Analysis of Neutrophil Proteins of Patients with Behçet’s Disease by Two-Dimensional Gel Electrophoresis

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Protein changes in the neutrophils of patients with Behçet’s disease were analyzed by high resolution two-dimensional gel electrophoresis to investigate the pathological features of Behçet’s disease. Two clear protein spots were found to be differentially expressed between healthy volunteers and patients with Behçet’s disease. One was a 53 kDa protein with pI 5.2 that was detected in healthy volunteers but was nearly absent in the patients. The other was a 40 kDa protein with pI 5.2 that was detected in the patients but nearly absent in the healthy volunteers. Analysis of the N-terminal amino acid sequence of the 40 kDa protein revealed that it was a truncated actin with an N-terminus of Met-44. The presence of the truncated actin in the neutrophils of patients was confirmed by Western blot analysis using an antibody to the C-terminus of actin. The 53 kDa protein could not be identified because its N-terminus was blocked. The presence of the truncated actin in the neutrophils of the patients may be important in understanding the pathology of Behçet’s disease.

Key words: Behçet’s disease; two-dimensional gel electrophoresis; neutrophil; actin

Behçet’s disease is a multisystem inflammatory disease, which was first described by Behçet in 1937.1 Its major symptoms are recurrent oral and genital ulcers and hypopyon, and other clinical signs of Behçet’s disease are relapsing iritis, erythema nodosum, nail fold thrombi, thrombophlebitis, arthritis, colitis and neurological alterations such as meningoencephalitic syndrome or peripheral nervous system dysfunction.2–5 The infiltration of lymphocytes and neutrophils in the lesions of patients with Behçet’s disease have been observed by histopathological studies,6–8 and neutrophils of patients with Behçet’s disease have been shown to be hyperfunctional,9,10 suggesting that the excessive neutrophil function plays a vital role in the development and clinical course of the disease. Therefore, it may be important to clarify the characteristics of hyperfunctional neutrophils for understanding their pathological roles.

In the present study, to investigate neutrophil characteristics related to the pathological features of Behçet’s disease, we applied high resolution two-dimensional gel electrophoresis (2-DE)13 in order to compare the neutrophil proteins of the patients with those of healthy volunteers. Electrophoresis was carried out by isoelectric focusing (IEF) in the first dimension, and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. 2-DE has a major advantage in analyzing highly complex protein mixtures such as cellular proteins, which could not be separated adequately with one-dimensional techniques. We found several proteins differently expressed in neutrophils between the patients and healthy volunteers.

MATERIALS AND METHODS

Materials Reagents used in this paper were purchased from Sigma Chemical Co. (U.S.A.) or Nacalai Tesque Inc. (Japan). All reagents were of analytical grade or guaranteed, except as noted. Two types of anti-actin antibodies were purchased from Sigma Chemical Co. (U.S.A.). One is a mouse monoclonal anti-beta-actin antibody, which recognizes an epitope located on the N-terminus of the actin. The other is a rabbit anti-actin antibody, which recognizes an epitope located on the C-terminus of the actin. Goat anti-mouse IgG (H+L) antibody conjugated with horseradish peroxidase and goat anti-rabbit IgG (H+L) antibody conjugated with horseradish peroxidase were purchased from Kirkegaard & Perry Laboratories, Inc. (Germany) and Caltag Laboratories, (U.S.A.), respectively.

Isolation of Human Neutrophils Human neutrophils were prepared from the venous blood of six patients with Behçet’s disease (incomplete type) who had not received steroids and who had given their informed consent for the study, and also from three healthy volunteers. Isolation of the neutrophils was performed according to the method of Beyer.14 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. To lyse contaminating erythrocytes, the cell pellet was immediately resuspended in 2 ml of a 0.2% w/v solution of NaCl. After 30 s of 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.

Sample Preparation for 2-DE Neutrophil proteins for 2-DE were solubilized according to the method of Garrels and Franz6 Briefly, neutrophils (1.0×10^7 cells) were added to two volumes (240 µl) of boiling buffer A containing 28 mM Tris–HCl, 22 mM Tris base, 0.3% w/v SDS, and 200 mM dithiothreitol (DTT), pH 7.8, and the mixture was boiled for another 5 min. Then, after cooling on ice, 24 µl of buffer B containing 476 mM Tris–HCl, 4 mM Tris base, 50 mM MgCl₂, 1 mg/ml DNase I (Boehringer Mannheim, Germany), 0.25 mg/ml RNase A (Wako Pure Chemical Industries, Ltd.), and 1% v/v glycerol (Nissui Seiyaku, Japan) were added to each sample.
Japan), pH 6.8, was added. After an additional 8 min of incubation on ice, the proteins were precipitated with acetone at a final concentration of 80% v/v by incubation for 20 min on ice, then pelleted by centrifugation (Beckman GS-15R centrifuge, U.S.A.) at 13500 rpm and 4°C for 10 min. The pellet was dried in a centrifugal evaporator and resuspended in 240 µl of a mixture of one volume of buffer A and four volumes of buffer C containing 9.9 M urea, 4% v/v Nonidet p-40 (NP-40), 2.2% v/v carrier ampholytes and 100 mM DTT. The final protein concentration in each sample was about 50 µg/100 µL.

