Plasma Dermatan Sulfate Proteoglycan in a Patient on Chronic Hemodialysis

By Michael A. Delorme, Niloufer Saeed, Andrea Sevick, Lesley Mitchell, Leslie Berry, Marilyn Johnston, and Maureen Andrew

A 68-year-old man on chronic hemodialysis for 6 years, presented with a spontaneous psoas muscle hemorrhage. Investigations showed intermittently elevated activated partial-thromboplastin time and thrombin time. Preliminary investigations suggested a heparin-like inhibitor in the patient’s plasma. However, no anti-Xa activity could be detected. Investigation of the ability of patient plasma to inhibit exogenous thrombin showed that most thrombin was inhibited by heparin cofactor II, in contrast to normal plasma in which most thrombin was inhibited by antithrombin III. Treatment of plasma with glycosaminoglycan-degrading enzymes suggested the presence of dermatan sulfate (DS) in patient plasma. This was confirmed in a heparin cofactor II-dependent antithrombin assay for DS that showed anticoagulant equivalent to 2.2 ± 0.3 µg/mL (mean ± SD) of porcine mucosal DS. Of this activity, approximately 90% was sensitive to enzymes that degrade DS. The glycosaminoglycan containing fraction of plasma was isolated and subjected to gel chromatography. Anticoagulant activity eluted from Sephadex G-100 (Pharmacia, Montreal, Quebec, Canada) as two peaks with Kav of 0.10 and 0.45. After treatment with base, the Kav of the higher molecular weight species was increased to 0.55. This activity was completely sensitive to enzymes that degrade DS. Thus, the active DS was present as a proteoglycan. The lower molecular weight material was not sensitive to enzymes that degrade DS or heparan sulfate and it was active in the heparin cofactor II-dependent antithrombin assay but not in an antithrombin III-dependent antithrombin assay. This activity was not degraded by heating. Subsequently, measurement of DS activity was performed in plasma obtained from eight other patients on hemodialysis before administration of heparin that showed that all patients had DS activity present that varied from 0.05 to 0.4 µg/mL. No enzyme-resistant activity could be shown in these patients. In summary, a circulating anticoagulant with properties of DS is present in patients requiring hemodialysis. © 1993 by The American Society of Hematology.

Although the association between renal failure and bleeding has been known for over a century,1 the mechanisms responsible for bleeding are not completely understood. The pathogenesis of the bleeding has been related to platelet dysfunction induced by molecules that are not cleared by the failing kidneys.2 In addition, abnormalities have been reported in various plasma hemostatic factors. However, other than increases in the concentrations of fibrinogen34 and factor VIII/von Willebrand factor,5,6 consistent abnormalities have not been identified. Similarly, global tests of the hemostatic system such as the prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin-clotting time (TCT) may be abnormal in some patients. Adequate hemodialysis or peritoneal dialysis ameliorate the bleeding complications.6 although abnormalities in laboratory tests of hemostasis may persist.

Once on dialysis, prevention of thrombosis becomes a concern. The hemostatic system of the patient on hemodialysis is challenged by intermittent exposure to the artificial surface of the dialysis membrane. Anticoagulation is required to prevent thrombotic occlusion of the dialyzer and occasionally to prevent thrombosis of a venous access site. Heparin is the standard agent for the former but its use may be complicated by bleeding.

In this report, we describe the investigation of a patient who developed bleeding complications after several years on hemodialysis. This was not related to heparin administration; indeed, he did not require heparin during his dialysis procedures. We have characterized an endogenous circulating anticoagulant in his plasma and found similar activity in the plasma of other patients on hemodialysis.

