Isolation of a Heparin-Like Anticoagulant From the Plasma of a Patient With Metastatic Bladder Carcinoma

By Ayalew Tefferi, Barbara A. Owen, William L. Nichols, Thomas E. Witzig, and Whyte G. Owen

A 73-year-old woman with metastatic transitional cell carcinoma of the bladder developed vaginal bleeding a few days after undergoing radical cystectomy. She had no other signs of mucocutaneous bleeding. Coagulation studies revealed a markedly prolonged thrombin time (>800 seconds), a slightly prolonged reptilase time (20 seconds), and mildly elevated fibrinogen (4.39 g/L), and fibrin D-dimer (200 to 500 ng/mL) levels. Treatment of the patient’s plasma in vitro with protamine or barium sulfate normalized the thrombin time. The anticoagulant activity corresponded to 0.15 heparin U/mL when measured by a thrombin time assay using normal plasma as substrate and standardized with porcine heparin. The anticoagulant was quantitatively bound to and subsequently eluted with 1 mol/L NaCl from quaternary aminoethyl (QAE) Sephadex, and then isolated by affinity chromatography on immobilized antithrombin III. The isolated anticoagulant was shown to be sensitive to heparinase digestion. Therefore, the inhibitor has functional and chemical properties similar to those of high-affinity heparin. Thus far, this is the only anticoagulant of this type isolated from the plasma of a patient bearing a tumor other than plasma cell myeloma.

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METHODS

Coagulation studies and measurements of plasma fibrin D-dimer and antithrombin III levels were performed according to published methods. Anticoagulant activity was assayed by a thrombin time method using bovine thrombin (Parke-Davis, Morris Plains, NJ) added in normal plasma to yield a clotting time of 20 to 22 seconds. Quaternary aminoethyl (QAE) Sephadex (Sigma Chemical Co, St Louis), swelled in 0.1 mol/L NaCl, was used as a thick slurry.

The anticoagulant activity in patient’s plasma was adsorbed with QAE Sephadex at 4°C for one hour. The anticoagulant was eluted from QAE Sephadex stepwise with increasing NaCl concentrations. The anticoagulant was isolated by affinity chromatography on a column of porcine antithrombin III noncovalently bound to concanavalin A-agarose. Polyacrylamide gel electrophoresis of glycosaminoglycans was performed with a Phast system (Pharmacia AB, Uppsala, Sweden) using 8% to 25% gradient polyacrylamide gels and native buffer strips. Gels were stained for glycosaminoglycans with Alcian Blue Silver.

The isolated anticoagulant was proteolytically digested with pronase (2 µg/mL) (Boehringer Mannheim Biochemicals, Indianapolis) or Heparinase (100 µ/mL) (Seikagaku Kogyo Co, LTD, Tokyo) in 0.1 mol/L NaCl, 20 mol/L Hepes, pH 7.0, overnight at room temperature.

Case report. A 73-year-old woman presented with macroscopic hematuria in April 1987. Investigation revealed a high-grade transitional cell carcinoma of the bladder. The patient underwent radical cystectomy and ileal conduit urinary diversion. Regional and paraaortic lymph node biopsies showed metastatic involvement. Ten days after surgery, the patient developed significant vaginal bleeding, with a drop in her hemoglobin from 14.0 g/dL to 7.5 g/dL. There were no other signs of mucocutaneous bleeding. The intravaginal suture lines were intact and blood was diffusely oozing out of the intravaginal operative site. Coagulation studies suggested a circulating heparin-like anticoagulant (Table 1). The patient was not receiving heparin by any route of administration, including low-dose subcutaneous heparin or heparin flushes to keep intravenous lines open. Treatment of the patient’s plasma in vitro with protamine or barium sulfate normalized the thrombin time (Table 2). Infusion of intravenous protamine sulfate (20 mg) had no effect on either the vaginal bleeding or the in vitro clotting times. Higher doses of intravenous protamine sulfate were not tried because acceptable control of the vaginal bleeding was achieved through local measures and RBC transfusion requirements were limited to <1 U/d on the average. Although the patient’s in vitro clotting abnormalities persisted, she did not show any other signs of bleeding during her illness. Serum immunoelectrophoresis revealed no monoclonal protein. The patient died a few days later from lactic acidosis and renal failure; autopsy was refused.

RESULTS

The results of the coagulation assays are summarized in Table 1. Notable was the marked increase in thrombin time, well out of proportion to the slightly increased reptilase time. The presence of an inhibitor was indicated by the finding that addition of an equal volume of normal plasma did not
correct the prolonged thrombin time. Plasma levels of factors V, VII, and X were within normal limits. The plasma factor VIII level was slightly elevated while prothrombin was mildly decreased (52%, normal 83% to 117%). The thrombin time was normalized after the in vitro addition of either protamine or barium sulfate to the patient's plasma (Table 2). A protamine concentration in vitro of 100 μg/dL was required to completely correct the thrombin time, while a protamine concentration of 10 μg/dL corrected the thrombin time of normal plasma containing an equivalent amount (by anticoagulant activity) of porcine heparin (Table 2). The anticoagulant activity in the patient's plasma corresponded to 0.15 heparin U/mL (Fig 1).

