Exocytosis of Neutrophil Formyl Peptide Receptor-Like 1 (fPRL1) Results in Downregulation of Cytoplasmic fPRL1 in Patients with Purulent Dermatitis

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N-Formyl peptide receptor-like 1 (fPRL1), which mediates chemotaxis, degranulation, and respiratory burst, is considered to be a member of the calcium-mobilizing G protein-coupled family of receptors for bacterial N-formyl peptides in phagocyte cells (16, 17, 18, 23). fPRL1 differs from N-formyl peptide receptor (fPR), with 69% identity at the amino acid level, and cDNA encoding fPRL1 is reported to be located on human chromosome 19 (4, 20, 21), but the biological implications of fPRL1 have remained poorly understood since it was first reported in 1999. fPRL1 was identified as one of the receptors to which an acute-phase reactant, acute-phase serum amyloid A (A-SAA), is bound, and fPRL1 mediates the chemotaxis of phagocyte cells such as neutrophils and monocytes (24). fPRL1 is also considered to be an agonistic receptor for mitochondrial proteins of either damaged host cells or invading pathogens. Therefore, fPRL1 is involved mainly in the host defense against infective organisms and in the clearance of damaged cells (17). Moreover, recent studies indicate that fPRL1 plays an important role in immune responses distinct from that of fPR: more than fPR, fPRL1 mediates the chemotactic migration of phagocytic cells and the resultant production of reactive oxygen species (16, 17, 23).

A-SAA is an ancient acute-phase reactant produced in response to trauma, infection, inflammation, and neoplasia (26, 27). A-SAA is synthesized not only in the hepatocytes, but also in the extrahepatic cells such as endothelial cells, monocytes, macrophages, vascular smooth muscle cells, and synovial cells (13, 14). A-SAA is also reported to be present in the skin (27). A-SAA is a ligand for fPRL1 which mediates the cellular calcium influx in neutrophils and results in the migration of neutrophils (2). Recent neutrophil studies in vitro have found that the mechanism of fPRL1 activation is complicated and that bacterial lipopolysaccharides (LPS) need to be primed for the activation of neutrophils through fPRL1 (1, 6, 7). However, the details of the physiological alteration and regulation of neutrophil fPRL1 in response to inflammation in vivo remain unclear. Moreover, the morphological changes in neutrophil fPRL1 in relation to the inflammatory process in vivo also remain unclear.

This study was undertaken to determine whether neutrophil fPRL1 is immunohistochemically altered and whether the active feature of neutrophil fPRL1 in patients with bacterial skin disease could be characterized by immunohistochemical analysis. To comprehend the morphological changes in neutrophils in inflammation, the alteration of neutrophil fPRL1 by stimulation with N-formylmethionyl-leucyl-phenylalanine (fMLP) was analyzed in vitro.

MATERIALS AND METHODS

Samples. We histologically investigated skin specimens from three patients with purulent dermatitis (two men and one woman, ages 46 to 70 years old); one suffered from necrotizing fasciitis (case 1, infection with Bacillus cereus; case 2, Streptococcus agalactiae infection, and case 3, Candida albicans infection). We also investigated bone marrow specimens, which were clinically and histologically determined to be normocellular bone marrow, from 10 patients without inflammatory diseases. All samples used in this study were obtained under the approval of the Hospital Ethics Committee of Kochi Medical School Hospital, Kochi.
University, Kochi, Japan, and were also given with the informed consent of participants.

**Reagents.** Tris-buffered saline (TBS; 0.05 M, pH 7.6), normal goat serum, goat anti-rabbit immunoglobulin-peroxidase conjugate, hematoxylin, and 3,3-diaminobenzidine tetrahydrochloride were purchased from Nichirei Corporation (Tokyo, Japan). Phosphate-buffered saline (PBS; 1/15 M, pH 7.4), hydrogen peroxide, sodium azide, formaldehyde, HEPES, and manganese chloride tetrahydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). fMLP, Histopaque 1083, dextran (average molecular weight, 520,000), acid citrate, and human serum were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide and glutaraldehyde were purchased from Nacalai Tesque Inc. (Kyoto, Japan). BlockAce was purchased from Snow Brand Co. (Sapporo, Japan). Precasting polyacrylamide gels and nitrocellulose membrane were purchased from Bio-Rad Laboratories (Hercules, CA). Goat anti-rabbit immunoglobulin G-fluorescein conjugate secondary antibody was purchased from CHEMICON International (Temecula, CA). All reagents were of the highest grade available. Stock solutions of phorbol myristate acetate were prepared in dimethyl sulfoxide and stored at −20°C until use.

