Plasma from Cancer Patients Featuring a Characteristic Protein Composition Mediates Protection against Apoptosis*

Susanne Vejda‡, Carsten Posovsky§, Sieglinde Zelzer¶, Barbara Peter‡, Editha Bayer‡, Dieter Gelbmann‡, Rolf Schulte-Hermann‡, and Christopher Gerner‡

By comparative proteome analysis we searched for characteristic alterations of human plasma accompanying neoplastic disease. We identified protein alterations in plasma of prostate-, lung-, and breast-cancer patients in comparison to controls, comprising elevated levels of fibrinogen γ-chain dimer, degradation products of antiplasmin and laminin γ-chain, and elevated levels of acute phase proteins. The latter proteins and laminin fragments have been described as anti-apoptotic factors. We raised the question whether these alterations may have any relevance for the regulation of apoptosis. In contrast to plasma derived from healthy donors, samples from prostate-, lung-, and breast-cancer patients selectively inhibited Fas- and staurosporine-induced apoptosis in Jurkat cells but remained ineffective upon UV light-induced apoptosis. These data suggested that inhibition occurred by extracellular interference with apoptosis induction. Supporting this hypothesis, we found that formation of the CD95 death-inducing signal complex was strongly inhibited in the presence of plasma from cancer patients. *Molecular & Cellular Proteomics* 1:387–393, 2002.

Evasion from apoptosis is one of the crucial features of malignant diseases (1). The following molecular mechanisms have been implicated in the insufficient occurrence of apoptosis in tumor cells: overexpression of anti-apoptotic proteins such as bcl-2 (2), mutations of tumor suppressors such as p53 (3), or inactivation of death-inducing signals by decoy receptors (4). In addition, apoptosis may be modulated by extracellular factors such as elevated levels of extracellular K⁺ ions (5) and lysosphingolipids (6). However, it remains unknown whether and to what extent these mechanisms can account for the clinical phenomenon of resistance to apoptosis displayed by many tumors.

Recently, we described a plasma marker for transglutaminase activity indicating cancer-associated fibrin deposition and plasmin-mediated fibrinolysis. This marker was identified in the course of a systematic comparative study of high resolution 2D² protein gels of human plasma samples derived either from healthy individuals or from patients suffering from inflammatory and neoplastic diseases (7). Under physiological conditions plasmin activates growth factors resulting in the generation of survival signals, as required during wound healing (8). Extending the proteome study, we identified laminin fragments in plasma of cancer patients. Laminin fragments have been described as anti-apoptotic factors (9). Elevated levels of a group of proteins associated with a so-called second-line acute phase response, a feature described to be characteristic for chronic diseases (10), were also observed in many cancer patients. Two of these proteins, α₁-acid glycoprotein and α₁-antitrypsin, are capable of preventing ischemia/reperfusion injury in the kidney (11) and tumor necrosis factor-induced apoptosis in hepatocytes *in vitro* and *in vivo* (12–14). Reflecting on these data raised the questions whether and by which mechanism these plasma proteins could interfere with the regulation of apoptosis in tumor patients.

Anti-apoptotic factors may act directly by interfering with death receptor activation or indirectly by triggering an intracellular response perturbing apoptotic signaling cascades. Crucial for the Fas-mediated apoptotic pathway is the formation of the DISC (15). Upon ligand binding, the CD95 receptor becomes engaged in complexes that consequently recruit FADD and caspase-8 (16). Recruited caspase-8 is processed autocatalytically and initiates the apoptotic cascade resulting in the activation of effector caspses and execution of apoptosis. CD95-receptor engagement may also induce apoptosis by an alternative pathway via mitochondrial membrane depolarization, making many different kinds of cells responsive for CD95-mediated apoptosis (17).

In the current study we describe that plasma of cancer patients exhibits characteristic alterations of the protein composition accompanied by the capability to interfere with apoptosis induction. We also demonstrate that this anti-apoptotic

---

The abbreviations used are: 2D, two-dimensional; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DISC, death-inducing signal complex; FADD, Fas-associating death domain; FCS, fetal calf serum.
Anti-apoptotic Plasma Proteins in Cancer Patients

effect is apparently mediated by interference with the DISC assembly, indicating that inhibition occurs at an early stage in the apoptotic pathway.