CASE REPORT

A 68-year-old man had been on chronic hemodialysis for 6 years for chronic renal failure secondary to focal glomerulosclerosis. He had a splenectomy for pancytopenia in 1984 and had peripheral blood and bone marrow findings suggestive of chronic myelomonocytic leukemia. He was noted to have a mildly elevated aPTT in late 1987 that was thought to be secondary to heparin contamination. In early 1988, he first noted oozing from venipuncture sites after hemodialysis and bruising unrelated to dialysis. During investigations into these minor bleeding episodes, he developed an apparently spontaneous psoas muscle hemorrhage. Shortly thereafter, he also developed a spontaneous hemarthrosis of the right knee. The aPTT remained elevated and the TCT (2U) was also prolonged. Addition of the heparin neutralizer, Heparisorb (Organon Technika, Durham, NC), to his plasma completely corrected the aPTT prolongation and partially corrected the TCT. The TCT did not correct with addition of protamine sulfate. Factor assays were normal (VIII 2.16 U/mL, IX 1.9 U/mL, XI 1.08 U/mL, XII 0.86 U/mL). His bleeding time was greater than 15 minutes on one occasion but platelet aggregation studies were normal. About 1 month later, his bleeding time was normal. Subsequently, he no longer required anticoagulation while on dialysis. The aPTT and TCT fluctuated (Table 1) with the aPTT more consistently prolonged. No cause for the presence of this anticoagulant became evident during his treatment. He continued to have intermittent episodes of...
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<td>25 [25]†</td>
<td>63 [25]†</td>
<td>—</td>
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</table>

* Normal ranges.
† Some samples also tested after in vitro addition of Heparosorb.

**MATERIALS AND METHODS**

Chemical reagents were purchased from Sigma (St Louis, MO) unless otherwise stated. Human α-thrombin was a kind gift of Dr J. Fenton II (New York State Department of Health, Albany, NY) or obtained from Affinity Biologicals (Yarker, ON). Na4HSO4, chondroitin AC lyase (ACase, EC 4.2.2.5), chondroitin ABC lyase (ABCase, EC 4.2.2.4) and heparitinase (EC 4.2.2.8) were purchased from ICN Biomedicals (Mississauga, Ontario, Canada). Ancrod (EC 3.4.21.28) was from Connaught Laboratories (Toronto, Ontario, Canada). Human heparin cofactor II (HC II) was purified by the method of Griffith et al.5 Purified human antithrombin III (AT III) was from Cutter Biologicals (Etbiockie, Ontario, Canada). The chromogenic substrate S-2238 was obtained from Kabi Diagnostica (Uppsala, Sweden). Sephadex G-100 and QAE-Sephadex were obtained from Pharmacia (Montreal, Quebec, Canada). Porcine mucosal dermatan sulfate (DS) was from Mediolamnum Pharmaceutici (MF 701, batch 48; Milan, Italy).

**Hemostatic tests.** Mixing studies using 50% normal pooled plasma were performed in aPTT, Xa, and Russell’s viper venom clotting-time studies. These clotting times and the reptilase time were performed according to standard techniques. Fibrin degradation products (FDP) were measured by latex agglutination. Anti-Xa activity was measured using a commercial kit (Asneracrom Heparin; Diagnostica Stago, Wellmark Diagnostics, Guelph, Ontario, Canada) and an ACL 300R (Fisher Scientific, Toronto, Ontario, Canada). Assays of AT III and HC II were performed using radial immunodiffusion.

**Thrombin inhibition.** The formation of thrombin-inhibitor complexes in plasma after addition of thrombin was performed as previously described.6 In brief, α-thrombin was radiolabeled with 125I using the Enzymobead reagent (Sigma, St Louis, MO) to a specific activity of 65,700 cpm/NIU U. Plasma was treated with ABCase or ACCase (final concentration 0.3 U/mL) or enzyme buffer for 1 hour at 37°C. In control experiments, it was shown that this concentration of enzyme was sufficient to degrade the amount of DS found in the patient’s plasma (see below). The treated plasma was then defibrinated with ancrod (final concentration 0.18 U/mL). Labeled thrombin was added to plasma to a final concentration of 25 mmol/L and incubated at 37°C. The reaction was terminated after 90 seconds by sampling into 3 vol of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heating in a boiling water bath for 2 minutes. Previous experiments in adult plasma had shown no difference in the proportion of thrombin complexed to each inhibitor, up to and including 90 seconds (this time point was chosen for convenience). The product was subjected to SDS-PAGE (≈10,000 cpm/lane) on a 5% to 15% gradient running gel using the buffer system of Laemmli.11 Gels were autoradiographed and thrombin-inhibitor complexes quantified by scanning densitometry (LKB Ultrascan XL; Pharmacia, Uppsala, Sweden).

