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Note

Chronic myelogenous leukaemia-associated polypeptide in platelets detected by two-dimensional polyacrylamide gel electrophoresis

EIJI KAJII*, SADAHIKO IWAMOTO and SHIGENORI IKEMOTO

Department of Legal Medicine and Human Genetics, Jichi Medical School, Minamikawachi-machi, Kawachi-gun, Tochigi-ken 329-04 (Japan)

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Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of various proteins has been developed by several authors [1–5]. This technique is useful for the mapping of normal and malignant cells, the identification of cell type-specific and tumour-specific proteins and the evaluation of tumour-specific proteins in a clinical setting [2–6]. These proteins are identified as tumour markers and applied to make diagnoses and to judge prognoses [2–9].

Chronic myelogenous leukaemia (CML) is a chronic myeloproliferative disorder [10]. Analytical isotachophoresis showed abnormalities in the composition of the platelet polypeptides in CML, but not in those in other chronic myeloproliferative disorders [11]. In order to analyse the abnormalities in more detail, the polypeptide patterns of platelets from healthy persons and patients with CML and other chronic myeloproliferative disorders were compared by 2D-PAGE. This paper describes the characterization of the platelet polypeptide associated with CML.

EXPERIMENTAL

Chemicals

Sodium citrate, tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), urea, sodium dodecyl sulphate (SDS), acrylamide, N,N′-methylenebisacrylamide (Bis), bromophenol blue, glycine and silver stain kit were purchased from Wako (Osaka, Japan). Nonidet P-40 (NP-40) and phenylmethyl-sulphonyl fluoride were purchased from Sigma (St. Louis, MO, U.S.A.). Phosphoric acid and sodium hydroxide were purchased from Kanto (Tokyo, Japan). Coomassie brilliant blue R-250 and 2-mercaptoethanol (2-ME) were purchased from Nakarai (Kyoto, Japan). Ampholine (pH 3.5–9.5) and low-molec-

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ular-mass calibration kit were purchased from LKB Instruments (Uppsala, Sweden).

Preparation of platelet samples for electrophoresis

Platelet concentrates were prepared by the method of Hanash *et al.* [12]. Whole blood was drawn from the cubital vein of seven patients with chronic myeloproliferative disorders, two patients with acute leukaemia and 80 healthy volunteers into 0.1 volume of 3.1% sodium citrate. The seven chronic myeloproliferative disorders consisted of five cases of CML, one case of essential thrombocythaemia and one case of polycythaemia vera. The two acute leukaemias were M2 and L1 in FAB classification. Platelet-rich plasma (PRP) was prepared by centrifuging the whole blood at 50 *g* for 15 min The PRP was added to 0.15 volume of acid–citrate–dextrose (ACD) solution. The mixture was then centrifuged at 1000 *g* for 15 min. The resulting platelet pellet was washed twice with Tris–EDTA saline (pH 7.3) containing 15% ACD. Tris–EDTA saline consisted of 10 m*M* Tris, 1 m*M* EDTA and 154 m*M* NaCl. One volume of the washed platelet pellet was solubilized in five volumes of a "urea mix" solution containing 8.5 *M* urea, 2% NP-40, 2% Ampholine (pH 3.5–9.5) and 5% 2-ME in water. All solubilized samples were frozen and rewarmed to room temperature before isoelectric focusing.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli [13]. The electrophoretic separation was carried out in a 5–15% gradient gel covered with a stacking gel of 3% acrylamide. The slab gel was 15 cm × 14 cm and 1 mm thick. A 10-μl volume of platelet pellet was denatured in 60 μl of 50 m*M* Tris–HCl (pH 6.8), 2% SDS, 5% 2-ME, 0.0025% bromophenol blue and 10% glycerol for 4 min at 100°C, and 10 μl of the SDS-treated sample were placed in each well of the slab gel. For SDS gel calibration, the low-molecular-mass calibration kit was used. Electrophoresis was performed at 12 mA constant current in the stacking gel and at 16 mA constant current in the separating gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 or silver. Coomassie staining was performed according to the method of Fairbanks *et al.* [14]. Silver staining was performed using silver stain kit as previously described by Inoue *et al.* [15].

