The Role of Sialic Acid in the Dysfibrinogenemia Associated With Liver Disease: Distribution of Sialic Acid on the Constituent Chains

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To further evaluate the role of sialic acid in the dysfibrinogenemia associated with liver disease, we studied the effect of removal of excess sialic acid residues from the fibrinogen of five patients with liver disease on the thrombin time and fibrin monomer aggregation. Patient fibrinogens containing 1.4–3.4 residues of sialic acid per molecule in excess of normal controls, with thrombin times 12–22 sec longer than normal and with abnormal fibrin monomer aggregation, were stripped of their excess sialic acid by incubation with Vibrio cholerae neuraminidase, followed by rapid removal of the enzyme by antineuraminidase antibody affinity chromatography. These partially desialylated patient fibrinogens, with a normal number of sialic acid residues remaining, exhibited normal thrombin times and normal fibrin monomer aggregation. Sodium dodecysulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of reduced normal, patient, and partially desialylated patient (sialyl-^3H)-fibrinogen exhibited 60% of the radioactivity in the Bβ chain and 40% in the γ chain. There was no radioactivity detectable in the Aα chain. These studies provide additional evidence that the increased sialic acid content of the acquired dysfibrinogenemia of liver disease is responsible for its functional defect and that the excess sialic acid is distributed on the Bβ chain and γ chains of the fibrinogen.

THE DYSFIBRINOGENEMIA associated with liver disease has been shown to be due to impaired fibrin monomer polymerization,1,2 which correlates with an increase in the sialic acid content of the protein.3,4 Complete desialylation of this abnormal fibrinogen results in an asialo-derivative whose thrombin time is shortened to that of normal asialofibrinogen.5 To further characterize the role of sialic acid in this functional abnormality, we studied the effects of the removal of excess sialic acid residues from this fibrinogen on its thrombin time and fibrin monomer polymerization. In addition, the distribution of tritium-labeled sialic acid on the Aα, Bβ, and γ chains of normal and abnormal fibrinogens and of the partially desialylated abnormal fibrinogens was studied on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the reduced fibrinogens.

MATERIALS AND METHODS

Patients

The patients chosen for the present study were selected by the same clinical and laboratory criteria employed in patient selection in our previously reported study of the dysfibrinogenemia of liver disease.1 Patient plasma thrombin times were at least 50% longer than normal controls in the absence of clinical and laboratory evidence of disseminated intravascular coagulation and/or fibrinolysis.3 Three patients had alcoholic liver disease and two had postnecrotic cirrhosis of undetermined etiology. The abnormal patient fibrinogens were functionally characterized as previously described and exhibited the biochemical and functional properties of the dysfibrinogenemia of liver disease.1,3

Purification of Fibrinogen

Blood was collected with plastic syringes into 1/9 vol 3.8% sodium citrate, with epsilon-aminocaproic acid (EACA) added to give a final concentration of 0.1 M in the anticoagulated plasma. Samples were centrifuged at 2,500 g and 4°C for 15 min. The platelet-poor plasma was separated and tested immediately or quick frozen and stored at −80°C. Fibrinogen was purified from normal and patient plasmas by the glycine precipitation method of Kazal et al.,3 modified by the addition of EACA to a final concentration of 0.1 M in plasmas and buffers.1 The concentration of fibrinogen present in the purified material was measured spectrophotometrically at 280 nm using an A_280 of 15.1.1 The recovery of fibrinogen from normal and abnormal plasmas ranged between 55% and 75%, and the purified fibrinogen was 96% clottable.

Sialic Acid Studies

Total sialic acid content of fibrinogen was measured by the thiobarbituric acid method after acid hydrolysis8 or after enzymatic cleavage with Vibrio cholerae neuraminidase (Behringwerke A.G., Marburg/Lahn, West Germany) in 0.15 M NaCl, 0.008 M CaCl₂, pH 6.2, at 37°C for 3 hr. To obtain partially desialylated patient fibrinogens from which only the excess sialic acid was removed, 3 mg of fibrinogen was incubated with 50 U of neuraminidase under these same conditions, except that the incubation was limited to 1.5–2.5 min, depending on the amount of sialic acid to be cleaved.8 We had previously demonstrated that the initial release of sialic acid by neuraminidase under these conditions was rapid.4 By varying the time of incubation and by employing antineuraminidase antibody affinity column chromatography to rapidly remove neuraminidase from the reaction mixture, we were able, by trial and error, to obtain the degree of fibrinogen desialylation desired. Rabbit antineuraminidase antibody was produced by injecting New Zealand white rabbits subcutaneously with Vibrio cholerae neuraminidase mixed with Freund’s adjuvant. The immunized rabbit’s serum was treated with 40% saturated (NH₄)₂SO₄ solution. The precipitate was dissolved in 0.15 M NaCl, precipitated a second time with (NH₄)₂SO₄, redissolved in 0.15 M NaCl, and dialyzed exten-
Preparation of [Sialyl-3H]-Fibrinogen

