Characterization of a Protease Responsible for Truncated Actin Increase in Neutrophils of Patients with Behçet’s Disease

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As described previously (Yamashita S. et al., Biol. Pharm. Bull. 23, 519—522 (2000)), high levels of a truncated actin with an N-terminus of Met-44 were detected in neutrophils of patients with Behçet’s disease. Since the increase of the truncated actin in neutrophils of patients may be important for understanding the pathology of Behçet’s disease, the mechanism of the truncated actin formation was studied. First, to investigate the presence of a specific protease, which cleaves the actin at the site between Val-43 and Met-44, a peptide with a partial amino acid sequence of actin from the N-terminal Pro-38 to Asp-51 was synthesized as the protease substrate. The synthesized peptide was digested with cytosolic fractions of neutrophils from patients and healthy volunteers, and digestion products were analyzed by C18-reverse phase HPLC. The chromatograms of these samples showed that an endoprotease, which cleaved the peptide at a specific site, was present in cytosolic fractions of neutrophils from patients with Behçet’s disease. Then, the effects of various kinds of protease inhibitors on the digestion of the peptide were investigated in order to identify the responsible endoprotease. The digestion of the peptide was suppressed by 4-(2-aminoethyl) benzenesulfonylfluoride (AEBSF, a serine protease inhibitor) and N-methoxy succinyl-Ala-Ala-Pro-Val chloromethylketone (CMK, a polymorphonuclear (PMN)-elastase inhibitor) in the presence of EDTA. Furthermore, PMN-elastase was found to cleave the substrate peptide and actin at the site between Val-43 and Met-44. These results lead to the conclusion that the PMN-elastase is responsible for cleavage of actin at the N-terminal site between Val-43 and Met-44 in neutrophils from patients with Behçet’s disease.

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

MATERIALS AND METHODS

Materials Reagents used in this paper were purchased from Sigma Chemical Company (U.S.A.) or Nacalai Tesque Inc. (Japan). All reagents were HPLC grade or analytical reagent grade unless otherwise noted. The substrate peptide with a partial amino acid sequence of actin from the N-terminal Pro-38 to Asp-51 (Pro-Arg-His-Gln-Gly-Val-Met-Val-Gly-Met-Gly-Gln-Lys-Asp), its N-terminal peptide (Pro-Arg-His-Gln-Gly-Val) and its C-terminal peptide (Met-Val-Gly-Met-Gly-Gln-Lys-Asp) were synthesized with a peptide synthesizer (MilliGen9050, Millipore, U.S.A.) and purified with an HPLC system. Protease inhibitors, 4-(2-aminoethyl)-benzenesulfonylfluoride, hydrochloride (AEBSF, a serine protease inhibitor, Calbiochem-Novabiochem, U.S.A.), 1-trans-epoxysuccinyl-leucylamido-(4-guanidino) butane (E-64, a cysteine protease inhibitor, Calbiochem-Novabiochem, U.S.A.), Ethylenediaminetetraacetic acid (EDTA · 4Na, a metalloprotease inhibitor, Calbiochem-Novabiochem, U.S.A.) and N-methoxy succinyl-Ala-Ala-Pro-Val chloromethylketone (CMK, a PMN-elastase inhibitor, Sigma, U.S.A.) were used at final concentrations of 0.1 mM, 10 μM, 10 mM and 0.3 mM, respectively. Cathespin-G and PMN-elastase were purchased from Elastin Products Co., U.S.A.

Isolation of Human Neutrophils After receiving informed consent, neutrophils were prepared from the venous blood of patients with Behçet’s disease (incomplete type) who had not received steroids. Neutrophils were isolated according to the method of Boyum.9) Briefly, neutrophils were separated from peripheral blood drawn into heparinized (100 U/ml Heparin Novo, Novo Industrie, Denmark) syringes by
dextran sedimentation (Dextran T2000, Pharmacia Biotech, Sweden) and Ficoll-Paque (Pharmacia Biotech, Sweden) density centrifugation. The cell pellet from 10 ml whole blood was immediately resuspended in 2 ml of a 0.2% w/v solution of NaCl to lyse contaminating erythrocytes. After 30 s incubation, 2 ml of a 1.6% w/v solution of NaCl was added to the cell suspension. More than 98% of the cells were viable as assayed by trypan blue dye exclusion and the percentage of neutrophils was greater than 95% as assayed by hematoxylin staining.