**Preparation of anti-fPRL1 antiserum.** Anti-fPRL1 antiserum was originally prepared by the repetitive immunization of rabbits with the synthetic peptide TVTIPNGDTYC, corresponding to amino acids 166 to 176 of fPRL1 (21), by a modification of previously described methods (5). The resulting fPRL1 antiserum was confirmed to be specific to human fPRL1 by Western blot analysis and electron microscopy and to be functionally active by the inhibition of migration of human vascular smooth muscle cells and synovial cells (14, 15).

**Immunohistochemical analyses.** For immunohistochemical analyses, tissues were fixed in 20% formaldehyde in PBS for 1 h at room temperature, dehydrated, and embedded in paraffin. Four-micrometer-thick sections were cut, dehydrated, and subsequently used for immunohistochemistry. Briefly, sections were deparaffinized, hydrated, and incubated for 10 min in 0.3% hydrogen peroxide diluted in methanol to reduce endogenous peroxidase activity. Immunostaining was performed using an automated immunostainer (OptiMax Plus; BioGenex, San Ramon, CA). The sections were incubated with 1:500-diluted primary antibody polyclonal anti-fPRL1 in TBS for 1 h at room temperature. After washing twice in TBS, the slides were incubated with 10% normal goat serum in TBS for 15 min to prevent nonspecific binding of the secondary antibody. After washing twice in TBS, sections were incubated with 1:1,000-diluted goat anti-rabbit immunoglobulin G-peroxidase conjugate for 30 min, followed by subsequent labeling with 3,3-diaminobenzidine tetrahydrochloride for 40 s at room temperature. Sections were counterstained with hematoxylin. The specificity of the immunohistochemical procedure was checked using negative and positive controls.

**Quantification of fPRL1 staining in neutrophils.** The immunostaining of neutrophils for fPRL1 was observed at high power (magnification, ×1,000) with a standard light microscope. With pictures of immunostained samples imported through the video camera, the intensity of fPRL1 immunostaining in neutrophils was quantitatively estimated using the NIH Image analysis program (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/nih-image/). In brief, the pictures of immunostained samples were automatically converted to monochrome images and then the spot intensities in neutrophil cytoplasm were arbitrarily graded from 0 for white to 100 for black. The intensities of the neutrophil staining were represented by the mean of the values for three randomly selected spots within the neutrophil cytoplasm. The intensity of neutrophil staining in the visual field was presented as the mean ± the standard deviation (SD) of the intensity values for 10 randomly selected neutrophils.

**Isolation of human neutrophils.** Neutrophils were purified from whole-blood samples (provided by Y.K.) as previously described (11). Briefly, whole-blood samples were intravenously obtained and collected in plastic syringes, followed by the separation of leukocytes from erythrocytes by sedimentation in 6% dextran (average molecular weight, 520,000) for 10 min at 4°C, followed by the hypotonic lysis of residual erythrocytes with cold distilled water for 30 s. Neutrophils were maintained in cold HEPES buffer (135 mM NaCl, 5 mM KCl, 1 mM CaCl2, 5 mM glucose, and 0.05) differences between the groups. Scale bars, 50 μm.

**Exocytosis of fPRL1 in vitro by stimulation.** fMLP has previously been identified as a potent leukocyte chemoattractant, and previous studies have demonstrated that fMLP binds to fPRL1 and induces intracellular signaling via fPRL1 (16, 17). Therefore, neutrophils were stimulated by treatment with fMLP. Neutrophils (10⁶ cells/ml) suspended in HEPES buffer were incubated with 10⁻⁸ M fMLP for 15 min at 37°C. Following stimulation, neutrophils were washed three times in cold HEPES buffer. Next, the cells were incubated with 1:500 anti-fPRL1 serum or preimmune serum in PBS for 15 min at 37°C. After being washed three times in cold HEPES buffer, neutrophils were incubated with 1:1,000 goat anti-rabbit immunoglobulin G-peroxidase conjugate in PBS for 15 min at 37°C. Following incubation, the immunostaining procedure was checked using negative and positive controls.