The sialic acid residues of normal, patient, and partially desialylated patient fibrinogens were labeled with tritium by the method of Van Lenten and Ashwell. Ten milligrams of fibrinogen in 0.1 M sodium acetate, 0.15 M NaCl, pH 6.0, were oxidized by the addition of sodium metaperiodate in 10 M excess with respect to sialic acid. The reaction was stopped by the addition of ethylene glycol, and the oxidized fibrinogens were reduced with tritiated sodium borohydride (specific activity 555 mCi/mmole, Amersham Corporation, Arlington Heights, IL) followed by the addition of excess cold sodium borohydride. The labeled fibrinogens were then precipitated with glycine at 20°C. The precipitates were dissolved in 0.055 M sodium citrate, pH 7.4, and dialyzed extensively against this buffer. To measure the amount of radioactivity incorporated, fibrinogen was clotted with bovine thrombin (Parke, Davis and Co., Detroit, MI) in the presence of 0.08% disodium ethylenediaminetetraacetate (EDTA), the clots were dissolved in 0.02 M acetic acid, and 10 µl of the fibrin monomer solution was added to 10 ml of Scintisol (Isolab, Akron, OH). Radioactivity was also measured in the supernatant after thrombin clotting of the fibrinogen and in the precipitates and supernatants after treatment of the labeled fibrinogens with a 3-vol excess of 20% trichloroacetic acid (TCA). A Packard tri-carb liquid scintillation spectrometer with a counting efficiency for tritium of 33% (Packard Instrument Co., Inc., Downers Grove, IL) was used.

To demonstrate restriction of the radioactive label to the sialic acid analogues of the fibrinogen, two methods were employed. In the first, the labeled fibrinogen was incubated with Vibrio cholerae neuraminidase and CaCl₂ for 18 hr. In the second, sialic acid analogues of the labeled fibrinogens were cleaved by acid hydrolysis. In both methods, the protein was precipitated by adding a 3-vol excess of ethanol:chloroform (9:1), and radioactivity was measured in the precipitates and supernatants.

The presence of labeled sialic acid analogues of the (sialyl-3H)-fibrinogen was also studied by resin chromatography. The supernatant obtained after acid hydrolysis of 30 mg of (sialyl-3H)-fibrinogen was evaporated under nitrogen and dissolved in deionized H₂O. The sample was then applied to a 20 x 1 cm Dowex 1-x8 column (200-400 mesh) (Bio-Rad Laboratories, Richmond, CA) equilibrated with water and developed with a linear gradient of H₂O and 1N formic acid. Absorbance at 280 nm, trinitroacetic acid, and conductivity were measured in the eluates. The radioactivity peaks were tested for the presence of sialic acid and its analogues by the thiobarbituric acid assay and by the resorcinol method.

Functional Studies of Fibrinogen

The thrombin times of the purified normal, patient, and partially desialylated patient fibrinogens, both unlabeled and tritium-labeled, were performed as previously described. All fibrinogens were diazylated extensively against 0.02 M sodium citrate, 0.15 M NaCl, pH 7.4, prior to determination of their thrombin times.

