CONCISE REPORT

Thrombospondin in Essential Thrombocythemia

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Essential thrombocythemia is a myeloproliferative disorder characterized by frequent bleeding and thrombotic complications. On a molecular level, two abnormalities of platelet thrombospondin have been identified: abnormal glycosylation of the intact 186,000-dalton chain has been detected and a shortened form of the thrombospondin chain is present. We have used two monoclonal antibodies and Lens culinaris lectin to probe the structure of thrombospondin in the platelets from three patients with essential thrombocythemia; one patient with polycythemia vera and two patients with secondary thrombocytosis. The presence of abnormal thrombospondin fragments with molecular weights of 160,000 and 30,000 was detected in the intact platelets and in the supernatant from thrombin-treated platelets, in all of the individuals except one of the secondary thrombocytosis patients. Monoclonal antibody binding studies indicate that both fragments are produced by proteolysis at a single site, which results in the removal of a 30,000-dalton fragment from the NH₂-terminal. Lens culinaris lectin-binding studies revealed that some of the carbohydrate moieties of thrombospondin are near this cleavage site. The results are consistent with the hypothesis that the abnormal thrombospondin fragments observed under conditions of increased platelet production are due to increased susceptibility to proteolysis, which, in turn, may be due to defective glycosylation.

METHODS

Clinical material. This study was conducted according to the principles in the Declaration of Helsinki. The patients studied were from the Department of Hematology/Oncology of St Elizabeth's Hospital of Boston. Four patients with myeloproliferative disorders were studied. Three of these individuals (M.S., D.R., and A.H.) had essential thrombocythemia and one (R.R.) had polycythemia vera. The platelet counts of these patients varied from 0.5 to 1.5 x 10¹¹ platelets/mL during the course of this study. Over the same period, two patients (E.K. and M.N.) with secondary thrombocytosis were also studied. One of these patients (M.N.) had a splenectomy for trauma and the other (E.K.) had temporal arteritis.

Preparation of platelets. Venous blood from the patients and control normal volunteers was collected into sterile tubes containing the anticoagulant citrate/dextrose (ACD, National Institutes of Health Formula A). Platelet-rich plasma was obtained by centrifuging whole blood at 500 x g for ten minutes. A one-fourth volume of ACD was added and the platelets were pelleted by centrifugation at 1,000 x g for 25 minutes. The platelets were washed once in 10 to 15 volumes of pH 6.5 buffer containing 0.102 mol/L NaCl, 3.9 mmol/L K₂HP0₄, 3.9 mmol/L Na₂HP0₄, 22 mmol/L NaH₂PO₄, and 5.5 mmol/L glucose. The platelets were resuspended in 15 mmol/L Tris-HCl (pH 7.6), 0.14 mol/L NaCl, and 5 mmol/L...
glucose at a cell count of $1 \times 10^8$ platelets/mL. The platelet suspension was treated with 1 mmol/L diisopropyl fluorophosphate (DFP) for ten minutes at 22 °C and was then solubilized with a one-fourth volume of electrophoresis sample buffer (pH 7.5) containing 0.25 mol/L Tris, 5% sodium dodecyl sulfate (SDS), 1.25 mol/L sucrose and 0.1 mol/L dithiothreitol at 100 °C for 60 seconds.

The supernatant from thrombin-treated platelets was prepared by exposing the platelet suspension, which had not received DFP treatment, to 0.25 U/mL of human thrombin (Sigma, St Louis) for two minutes at 22 °C. DFP (1 mmol/L, final concentration) was then added and the sample was spun at 1200 x g for five minutes. The supernatant was decanted and spun at 50,000 x g for 30 minutes.

**Protein electrophoresis and blotting.** SDS-Polyacrylamide gel electrophoresis (PAGE) was carried out on the discontinuous system of Laemmli as described previously. After SDS-PAGE, the proteins were electrotherochemically transferred to nitrocellulose paper (BioRad, Richmond, Calif) at 50 volts for 24 to 72 hours in pH 8.3 buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol. For monoclonal antibody binding studies, the nitrocellulose paper containing a replica of the gel was washed briefly in 0.15 mol/L NaCl and incubated in 15 mmol/L Tris-HCl (pH 7.6), 0.14 mol/L NaCl, and 5% bovine serum albumin for one hour at 22 °C. The protein blot was washed with five changes of 0.15 mol/L NaCl within a 30-minute interval and incubated with TBS-BSA containing monoclonal anti-thrombospondin antibodies (10 to 20 µL/10 mL TBS-BSA) for 16 hours at 22 °C. The production and epitope mapping of the monoclonal antibodies has been described previously.

