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APPLICATION OF ANALYTICAL ISOTACHOPHORESIS IN THE STUDY OF PLATELET MEMBRANE PROTEINS IN PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISORDERS

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SUMMARY

The supernatants of dispase-treated platelets from normal persons and patients with myeloproliferative disorders were analysed by capillary isotachophoresis. The examined myeloproliferative disorders were three cases of chronic myelogeneous leukemia, one case of chronic neutrophilic leukemia, one case of essential thrombocythemia and one case of polycythemia vera. An additional peak was revealed in the samples from the patients with chronic myelogeneous leukemia. Platelets from the other three patients showed no additional bands. Isotachophoresis will be useful not only to analyse the superficial peptide on the cell membrane but also to define the peptide associated with malignant transformation.

INTRODUCTION

Among all electrophoretic techniques, capillary isotachophoresis offers the unique advantages of short separation times, high sensitivity, reproducibility and high resolution [1–3]. A further advantage is that no pretreatment of samples is necessary. The commercial isotachophoresis equipment has built-in detectors, providing an immediate picture of protein patterns. Isotachophoresis

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can be used to investigate various proteins in serum, cerebrospinal fluid and urine [1–6], which makes it useful for the clinical laboratory.

In this study, we used isotachophoresis to try to prove the abnormal composition of platelet membrane proteins in patients with chronic myeloproliferative disorders. The chronic myeloproliferative disorders are thought to be stem-cell disorders characterized by a striking overaccumulation of erythrocytes, granulocytes or platelets [6]. Carbohydrate-specific surface labelling and ^{125}I-labelled lectin binding techniques, in combination with one- or two-dimensional (non-reduced/reduced) sodium dodecyl sulphate polyacrylamide gel electrophoresis, showed the abnormality of platelet membrane glycoproteins in patients with certain disorders [7]. Although this method is very useful for in-depth studies, it is too complicated to be suitable for routine screening. We wish to demonstrate an available method using isotachophoresis for analyses of cell membrane proteins.

EXPERIMENTAL

Chemicals

2-Amino-2-methyl-1,3-propanediol, 6-aminocaproic acid, hydrochloric acid, barium hydroxide, asparagine, glutamate, valine, leucine, β-alanine, glycine and *n*-tris-methylglycine were purchased from Wako (Osaka, Japan). Hydroxypropylmethylcellulose was purchased from Aldrich (Milwaukee, WI, U.S.A.). Ampholine was purchased from LKB (Bromma, Sweden). Trypsin and chymotrypsin were purchased from Sigma (St. Louis, MO, U.S.A.). Dispase was purchased from Godo Shusei (Tokyo, Japan).

Capillary isotachophoresis

Analyses were performed with the Shimadzu capillary isotachophoresis IP-2A fitted with two migration tubes (100 mm × 1.0 mm I.D. and 300 mm × 0.5 mm I.D.) in serial connection. The method was based on that of Yagi and Kojima [4]. The migration current was maintained at 100 μA for 12 min and then decreased to 50 μA. The leading electrolyte consisted of 10 m*M* 2-amino-2-methyl-1,3-propanediol and 0.1% hydroxypropylmethylcellulose (15 000 cp), and was adjusted to pH 8.95 by the addition of hydrochloric acid. The terminating electrolyte consisted of 10 m*M* 6-aminocaproic acid and 10 m*M* 2-amino-2-methyl-1,3-propanediol and was adjusted to pH 10.8 by the addition of barium hydroxide. An amino acid mixture of 10 mg each of asparagine, glutamate, valine, leucine, β-alanine, glycine and *n*-tris-methylglycine was made up to 10 ml with distilled water. For the spacer-ion solution, 0.4 ml of the amino acid solution and 0.05 ml of pH 3.5–9.5 Ampholine were mixed and made up to 1.0 ml with distilled water.

Protease treatments

Three proteases of trypsin, chymotrypsin and dispase were used: 8 mg of trypsin, 8 mg of chymotrypsin and 33 mg of dispase were dissolved in 1 ml of 0.1 *M* phosphate-buffered saline (PBS) at pH 7.3. The 50 μl of pellet of platelet were mixed and incubated with 50 μl of each of the enzyme solutions for 4 h at 37°C.

Samples

Platelets were obtained from twenty normal persons and six patients with myeloproliferative disorders [three cases of chronic myelogeneous leukemia (CML) with Ph^1 chromosome, one case of chronic neutrophilic leukemia (CNL) without Ph^1 chromosome, one case of essential thrombocythemia (ET) and one case of polycythemia vera (PV)]. Platelets were prepared according to the method of Hanash et al. [8]. Platelets were treated with proteases, and the supernatants were separated by centrifugation at 1000 *g* for 10 min. A 10-μl volume of the supernatant was mixed with 30 μl of the spacer-ion solution, and 3 μl of the mixture were used for analysis by the capillary isotachophoresis.