**EXPERIMENTAL PROCEDURES**

**Plasma Samples**—Plasma samples of untreated cancer patients were kindly provided by Andrea Gsur (Institute of Cancer Research, University Vienna, Vienna, Austria) in the course of ongoing molecular epidemiology of lung cancer and prostate cancer studies (approved by the local ethic committees). All included subjects gave written informed consent for participation in this study. Plasma samples from patients suffering from systemic inflammation were obtained in the course of the clinical trial “Procalcitonin and myeloperoxidase as diagnostic and prognostic parameters by febrile conditions in surgical patients caused by bacterial infections.” Plasma samples from healthy donors (controls) were obtained from the Otto-Warburg Society for Research in Immunology and Oncology, Vienna, Austria. After obtaining informed consent from patients and healthy volunteers, peripheral blood samples were collected by aspiration in Vacutainer tubes containing 0.105 mol/liter sodium citrate. Plasma samples were centrifuged and kept at −70 °C until use.

**Cell Culture and Induction of Apoptosis**—Jurkat cells were cultivated routinely in RPMI 1640, supplemented with 10% FCS at 37 °C in a humidified atmosphere containing 5% CO2. For induction of apoptosis, Jurkat cells were washed in serum-free medium and reseeded at a density of 106 cells/ml in RPMI 1640 supplemented with either 1% FCS or 1% plasma as indicated. Antichymotrypsin, α1-antitrypsin, and haptoglobin were purchased from Sigma. Apoptosis was induced 15 min after seeding by addition of either anti-Fas (CD95) antibody (50 ng/ml, clone CH-11; Upstate Biotechnology, Lake Placid, NY) or staurosporine (Calbiochem) at 1.25 μM. Alternatively, cells at a density of 105 cells/ml were preincubated for 30 min in medium supplemented with 1% plasma as indicated and treated with 30 J/m2 UV light (254 nm).

**Scoring of Apoptosis**—Cells were harvested by combining floating and weakly adhering cells, washed twice in phosphate-buffered saline, fixed for 10 min in 2% formaldehyde/phosphate-buffered saline at room temperature, permeabilized with phosphate-buffered saline/0.2% Triton X-100 for 1 min, and stained in 1 μg/ml Hoechst-33258 (Calbiochem) for 2 min. The percentage of cells displaying typical apoptotic nuclear morphology ( crescent-shaped condensed chromatin lining nuclear periphery; apoptotic bodies), referred to as the apoptotic index, was then assessed using the fluorescence microscope (Nikon Eclipse TE300). Each time triple determinations were performed counting at least 100 cells.

**Statistical Analysis**—Apoptosis quantification data were evaluated using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA) with the one-way analysis of variance test, and p values for the patient groups with respect to the control were obtained with the Dunnet’s multiple comparison test.

**Two-dimensional Electrophoresis and Evaluation of 2D Data**—Isoelectric focusing, SDS-PAGE (using 10% or 13% polyacrylamide concentrations), and silver staining were performed essentially as described (18). In case of plasma samples, 1 μl was diluted up to 30 μl with sample buffer (10 m urea, 4% CHAPS, 0.5% SDS, 100 mM dithiothreitol) and loaded. Scanning of gels and comparative gel analysis and determination of integrated optical spot densities were accomplished with the BioImage Investigator system (Biologic, Ann Arbor, MI), using the 2-D AnalyzerTM V 6.1 software package as described (18). Comparison of the obtained 2D protein patterns with published human plasma protein patterns of the SWISS-2DPAGE database (19) allowed identification of the indicated plasma proteins, and the identity of albumin and haptoglobin were confirmed by 2D gel analysis of purchased protein samples (not shown).

**Immunoprecipitation**—The laminin γ-chain degradation product was immunoprecipitated with Sepharose G beads (Sigma) from precleared plasma that has been allowed to react with laminin B2/γ1 Ab-1 rat monoclonal antibody Clone A5 (NeoMarkers, Fremont, CA) at a dilution of 1:100.