IEF The first-dimensional IEF was performed using the Investigator 2-D Electrophoresis System™ (Millipore, U.S.A.). Capillary gels (18 cm in length and 1 mm in diameter) containing 4.1% w/v polyacrylamide (stock solution: 30% w/v acrylamide, 0.8% w/v N,N-methylenebisacrylamide), 9.5 M urea, 2% v/v NP-40, 5 mM 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), and 2.3% v/v carrier ampholytes, pH 3-10/2D (Millipore, U.S.A.), were used for IEF. Using a programmable multiple-output power supply, IEF gels were prefocused to 2000 V over a 2 h period with current limited to 110 µA/gel with a sample overlay buffer (0.5 M urea, 0.2% v/v NP-40, 0.1% v/v carrier ampholytes and 50 mM DTT). The electrode solutions were 100 mM NaOH at the cathode and 100 mM H3PO4 at the anode. Protein samples (50 µg for silver staining) were applied onto the cathodic side of the gel and focused for a total of 18000 V h (17 h electrophoresed at 1000 V, followed by electrophoresis at 2000 V for 30 min). All steps were run at room temperature. After isoelectric focusing, the gels were equilibrated for 10 min in a buffer containing 300 mM Tris base, 75 mM Tris–HCl, 3% w/v SDS, 50 mM DTT, and 0.01% w/v bromophenol blue.

SDS-PAGE SDS-PAGE without a stacking gel was performed in the second dimension of 2-DE. The IEF gels were applied onto the SDS-PAGE gels (20 cm width, 20 cm length and 1 mm thickness) containing 12.5% w/v polyacrylamide. They were run with a cathode buffer containing 25 mM Tris base, 192 mM glycine and 0.2% w/v SDS, pH 8.3, and an anode buffer containing 25 mM Tris base, 192 mM glycine and 0.1% w/v SDS, pH 8.3, at a constant power of 1000 mW/gel for 5 h.

Silver Staining Silver staining was performed using a silver staining kit (2D-Silver Stain Daiichi, Daiichi Pure Chemicals, Japan).

2-DE Gel Profile Analysis Silver stained 2-DE gels were scanned by a Molecular Dynamics computing densitometer. Gel profiles were analyzed with the Pdquest™ Version 5.0 Software (Protein and DNA Imagemage Systems, U.S.A.) on a Sun IPX computer (Sun Microsystems, U.S.A.). Gel spots in the 2-DE gels from the patients and healthy volunteers were compared by a matchset constructed on Pdquest software.

Amino Acid Sequencing For amino acid sequencing, 10 times the normal amount of protein was applied onto 2-DE. Proteins on the 2-DE gels were electroblotted onto polyvinyl difluoride (PVDF) membranes (Perkin Elmer, U.K.) according to the method of Matsudaia. Then, 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.).

Western-Blot and Enhanced Chemiluminescence (ECL) Detection Aliquots of neutrophil lysates were subjected to SDS-PAGE by the method of Laemmli on a 10% w/v polyacrylamide gel (90 mm wide, 80 mm long and 1 mm thick, pH 8.8; proteins from 2–6×10⁶ cells were loaded per lane) at 25 mA for 90 min. Protein concentrations loaded on the gels were adjusted to give equivalent Coomassie Brilliant Blue stained profiles in each lane.

Proteins were electrotransferred onto a nitrocellulose membrane (Amersham, U.K.) in a 0.1 M Tris base, 0.192 M glycine, 0.02% SDS and 20% methanol at 100 mA for 1 h. The western transferred membrane was blocked with Block Ace™ (Dainippon Seiyaku, Japan) and immunoblotted with antibody to the N-terminus or C-terminus of actin in Block Ace. This membrane was washed with 0.05% Tween-20/ phosphate buffered saline (PBS) three times and was immunoblotted with a secondary antibody, either goat anti-mouse IgG (H+L) antibody conjugated with horseradish peroxidase or goat anti-rabbit IgG (H+L) antibody conjugated with horseradish peroxidase. The detection was performed using the ECL system (Amersham, U.K.).