RESULTS

The solutions of trypsin, chymotrypsin and dispase, and the supernatants of platelets treated with these proteases, were analysed with the capillary-type isotachophoretic analyser in the spacer-ion solution containing seven amino acids. Figs. 1, 2 and 3 show isotachopherograms of these samples. Trypsin, chymotrypsin and dispase give ca. 22, 28 and 26 peaks, respectively. Peak heights increased in proportion to the sample volumes. Also, there was obviously a potential difference between the samples and carbonate. These data ruled out the adsorption of proteins on the capillary wall and the carbonate interference, and showed that this system was reproducible. The supernatants of normal platelets treated with trypsin, chymotrypsin and dispase exhibit three, four and four peaks, respectively, in addition to the peaks from the enzymes. In Fig. 3, the dispase-related samples are fractionated into seven amino acids. The numbers at the bottom show the numbers of peptide fractions. Four peptides were obtained from normal platelets by dispase treatment. These peptides are located in the fractions 3, 4 and 5.

Platelets from patients with myeloproliferative disorders were treated with dispase, and the supernatants were analysed by isotachophoresis. The isotachopherograms are shown in Figs. 4 and 5. All samples from the three patients with CML give an additional peak in fraction 2. The mixture of a sample from normal platelets and one of these samples shows the additional peak. The other samples from patients with CNL, PV and ET show the same isotachopherograms as those from normal persons.

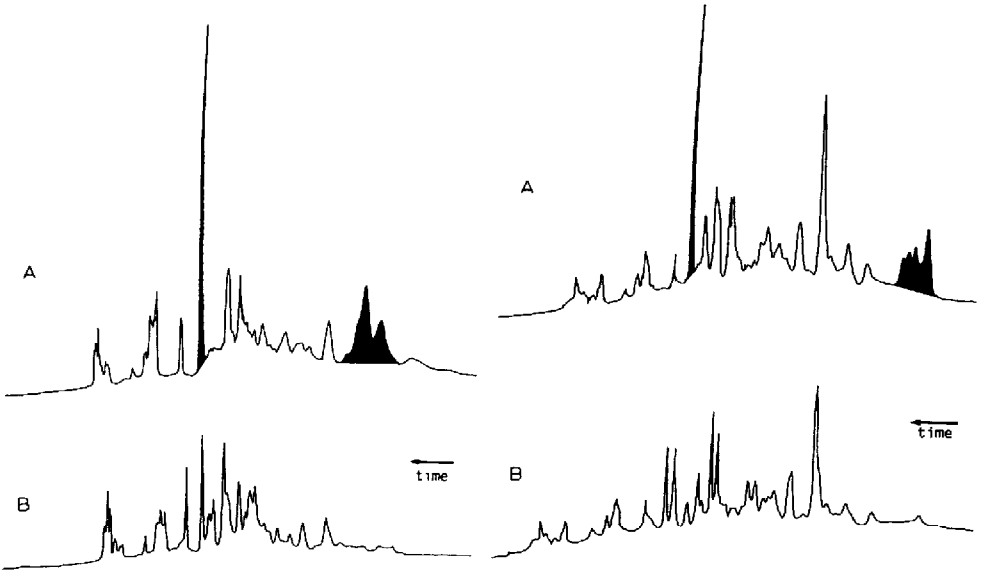

Fig. 1. Isotachopherograms of (A) a supernatant of trypsin-treated platelets from a normal person and (B) a trypsin solution. The shaded zones in (A) show peptides released from platelet membranes by trypsin.

Fig. 2. Isotachopherograms of (A) a supernatant of chymotrypsin-treated platelets from a normal person and (B) a chymotrypsin solution. The shaded zones in (A) show peptides released from platelet membranes by chymotrypsin.

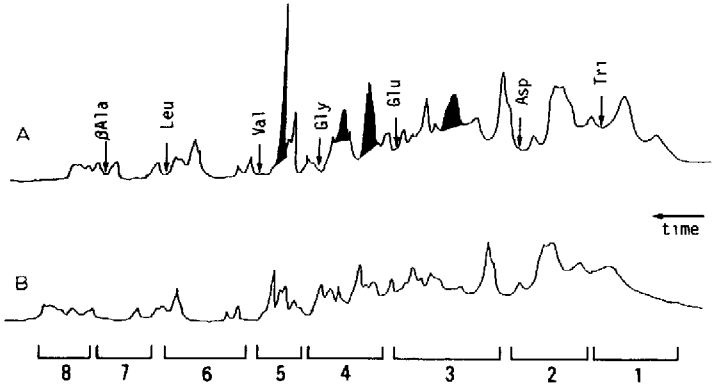

Fig. 3. Isotachopherograms of (A) a supernatant of dispase-treated platelets from a normal person and (B) a dispase solution. The spacing zones are marked by seven amino acids, corresponding to the following: Tri = *n*-tris-methylglycine; Asp = asparagine; Glu = glutamate; Gly = glycine; Val = valine; Leu = leucine; βAla = β-alanine. The numbers at the bottom show the numbers of peptide fractions. The shaded zones of No. 3, 4 and 5 in (A) show peptides released from platelet membranes by dispase.