The protein blots were washed again and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (10 µL/30 mL TBS-BSA: N.L. Cappel Laboratories, Cochranville, Pa) for one hour at 22 °C. After the final incubation, the protein blots were washed and incubated with the substrate solution containing 40 mmol/L Tris-HCl (pH 7.6), 0.16 mol/L NaCl, 0.015% H$_2$O$_2$, 20% (vol/vol) methanol and 3.36 mmol/L 4-chloro-l-naphthol for one to five minutes for the detection of horseradish-peroxidase-conjugated anti-mouse IgG. For Lens culinaris lectin (Polysciences, Warrington, Pa) binding studies, the nitrocellulose paper containing a replica of the gel was washed briefly in 0.15 mol/L NaCl and incubated in 15 mmol/L Tris-HCl (pH 7.6), 0.14 mol/L NaCl, 2 mmol/L CaCl$_2$, and 3% periodic acid-treated BSA (TBS-P-BSA) for one hour at 22 °C. The protein blots were washed as described above and incubated with TBS-P-BSA containing horseradish-peroxidase-conjugated Lens culinaris lectin (150 µL/30 mL TBS-P-BSA) for 16 hours at 22 °C. The blots were washed and incubated with the substrate solution described above.

**RESULTS**

The identification of abnormal thrombospondin fragments. The peptide compositions of the platelets from the essential thrombocythemia patient M.S. and a control normal volunteer are shown in Fig 1. At the protein loadings used, no differences are detectable by staining with Coomassie blue. We have electrophoretically transferred this pattern to nitrocellulose paper and have probed the replica with two murine monoclonal anti-thrombospondin antibodies, designated MA-I and MA-II. The epitope for MA-I is contained in a 47,000-dalton fragment of thrombospondin that is near the COOH-terminal of the 185,000-dalton thrombospondin chains. The epitope for MA-II is contained in the NTerminal, heparin-binding peptide. This latter portion of the molecule is susceptible to proteolysis and is released as a 23,000- to 30,000-dalton fragment, depending on the enzyme used.

When the platelet proteins from control normal volunteers or the essential thrombocythemia patients M.S., D.R., or A.H. were probed with MA-I or MA-II, the 185,000-dalton reduced chain of thrombospondin, was the principal band detected (Figs 1 and 2). When compared with the control samples, an increase in a 160,000-dalton band that binds MA-I and MA-II was observed in these patients (Figs 1 and 2). The nearest band in the control samples had decreased electrophoretic mobility and staining intensity as compared with the 160,000-dalton band. When the blots were probed with MA-II, an increase in the 30,000-dalton band was observed in the platelets from the essential thrombocythemia patients, as compared with controls (Figs 1 and 2). Comparable levels of a 25,000-dalton band are observed for the controls and the essential thrombocythemia patients, with MA-II. The 160,000-, 30,000-, and 25,000-dalton fragments comigrate.

![Peptide composition of the platelets from a control (C) normal volunteer and the essential thrombocythemia patient M.S. Solubilized platelets (P) and the supernatant (S) from thrombin-treated platelets were electrophoresed on 10% gels and stained with Coomassie blue (Cb) or electrophoretically transferred to nitrocellulose paper and probed with MA-I or MA-II. The electrophoretic mobility of the intact thrombospondin chain (TSP) and the 160,000-, 30,000-, and 25,000-dalton fragments are indicated on the left. The arrows indicate the position of the abnormal 160,000- and 30,000-dalton fragments detected by MA-I and MA-II, respectively.](image-url)
Fig 2. Peptide composition of platelets from a control (C) normal volunteer, the essential thrombocythemia patient D.R. and the secondary thrombocytosis patient M.N. The gels were stained with Coomassie blue (Cb) or electrophoretically transferred to nitrocellulose paper and probed with MA-II. The electrophoretic mobility of the intact thrombospondin chain (TSP) and the 30,000-dalton fragment are indicated on the left. The arrow indicates the position of the abnormal 30,000-dalton fragment detected by MA-II.

with fragments of thrombospondin produced by mild tryptic or chymotryptic digestion (data not shown). Similar results are obtained when the supernatants from thrombin-treated platelets are prepared from patients with essential thrombocythemia and control volunteers. That is, an increase in the 160,000-dalton fragment is detected with MA-I and an increase in the 30,000-dalton fragment is detected with MA-II in the essential thrombocythemia patients, relative to the controls (Fig 1).

To determine if the presence of the abnormal 160,000- and 30,000-dalton bands was specific to essential thrombocythemia, we also studied one patient (R.R.) with polycythemia vera and two patients (E.K. and M.N.) with secondary thrombocytosis. One of the secondary thrombocytosis patients (E.K.) was indistinguishable from control normal volunteers (data not shown). In contrast, patients R.R. and M.N. showed an increase in the 160,000-dalton and the 30,000-dalton bands detected by MA-I and MA-II, respectively (Fig 2).

