Possible Physiological Roles of Proteolytic Products of Actin in Neutrophils of Patients with Behçet’s Disease

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A truncated actin with an N-terminus of Met-44 is known to be selectively increased in neutrophils of patients with Behçet’s disease and to be generated proteolytically by PMN-elastase (Yamashita S. et al., Biol. Pharm. Bull., 23, 519—522 (2000); Biol. Pharm. Bull., 24, 119—122 (2001)). In this study, the functions of the N-terminal peptide consisting of Asp-2 to Val-43 (β-actin (42-merP)) and the truncated actin with an N-terminus of Met-44 were examined. We first confirmed that the 42-merP existed in the patient plasma. The motility of human peripheral blood neutrophils and neutrophilic granulocytes differentiated from HL-60 cells was suppressed by the 42-merP. Furthermore, when neutrophil-like cells from HL-60 cells were preincubated with 10 nm 42-merP, migration of the cells induced by chemotactic factors such as fMLP and IL-8 was suppressed. The release of PMN-elastase, which is a neutrophil granular enzyme that is responsible for the production of the 42-merP and truncated actin, was suppressed by pretreating the neutrophils with 42-merP before fMLP-stimulation. The truncated actin was unable to polymerize in 0.1 M KCl, suggesting that the increase of truncated actin damages the reconstitution capacity of actin in neutrophils of the patients. These results suggest that the increase of 42-merP and truncated actin in patients with Behçet’s disease changes functions of neutrophils.

Key words  Behçet’s disease; neutrophil; actin; chemotaxis; chemokinesis; elastase

Behçet’s disease is thought to be an immune disorder. The patients have repeated exacerbations and remissions of the symptoms. It is possible that the inflammatory symptoms are related to the fact that patients with Behçet’s disease have hyperfunctional neutrophils.1–4 In a previous study,5 we applied high resolution two-dimensional gel electrophoresis (2-DE) in order to compare the neutrophil proteins of patients with those of healthy volunteers and reported an increase of a truncated actin with an N-terminus of Met-44 in neutrophils of patients with Behçet’s disease. Furthermore, we have found that polymorphonuclear (PMN)-elastase specifically cleaves actin at the site between Val-43 and Met-44 in neutrophils of patients with Behçet’s disease.5 PMN-elastase is an enzyme localized in the azurophil granules of neutrophils and is thought to take part in the degradation of materials that have been engulfed by phagocytes.5,7 Chymotrypsin,9 subtilisin10 and protease from E. coli11,12 have been reported to cleave actin at sites that are different from that of PMN-elastase. However, the physiological significance of actin hydrolysis by these proteases is not clear.

The truncated actin, which is generated by hydrolysis of actin by PMN-elastase, may be defective in its ability to polymerize because the N-terminal peptide consisting of Asp-2 to Val-43 of β-actin (42-merP) is one of responsible sites for protein–protein interaction that are involved in the actin helix.12,13 In addition, the 42-merP may contain a site for actin-binding proteins and regulate actin function.14–16 These observations suggest that the truncated actin may impair neutrophil function. In addition, the 42-merP itself may have adverse effects on neutrophil function.

In the present study, we (1) determined whether the 42-merP was present in the plasma of Behçet’s patients, (2) examined the effects of 42-merP on the motility and degradation of neutrophils, and (3) determined the ability of the truncated actin to polymerize.

MATERIALS AND METHODS

Materials  Reagents used in this paper were purchased from Sigma Chemical Company Co. (U.S.A.) or Nacalai Tesque Inc. (Japan). All reagents were HPLC grade or analytical reagent grade unless otherwise noted. A 42-mer peptide with partial amino acid sequence of actin from N-terminal Asp-2 to Val-43 (42-merP) was synthesized by and purchased from Swady Technology Co. (Japan). PMN-elastase was purchased from Elastin Products Co. (U.S.A.). Human recombinant interleukin (IL)-817 and N-formyl-methionyl-leucyl-phenylalanine (fMLP)18 were purchased from Sigma (U.S.A.). Rabbit skeletal muscle actin was purchased from Calzyme Laboratories Inc. (U.S.A.).

Isolation of Neutrophils  After receiving informed consent, blood samples were drawn using evacuated blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) disodium salt (Terumo, Japan). Neutrophils were isolated from the venous blood of healthy volunteers as described previously.5

Cell Culture  Promyelocytic HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% v/v heat-inactivated fetal calf serum, 2 mm L-glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin. The cell culture medium was obtained from Gibco BRL (U.S.A.), and the plastic flasks and the petri dishes were from Becton Dickinson (U.S.A.) or Corning Costar Corporation (U.S.A.). The cells were maintained in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Before use, the cells were differentiated into neutrophilic granulocytes (HL-60G) by exposure to 1.25% v/v dimethylsulfoxide (DMSO) for 5 d.

