Role of Plasmin in the Degradation of the Stroma-Derived Fibrin in Human Ovarian Carcinoma

Olaf Wilhelm, Reimar Hafter, Agnes Henschen, Manfred Schmitt, and Henner Graeff

The aim of this study was to evaluate the type of enzymes involved in tumor-associated fibrinolysis of the stroma component fibrin in ovarian cancer patients. For this purpose, the high-molecular-mass fibrin degradation products (HMM-XDP) were isolated from malignant ascitic fluid by protamine sulfate precipitation and further purified by gel filtration and acid precipitation. After reduction with 2-mercaptoethanol, the peptide chain components were separated by reverse-phase high-performance liquid chromatography (RP-HPLC). The nature of these components was elucidated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal amino acid sequence analysis and compared with fibrin-derived fragments formed in vitro. The results indicate that plasmin is the essential protease involved in the degradation of the stroma-derived fibrin portion found in ovarian cancer ascites.

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Biological evidence has been presented that procoagulants and plasminogen activators regulating coagulation and fibrinolysis are involved in the growth and metastasis of solid tumors. As a result, high concentrations of fibrin degradation products can be detected in malignant ascitic fluid, in contrast to peritoneal exudates and liver cirrhosis ascites. In ovarian cancer these degradation products most probably are derived from fibrin deposits in the tumor stroma surrounding the tumor nests. The level of total crosslinked fibrin degradation products (XDP) in ascitic fluid and in plasma is a sensitive indicator for monitoring the course of tumor disease and has found clinical application. Part of the products consist of degraded soluble crosslinked high-molecular-mass fibrin (HMM-XDP).

It was the aim of the study to evaluate the type of protease involved in the degradation of the tumor stroma component fibrin. For this purpose, HMM-XDP were obtained by protamine sulfate precipitation from ascitic fluid. After further purification by gel filtration and acid precipitation, HMM-XDP were separated under reduced conditions by reverse-phase high-performance liquid chromatography (RP-HPLC). Purified HMM-XDP subunits were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal amino acid sequence determination. This approach allowed a detailed comparison of the HMM-XDP isolated from ascitic fluid with HMM-XDP obtained by the action of thrombin, factor XIIIa, and plasmin on fibrinogen in vitro.

Materials and Methods

The suppliers of reagents and antibodies are indicated in parentheses: acetonitrile and trifluoroacetic acid (Sigma, München, FRG); 2-mercaptoethanol and 4-vinylpyridine (Merck, Darmstadt, FRG); Ultrogel AcA 34 (LKB-Pharmacia, Freiburg, FRG); Nacokisel 300-10 (C-18) (Macherey & Nagel, Düren, FRG); protamine sulfate (Novo, Mainz, FRG); monoclonal mouse antibody directed to the crosslinking region of XDP (DD-3B6/22) and horseradish peroxidase-conjugated monoclonal antibody directed to fibrinogen (DD-4D2/182-HRP) (Agen Biomedical Ltd, Brisbane, Australia); aprotinin (TrasyloL Bayer, Leverkusen, FRG); human fibrinogen (Deutsche Kabi, München, FRG); and bovine thrombin and streptokinase (Behring-Werke, Marburg, FRG).

Preparation of HMM-XDP from ascitic fluid. Freshly collected ascitic fluid from patients with advanced ovarian cancer (FIGO III/IV; three cases of moderately differentiated cystadenocarcinomas) was centrifuged for 20 minutes at 3,000g, 4°C, and the cell pellet was discarded. The supernatant was anticoagulated by the addition (1:10) of a solution containing 0.129 mol/L trisodium citrate, 0.06 mol/L tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES), 0.05 mol/L Na2EDTA, and 1,000 kIU/mL aprotinin adjusted to pH 7.5 with sodium hydroxide. To 100 mL of this ascitic fluid, 20 mL of 0.2% aqueous protamine sulfate was added at 4°C. A flocculos precipitate formed within 10 minutes and was harvested by centrifugation. The pellet was washed with 6 mL of 12% aqueous ammonium sulfate, centrifuged, and dissolved in 6 mL of a solution containing 0.06 mol/L TES, 0.081 mol/L sodium chloride, 0.0129 mol/L trisodium citrate, and 100 kIU/mL aprotinin, pH 7.6, adjusted with sodium hydroxide. The yield was 34 mg protein in 9 mL, calculated as fibrin by measuring the optical density, assuming Extinction coefficient = 16.2.