Preparation of Cytosolic Fractions from Neutrophils
Neutrophils were suspended at a concentration of $1.0 \times 10^5$ cells/ml in Milli-Q water, and then disrupted by three cycles of freezing and thawing with liquid nitrogen. The neutrophil cytosol was obtained by centrifugation (Beckman, GS-15R centrifuge, U.S.A.) at 13500 rpm and 4 °C for 10 min. The resulting supernatant was used as the cytosolic fraction for peptide digestion.

Analysis of Peptides Cleaved with the Cytosolic Fraction
The standard peptides (Fig. 1) were separated on the HPLC system. Figure 2A shows the chromatogram of a mixture of standard peptides and Fig. 2B shows the chromatogram of the substrate peptide (SP) after incubation with the cytosolic fraction. Peaks 1, 2, 3 and 4 correspond to the C-terminus peptide of SP (CP), the N-terminus peptide of SP (NP), SP and sulindac (internal standard), respectively. CP and NP were not detected in the chromatogram of Fig. 2B and the original SP peak was almost disappeared. This result suggests that several kinds of proteases, which may almost completely digest SP, are activated in the neutrophil cytosol of patients with Behçet’s disease. Since many proteases are known to be dependent on a metal ion, the effect of a metalloprotease inhibitor on the digestion of SP with the cytosolic fraction of neutrophils was then tested.

SDS-PAGE Analysis of Actin Cleaved with PMN-Elastase
A 0.5 mg/ml actin solution was prepared with 2 mM Tris–HCl (pH 7.6) containing 0.2 mM CaCl2, 0.2 mM ATP and 0.2 mM DTT (G-Buffer). For PMN-elastase digestion of actin, the actin solution was mixed with 2 µl PMN-elastase solution containing 1 mg of protein per milliliter in Milli-Q water. The digestion was carried out for 3 h at 37 °C and was stopped by boiling for 5 min or by addition of 100 mM CMK solution. Aliquots of the samples were subjected to SDS-PAGE by the method of Laemmli10) on a 10% w/v polyacrylamide gel (90 mm width, 80 mm length and 1 mm thickness, pH 8.8) at 25 mA for 90 min. Truncated actins were detected by staining with Coomassie Brilliant Blue R250.

Amino Acid Sequencing
First, SDS-PAGE was performed and then proteins on the gel were electrophoresed onto PVDF membranes (Amersham, U.K.) according to the method of Matsudaira.11) Second, after Coomassie Brilliant Blue staining, proteins on the PVDF membranes were directly applied to a protein sequencer (Model 473 A, Perkin Elmer, U.S.A.).

RESULTS AND DISCUSSION
Digestion of Substrate Peptide with the Cytosolic Fraction of Neutrophils and HPLC Analysis of the Digested Products
The standard peptides (Fig. 1) were separated on the HPLC system. Figure 2A shows the chromatogram of a mixture of standard peptides and Fig. 2B shows the chromatogram of the substrate peptide (SP) after incubation with the cytosolic fraction. Peaks 1, 2, 3 and 4 correspond to the C-terminus peptide of SP (CP), the N-terminus peptide of SP (NP), SP and sulindac (internal standard), respectively. CP and NP were not detected in the chromatogram of Fig. 2B and the original SP peak was almost disappeared. This result suggests that several kinds of proteases, which may almost completely digest SP, are activated in the neutrophil cytosol of patients with Behçet’s disease. Since many proteases are known to be dependent on a metal ion, the effect of a metalloprotease inhibitor on the digestion of SP with the cytosolic fraction of neutrophils was then tested.
fraction was investigated. Figure 3A shows a chromatogram of mixtures of standard peptides and Fig. 3B shows a chromatogram of SP after incubation with the cytosolic fraction in the presence of a metalloprotease inhibitor, EDTA. Peaks 5 and 6 in Fig. 3B corresponded to peak 1 (CP), and peak 2 (NP), in Fig. 3A, respectively. These results suggest that metalloproteases are not responsible for the production of the truncated actin, but they do participate in its further digestion.