Two-dimensional polyacrylamide gel electrophoresis

The 2D-PAGE technique was described initially by O'Farrell [1]. The first dimension was isoelectric focusing of the protein mixture, and the second dimension was electrophoresis on a slab gel containing SDS. Tube gels (2 mm) for isoelectric focusing were made of 4% acrylamide, 0.2% Bis, 8.5 *M* urea, 2% NP-40 and 2% Ampholine. For each sample, 40-μl aliquots of the platelet protein solution were immediately applied to isoelectric focusing gels. Electrode buffers

for the anode and cathode were 0.01 *M* phosphoric acid and 0.02 *M* sodium hydroxide, respectively. Isoelectric focusing was performed at 200 V for 2 h, at 300 V for 15 h and at 500 V for 1 h. After focusing, the gels were removed, equilibrated with a buffer containing 1% SDS and 5% 2-ME at 40°C for 30 min and placed on top of SDS slab gel. SDS slab gels were made of a 5–15% continuous gradient separating gel and a 3% stacking gel, as previously reported by Laemmli [13] A 40-μl volume of 0.1% bromophenol blue was added to the upper reservoir. The electrode buffer (pH 8.4) consisted of 0.025 *M* Tris, 0.192 *M* glycine and 0.1% SDS. The stacking gels were run at 12 mA constant current and the separating gels were run at 16 mA constant current until the dye front reached the bottom of the gels.

Computer image analysis

The two-dimensional electrophoresis patterns of platelet polypeptides were analysed with an image analyser (Immuno Medica, Shizuoka, Japan). This computer program processes and compares 2D-PAGE images.

RESULTS

Platelet samples from various patients and healthy persons were analysed on SDS-PAGE. No disease-specific polypeptide was detected in SDS-PAGE gels (Fig. 1).

Approximately 350 polypeptide spots were visualized on each 2D-PAGE gel (Fig. 2). Of these, those that were deeply stained and large were termed "major spots" The total number of major spots was 87. The patterns did not include contaminating polypeptides, as might be derived from plasma or erythrocytes [15]. Most polypeptides were located within an effecting focusing range of isoelectric point (p*I*) 3.8–6.7. Actin was readily identifiable as a major polypeptide owing to its molecular mass (43 000), its characteristic location and its relative abundance in the gels. The high-molecular-mass ($>$ 100 000) glycoproteins, such as Gp I, II and III, were poorly visualized owing to the inhibition of their entry into the gel. Much better resolution and separation were seen for polypeptides in the 100 000 to 10 000 molecular mass range. No significant variation in relative spot intensity was observed between patterns obtained from $1.4 \cdot 10^8$ to $3.8 \cdot 10^8$ cells.

We compared the 2D-PAGE pattern of gels for platelets from 9 patients with chronic myeloproliferative disorders and acute leukaemia, and 70 unrelated healthy Japanese. A major polypeptide with molecular mass 24 000 is a constant constituent of all patients and healthy persons and serves as a reference marker in the gel (Fig. 2A and B). The upper right spot was observed in all platelets. The upper left spot marked in Fig. 2B by the arrow was characteristic of platelets from all patients with CML (Fig 3). This is a polypeptide of molecular mass 25 000 and p*I* 4.6. This polypeptide is henceforth called the 25 000 polypeptide. It was