Protamine sulfate precipitates fibrin degradation products (XDP) predominantly of the high-molecular-mass type (HMM-XDP). In the ascitic fluid preparation, XDP with a relative molecular mass (M,) greater than 200,000 (HMM-XDP) were enriched in the precipitate. Entities with M, less than 200,000, including DD, remain mostly in the supernatant.

Preparation of HMM-XDP from fibrinogen in vitro. To 2 mL of fibrinogen (2 mg) in 1% sodium citrate-0.4% NaCl solution containing sufficient residual plasminogen and factor XIII, 2 mL of 0.9% NaCl, 2 mL 0.025 mol/L CaCl2, and 0.1 mL thrombin (3 IU) were added at 23°C. Twenty seconds later, 0.2 mL streptokinase (1,000 kIU) was added, and immediately, a gel was formed. The gel was completely dissolved within 20 minutes at 37°C. The reaction was stopped by addition of 0.2 mL aprotinin (2,000 kIU). HMM-XDP were precipitated by the addition of 1.3 mL 0.2% protamine sulfate. The precipitate was washed and dissolved as described for ascitic fluid.

Gel filtration chromatography. A gel filtration column (80 × 1.5 cm) was packed with Ultrogel AcA 34, equilibrated, and eluted with a solution of 0.05 mol/L Tris-HCl, 0.116 mol/L NaCl, 0.0129 mol/L trisodium citrate, and 0.2 mol/L epsilon-aminoacproic acid, pH 7.6. The flow rate was 21 mL/h. A sample of 1 mL of protamine...
sulfate-precipitated ascitic fluid preparation (3.75 mg of protein) was applied to the column, and fractions (3 mL) were collected. The absorbance was monitored at 280 nm. One large and two small peaks were obtained. XDP were quantified by XDP-enzyme linked-immunosorbent assay (ELISA) and found only in the large peak. This peak was pooled, and HMM-XDP was precipitated by adjusting the pH to 3.7 with HCl.15 The precipitate was collected by centrifugation and then dissolved in 6 mol/L aqueous guanidine hydrochloride (for HPLC) or 8 mol/L urea (for SDS-PAGE), respectively. By the acid precipitation step, HMM-XDP were recovered in a highly purified and concentrated form.12

Preparation of S-pyridylethylated HMM-XDP. Samples were incubated with 2-mercaptoethanol (5% final concentration) and 6 mol/L guanidine hydrochloride in 0.1 mol/L Tris-HCl, pH 8.5 (under nitrogen) for 16 hours at 37°C. Free sulfhydryl groups were blocked by the addition of 4-vinylpyridine (7.5% final concentration). The samples were then incubated at 23°C in the dark for 90 minutes. The reaction was stopped by the addition of formic acid to a final concentration of 20%. The material was immediately subjected to RP-HPLC.13,14

RP-HPLC. The subunits of HMM-XDP were filtered through a 0.45 μm filter (Spartan filter; Schleicher & Schüll) and then separated on a Nucleosil 300-10 (C-18) preparative column (250 × 8 mm). A gradient elution system was used: solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile. The elution (210 nm) was performed by using a linear gradient between 34% and 46% of solvent B within 75 minutes at a flow rate of 4 mL/min.13,15,16

N-terminal amino acid sequence analysis. Edman degradation was performed in the sequenator version.17 The phenylthiohydantoines of the N-terminal amino acid residues of each peptide were identified by the method outlined.14,17

Determination of XDP by ELISA. XDP containing the dimeric γ-chain (γ-γ) configuration were measured by XDP-ELISA as described18 using monoclonal antibody DD-3B6/22 as capture antibody and DD-4D2/182-HRP as the tagging antibody. This XDP-ELISA recognizes the HMM-XDP (X-oligomers, DY), as well as low-molecular-mass-XDP (DD).19,20

SDS-PAGE. SDS-PAGE of XDP was performed as described.3,11 For investigation under reducing conditions, samples were treated with 2-mercaptoethanol in the presence of 8 mol/L urea.