**Anticoagulant activity assays.** A HC II-dependent antithrombin assay was used to measure anticoagulant DS in plasma and purified samples. This is a modification of the method of Dupouy et al2 and a commercially available assay (Stachrom DS; Diagnostica Stago, Asnieres, France). In brief, fibrinogen, AT III, and HC II were removed from plasma by mixing with 1 vol of 50 g/L bentonite in 0.15 mol/L NaCl/0.02 mol/L Tris pH 7.4. This step was eliminated in the assay of purified samples. An 80-μL sample was incubated at 37°C for 2 minutes with 80 μL of human HC II (≈100 μg/mL) in 0.15 mol/L NaCl/0.02 mol/L Tris pH 7.4. To this was added 80 μL of thrombin (5 NIH U/mL) in 0.15 mol/L NaCl/0.1% polyethylene glycol/0.02 mol/L Tris pH 7.4) and incubated for 2 minutes. S-2238, 80 μL (1 mmol/L in 0.8 mol/L Tris pH 8.3), was added and incubated for a further minute at which time the reaction was stopped by addition of 80 μL of 50% acetic acid. Absorbance of the product at 405 nm was determined. A standard curve was generated by addition of porcine mucosal DS at varying concentrations to pooled normal adult plasma before bentonite treatment or to 0.15 mol/L NaCl. This assay is only linear up to a DS concentration in plasma of 0.8 μg/mL. Therefore, it was modified to be less sensitive in order to directly measure the DS concentration in the plasma of the index patient. To achieve this, the HC II concentration was decreased to 40 μg/mL and the incubation time with thrombin was increased to 4 minutes. This assay was linear to greater than 3 μg/mL DS. Because the assay is also sensitive to heparin and heparan sulfate, samples showing activity were confirmed to contain DS by incubation of the sample with ABCase (final concentration 5 μU/mL at 37°C for 1 hour before assay. If ABCase failed to eliminate anticoagulant activity, the sample was incubated at 37°C with heparitinase (final concentration 5 μU/mL) for 1 hour. In addition to the index patient, measurements were performed on plasma samples from 11 patients on chronic hemodialysis taken just before each of one to three dialysis procedures before administration of heparin. This assay was modified to sensitively detect AT III-dependent activity by substituting purified AT III (final concentration 0.15 U/mL) for HC II, increasing the thrombin concentration to 7 NIH U/mL and decreasing the incubation time with thrombin to 1 minute. This assay could detect significantly less than 0.01 U/mL of heparin.

**Isolation and characterization of plasma dermatan sulfate.** Polyethylene glycol was added to plasma to a final concentration of 35 g/L and allowed to mix for 30 minutes at 4°C. The mixture was centrifuged at 1,700g for 15 minutes, the supernatant recovered.
and diluted with 1 vol of water. This was applied to a 3-mL column of QAE-Sephadex equilibrated with 0.075 mol/L NaCl/0.02 mol/L Tris pH 7.4. The column was washed with equilibration buffer, then with 0.075 mol/L NaCl/0.02 mol/L acetate pH 4.0. The column was eluted with 2 mol/L NaCl/0.02 mol/L acetate pH 4.0. Fractions were analyzed for glycosaminoglycan (GAG)/proteoglycan by alcian blue staining and those showing staining were Fractions containing ABCase sensitive activity were pooled and concentrated by pressure dialysis against water. The volume was increased to a total of 25 µL of 50% acetic acid. The volume was increased to a total of 500 µL with 0.05 mol/L NaCl and the mixture dialyzed against 0.05 mol/L NaCl. This was loaded onto a column of Sephadex G-100 (1 x 49 cm), eluted with water. Fractions were assayed for activity in the HC II-dependent antithrombin assay as above. Fractions containing ABCase sensitive activity were pooled and lyophilized. The lyophilized material was resuspended in 200 µL of water. To this was added 20 µL 2N KOH and solid NaBH₄ to a final concentration of 1 mol/L. This mixture was allowed to react at room temperature for 18 hours at which time it was neutralized with 25 µL of 50% acetic acid. The volume was increased to a total of 500 µL with 0.05 mol/L NaCl and the mixture dialyzed against 0.05 mol/L NaCl. This was loaded onto a column of Sephadex G-100 (1 x 49 cm), eluted with 0.05 mol/L NaCl, and fractions collected and assayed as above.