Detection of 42-merP in Plasma  After receiving in-
formed consent, three patients with Behçet’s disease and three healthy volunteers were bled. The diagnosis of Behçet’s disease was made according to the criteria of the International Study Group for Behçet’s disease. The blood samples were immediately centrifuged at 1500 × g for 10 min to obtain plasma. Plasma samples were dialyzed in 12000 Da molecular weight cut-off tubing against 3 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 0.1 mM dl-dithiothreitol (DTT) for one day at 4 °C. After dialysis, the buffer outside of the dialysis membrane was concentrated and applied to a blotting analysis using an antibody to the N-terminus of actin as described previously. The antibody to the N-terminus of actin was prepared by immunizing rabbits with a KLH-conjugated peptide (Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys), which was involved in a amino acid sequence of 42-merP and had no cross-reactivity with the truncated actin.

Effect of 42-merP on Motility of Neutrophils and HL-60G Cells Chemotaxis and chemokinesis were assayed by a modified Boyden chamber technique using a 96-well microchemotaxis chamber (Neuro Probe Co., Model MBB96, U.S.A.) with polyvinyl propylene-free polycarbonate membranes with 3 μm pores for neutrophils or 5 μm pores for HL-60G cells. This assay was performed to determine whether 42-merP has chemotactic or chemokinetic activity for human neutrophils. Various concentrations of 42-merP (0, 1, 10, 100 nM) were added into the upper wells with cells (4.0 × 10⁶ cells/well) and into the lower wells of a chemotaxis chamber. The chamber was incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C for 90 min. Then, fluid and non-migration cells were removed from the upper well. After washing twice with Hank’s buffer (Gibco BRL, U.S.A.), 2 mM EDTA in Hank’s buffer was added into upper wells and the chemotaxis chamber was further incubated at 4 °C for 20 min and the fluid in the upper wells was removed. The chemotaxis chamber was capped with a membrane filter was centrifuged (200 × g, at 4 °C for 10 min) to remove fluid from lower wells. The cells that migrated from the upper wells to the lower wells were stained with 10% v/v Alamar blue® (Dainipponseiyaku, Japan) in 1% BSA RPMI 1640 medium and then the fluorescence intensity was measured with a spectrofluorophotometer (Fluoroskan, Dainipponseiyaku, Japan) with excitation at 544 nm and emission at 590 nm. Experiments were performed in triplicate. Cell viability was estimated by a trypsin blue dye exclusion test on HL-60G cells incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C for 90 min or 12 h with or without 42-mer P.

Effect of 42-merP on Chemotaxis of HL-60G Cells Induced by IL-8 or fMLP HL-60G cells were preincubated with or without 10 nM 42-merP in 1% v/v BSA RPMI 1640 medium for 20 min at 37 °C, and the chemotaxis induced by IL-8 (25, 50, 100 ng/ml) or fMLP (1, 10, 100 nM) was assayed by a 96-well microchemotaxis chamber as described above. The results were expressed as the chemotactic index calculated by the ratio of the fluorescence intensity of cells that migrated by chemotaxis induced by IL-8 or fMLP to the fluorescence intensity of cells that migrated by random movement (medium alone).

Effect of 42-merP on PMN-Elastase Release from Neutrophils Stimulated by fMLP Neutrophils were suspended in Hank’s buffer (Gibco BRL, U.S.A.) including 25 mM 2-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (pH 7.4) at 2×10⁶ cells/ml. The cells (4×10⁵ cells/tube) were preincubated with or without 100 nM 42-merP in Hank’s buffer for 20 min at 37 °C. After preincubation, the cells were stimulated with 100 nM fMLP for 30 min at 37 °C and then centrifuged at 400 × g for 5 min at 4 °C. The supernatants were stored at −80 °C. The amounts of PMN-elastase in the supernatants were determined by a rapid homogeneous immunonucleation (IMAC) assay using latex particles coated with antibody fragments F(ab)’2 against human PMN-elastase. Test kits (ECole® PMN elastase; detection limit of 4 μg/l) were purchased from Merck (U.S.A.). The turbidity which indicates the degree of latex particle agglutination, was measured photometrically with a Cobas Mira™ (Roche, U.S.A.).