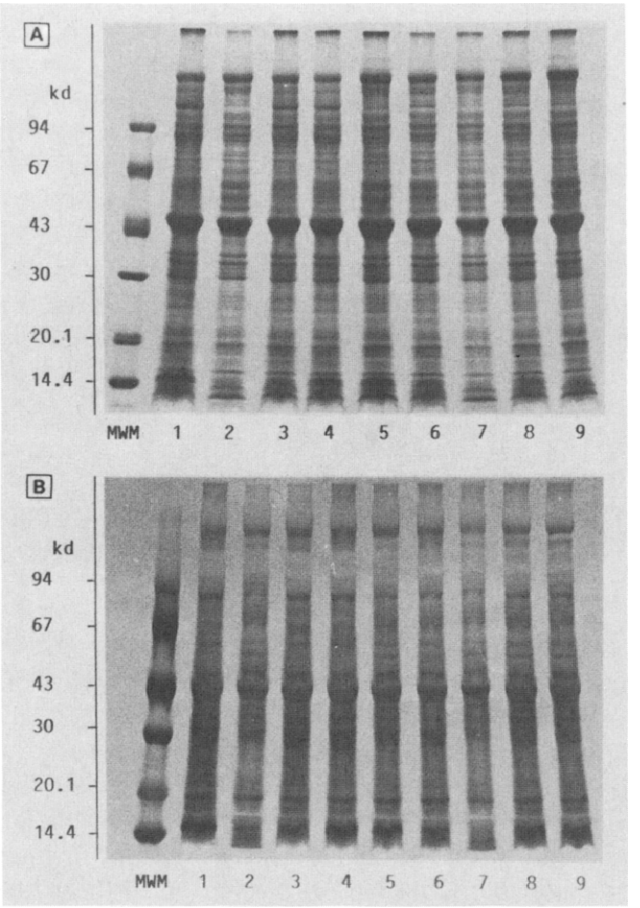

Fig 1 SDS-PAGE of platelet polypeptides from a healthy person and eight patients with haematological disorders Electrophoresis was performed in a slab containing 5–15% (w/v) gradient acrylamide with an overlay of 3% acrylamide in the buffer system of Laemmli [9] The gel was stained with Coomassie brilliant blue R-250 (A) or silver reagent (B) MWM indicates molecular weight markers. Lane 1, a healthy person, lanes 2–4, patients with M2-acute leukaemia (2), essential thrombocythaemia (3) and polycythaemia vera (4); lanes 5–9 patients with chronic myelogenous leukaemia

not detected in gels for platelets from healthy persons and patients without CML, but its spot was observed in the gel for the mixture of two samples from a healthy person and a patient with CML. Virtually identical patterns were observed for the same patient with freshly prepared platelets and with ones that were frozen at −20°C for one year. Addition of the proteolytic inhibitor phenylmethylsulphonyl fluoride to the solubilization had no effect on the 2D-PAGE pattern.

The three-dimensional map of the closed sections in Fig. 2A and B, obtained with the image analyser, is shown in Fig. 4. The 25 000 polypeptide occupied *ca.* 30% of the volume of the region circled in Fig. 4.

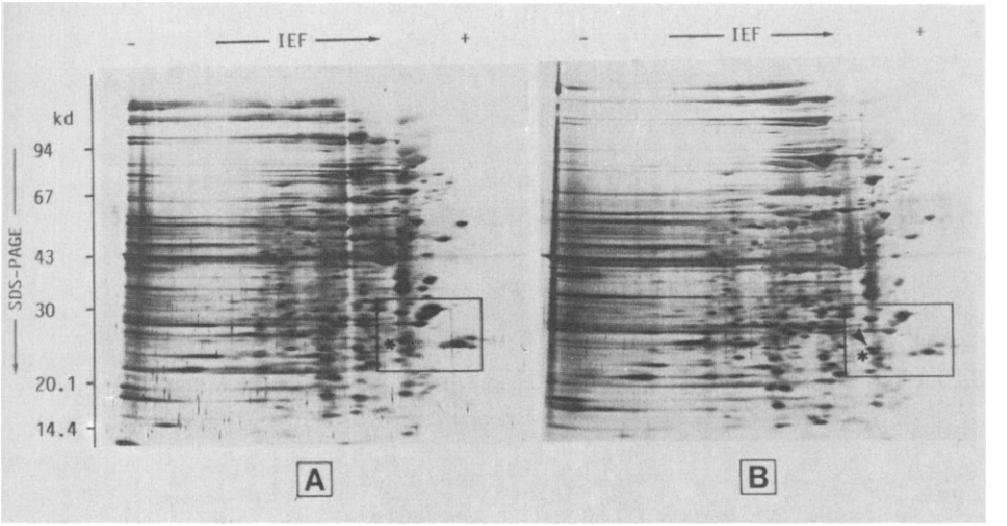

Fig 2 Two-dimensional electrophoresis patterns of total cellular polypeptides of platelets from a healthy person (A) and a patient with chronic myelogenous leukaemia The electrophoresis was performed according to the method of O'Farrell [1] The first dimension was isoelectric focusing and the second dimension was SDS-PAGE The asterisk in the outlined area shows a major polypeptide spot The upper right spot was observed in all platelets The upper left spot, marked with an arrow, was characteristic of platelets from patients with chronic myelogenous leukaemia