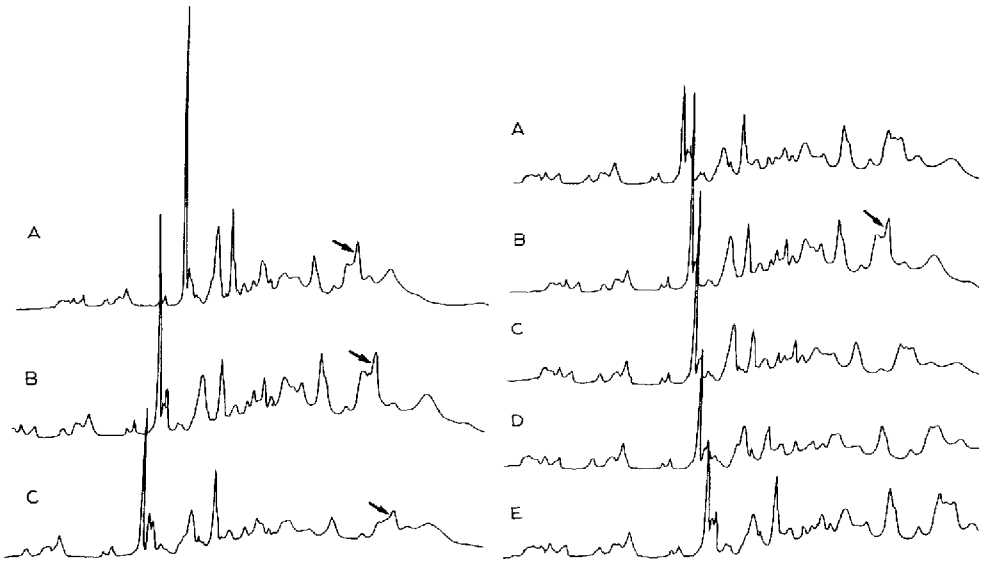

Fig. 4. Isotachopherograms of the supernatants of dispase-treated platelets from three patients with chronic myelogeneous leukemia. The arrow shows an additional peak that was not found in the sample from normal persons. This peak appears between *n*-tris-methylglycine and asparagine in fraction 2.

Fig. 5. Isotachopherograms of the supernatants of dispase-treated platelets from a normal person and four patients with chronic myeloproliferative disorders. (A) Normal person; (B) patient 2 with chronic myelogeneous leukemia; (C) patient 4 with chronic neutrophilic leukemia; (D) patient 5 with polycythemia vera; (E) patient 6 with essential thrombocythemia. The arrow shows a peak associated with CML.

DISCUSSION

The abnormality of membrane proteins in platelets from patients with chronic myeloproliferative disorders has been detected using surface labelling and ^{125}I-labeled lectin-binding technique [7]. The chronic myeloproliferative disorders consist of CML, CNL, ET, PV and primary myelofibrosis. In ET, platelet membrane glycoproteins were significantly less sialylated than in normals [7]. In CML, there was increased labelling of the penultimate galactose/N-acetylgalactosamine residues of glycoprotein Ib, IIb, IIIa and IIIb [7]. Conversely, secondary thrombocytosis showed no significant changes [7]. These data show that the analysis of platelet membrane glycoproteins may be the basis per differential diagnosis in chronic myeloproliferative disorders. However, the reported method is too complicated for use as a screening test.

It has been reported that the supernatant of dispase-treated red blood cells from a patient with autoimmune hemolytic anemia was analysed by isotachophoresis, and an abnormality in the composition of red blood cell membrane

proteins was found [9]. Dispase is a proteolytic enzyme that is produced from the culture filtrate of *Bacillus polymyxa*. This enzyme is available for digesting the glycoproteins on red blood cell membranes [10,11].

This analytical method for red blood cells was applied to the analysis of platelet membrane proteins. An attempt was made to separate peptides in the supernatant of dispase-treated platelets from normal persons and patients with various myeloproliferative disorders by isotachophoresis. In all patients with CML an additional peak was observed in the isotachopherogram. This peak was not detected in other myeloproliferative disorders. The additional peak may indicate the production of an abnormal protein on platelet membranes in CML. Analytical isotachophoresis needs not only very small sample volumes but also short separation times, and is thus suitable for studying abnormalities in platelet membrane proteins. It may be applicable to the analyses of proteins in various cell membranes.

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