Polymerization Ability of Truncated Actin and Effect of 42-merP on Polymerization of Native Actin For PMN-elastase digestion, the actin was dissolved in 2 mM Tris–HCl (pH 7.6) containing 0.2 mM CaCl₂, 0.2 mM ATP and 0.2 mM DTT (G-Buffer) at a concentration of 10 μM. The actin solution (10 μl) was mixed with 2 μl PMN-elastase solution (1 mg protein/ml). The digestion was carried out for 1 h at 37 °C and stopped by the addition of 100 mM N-Methoxysuccinyl- Ala-Ala-Pro-Val chloromethylketone (a PMN-elastase inhibitor, Sigma, U.S.A.). Actin and truncated actin with an N-terminus of Met-44 were detected by SDS/PAGE analysis as reported previously. Polymerization of actin after digestion was induced by incubation at 25 °C for 60 min in 0.1 M KCl solution containing 0.2 mM MgCl₂. Then, polymerized actin was sedimented by centrifugation at 198000 × g for 60 min. The pellet was homogenized with G-buffer. Aliquots of the supernatants and the homogenized pellets were subjected to SDS/PAGE. To examine the effect of 42-merP on actin polymerization, the actin solution (10 μM) was added to an equal volume of 42-merP (10 μM) solution and incubated under polymerizing conditions as mentioned above. After polymerization, the reaction mixture was centrifuged at 198000 × g for 60 min. Aliquots of the supernatants and the homogenized pellets were subjected to SDS/PAGE analysis.

Statistical Calculations All results are expressed as mean ± S.D. Statistical analysis was performed using Student’s t-test or Welch’s t-test for comparison of two groups. Differences were considered significant if p values were 0.01, 0.05 or less.

RESULTS AND DISCUSSION

Detection of 42-merP in Plasma The N-terminal peptide of β-actin (42-merP) in plasma from patients with Behçet’s disease and healthy volunteers was detected by immunoblot analysis using an antibody to the N-terminus of β-actin as previously reported. It was confirmed that 42-merP exists in the blood plasma of Behçet’s disease patients (Fig. 1 BD, n=3) and only small amounts were detected in the plasma of healthy volunteers (Fig. 1 HV, n=3). It is undoubtedly that 42-merP is further digested with various proteases present in the plasma. However, 42-merP was detected in the plasma of patients with Behçet’s disease as shown in Fig. 1. Therefore, it is possible that 42-merP exerts some effects on neutrophil function in the patients.

Effect of 42-merP on Neutrophil Motility Migration of
The 42-merP in plasma from patients with Behçet’s disease and healthy volunteers detected by immunoblot analysis using an antibody to the N-terminus of β-actin as described in Materials and Methods.

To examine whether 42-merP has chemotactic activity, various concentrations of 42-merP (0, 1, 10, 100 nM) were added to the lower wells of a chemotaxis chamber with neutrophils or HL-60G cells (—). The number of cells migrating to the lower wells were measured by fluorometric assay as described in Materials and Methods. All results are expressed as mean ± S.D. Statistical analysis was performed using Student’s t-test for comparisons of two groups. Differences were considered significant if p values were 0.05*, 0.01** or less.

Table 1. Effect of 42-merP on Motility of HL-60G Cells

<table>
<thead>
<tr>
<th>42-merP added (lower well)</th>
<th>42-merP added (upper well)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 nM</td>
</tr>
<tr>
<td>Fluorescence intensity</td>
<td></td>
</tr>
<tr>
<td>0 nM</td>
<td>399.4 ± 31.6</td>
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<tr>
<td>1 nM</td>
<td>385.7 ± 17.9</td>
</tr>
<tr>
<td>10 nM</td>
<td>399.2 ± 11.6</td>
</tr>
<tr>
<td>100 nM</td>
<td>389.5 ± 23.8</td>
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</tbody>
</table>

Various concentrations of 42-merP (0, 1, 10, 100 nM) were added to the upper and lower wells of a chemotaxis chamber. The upper wells each contained 3 × 10^5 HL-60G cells, and the lower wells contained no cells. The number of cells migrating to the lower wells was measured by a fluorometric assay as described in Materials and Methods. All results are expressed as mean ± S.D. Statistical analysis was performed using Student’s t-test for comparisons of two groups. Differences were considered significant if p values were 0.05*, 0.01** or less.
ever, the present results show that hydrolytic products of actin by PMN-elastase have suppressing effects on neutrophilic function. Accordingly, it is expected that the increase in 42-merP and truncated actin in the patients leads the neutrophil hyperfunction in the inflammatory process in Behçet’s disease which consists of an acute severe phase and a remission phase. The major symptom of Behçet’s disease is the infiltration of neutrophils into specific tissues.21) The effect of 42-merP on this process has not been examined, but the present results raise the possibility that 42-merP depresses the hyperfunction of neutrophils in the inflammatory site of the patients.

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REFERENCES