DISCUSSION

The synthesis or the regulation of platelet proteins has been demonstrated to be clinically important in the detection and diagnosis of a variety of diseases [17]. Over the past decade, it has been possible to associate the defect in a variety of bleeding disorders with the absence of, or changes in, specific platelet glycoproteins using one-dimensional gel electrophoresis. Two major congenital disorders, Glanzmann's thrombasthenia and Bernard–Soulier's syndrome, where the glycoprotein defects are fairly well defined, have provided great impetus to the study of platelet glycoproteins [17].

The development of highly sensitive gel electrophoretic methods for the analysis of various proteins has opened up new possibilities for the definition of genetic variation and molecular changes. Two-dimensional gel analyses of abnormal leukocytes from patients with leukaemia, infectious mononucleosis and rheumatoid arthritis have been shown to be capable of detecting reproducible abnormalities in these patterns, which are characteristic of the specific disease [2–5,18,19]. Recently this high-resolution method has been applied to the analysis of platelets [12,20]. Consequently, several polymorphisms in platelet proteins have been reported [12,21,22]. In this report, we described a CML-specific polypeptide in platelets from patients with CML.

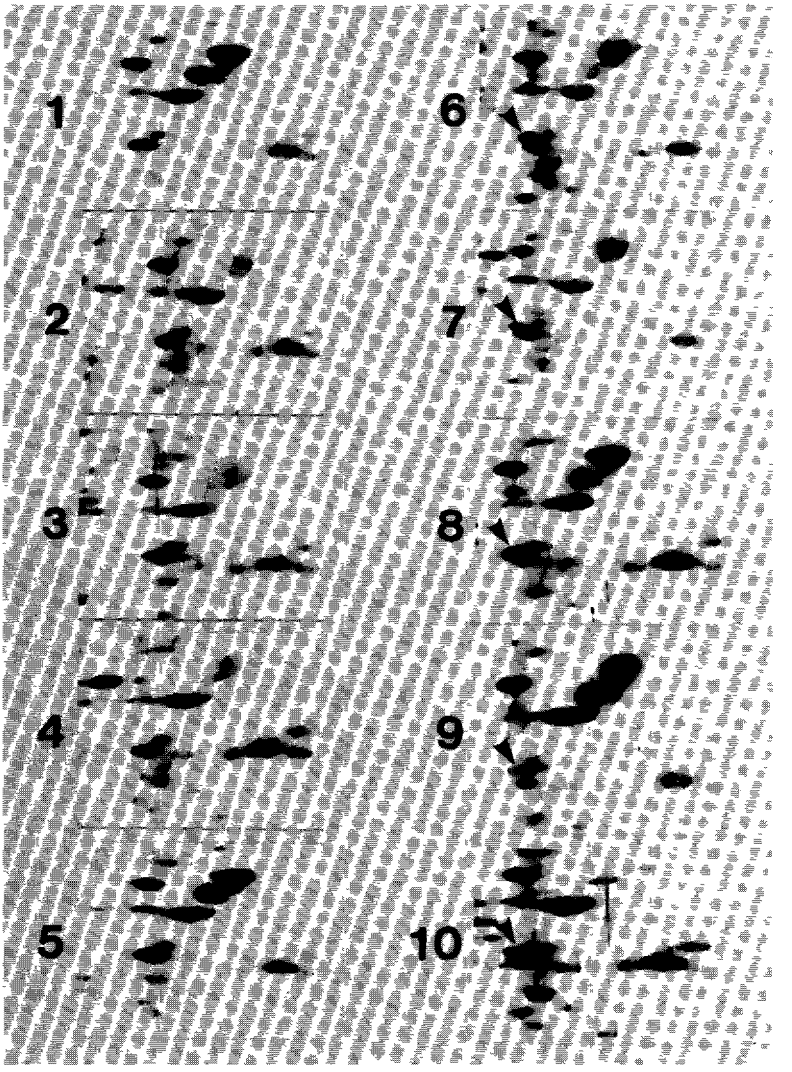

Fig. 3. Close-up comparison of the patterns of chronic myelogenous leukaemia (right) and other haematological disorder (left), corresponding to the area outlined on the acidic end of the bottom gel in Fig 2 Patterns 1 = a healthy person, 2–5 = patients with acute leukaemias (2,3), essential thrombocythaemia (4) and polycythaemia vera (5), 6–10 = patients with chronic myelogenous leukaemia (CML) The arrows point to a unique spot in CML gels