RESULTS

Hemostatic tests. The index patient’s aPTT and TCT at various times during his course and the effect of Heparins on these tests are summarized in Table 1. Mixing studies showed incomplete correction of the prolongation in aPTT, Xa, and Russell’s viper venom clotting times. The reptilase time was prolonged to 27 seconds (control 19 seconds). FDP were normal (0.02 g/L). As noted previously, concentrations of procoagulants were normal as were concentrations of the inhibitors, AT III (0.81 U/mL) and HC II (0.79 U/mL). No increase in anti-Xa activity over normal plasma could be detected (lower limit of sensitivity 0.1 U/mL).

Thrombin inhibition. Plasma from the index patient inhibited significantly more exogenous thrombin than normal adult plasma (77% vs 57%, Fig 1). The majority of the inhibited thrombin was present as thrombin-HC II complexes (15.2 nmol/L) with less complexed to AT III (3.8 nmol/L). This contrasted with the pattern seen in normal adult plasma in which the majority of thrombin was complexed to AT III (11.8 nmol/L) whereas thrombin-HC II and thrombin-α₂M complexes were 0.7 nmol/L and 1.6 nmol/L, respectively. This observation could not be accounted for by an increased plasma concentration of HC II.

These findings suggested the presence of a substance that accelerated the thrombin-HC II interaction. Treatment of the patient’s plasma with ABCase caused a decrease in the concentration of thrombin-HC II complexes generated (6.7 nmol/L) whereas the concentration of thrombin-AT III complexes returned toward normal (7.9 nmol/L). Treatment of plasma with ABCase decreased thrombin-HC II complexes slightly (13.8 nmol/L) whereas treatment with denatured ABCase had no effect.

Anticoagulant activity assays. Assay of the DS activity in the patient’s plasma from six predialysis samples taken over a 7-week period showed the presence of activity equivalent to 2.3 ± 0.4 µg (mean ± SD) porcine mucosal DS/mL. We have previously shown that normal plasma does not contain activity detectable by this assay (sensitive to 0.05 µg/mL DS). Consistently, only approximately 90% of the activity in each sample was destroyed by ABCase. In control plasma containing 3.2 µg/mL DS, this concentration of enzyme eliminated all activity. Remaining activity was also resistant to heparitinase. Paired postdialysis samples were available for six of the above predialysis samples. Concentrations postdialysis (2.2 ± 0.3 µg/mL) were not significantly different using a paired t-test (P > 0.05).

Characteristics of the other hemodialysis patients tested are presented in Table 2. Of the 11 patients on hemodialy-

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**Table 2. Characteristics of Hemodialysis Patients Tested for the Presence of Plasma Dermatan Sulfate Anticoagulant Activity**

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</tr>
<tr>
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<tr>
<td>7</td>
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<tr>
<td>11</td>
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<td>Polycystic kidneys</td>
<td>2</td>
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</table>
PLASMA DERMATAN SULFATE IN A DIALYSIS PATIENT

Patients. Samples were taken on one to four occasions predialysis and before heparin administration. DS was measured by an HC II-dependent antithrombin assay and expressed as activity equivalent to a porcine mucosal DS. "PATIENT #" refers to patient number in Table 2.