The anticoagulant was quantitatively bound to and subsequently eluted from QAE-Sephadex. All the anticoagulant activity in the patient's plasma corresponded to 0.15 heparin U/mL (Table 2). A protamine concentration in vitro of 100 μg/dL was required to completely correct the thrombin time, while a protamine concentration of 10 μg/dL corrected the thrombin time of normal plasma containing an equivalent amount (by anticoagulant activity) of porcine heparin (Table 2). The anticoagulant activity in the patient’s plasma corresponded to 0.15 heparin U/mL (Fig 1).

The anticoagulant was quantitatively bound to and subsequently eluted from QAE-Sephadex. All the anticoagulant activity in 5 mL of patient’s plasma was adsorbed with 300 μL of QAE-Sephadex, while 100 μL were required to adsorb an equivalent amount (by anticoagulant activity) of porcine heparin added to 5 mL of normal, platelet-poor plasma. Stepwise elution of the patient’s anticoagulant from the QAE-Sephadex with buffers of increasing salt concentration yielded minimal elution of 0.5 mol/L and near-complete elution at 1 mol/L NaCl. The anticoagulant in the eluate was then isolated by affinity chromatography on a column of antithrombin III immobilized by concanavalin A- sepharose (Fig 2).

Porcine heparin at a concentration as low as 2 U/mL (15 μg/mL) was detectable when subjected to electrophoresis on native polyacrylamide gels and stained with Alcian Blue-Silver. The isolated anticoagulant was not stained when samples containing anticoagulant activity as high as 10 heparin equivalent U/mL were analyzed by this method.

Limited material prevented more exhaustive structural analysis. Nevertheless, the isolated anticoagulant activity in side fractions from the affinity column was not affected by digestion with pronase but was neutralized by digestion with heparinase (Table 3).

### DISCUSSION

The prolonged thrombin time with a near-normal reptilase time in the patient’s plasma is a characteristic effect of circulating heparin-like substances. The resistance of the anticoagulant to neutralization by protamine is a feature ascribed to heparan sulfate in contrast to unfractionated or low molecular weight heparin. An unfractionated heparin of equivalent activity required approximately one tenth of the protamine sulfate needed to correct completely the patient’s thrombin time (Table 2). However, the reduction in the anticoagulant activity obtained by heparinase digestion suggests properties of the anticoagulant more to resemble those of heparin. Upon electrophoresis of the patient's

### Table 1. Coagulation Studies

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin time</td>
<td>27 s</td>
<td>17-19 s</td>
</tr>
<tr>
<td>Partial thromboplastin time</td>
<td>115 s</td>
<td>25-40 s</td>
</tr>
<tr>
<td>Thrombin time</td>
<td>&gt;600 s</td>
<td>21 s</td>
</tr>
<tr>
<td>Reptilase time</td>
<td>90 s</td>
<td>16 s</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>4.39 g/L</td>
<td>1.90-3.65 g/L</td>
</tr>
<tr>
<td>Fibrin split products</td>
<td>20 μg/mL</td>
<td>&lt;3 μg/mL</td>
</tr>
<tr>
<td>Fibrin D-dimer</td>
<td>200-500 ng/mL</td>
<td>&lt;200 ng/mL</td>
</tr>
<tr>
<td>Protamine gel test</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>32%</td>
<td>77% to 105%</td>
</tr>
</tbody>
</table>

### Table 2. Thrombin Times After In Vitro Addition of Protamine Sulfate

<table>
<thead>
<tr>
<th>Protamine Concentration (μg/mL)</th>
<th>Patient Plasma (s)</th>
<th>Heparinized Normal Plasma* (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>10</td>
<td>&gt;200</td>
<td>26</td>
</tr>
<tr>
<td>50</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>100</td>
<td>22</td>
<td>23</td>
</tr>
</tbody>
</table>

*Concentration = 0.15 units of porcine heparin per mL of normal plasma.
isolated anticoagulant containing five times the equivalent activity of the minimum detectable sample of porcine heparin, no material was detected by metachromatic or silver staining. Thus, either the anticoagulant expresses an exceptional specific activity or, more likely, is a conjugated proteoglycan that stains poorly. We have noted (unpublished observation, July 1988) that noncovalent heparin-antithrombin III complexes stain relatively weakly as compared with heparin or antithrombin III alone.

In the last 10 years at the Mayo Clinic, we have detected a total of five patients with circulating heparin-like anticoagulant activity. With the exception of the current report, all have been associated with plasma cell proliferative disorders: three with plasma cell myeloma and one with monoclonal gammopathy of undetermined significance (MGUS). All the patients manifested a thrombin time of >600 seconds that corrected in vitro by the addition of protamine and/or barium sulfate. Bleeding was severe in the patient with MGUS and in two of the patients with plasma cell myeloma. In one of the patients, high doses of continuous intravenous protamine sulfate (15 mg/h) were found to improve clinical bleeding and partially correct the in vitro clotting studies.

Although circulating heparin-like anticoagulant activity in humans has been associated mostly with plasma cell proliferative disorders, it can occur in other malignancies.

ACKNOWLEDGMENT

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