CML is a clonal disorder involving a pluripotent stem cell from which neutrophils, eosinophils, basophils, erythrocytes, monocytes/macrophages and B lymphocytes, megakaryocytes arise [23,24]. Circulating mature granulocytes in patients with CML appear to be morphologically normal. However, the specific changes in the polypeptide pattern in the mature CML granulocytes were re-

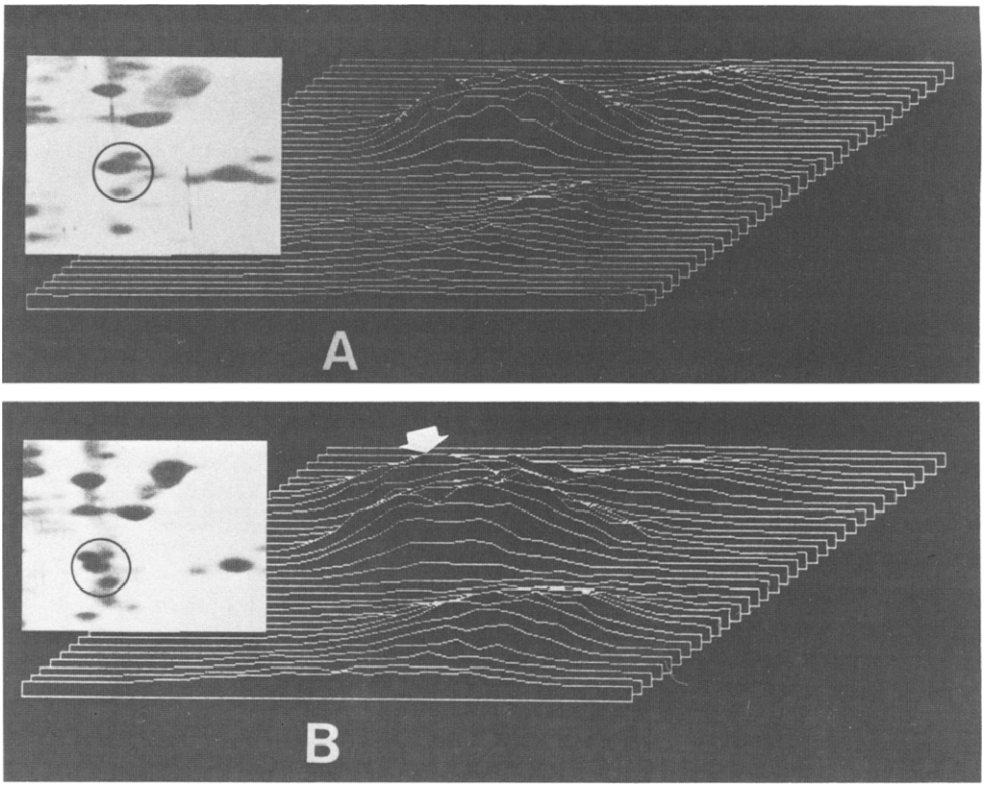

Fig. 4 Two-dimensional electrophoresis and computer analysis of the CML-associated polypeptide The region shown is that circled on the gel patterns from a healthy person (A) and a patient with CML (B) The arrow shows the CML-associated polypeptide

vealed by 2D-PAGE [25]. The evidence of the CML-associated polypeptide in platelets from patients with CML supports the molecular-biological abnormality of CML platelets as well as CML granulocytes. The CML-associated polypeptide was not detected in other chronic myeloproliferative disorders. This may reflect the malignant transformation, although further investigations will be necessary because the number of examined cases was so few. This method of 2D-PAGE could be clinically applied for the analysis of platelet disorders in CML.

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