Characterization of plasma anticoagulant activity. Anticoagulant active material that was isolated from plasma contained both ABCase sensitive and resistant material, similar to the starting plasma. These were separated by chromatography on Sephadex G-100 (Fig 2). The ABCase-sensitive fraction eluted with K_{av} 0.10 whereas the ABCase resistant material eluted later with K_{av} 0.45. After treatment of the ABCase-sensitive fraction with alkali, the activity was found on molecules eluting from Sephadex G-100 at a position corresponding to an M_r ranging from 80,000 daltons (for a globular protein) to 30,000 daltons for an isolated GAG chain. Thus, it was not obvious whether the activity resided on a proteoglycan or isolated GAG. Therefore, we treated the intact molecule with base. This cleaves the O-linkage between the GAG and core protein by a β-elimination reaction. The product eluted from Sephadex G-100 at a position corresponding to an M_r of ~11,000 daltons (based on elution patterns of dextran). This decrease in size indicates that the plasma DS exists as a proteoglycan. The source of the proteoglycan remains speculative as small DS proteoglycans are components of many connective tissues. Unfortunately, Thrombin-HC II complexes were decreased by 56% after treatment of the patient’s plasma with ABCase but only by 9% following ACase treatment. These findings are characteristic of DS. Similar activity and response to GAG-degrading enzymes could be measured in other patients on hemodialysis, though at lower concentrations. Subsequent purification isolated two fractions with anticoagulant activity. The fraction with low amounts of activity was insensitive to ABCase, providing a possible explanation for the failure to eliminate all activity in the thrombin inhibition and HC II-dependent antithrombin assays.

DS is a GAG with repeating disaccharide subunits of iduronic acid (some O-sulfated at C-2 or -3) and N-acetylgalactosamine-4-O-sulfate. In vivo, it exists as a proteoglycan attached to the serine residue of a core protein. We characterized the molecular weight of the plasma DS from the index patient by gel chromatography. The intact molecule eluted from Sephadex G-100 at a position corresponding to an M_r ranging from 80,000 daltons (for a globular protein) to 30,000 daltons for an isolated GAG chain. Thus, it was not obvious whether the activity resided on a proteoglycan or isolated GAG. Therefore, we treated the intact molecule with base. This cleaves the O-linkage between the GAG and core protein by a β-elimination reaction. The product eluted from Sephadex G-100 at a position corresponding to an M_r of ~11,000 daltons (based on elution patterns of dextran). This decrease in size indicates that the plasma DS exists as a proteoglycan. The source of the proteoglycan remains speculative as small DS proteoglycans are components of many connective tissues. Unfortunately, Thrombin-HC II complexes were decreased by 56% after treatment of the patient’s plasma with ABCase but only by 9% following ACase treatment. These findings are characteristic of DS. Similar activity and response to GAG-degrading enzymes could be measured in other patients on hemodialysis, though at lower concentrations. Subsequent purification isolated two fractions with anticoagulant activity. The fraction with low amounts of activity was insensitive to ABCase, providing a possible explanation for the failure to eliminate all activity in the thrombin inhibition and HC II-dependent antithrombin assays.

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DISCUSSION

This report describes a patient on chronic hemodialysis who presented with bleeding and was found to have two circulating anticoagulants. These anticoagulants accelerated the inhibition of exogenous thrombin by HC II. Although heparin, heparan sulfate, and DS all catalyze thrombin inhibition by HC II, heparin, and heparan sulfate preferentially catalyze thrombin inhibition by AT III.
insufficient material was available on the other patients tested to characterize the DS in their plasmas.

The low-activity anticoagulant was difficult to characterize. Neither ABCase nor ACase treatment would be expected to eliminate the activity of heparin or heparan sulfate; however, the material was also resistant to heparitinase and did not catalyze the inhibition of thrombin by AT III. It does not directly inhibit thrombin and does not appear to be a heat-labile protein.