Fibrin monomer aggregation was studied by a modification of the method of Belitsier et al. and one-twentieth milliliter of a solution of fibrinogen at a concentration of 1.0 mg/ml in 0.06 M sodium phosphate, pH 6.8, with EDTA at a final concentration of 0.08%, was clotted with 4 U of human thrombin for 3 hr at 37°C. Highly purified human thrombin was obtained through the courtesy of Dr. John Fenton, New York State Department of Health Laboratories, Albany, NY. The clots were recovered on a glass rod, washed 3 times with 0.15 M NaCl, and dissolved in 0.02 M acetic acid. One volume of monomer was mixed with 10 vol of 0.06 M sodium phosphate, pH 6.8, and allowed to aggregate for 3 hr at 4°C. The clots were then recovered, washed, and dissolved in acetic acid as described above. To 0.1 ml of the fibrin monomer solution at a concentration of 1.2 mg/ml in a cuvette was added 1.1 ml of 0.06 M sodium phosphate, pH 6.8, ionic strength 0.12, and the aggregation of the fibrin monomers was followed by measuring absorbance at 350 nm over 30 min in a Gilford spectrophotometer with an automatic cuvette positioner (Gilford Instrument Laboratories, Inc., Oberlin, OH). Fibrin monomer aggregation was also studied at an ionic strength of 0.24 by adjusting the ionic strength of the phosphate buffer with NaCl.

Electrophoretic Studies

SDS-PAGE of 5 normal and 5 patient purified fibrinogens reduced with β-mercaptoethanol was performed as previously described. Densitometric scans of the Coomassie-blue-stained gels were made with a Gilford gel scanner. Gels of the reduced normal and patient neuraminidase-trated fibrinogens were also scanned. Normal, patient, and partially desialylated patient (sialyl-3H)-fibrinogens were also reduced and run in SDS-PAGE. The gels were cut into 1-mm slices, and the protein was eluted with 6M guanidinium chloride at 90°C overnight. The eluates were mixed with 15 ml of Triton X-100-toluene scintillation cocktail and radioactivity was measured in a Packard liquid scintillation spectrometer.

RESULTS

All patient fibrinogens studied had increased sialic acid contents ranging from 1.4 to 3.4 residues per molecule in excess of normal fibrinogen (Fig. 1).

Neutralization Studies

A 0.1-ml aliquot of the rabbit antineuraminidase antibody fraction neutralized the activity of 50 U of Vibrio cholerae neuraminidase in incubation studies. Vibrio cholerae neuraminidase treated with nonimmunized rabbit serum antibody fraction, on the other hand, retained its enzymatic effect, releasing 5.2 sialic acid residues from fibrinogen over the period of incubation. In addition, patient fibrinogen–neuraminidase
incubation mixtures retained no detectable enzymatic activity after one passage through antineuraminidase antibody affinity columns. Samples tested immediately after chromatography and again 24 hr following chromatography had the same number of sialic acid residues per molecule. Incubation of patient fibrinogens with neuraminidase followed by rapid removal of the enzyme by antineuraminidase antibody affinity chromatography produced partially desialylated derivatives with normal numbers of sialic acid residues (Fig. 1).

Functional Studies of Fibrinogen

The thrombin times of the patient fibrinogens with full sialic acid complements ranged from 12 sec to 22 sec longer than the normal controls. Patient 3 fibrinogen, the most abnormal fibrinogen studied, had a thrombin time of 44 sec. Removal of 3.1 residues of sialic acid per molecule from this fibrinogen resulted in a derivative with a normal number of sialic acid residues and a thrombin time of 23 sec (Fig. 1). In like manner, removal of excess sialic acid residues of the other four patient fibrinogens studied resulted in normalization of their thrombin times (Fig. 1). When partial desialylation resulted in removal of more than the excess number of sialic acid residues, the thrombin times of these partially desialylated fibrinogens were correspondingly shorter than the normal controls. Normal fibrinogen incubated as above, except that neuraminidase was eliminated from the incubation mixture, had a normal thrombin time before and after immunoadsorbent column chromatography.

The aggregation of fibrin monomers prepared from normal, patient, and partially desialylated patient fibrinogens is shown in Fig. 2. Patient fibrinogens with full sialic acid complements demonstrated impaired rate of onset and total extent of monomer aggregation as compared to controls (Fig. 2A). Removal of the excess sialic acid residues resulted in normalization of the monomer aggregation (Fig. 2B). Similar results were obtained in studies done at an ionic strength of 0.24. The sialic acid content of two partially desialylated patient fibrin monomer preparations was measured, and the monomers were found to have the same sialic acid content as the partially desialylated fibrinogen from which they were derived.