RESULTS

Purification of HMM-XDP from ovarian cancer ascitic fluid. The steps of purification of HMM-XDP from ascitic fluid and fibrinogen prepared in vitro (see Materials and Methods) were monitored by SDS-PAGE under nonreducing (Fig 1A) and reducing conditions (Fig 1B). The recovery rate of total XDP in ascitic fluid was determined by XDP-ELISA before and after the precipitation with protamine sulfate. The yield was 64% ± 23% (mean of five determinations). Most of the low-molecular-mass portion of XDP (mainly DD) remained in the supernatant (Fig 1A, lane 3).

By SDS-PAGE, at least six polypeptide bands were detected in the ascitic fluid preparation (Fig 1A, lane 4), confirming the presence of DY, DX, XY, DXD, and other X-oligomers.10 Earlier investigations11 have also shown that the fragment X is present in ascitic fluid. This fragment has the same electrophoretic behavior as the fragment DY and is hidden in the DY band.

Under reducing conditions, the subunits γ-γ, γ-γ', γ-β, β-β', γ, β, α', and α'' could be identified by migration behavior (Fig 1B). Intact γ-γ-chain represents a major component of both preparations. This subunit can be derived from HMM-XDP entities (XY, DXY, DXD, etc, X-oligomers).19,20 Intact

Fig 1. SDS-PAGE analysis of HMM-XDP. (A) Nonreducing conditions, 4% acrylamide; and (B) reducing conditions, 7.5% acrylamide. Lane 1, total in vitro preparation (XDP); lane 2, protamine sulfate precipitate of in vitro preparation; lane 3, protamine sulfate precipitate from ascitic fluid; lane 4, acid precipitate after gel filtration (large peak) of material shown in lane 3; lane 5, fibrinogen (in B: αα-, ββ-, γ-chain); and lane 6, purified DD (in B: γ-γ', β-β'-chain remnants). Amount of protein applied per lane was 75 μg. Data represent three preparations from different patients. Gels were stained with Coomassie Brilliant Blue G 250 (Serva, Heidelberg, FRG) and destained in 7% acetic acid. The strong polypeptide band in position of α-chain represents albumin (B, lane 3).17 In vitro material in lane 1 represents extensively degraded crosslinked fibrin. Therefore, less bands were observed. This is a matter of preparation and depends on the lysis conditions. Clearly identified is DY fragment and DD. As shown earlier,4,10 preparations with more fragments can also be obtained.
single γ-chains predominantly found in the ascitic fluid preparation originate from fragment X and intact or slightly degraded noncrosslinked fibrin.

Albumin, which runs at the position of the α-chain (Fig 1B, lane 3), was removed by gel filtration and acid precipitation (Fig 1B, lane 4). Albumin is a complex partner of HMM-XDP. The protamine sulfate precipitate from ascitic fluid (Fig 1A, lane 3) contained little DD-fragment. Its content was further diminished after gel filtration and acid precipitation (Fig 1A, lane 4). Accordingly, virtually no γ'-γ'-chain remnants (Fig 1B, lanes 3 and 4) could be demonstrated, indicating that the DD-fragment was gradually lost during the purification steps. As a result, only the HMM-XDP remained in the final samples to be fractionated by RP-HPLC.

**Separation of HMM-XDP subunits by RP-HPLC.** The HMM-XDP purified from ascitic fluid (Fig 1B, lane 4) and those obtained by plasmic degradation of crosslinked fibrin in vitro (Fig 1B, lane 2) were fractionated by RP-HPLC. Five major peaks were separated in the in vitro preparation (Fig 2A). In the ascitic fluid preparation (Fig 2B), six peaks were detected; five of those (I, III through VI) correspond to the peak positions in the in vitro preparation. The peak fractions were pooled and analyzed by SDS-PAGE (Fig 3A and B). The subunit chains of HMM-XDP were tentatively designated according to their electrophoretic migration behavior and their retention rate on HPLC. Table 1 summarizes the results. All subunit chains from the in vitro preparation (γ-γ, γ-γ', γ, β, β', α', and α) were also detected in the ascitic fluid preparation. In addition, the HMM-XDP preparation (Figs 2B and 3B) from ascitic fluid contained β' and a small amount of intact α-chain (HPLC-peak II). Two other peptides in HPLC-peak II, which run at the positions of 45,000 and 48,000 dalton, could not be identified (Fig 3B and Table 1).