**Quantification of fPRL1 staining in neutrophils** (A) and expression of fPRL1 staining intensities in the neutrophil cytoplasm as measured in digitally remastered images (B) (case 1). In panel A, immunostaining pictures show that neutrophil fPRL1 stained strongly in the blood (a) and weakly stained in the central area of the dermatitis tissue (d). In panel B, the intensities of fPRL1 staining in the cytoplasm of neutrophils from the indicated areas were expressed in arbitrary units (EU) and plotted. The fPRL1 intensities decreased significantly in successions (one-way analysis of variance) from that in the blood (area a, 74.3 ± 2.7 arbitrary units) to those in the peripheral margin (area b, 54.3 ± 2.6 arbitrary units), the intermediate area (area c, 48.4 ± 5.6 arbitrary units), and the central area (area d, 40.1 ± 3.1 arbitrary units) of the dermatitis tissue. Asterisks indicate significant (P < 0.05) differences between the groups. Scale bars, 50 μm.
RESULTS

Intensity of fPRL1 staining in neutrophils in tissues. As the intensity of the immunohistochemical staining of the cytoplasmic fPRL1-invaded neutrophils varied from sample type to sample type, the fPRL1 staining intensity in the neutrophil cytoplasm as measured in photographs was expressed numerically. The intensity of fPRL1 staining in the cytoplasm of the neutrophils in blood was high, but that of the neutrophils in dermatitis tissue was low (Fig. 1A). In the dermal tissue, the neutrophils located in the central area (Fig. 1A, area a) of the inflammation stained less intensely for fPRL1 than those in the peripheral margin (Fig. 1A, area b). Values expressing the staining intensities in neutrophil cytoplasm in digitally remastered images are shown in Fig. 1B. Values for fPRL1 staining intensity in blood (74.3 ± 2.7 arbitrary units) (Fig. 1A, area a) were significantly higher than those for staining intensity in the dermal tissue. Values for fPRL1 staining in the peripheral margin of the dermal tissue (54.3 ± 2.6 arbitrary units) (Fig. 1A, area b) were significantly higher than those for staining in the intermediate area (48.4 ± 5.6 arbitrary units) (Fig. 1A, area c) and in the central area (40.1 ± 3.1 arbitrary units) (Fig. 1A, area d). fPRL1 values for the intermediate area (between the central area and the peripheral margin) were intermediate, and the distribution pattern in Fig. 1A, area c, shows a mix of patterns from area b and area d. As shown in Fig. 2, the intensity of the fPRL1 staining in neutrophil cytoplasm in case 1 specimens was identical to the intensities in samples from case 2 and case 3. We also investigated the intensity of the staining of neutrophils in bone marrow (Fig. 3), and bone marrow neutrophil fPRL1 (intensity, 66.4 ± 6.4 arbitrary units) stained as that in blood. The fPRL1 staining intensity in the cytoplasm of the bone marrow neutrophils was statistically different from that of neutrophils in the blood (case 1, 74.3 ± 2.7 arbitrary units; case 2, 70.6 ± 2.6 arbitrary units; and case 3, 74.2 ± 2.9 arbitrary units).

FIG. 2. The intensities of immunohistochemical fPRL1 staining in the neutrophil cytoplasm in dermatitis tissues from case 2 and 3 patients were expressed in arbitrary units (EU) and plotted. The fPRL1 staining intensities in the dermatitis tissues were significantly lower than those in the blood (in both cases 2 and 3). Values of fPRL1 staining intensity were as follows: case 2, 70.6 ± 2.6 (area a, corresponding to area a in Fig. 1A), 54.1 ± 2.6 (area b), and 40.2 ± 3.0 (area c); case 3, 74.2 ± 2.9 (area a), 57.5 ± 2.4 (area b), and 41.7 ± 3.7 (area c). Asterisks indicate significant (P < 0.05) differences between the groups.
We further investigated the morphological changes in neutrophils in relation to fPRL1 staining. As shown in Fig. 4 (case 1), we found that the fPRL1 staining intensity in the neutrophil cytoplasm decreased as neutrophils from the blood infiltrated the inflamed tissue. In the dermatitis tissue, the neutrophil cellular membranes stained strongly for fPRL1, but the cytoplasm was less stained. These data suggest that the shift of neutrophil fPRL1 from the cytoplasm to the cellular membrane during inflammation is due to exocytosis. There were no morphological changes in neutrophils indicative of apoptosis, which is characterized by shrinkage, the compaction of chromatin, or the loss of the multilobed shape of the nucleus.