(Sialyl-\(^{3}\)H)-Fibrinogen Studies

Normal and patient fibrinogens were labeled with \(^{3}\)H-borohydride, and the radioactivity was expressed as cpm/mg of protein. Partially desialylated patient fibrinogens with sialic acid contents approximating that of normal fibrinogen were also labeled. The results of these studies are shown in Table I. Normal (sialyl-\(^{3}\)H)-fibrinogen contained \(8.7 \times 10^3\) cpm/mg of protein, while patient fibrinogens that had 1.4–3.4 residues of sialic acid per molecule of protein in excess of normal controls incorporated more of the radioactive label. Partial desialylation resulted in a decrease in incorporation of the radioactive label by patient fibrinogens.

The thrombin clottability of the labeled control and patient fibrinogens was normal, with 96% of the radioactivity present in the clot. Similar results were obtained with TCA precipitation. The radioactive label was shown to be restricted to the sialic acid analogues of fibrinogen by acid hydrolysis and enzymatic cleavage studies. Ninety-five to ninety-six percent of the radioactivity was released by acid hydrolysis with only 4%–5% of the radioactivity remaining with the protein. Enzymatic cleavage resulted in release of 85% of the protein's radioactivity.

The \(^{3}\)H-labeled sialic acid analogues released from (sialyl-\(^{3}\)H)-fibrinogen by acid hydrolysis were studied
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Fig. 2. Aggregation of fibrin monomers of purified normal and patient fibrinogens. Shaded areas represent the range of aggregation of five normal controls. The dashed lines represent the fibrin monomer aggregation of five patient fibrinogens with their full sialic acid complement (A) and after removal of their excess sialic acid (B) (ionic strength).

Reduced normal, patient, and partially desialylated patient (sialyl\(^1\)H)-fibrinogens run in SDS-PAGE demonstrated the presence of radioactivity in the \(\beta\) and \(\gamma\) chains only, with no radioactivity detectable in the \(\alpha\) chain (Fig. 3). In both normal and patient fibrinogen, 60% of the radioactivity was present in the \(\beta\) chain and 40% was present in the \(\gamma\) chain. The partially desialylated patient fibrinogen showed a similar distribution of radioactivity between the \(\beta\) and \(\gamma\) chains. Gels run in parallel and stained with Coomassie blue revealed normal mobility and amount of \(\alpha\), \(\beta\), and \(\gamma\) chains, with no evidence of proteolysis.

Biologic properties of normal, patient, and partially desialylated patient fibrinogens appeared to be unaffected by the \(^1\)H-borohydride labeling process, as the thrombin times of the labeled proteins were the same as those of the respective unlabeled proteins.

DISCUSSION

The dysfibrinogenemia associated with liver disease has been functionally characterized by a prolongation of the thrombin time due to impaired fibrin monomer polymerization.\(^1\) This abnormal fibrinogen has an increased sialic acid content,\(^1\) and this increase in sialic acid correlates with the prolongation of the thrombin time.\(^1\) In addition, complete desialylation of the protein results in an asialoderivative whose thrombin time is the same as that of control normal asialo fibrinogen.\(^1\) In the present study, partially desialylated fibrinogens from patients with liver disease were prepared by incubating the proteins with Vibrio cholerae neuraminidase and rapidly removing the enzyme from the incubation mixture by passing it through an antineuraminidase antibody immunoadsorbent column. Employing this technique, we were able to prepare patient fibrinogens from which only the excess sialic acid residues were removed. Immunoadsorbent column chromatography per se had no effect on the biologic properties of fibrinogen in control studies. Removal of the excess sialic acid from patient fibrinogens resulted in normalization of their thrombin times.

### Table 1. Sialic Acid Labeling Studies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sialic Acid Residues per Molecule Fibrinogen*</th>
<th>(^{1})H-Sialyl Fibrinogen (cpm) per mg Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>6.0 \pm 0.3 (\times 10^5)</td>
<td>87 (\pm 15 \times 10^5)</td>
</tr>
<tr>
<td>Patient 1</td>
<td>8.0</td>
<td>120 (\times 10^5)</td>
</tr>
<tr>
<td>Patient 2†</td>
<td>5.4</td>
<td>83 (\times 10^5)</td>
</tr>
<tr>
<td>Patient 3</td>
<td>9.0</td>
<td>135 (\times 10^5)</td>
</tr>
<tr>
<td>Patient 4†</td>
<td>5.0</td>
<td>95 (\times 10^5)</td>
</tr>
<tr>
<td>Patient 5</td>
<td>7.4</td>
<td>120 (\times 10^5)</td>
</tr>
<tr>
<td>Patient 6†</td>
<td>5.0</td>
<td>65 (\times 10^5)</td>
</tr>
</tbody>
</table>