Identification of the Protease Responsible for the Truncation of Actin To identify the protease responsible for cleavage of actin at the site between Val-43 and Met-44, the effects of various protease inhibitors on the digestion of SP were investigated. As described above (Figs. 2B and 3B), metalloproteases in the cytosolic fraction further digested CP and NP, which made it impossible to detect SP, CP and NP. The protease inhibitors did not suppress the further digestion of CP and NP (Fig. 4). Therefore, to study the effects of various protease inhibitors on SP digestion, they were added to the assay mixture in the presence of a metalloprotease inhibitor, EDTA (Fig. 5). Peaks 1, 2, 3 and 4 in Fig. 5A corresponded to CP, NP, SP and sulindac, respectively. There was a peak corresponding to SP but not one corresponding to CP or NP in Figs. 5B and 5C, indicating that AEBSF, a serine protease inhibitor, and CMK, an inhibitor of PMN-elastase (a kind of serine protease), suppressed the cleavage of SP to CP and NP. On the other hand, small peaks of CP, NP and SP were observed in Fig. 5D, indicating that E-64, a cysteine protease inhibitor, did not suppress the cleavage of SP. These results suggest that a neutrophil serine protease, especially a PMN-elastase, is responsible for the cleavage of SP to CP and NP. Therefore, the effects of known neutrophil-serine proteases, cathepsin-G and PMN-elastase, on the cleavage of SP were investigated (Fig. 6). As shown in Fig. 6B, PMN-elastase digested SP to CP and NP. However, cathepsin-G did not digest SP (data not shown). Since subtilisin and chymotrypsin are known to cleave actin between Met-47 and Gly-48 and between Met-44 and Val-45, respectively, the amino acid sequences of peaks 5 and 6 in Fig. 6B were determined and compared with those of CP and NP. They were shown to correspond to those of CP and NP (data not shown). These results suggest that a PMN-elastase in neu-
trophils of patients with Behçet’s disease cleaves actin at the site between Val-43 and Met-44 and produces the truncated actin.

Cleavage of Actin with PMN-Elastase

In order to confirm the above supposition, the effect of PMN-elastase on the cleavage of native actin was investigated. The actin treated with PMN-elastase was analyzed with SDS-PAGE. A truncated actin band with a relative molecular mass of 38 kDa was observed (lane 3 in Fig. 7). The N-terminal amino acid sequence of this 38 kDa protein was determined and found to correspond with that of the truncated actin observed in neutrophils of patients with Behçet’s disease. These results lead to the conclusion that PMN-elastase is activated in neutrophils of patients with Behçet’s disease, which results in the increase of the truncated actin with Met-44 as the N-terminal amino acid.

It has been reported that subtilisin and chymotrypsin cleave actin at sites that are different from the cleavage site of PMN-elastase and produce truncated actins which fail to polymerize in 0.1 M KCl.12,13) Brown et al.14) also reported that actin is cleaved between Val-43 and Met-44 during constitutive apoptosis of neutrophils by an unknown protease. Furthermore, Ishihara et al.15) reported that rat peritoneal macrophages stimulated with PMN-elastase showed increased production of chemokines for neutrophils. These observations suggest that the peptide with an C-terminus of Val-43 in neutrophils of patients with Behçet’s disease may have some biological activity and be related to the pathological features of Behçet’s disease. Therefore, it may be important to study whether the products of actin cleaved with PMN-elastase have biological activity. Such studies are now under investigation in our laboratory.

On the other hand, it has been reported that the PMN-elastase level in blood plasma reflects disease conditions of patients with Behçet’s disease and is a good biological marker for diagnosis of Behçet’s disease.16) Therefore, it may be important to investigate the mechanism controlling the increase of PMN-elastase activity in neutrophils of patients with Behçet’s disease in order to understand the pathological features of the disease.

REFERENCES