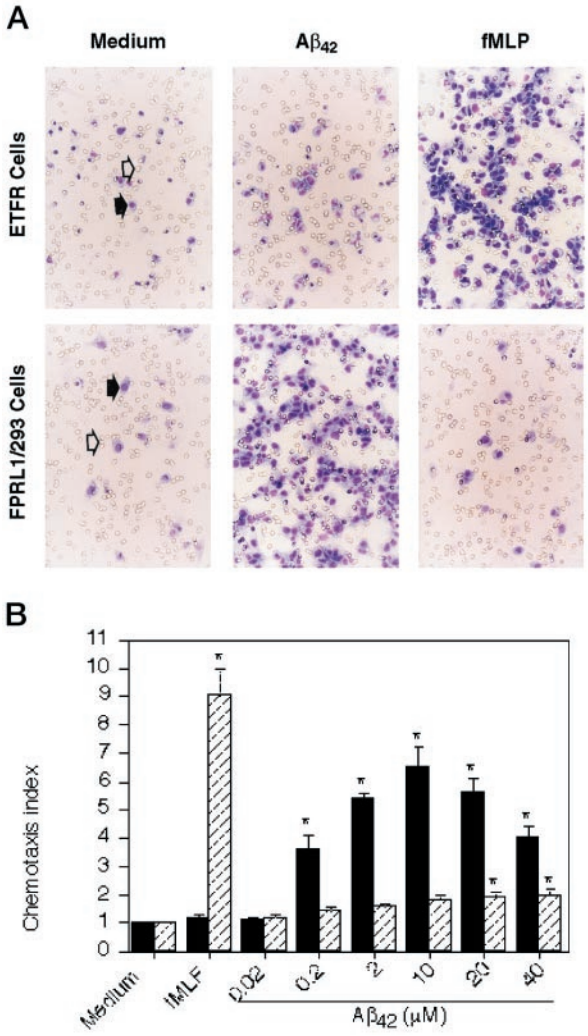

Figure 3. Cell migration induced by $A\beta_{42}$ via FPR and FPRL1. *A,* Migration of FPRL1/293 or ETFR cells to medium (control), $A\beta_{42}$ (1 μM), or fMLF (fMLP, 1 μM). *Solid arrows* denote cells migrating across the filters, and *open arrows* indicate the micropores in the filter. *B,* Dose-dependent migration of FPRL1/293 (*black bars*) and ETFR cells (*diagonally hatched bars*) toward $A\beta_{42}$. fMLF at 100 nM was used as a control. $^*p < 0.05$ compared with cell migration to medium.

for FPRL1 in monocyte activation. Because directional cell migration is considered an initial step for cell infiltration and accumulation at sites of inflammation, we propose that FPRL1 is a functionally relevant receptor used by $A\beta_{42}$.

Expression of FPRL1 gene in AD brain tissue

To gain insight into the pathophysiological relevance of FPRL1 to AD, we examined FPRL1 gene expression in normal versus AD brain tissues. Multiple senile plaques were readily visible with Congo Red staining in sections of brain tissues from AD patients (Fig. 4*A,C, red*), but not from normal brain (data not shown). All senile plaques, but not surrounding brain tissue, were infiltrated by cells expressing considerable levels of FPRL1 as determined by *in situ* hybridization with antisense FPRL1 probe (Fig. 4*B,D*). Hybridization signals were not detected with FPRL1 sense probe in serial sections of senile plaques (data not shown). The cells infiltrating plaques were positively stained with monoclonal antibody against CD11b, a marker for microglial cells (Fig. 4*A,D, brown*; 400×). These results confirm the microglial cell infiltration in AD lesions, and the infiltrating cells express FPRL1.

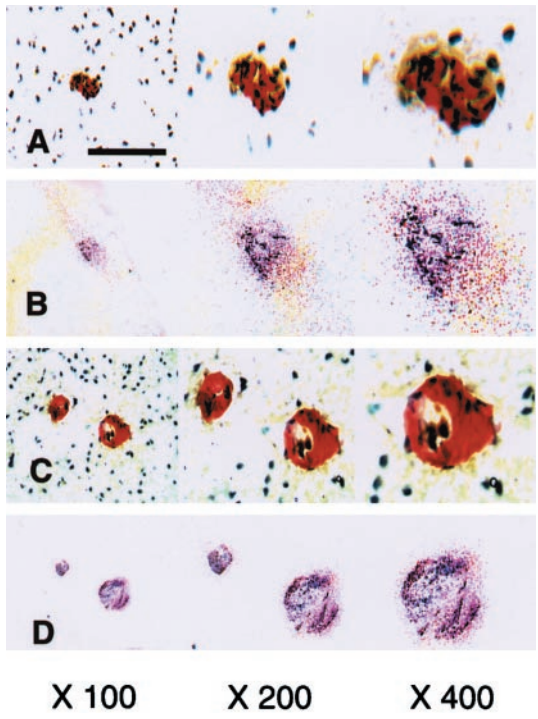

Figure 4. Expression of FPRL1 gene in cells infiltrating AD plaques. Frozen brain tissues (frontal cortex) of an AD patient (NSP 2678) were sectioned and stained with Congo Red (*red*) and anti-CD11b antibody (*brown*). *A, C*, Magnifications from 100 to 400×. Serial sections of the same brain tissues were hybridized with antisense FRPL1 probe (*B, D*). Results obtained from two separate tissue areas are shown. Scale bar, 200 μm.