* Sialic acid residues were measured after acid hydrolysis by the thiobarbituric acid assay, as previously described.
† Six normal fibrinogens. The control is expressed as the mean \(\pm 1\) SD.
† Indicates partially desialylated patient fibrinogens.
and their fibrin monomer polymerization. These studies indicate that the excess sialic acid content is responsible for the functional defect of this dysfibrinogenemia.

Normal and patient fibrinogens were also labeled with $^3$H-borohydride. The label was shown to be incorporated into sialic acid analogues on the fibrinogen as acid hydrolysis, which is known to cleave sialic acid under the conditions employed, and caused release of 95% of the protein’s radioactivity. A lesser amount of radioactivity was released by neuraminidase treatment, because the sialic acid analogues present after labeling are a less suitable substrate for cleavage by neuraminidase. The radioactivity was recoverable as labeled sialic acid analogues as shown by resin chromatography and resorcinol assay of the radioactive peaks. The labeling procedure did not alter the biologic properties of the proteins as reflected in their thrombin times. SDS-PAGE of the reduced normal and patient (sialyl-$^3$H)-fibrinogens demonstrated restriction of the label to the $\beta\beta$ and $\gamma$ chains, with no radioactivity present on the $\alpha\alpha$ chain. A similar distribution of radioactivity between the $\beta\beta$ and $\gamma$ chains was also observed when the partially desialylated patient fi-
brinogens were labeled with tritium and reduced, indicating that neuraminidase removed proportional amounts of sialic acid from the Bβ and γ chains. These findings are consistent with previous reports that indicate that the carbohydrate moiety of fibrinogen is attached to Asn 364 of the B chain and to Asn 52 of the γ chain. The tripeptide sequence Asn-X-Thr/Ser, which serves as the site of carbohydrate attachment in plasma glycoproteins, occurs only once in the Bβ and γ chains. The increased sialic acid content of the Bβ chain as compared to the γ chain found in our studies is consistent with previous reports. The increased sialic acid present in the Bβ and γ chains of patients with liver disease may reflect an alteration in the primary structure of the peptide sequence of these chains, resulting in the presence of additional sites for carbohydrate attachment. However, it is more likely that the alteration occurs in the branching of the oligosaccharide chain itself, resulting in an increased number of sialic acid as well as galactose residues per chain.

Subpopulations of Bβ and γ chains, which differ in their sialic acid contents, have been demonstrated in studies with reduced and carboxymethylated human fibrinogen. In addition, studies of the isolated γ chains of bovine fibrinogen demonstrate heterogeneity based on differences in carbohydrate content. While earlier studies indicated that carbohydrate was present on the Bβ chain, more recent studies, including our own, failed to demonstrate the presence of carbohydrate on the Bβ chain by periodic acid-Schiff reagent staining of the reduced fibrinogens run in SDS-PAGE. Furthermore, after labeling of fibrinogen sialic acid with tritium, no or negligible radioactivity was detectable in the Aα chain after reduction and separation of the chains by electrophoresis, as in the present study, or by chromatographic techniques. A recent report of the completed sequence of the Aα chain indicates that two asparagine residues, Asn 269 and Asn 400, are in proper tripeptide sequence, i.e., Asn-X-Ser, to theoretically serve as possible sites for attachment of carbohydrate. Not all of the asparagine residues in glycoproteins that are in the appropriate tripeptide sequence, however, have attached carbohydrate. In such cases, it may be that the folding of the nascent polypeptide prevents the availability of these sites for carbohydrate attachment.

Similar alterations of the carbohydrate content of several plasma glycoproteins have been described in patients with hepatoma. The dysfibrinogenemia associated with hepatoma has been demonstrated to have an increased sialic acid content that is probably responsible for its defective fibrin monomer polymerization. An R-type B1 binding protein with increased sialic acid content has also been described in patients with hepatoma. Further studies are necessary to determine the mechanism whereby the carbohydrate content of these plasma glycoproteins is altered in patients with liver disease.

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