Circulating GAG inhibitors have been previously described\textsuperscript{16-20} but have generally been characterized as heparan sulfate or heparinlike molecules. We have recently described the presence of a circulating DS proteoglycan in the plasmas of pregnant women at term and their newborn babies.\textsuperscript{14} An ABCase-sensitive GAG inhibitor has recently been identified in the plasma of a patient with prostatic carcinoma\textsuperscript{27} but this was not extensively characterized.

The present report is the first to describe DS circulating in a patient with a bleeding disorder. However, the role of the plasma DS in the index patient’s bleeding is unclear. In an animal model, DS has been shown to produce less bleeding than standard heparin at equivalent antithrombotic doses.\textsuperscript{21,22} A recently reported clinical trial of DS in prevention of deep venous thrombosis in patients with hip fracture\textsuperscript{23} achieved concentrations of 6.2 $\mu$g/mL with no increase in bleeding complications over placebo-treated controls even during surgery. Thus, it would appear that the presence of the inhibitor alone would not account for the bleeding problems seen. Possible explanations for this discrepancy include: (1) increased sensitivity of the patient to the bleeding effects of DS; (2) a sole or synergistic effect of the unidentified material with DS; (3) DS proteoglycan has different bleeding effects than the DS GAG used in antithrombotic studies; or (4) a combined effect of one or both inhibitors with another abnormality such as dysfibrinogenemia (suggested by the prolonged reptilase time) or platelet dysfunction, which may be seen in chronic renal failure (suggested by the prolonged bleeding time). In addition, an undetected abnormality of the vessel wall might account for the bleeding and prolonged bleeding time. Recently, a DS proteoglycan with similar characteristics has been isolated from the intima/media of the human aorta.\textsuperscript{24} Thus, a vessel-wall abnormality might also account for the presence of DS proteoglycan in the plasma.

Similarly, the role of the DS in allowing the patient to undergo dialysis without anticoagulation is not clear. Clinical trials of DS as an antithrombotic in hemodialysis have been started. One group reported successful dialysis after a bolus IV dose of 4.5 or 6 mg/kg\textsuperscript{25}; however, another found doses of 2 to 4 or 5 to 6 mg/kg to be ineffective.\textsuperscript{26} This difference may relate to factors in the dialysis procedure itself such as type of dialysis membrane, flow rate, and time on dialysis or differences in the DS preparation used. The latter study achieved peak DS concentrations of 40 $\mu$g/mL and 80 $\mu$g/mL, respectively, with the two doses and the half-life of DS in dialysis patients is reported to be prolonged.\textsuperscript{27} These are again more than an order of magnitude higher than the concentrations measured in the index patient. Doses lower than 2 mg/kg have not been reported. The apparent efficacy of the DS concentration measured in this patient might be explained by the same factors as postulated to account for the increased bleeding.

The finding of similar activity at lower levels in other patients on dialysis is interesting but of uncertain significance. DS and standard (but not low molecular weight) heparin have been found to be synergistic in their antithrombotic effects.\textsuperscript{28} The intriguing observation that a subgroup of patients on hemodialysis with low HC II concentrations were found to require more heparin on dialysis\textsuperscript{29} may provide indirect evidence that this mechanism operates in these patients. Significant concentrations of DS may be present in plasma without detectable abnormalities in screening coagulation tests and may play a role in variability of heparin requirements. The source of the DS in these patients remains to be determined. Although no ABCase-resistant activity was measured in these patients, it may be present at very low levels that are below the sensitivity of the assay. It will be interesting to determine the relationship, if any, between DS concentration and underlying disease, such as diabetes, and complications of dialysis such as accelerated vascular disease.

REFERENCES

Plasma dermatan sulfate proteoglycan in a patient on chronic hemodialysis

MA Delorme, N Saeed, A Sevcik, L Mitchell, L Berry, M Johnston and M Andrew