**N-terminal amino acid sequence analysis of HMM-XDP purified from ascitic fluid.** The fractions separated by RP-HPLC (Fig 2B, see also SDS-PAGE in Fig 3B) were subjected to N-terminal amino acid sequence analysis of the
Table 1. Identity of Subunits of HMM-XDP Separated by RP-HPLC

<table>
<thead>
<tr>
<th>Peak</th>
<th>Position (% Acetonitrile)</th>
<th>Componentss</th>
<th>Mol Wt† ‡</th>
<th>identity† ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro HMM-XDP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>38.5</td>
<td>1</td>
<td>12,300</td>
<td>a2</td>
</tr>
<tr>
<td>III</td>
<td>41</td>
<td>1</td>
<td>22,600</td>
<td>a'</td>
</tr>
<tr>
<td>IV</td>
<td>42</td>
<td>2</td>
<td>53,000</td>
<td>b</td>
</tr>
<tr>
<td>Vb</td>
<td>44.5</td>
<td>1</td>
<td>48,700</td>
<td>γ-γ</td>
</tr>
<tr>
<td>VI</td>
<td>45.5</td>
<td>1</td>
<td>97,400</td>
<td>γ-γ</td>
</tr>
<tr>
<td>HMM-XDP from ascitic fluid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>39</td>
<td>1</td>
<td>12,300</td>
<td>a2</td>
</tr>
<tr>
<td>II</td>
<td>40.5</td>
<td>3</td>
<td>70,000</td>
<td>α</td>
</tr>
<tr>
<td>III</td>
<td>41</td>
<td>1</td>
<td>22,600</td>
<td>α'</td>
</tr>
<tr>
<td>IV</td>
<td>41.5</td>
<td>3</td>
<td>53,000</td>
<td>b</td>
</tr>
<tr>
<td>Vb</td>
<td>44</td>
<td>1</td>
<td>48,700</td>
<td>γ</td>
</tr>
<tr>
<td>VI</td>
<td>45.5</td>
<td>1</td>
<td>97,400</td>
<td>γ-γ</td>
</tr>
</tbody>
</table>

Abbreviation: NI, not identified.

*By RP-HPLC (Fig 2).
†By SDS-PAGE (Fig 3).
‡Calculated from the known amino acid sequences.

First 10 amino acid residues. The findings are summarized in Table 2 together with the previous identifications by SDS-PAGE and HPLC retention behavior. Sequences were observed that are in agreement with known amino acid sequences of fibrin chains and remnants. The identified N-terminal amino acid sequence of each subunit proves that the designation for the fibrin subunits (SDS-PAGE in Fig 3, Tables 1 and 2) is in accordance with published data. The N-terminal amino acid residues determined also indicate that thrombin or plasmin acted on crosslinked fibrin or noncrosslinked fibrinogen: thrombin (α, α', β); plasmin (α2, β1, β2, γ-γ1, and γ-γ1-variants). In addition, intact single and dimeric γ-chains were present, indicating that undegraded noncrosslinked fibrin (fragment X) and some selected X-oligomer fragments were present. Since no α- or β-N–termini were observed, it can be assumed that no intact fibrinogen was present in the investigated material. There were no cleavage sites identified by N-terminal amino acid sequence analysis, which indicates that other proteases, such as leukocyte elastase or cysteine proteinases, did not act on crosslinked fibrin to generate HMM-XDP.

DISCUSSION

Ovarian cancer cells synthesize and secrete the urokinase-type plasminogen activator (uPA) as an enzymatically inactive single-chain proenzyme (pro-uPA) that can be bound to specific receptors on the tumor cell surface. After binding, pro-uPA may be converted by trace amounts of...