Alteration of cellular neutrophil fPRL1 in vitro by fMLP stimulation. The alteration of neutrophil fPRL1 present on cellular membranes by fMLP stimulation for up to 15 min was monitored using flow cytometry (Fig. 5). Upon stimulation with 1 μM fMLP, M1 area values for neutrophil fPRL1, expressed as percentages of the area under the curve, increased throughout the time course (1.6% ± 0.03% at time 0, 7.1% ± 0.39% at 5 min, 10.4% ± 1.28% at 10 min, and 20.1% ± 3.19% at 15 min).

We investigated microscopically the morphological changes in neutrophil fPRL1 in vitro after incubating the neutrophils with fMLP. As shown in Fig. 6, the neutrophils shone brightly after fMLP stimulation, suggesting that the levels of neutrophil fPRL1 on the cellular membranes increased after fMLP stimulation.

**DISCUSSION**

Neutrophils are considered to be part of the first line of defense against bacterial infections, and the activation of neutrophils is induced by chemotactic receptors of fPR and fPRL1. fPRL1 is expressed not only in the neutrophils, but also in a variety of cells including phagocytes, epithelial cells, T lymphocytes, vascular endothelial cells, vascular smooth muscle cells, and synovial cells (14, 15, 16, 17, 18, 23), all of which are considered to be capable of participating in inflammation. The mobilization of neutrophil fPRL1 to the cellular membrane by LPS and proinflammatory mediator stimuli in vitro has been reported previously (1, 6, 7), but there is no in vivo study of neutrophil fPRL1. This is the first clinical report describing the pathological changes in human neutrophil fPRL1 in the inflamed tissue in vivo. We found that (i) the fPRL1 staining intensity in neutrophil cytoplasm in the inflamed tissue was lower than that in the blood and that (ii) the fPRL1 staining intensity on neutrophil cellular membranes increased in contrast to the decrease of intensity in the neutrophil cytoplasm. This fact is conceivably explained by the exocytosis of neutrophil fPRL1 (8).
In dermatitis tissue, the level of cytoplasmic neutrophil fPRL1 was clearly different from that in the blood, which was characterized by decreased fPRL1 staining intensity in neutrophil cytoplasm and increased fPRL1 staining intensity on neutrophil membranes. We also found that neutrophil cytoplasm in the dermatitis tissue was less stained by Naphtol AS-D chloroacetate esterase than that in the blood (data not shown). It has been reported previously that the alkaline phosphatase in neutrophils is located intracellularly while neutrophils are unstimulated but moves to the cellular surface after stimulation (11). The distinctive staining features of neutrophil fPRL1 in the dermatitis tissue indicate that fPRL1 acts like alkaline phosphatase.

FIG. 5. The alteration of fPRL1 on neutrophil membranes throughout the time course of fMLP stimulation was investigated using flow cytometry. Neutrophils (10⁶ cells/ml) were incubated with 1 μM fMLP for 5, 10, and 15 min at 37°C, followed by flow cytometric analysis. The levels of membranous fPRL1-bearing neutrophils, expressed as percentages of the area under the curve (MI), markedly increased after fMLP stimulation (1.6% ± 0.03% at time 0, 7.1% ± 0.39% at 5 min, 10.4% ± 1.28% at 10 min, and 20.1% ± 3.19% at 15 min). The fPRL1 fluorescence intensity is expressed in arbitrary units. Representative patterns of neutrophil membranous fPRL1 are shown. Data are means ± SD of results from three independent experiments yielding similar results. SSC-H, side scatter-height; FSC-H, forward scatter-height.