Lens culinaris lectin binding to thrombospondin fragments. The results of limited chymotryptic digestion of thrombospondin at low enzyme-to-substrate ratios (1:10,000 and 1:1000) are shown in Fig 3. At an enzyme-to-substrate ratio of 1:10,000, a 30,000-dalton fragment is produced that contains the epitope for MA-II and a binding site for Lens culinaris lectin (Fig 3, lanes A). A 25,000-dalton fragment that contains the epitope for MA-I is also produced; however, this fragment does not bind Lens culinaris lectin. When the enzyme-to-substrate ratio is increased to 1:1000, a decrease in the 30,000-dalton fragment and a concomitant increase in the 25,000-dalton fragment is observed (Fig 3, lanes B). Despite the increase in the concentration of the 25,000-dalton fragment, no binding of Lens culinaris lectin could be detected under these conditions. At an enzyme-to-substrate ratio of 1:1000, a 70,000-dalton fragment is the principal Lens culinaris lectin binding fragment.

DISCUSSION

The data presented here corroborates the data of Booth et al in that there is an increase in a high molecular weight fragment of thrombospondin in the platelets from patients with essential thrombocythemia. The difference of 10,000 daltons between the reported molecular mass and the value of 160,000 daltons determined in this study is probably due to subtle differences in the calibration of the gel systems in the two laboratories. We have shown that the 160,000-dalton fragment includes the epitope for MA-I, but not the epitope for MA-II. In addition, we have detected the increase in a 30,000-dalton fragment that binds MA-II. Collectively, these data indicate that there is increased proteolysis of thrombospondin at a site that results in the release of a 30,000-dalton fragment from the NH₂-terminal of the thrombospondin chains. This corresponds to site I, which is between segments I and II described in our previous report. The observation that both the 30,000- and 160,000-dalton fragments are present in the supernatant of thrombin-treated platelets suggest that both fragments are present in the α-granules. In the normal platelet, there seems to be proteolytic activity that results in the production of a 25,000-dalton fragment, which binds MA-II and is therefore at or near the NH₂-terminal of thrombospondin. The abnormal 160,000- and 30,000-dalton fragments were also detected in the
polycythemia vera patient (R.R.) and one of the two patients (M.N.) with reactive thrombocytosis. Thus, these changes may generally occur under conditions of increased platelet production and are not specific to essential thrombocytopenia.

Abnormal glycosylation of thrombospondin, in addition to other glycoproteins, has been reported in essential thrombocytopenia.\textsuperscript{8-11} We have used peroxidase-conjugated Lens culinaris lectin to locate the carbohydrate side chains that bind this lectin. In the chymotryptic digests, the 30,000-dalton fragment of the NH\textsubscript{2}-terminal region binds Lens culinaris lectin, but the 25,000-dalton fragment does not. The 25,000-dalton fragment is probably derived from the 30,000-dalton fragment because both fragments contain the epitope for MA-II and an increase in the 25,000-dalton fragment occurs concomitant to the decrease in the 30,000-dalton fragment. The NH\textsubscript{2}-terminal amino acid sequence of the 25,000-dalton fragment is identical to that of the intact thrombospondin chain.\textsuperscript{1,12} Thus, the conversion from the 30,000-dalton fragment to the 25,000-dalton fragment probably occurs as a result of the proteolytic removal of 5,000 daltons from the COOH-terminal of the 30,000-dalton fragment. Since the 30,000-dalton fragment binds Lens culinaris lectin and the 25,000-dalton fragment does not, at least one carbohydrate moiety is probably located in the 5,000-dalton C-terminal portion of the 30,000-dalton fragment. A second binding site for Lens culinaris lectin is located in the 70,000-dalton fragment, which is derived from a portion of the molecule that is adjacent to the 30,000-dalton fragment.\textsuperscript{1,13}

The data presented here indicates that there is increased susceptibility of thrombospondin to proteolysis in some of the myeloproliferative disorders and in secondary thrombocytopenia. The site for proteolysis is in the immediate vicinity of a carbohydrate side chain. Abnormal glycosylation of thrombospondin in essential thrombocytopenia has been reported.\textsuperscript{14} Furthermore, carbohydrate moieties on proteins have been reported to confer resistance to proteolysis.\textsuperscript{15} Thus, we propose that the increased susceptibility of the thrombospondin to proteolysis in primary and secondary thrombocytopenia is a direct result of defective glycosylation.

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