RESULTS

Separation of Neutrophil Proteins by High Resolution 2-DE Figure 1 shows typical 2-DE profiles of neutrophil proteins from healthy volunteers (A) and patients with Behçet's disease (B) that were obtained under the conditions used, within the experimental window of a molecular mass of 10—200 kDa and pf of 3—10, and which were visualized by silver staining. About one thousand protein spots were counted on the 2-DE gels of the healthy volunteers and about seven hundreds protein spots were counted on the 2-DE gels of the patients by the Pdquest software. Almost all of protein spots on the 2-DE gels of the patients overlapped with those on the 2-DE gels of the healthy volunteers. For further comparison of neutrophil proteins from the patients to those from the healthy volunteers, the sections enclosed by rectangles (Fig. 1) were expanded, as shown in Fig. 2. Several protein spots were differently expressed between the healthy volunteer and the patient. Especially, two protein spots were clearly different. One was a 53 kDa protein with pf 5.2 that was clearly detected in the volunteers but nearly absent in the patients (spots enclosed in boxes in Fig. 2). The other was a 40 kDa protein with pf 5.2 that was clearly detected in the patients but nearly absent in the healthy volunteers (spots enclosed in circles in Fig. 2).

Identification of the 40 kDa Protein A clear spot for the 40 kDa protein was observed only on the 2-DE gels of the patients, suggesting that the increase in the 40 kDa protein may correlate with the pathological features of Behçet's disease. Therefore, to identify the 40 kDa protein, the N-terminal amino acid sequence of the 40 kDa protein was determined by microsequencing and a search for identity or homology between the microsequencing data of the 40 kDa protein and N-terminal sequences of known proteins was performed. The N-terminal sequence of sixteen amino acids, except the 9th amino acid of the 40 kDa protein, was determined and was found to have 100% identity with 44th—59th amino acids from the N-terminus of actin (Fig. 3). This result suggests that the 40 kDa protein would be a truncated actin
composed of 332 amino acid residues with an N-terminus of Met-44. Microsequencing of the 53 kDa protein was unsuccessful, suggesting that its N-terminus is blocked.

**Immunoblot Analysis** To confirm the extensive expression of the 40 kDa protein in patients with Behçet’s disease, immunoblot analysis using an antibody specific for the N-terminal or C-terminal sequence of actin was performed. Figure 4 shows the results of an immunoblot analysis using the antibody to the C-terminus of actin. The arrow indicates the position of the 40 kDa protein, which is a truncated actin with...
**40kDa Protein**

**Met-Glu-Gly-Gly-Lys-Asp-xxx-Tyr-Val-Gly-Asp-Glu-Ala-Gln**

**Actin**


Fig. 3. N-Terminal Amino Acid Sequence of 40 kDa Protein and the Partial Amino Acid Sequence of Actin

![Image of Amino Acid Sequence](image)

an N-terminus of Met-44. The 40 kDa protein was intensely detected in the patients (Fig. 4). On the other hand, the 40 kDa protein was not detected in the healthy volunteers or in the patients by the antibody to the N-terminus of actin (data not shown).

**DISCUSSION**

It has been known that various functions of neutrophils in peripheral blood, such as chemotaxis, phagocytosis and superoxide radical anion generation, are increased in Behçet’s disease, and the neutrophils infiltrate lesions in the patient. Since the increases in neutrophil function may be accompanied by protein changes in the neutrophils of the patients, we analyzed the protein composition of neutrophils from the patient as well as from healthy volunteers by high resolution 2-DE (Fig. 1) Almost all of the proteins present on 2-DE gel of neutrophils from the patients could be detected on that of neutrophils from the healthy volunteers (Fig. 2). However, several proteins, differently expressed in the neutrophils between the patients and healthy volunteers, were found in gel sections of a molecular mass of 30—55 kDa and pl of 4.8—6.2. Especially, 40 kDa protein was detected as a clear spot only in neutrophils from the patients, but an unclear spot in those from the healthy volunteers (Fig. 2).

Since it seemed that the high level expression of the 40 kDa protein in the patient was correlated with the disease, to identify the 40 kDa protein, we determined its N-terminal amino acid sequence by microsequencing. The 40 kDa protein was identified as a truncated actin with an N-terminus of Met-44 (Fig. 3). The extensive expression of the 40 kDa protein in the patients was confirmed by immunoblot analysis using an antibody to the C-terminus of actin (Fig. 4).

Actin, a major component of cytoskeletons, plays an important role in the process of cell growth and differentiation and many other cellular functions such as phagocytosis and chemotaxis. Furthermore, it has been reported that the cleavage of actin with different proteases modifies its polymerizability to various degrees. The C-terminal 36 kDa peptide of actin, when cleaved with subtilisin between Met-47 and Gly-48, polymerizes but its polymerization rate is slower and the critical concentrations for this process are higher than they are with the intact protein. On the other hand, the C-terminal 36 kDa peptide of actin cleaved with *E. coli* protease between Gly-42 and Val-43 cosedimented with native actins, although the main portion was found in the supernatant fraction. These observations suggest that the increase in truncated actin with an N-terminus of Met-44 affects the neutrophil function of patients with Behçet’s disease.

The precise role and mechanism of actin cleavage between Val-43 and Met-44, which may be important for understanding the pathology of Behçet’s disease, are now under investigation.

**REFERENCES**