FIG. 6. The change in fPRL1 on neutrophil surfaces after 15 min of stimulation with 10⁻⁶ M fMLP is shown. Neutrophils were visualized by fluorescence microscopy. (a) Unstimulated neutrophils stained with anti-fPRL1 antiserum; (b) fMLP-stimulated neutrophils stained with preimmune serum; and (c) fMLP-stimulated neutrophils stained with anti-fPRL1 antiserum. fMLP markedly induced fPRL1 presentation on the cellular membrane (c), suggesting the exocytosis of fPRL1-containing granules.
phosphatase. These facts indicated that the activated neutrophils infiltrate the inflamed tissue in proportion to the increase in the grade of inflammation (Fig. 1A, areas c and d). This pathognomonic feature of neutrophil fPRL1 was confirmed by in vitro (Fig. 5 and 6). The life span of neutrophils in circulation is reported to be 6 to 8 h (29). However, it is prolonged when the neutrophils are incubated with proinflammatory cytokines and mediators (22). Neutrophil fPRL1, depending on the ligands, is also reported to play an important role in activating and inactivating inflammation (16, 17, 18, 23). Therefore, whether these morphological characteristics of neutrophils are related to the neutrophil function (phagocytosis) or to the near-future outcome for the neutrophils (apoptosis) is worthy of consideration.

The upregulation of fPRL1 on neutrophil membranes, regarded as the activation of neutrophils, is induced by preincubation with bacterial LPS and/or cytokines but is hardly induced without this priming condition (1, 6, 7). There is no previous report of neutrophil activation in the bloodstream during inflammation. Contrary to our expectations, we found a significant difference in fPRL1 staining intensities in neutrophil cytoplasm, with higher intensities in blood and lower intensities in bone marrow. This pattern is hard to understand in theory, because neutrophils activated in the bloodstream should have already been stimulated before infiltrating the inflamed tissue in patients with purulent dermatitis. Our finding cannot be explained definitively, but we can raise possible reasons as follows. First, the level of neutrophil fPRL1 in bone marrow is possibly underestimated. The maturation of bone marrow neutrophils proceeds heterogeneously, and immature but not mature neutrophils in bone marrow are reported to have secretory vesicles poor in fPRL1 (8). Second, the level of neutrophil fPRL1 in the bloodstream may be increased by some mechanisms induced by inflammation, including the transcriptional upregulation of the fPRL1 gene. We do not know whether this discrepancy has any significance, because we did not directly investigate the neutrophil fPRL1 staining intensity in bone marrow from patients with purulent dermatitis (cases 1 to 3). Moreover, the precise mechanism related to the upregulation of cytoplasmic neutrophil fPRL1 is yet to be determined, but there is a possibility that the production of neutrophil fPRL1 may have been upregulated by proinflammatory cytokines.

Aside from fMLP, T21/DP107, and the W peptide (16, 17, 23), several endogenous agonists and antagonists involved in the inflammatory process have been reported to interact with fPRL1. Lipoxin A4, a metabolite of prostaglandins, has been reported to be a potent inhibitor of acute inflammation through the suppression of chemokine production by fPRL1-positive cells (9, 25). A-SAA, another endogenous ligand, has also been reported to promote or inhibit the migration of neutrophils through fPRL1 (3, 10) and to inhibit the oxidative burst response of neutrophils stimulated by fMLP (19). However, these previous results are all different and depend on the condition of the cells, the culture conditions, or the type of experiment. A-SAA is chemotactic for the migration of human aortic smooth muscle cells and synovial fibroblasts, and the A-SAA-induced migration of human aortic smooth muscle cells and synovial fibroblasts is inhibited by anti-fPRL1 (14, 15). In addition to the production of proinflammatory cyto-

kines after stimulation with bacterial LPS, A-SAA locally synthesized in the skin (27) and additionally produced by the infiltrated monocytes and macrophages (28) may be implicated in neutrophil migration in dermatitis.

The chemotactic migration of neutrophils and vascular smooth muscle cells via fPRL1 is mediated by the activation of protein kinase C, phosphatidylinositol 3-kinase, Ras of small G protein, and p38–mitogen-activated protein kinase (12, 18). Although the major pathway of fPRL1 signal transduction and the relationships of fPRL1 signaling to the neutrophil functions including the exocytosis of granules are not known, the tertiary gelatinase granules of the neutrophils are reported to be the potential stores of fPRL1 (1, 6). These data indicate a clinical linkage between gelatinase and fPRL1 in the inflammatory process.

In conclusion, neutrophil fPRL1 staining of the purulent dermatitis tissue revealed lower fPRL1 staining intensity in the cytoplasm and higher intensity on the cellular membranes of neutrophils, indicating the exocytosis of fPRL1 during neutrophil invasion.

REFERENCES