DISCUSSION

Aβ peptides have previously been shown to elicit a diverse proinflammatory responses in mononuclear phagocytes, including microglial cells, monocytes, and monocytic cell lines. These include induction of cell adhesion, migration (Davis et al., 1992; El Khoury et al., 1996; Yan et al., 1996; Nakai et al., 1998), accumulation at sites of injection in the brain (Scali et al., 1999), Ca^{2+} mobilization (Combs et al., 1999), phagocytosis (Kopec and Carroll, 1998), release of reactive oxygen intermediates, and increased production of neurotoxic or proinflammatory cytokines (Bonaiuto et al., 1997; Klegeris and McGeer, 1997; McDonald et al., 1997; Fiala et al., 1998). Aβ signal transduction in monocytes involves activation of G-proteins, protein kinase C (Zhang et al., 1996; Klegeris et al., 1997; Lorton, 1997; Nakai et al., 1998), and tyrosine kinases (Zhang et al., 1996; McDonald et al., 1997, 1998; Combs et al., 1999), which are known to be activated by STM receptors including FPR and FPRL1 (Murphy, 1994; Prossnitz and Ye, 1997; Le et al., 1999), but not by the previously reported Aβ receptors SR or RAGE. A recent study reported that the bacterial fMLF and antagonists against the high-affinity fMLF receptor FPR attenuated the production of proinflammatory cytokines induced by Aβ in microglial and THP-1 monocytes, suggesting that Aβ may activate an FPR-like cellular receptor (Lorton et al., 2000). We now have shown that Aβ_{42} is able to activate FPR, however, the efficacy of this receptor to mediate cell migration and activation is much lower than that of FPRL1. Because Aβ_{42} induces high levels of chemotaxis and Ca^{2+} flux via FPRL1 on monocytes, and furthermore, the concentrations of Aβ required for cell activation can be detected in AD brain and plasma (Kuo et al., 1999; McLean et al., 1999), it is likely that *in vivo* Aβ_{42} activates mononuclear phagocytes mainly via FPRL1. The preferential activation of FPRL1 by Aβ_{42} was also confirmed by using HEK 293 cells transfected to express the mouse homolog of FPRL1 (H. L. Tiffany and P. M. Murphy, unpublished data), which will facilitate studies in mouse models of AD.

FPRL1 was originally cloned as an orphan receptor, and no clear biological roles and disease associations have been described (Murphy, 1994; Prossnitz and Ye, 1997; Le et al., 1999). The bacterial chemotactic peptide fMLF is a weak agonist for FPRL1 and induces calcium flux, but not chemotaxis, through this receptor at high concentrations (Su et al., 1999). Recently, several highly efficacious chemotactic agonists have been identified for FPRL1, including synthetic peptide domains derived from HIV-1 envelope proteins (Deng et al., 1999; Le et al., 1999), and two endogenously produced ligands, the eicosanoid lipoxin A4 (LXA4) (Fiore et al., 1994) and SAA (Su et al., 1999). Identification of FPRL1 as a common receptor for Aβ and SAA raises the possibility of involvement of this receptor in other amyloidogenic diseases. It should be noted that Aβ_{42} and SAA do not bear significant sequence homology. We therefore are currently investigating the structural requirements for these diverse ligands to activate this receptor.

FPRL1 is expressed by a variety of cell types such as phagocytic leukocytes, lymphocytes, epithelial cells, microvascular endothelial cells, and astrocytes (Y. Le, unpublished data). Human neutrophils also express FPRL1 and can be activated by agonists known for this receptor (Deng et al., 1999; Le et al., 1999) as well as Aβ_{42} (Y. Le, W. Gong, and J. M. Wang, data not shown). Neutrophils do not appear to play a significant role in the pathogenesis of AD, although activation of these cells by Aβ peptides *in vitro* has been reported recently (Bianca et al., 1999). It is therefore of interest to determine whether the blood–brain barrier may limit migration and extravasation of neutrophils in response to elevated Aβ in the brain. Moreover, the role of FPRL1 in Aβ-induced direct neurotoxicity (Lambert et al., 1998), vasculopathy (Thomas et al., 1996), or activation of astrocytes (Johnstone et al., 1999) is presently unknown and merits investigation.

The identification of FPRL1 as a functional receptor for Aβ_{42} and detection of FPRL1 mRNA in mononuclear phagocytes infiltrating senile plaques provide a molecular basis for inflammation in AD and suggest an additional target for development of therapeutic agents.

The pathophysiological relevance of our finding to precipitated and soluble forms of Aβ_{42} is of considerable concern. It should be noted that Aβ_{42} "aged" at 37°C showed a reduced potency in inducing cell migration, suggesting that aggregated peptide is still recognized by FPRL1 yet with lower efficacy. However, it has been reported that only aggregated Aβ_{42} triggers certain monocyte functions such as mediator release and tyrosine kinase activation (McDonald et al., 1997, 1998). This raises the question as to whether soluble and aggregated Aβ_{42} may activate different signal molecules coupled to FPRL1, thereby eliciting a diverse pattern of cell responses. Although our observations showed chemotactic activity of both soluble and aggregated Aβ_{42}, further research is underway to fully address the consequences of FPRL1 activation by Aβ_{42} in soluble versus aggregated forms.

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