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 amine (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.

© 1989 by Grune & Stratton, Inc.

CIRCULATING heparin-like anticoagulants have been described in dogs with experimentally induced anaphylactic shock. In humans, heparin-related compounds (glycosaminoglycans) have been isolated and characterized from mast cells, fibroblast cultures, basophils, eosinophils, and platelets. Although endogenous glycosaminoglycans are normally present in human plasma in minute amounts, a significant circulating heparin-like anticoagulant activity is rarely associated with plasma cell myeloma, acute monoblastic leukemia, systemic mastocytosis, suramin (anti-protozoan) therapy, and the acquired immune deficiency syndrome (AIDS).

Clinically significant circulating heparin-like anticoagulants in human plasma have only been isolated from two patients with plasma cell myeloma and were shown to have the physical and biochemical properties ascribed to heparan sulfate. In this report, we describe the isolation of an anticoagulant, having properties similar to those of heparin, from the plasma of a patient with transitional cell carcinoma of the bladder.

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. Polycrylamide 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.
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 (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 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 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 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

correct the prolonged thrombin time. Plasma levels of factors V, VII, and X were within normal limits. The plasma factor VIII level was slightly 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 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 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>20 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 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.

Fig 1. Anticoagulant assay. Thrombin times on samples of normal plasma containing increasing dilutions of porcine heparin (Elkins-Sinn, Inc, Cherry Hill, NJ) were used to construct a standard curve (A). The same pool of normal plasma containing increasing dilutions of patient plasma was used to assay anticoagulant activity in patient plasma (Δ).

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

Fig 2. Affinity chromatography of the anticoagulant (QAE-Sephadex eluate) on antithrombin III agarose. Anticoagulant activity is derived from thrombin time assays and expressed in porcine heparin units having equivalent anticoagulant activity. The column (0.9 mL) was washed with 2 mL of 0.1 mol/L NaCl before elution with 1 mol/L NaCl. Single-drop fractions contained approximately 50 µL of eluate.
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

We thank Pam K. Fisher for her technical assistance.

REFERENCES

19. Griffith MJ, Marbet GA: Dermatan sulfate and heparin can be fractionated by affinity for heparin cofactor II. Biochem Biophys Res Commun 112:663, 1983
Isolation of a heparin-like anticoagulant from the plasma of a patient with metastatic bladder carcinoma

A Tefferi, BA Owen, WL Nichols, TE Witzig and WG Owen