Table 2. N-Terminal Amino Acid Sequence Analysis of the HPLC-Separated Subunits of HMM-XDP Purified From Ovarian Cancer Ascites

<table>
<thead>
<tr>
<th>Designation</th>
<th>Peak</th>
<th>Peptide Chain (Segment)</th>
<th>N-Terminus (10 Amino Acids)</th>
<th>Enzyme</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>II</td>
<td>17-210</td>
<td>GPRVERHGS</td>
<td>Thrombin</td>
<td>Fb</td>
</tr>
<tr>
<td>α'</td>
<td>III</td>
<td>17-210</td>
<td>GPRVERHGS</td>
<td>Thrombin</td>
<td>X, Y, XDP</td>
</tr>
<tr>
<td>α2</td>
<td>I</td>
<td>106-208</td>
<td>DNTYRVSED</td>
<td>Plasmin</td>
<td>XDP, D</td>
</tr>
<tr>
<td>β1</td>
<td>IV</td>
<td>15-461</td>
<td>GHRPLDDKRE</td>
<td>Thrombin</td>
<td>X, XDP, Fb</td>
</tr>
<tr>
<td>β2</td>
<td>IV</td>
<td>134-461</td>
<td>DENVVNEYS</td>
<td>Plasmin</td>
<td>X, Y, XDP</td>
</tr>
<tr>
<td>γ1</td>
<td>Vb</td>
<td>1-411</td>
<td>YVATRDNC1I</td>
<td>—</td>
<td>X, Fb</td>
</tr>
<tr>
<td>γ-γ1</td>
<td>VI</td>
<td>(1-411)</td>
<td>YVATRDNC1I</td>
<td>—</td>
<td>XDP</td>
</tr>
<tr>
<td>γ-γ1</td>
<td>Va</td>
<td>(1-411)-(54-411)</td>
<td>YVATRDNC1I/TSEVQKLKA</td>
<td>Plasmin</td>
<td>DY, XDP</td>
</tr>
<tr>
<td>γ-γ1</td>
<td>Va</td>
<td>(1-411)-(63-411)</td>
<td>YVATRDNC1I/AICLTYNPOE</td>
<td>Plasmin</td>
<td>DY, XDP</td>
</tr>
<tr>
<td>γ-γ1</td>
<td>Va</td>
<td>(1-411)-(86-411)</td>
<td>YVATRDNC1I/SKMLLEEIMK</td>
<td>Plasmin</td>
<td>DY, DXP</td>
</tr>
</tbody>
</table>

Abbreviation: Fb, fibrin.

*HPLC in Fig 2.
†The peptide segment size was deducted from SDS-PAGE in Fig 3 and the known amino acid sequences and plasmin cleavage sites.
plasmin into the enzymatically active form (uPA). Receptor-bound uPA converts plasminogen into plasmin, which also binds to cell surface receptors. Thus an uninhibitable protease system is established that is focused on the tumor cell surface. Plasmin can degrade proteins (e.g., fibrin, fibronectin) of the tumor stroma.

Dvorak suggested that fibrin accumulation and degradation in the tumor stroma affects tumor growth. Fibrin accumulation in tumors may result from enhanced local permeability of tumor-related blood vessels, leading to extravasation of plasma proteins, including fibrinogen and plasminogen, followed by extravascular clotting and crosslinking of fibrin by factor XIII.

In ovarian cancer ascites, high amounts of degradation products of crosslinked fibrin were identified. In the present investigation, it could be demonstrated that HMM-XDP in this type of ascites consist mainly of fragments DY and X-oligomers. Fragment X and/or intact or slightly degraded noncrosslinked fibrin were also present.

The separation of the various HMM-XDP subunits was achieved by RP-HPLC after mercaptoethanol and sulfhydryl group modification of HMM-XDP. The subunits were subjected to N-terminal amino acid sequence analysis of the first 10 amino acid residues. The results obtained provide strong evidence that the investigated HMM-XDP in ascites from ovarian cancer patients is mainly derived from crosslinked fibrin by the action of plasmin only. A small portion of the ascitic fluid preparation consisted of noncrosslinked fibrin, as judged by the presence of N-terminally intact α-chain and single γ-chain. Noncrosslinked fibrin can remain as soluble fibrin monomer complexes in the presence of suitable plasmic fragments.

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