**DISC Analysis**—2 × 107 Jurkat cells were preincubated in RPMI 1640 supplemented with 1% plasma derived from healthy donors (controls), cancer patients, or patients with systemic inflammation, respectively, and treated with 2 μg/ml anti-Apo 1 antibody (clone CH-18) (20) for 10 min at 37 °C. Subsequently, cells were lysed in lysis buffer (30 mM Tris/HCL, pH 7.5, 150 mM NaCl, 1% Triton X-100 (Serva), 10% glycerol, proteinase inhibitors). As control, 2 × 107 cells were treated alike, and 1 μg/ml anti-Apo 1 antibody was added to the unstimulated cells after lysis (unstimulated conditions). The CD95-DISC was then precipitated using Dynabeads M-450 (goat anti-mouse IgG) (Dynal). After immunoprecipitation the supernatant was collected (lysate), and the precipitated proteins were analyzed by Western blotting.

**Western Blotting**—For the detection of FADD and caspases-8, -9, and -9, immune complexes and the lysates collected after immunoprecipitation were separated by 10% SDS-PAGE, transferred to Hybond P membranes (Amersham Biosciences), blocked with 5% non-fat dry milk in TBST (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h and incubated with either anti-FADD (1:1000) (Calbiochem) or anti-caspase-8 Ab-3 (1:2000) (Oncogene) diluted in TBST containing 10% non-fat dry milk. The ECL Plus Western blotting detection system (Amersham Biosciences) was used according to the instructions from the manufacturer.

**Mass Spectrometry Analysis**—Preparation of tryptic protein hydrolysates for mass fingerprints was carried out essentially as described by Fountoulakis and Langen (21). Matrix-assisted laser desorption ionization-time-of-flight analysis was performed with 2,5-dihydroxybenzoic acid as crystallization matrix, using the Voyager-DE™ STR biospectrometry workstations (PerkinElmer Life Sciences) by TopLab (Martinsried, Germany). Protein identification was accomplished by means of PeptIdent software (22) made accessible by ExPASy at www.expasy.org/. Calculation of molecular weight and pl data of proteins and peptides was performed by means of Compute pl/ molecular weight software (23) made accessible by ExPASy.

**RESULTS**

**Alterations in Plasma Protein Composition of Cancer Patients**—We compared the plasma protein composition of 16 human healthy donors, 8 patients suffering from inflammatory diseases, and 24 untreated patients suffering from prostate, lung, or breast cancer by high resolution two-dimensional gel electrophoresis. Three specific features were found intimately associated with neoplastic disease. First, we identified fibrinogen γ-chain dimers as a plasma marker for fibrin deposition and fibrinolysis accompanying neoplastic disease (Table I), confirming data published recently (7). Second, plasma from cancer patients displayed significantly elevated levels of the acute phase proteins α1-acid glycoprotein, α1-antitrypsin, antichymotrypsin, antiplasmin, haptoglobin, and fibrinogen (see Fig. 1 and Table I), indicating a second-line acute phase response (10). Moreover, a decreased concentration of some proteins during acute phase response became evident compared with healthy controls. This group of proteins, designated as negative acute phase proteins, included albumin, prealbumin, apolipoprotein A1, fetuin, and serotransferrin (Fig.
1) (10). Third, two protein spots, highly elevated in plasma of cancer patients, were identified by mass spectrometry as C-terminal fragments of laminin α9253-chain and N-terminal fragments of antitrypsin, respectively (see Fig. 1 and Table II). To our knowledge, these fragments have not been described previously in plasma of cancer patients. However, it has been shown that laminin fragments are capable of inhibiting apoptosis (9). To confirm the presence of laminin fragments in plasma, we immunoprecipitated the laminin α-chain from plasma samples. Indeed, polypeptides with molecular weight and isoelectric points similar to the spots identified in plasma as laminin α-chain fragments were immunoprecipitated successfully from plasma of carcinoma patients but hardly from plasma derived from healthy controls (Fig. 2). Plasma from patients suffering from inflammatory diseases expectedly displayed highly elevated levels of acute phase proteins but almost no laminin α-chain fragments and no elevated levels of fibrinogen α-chain dimer (Table I). These observations indicated elevated levels of anti-apoptotic factors such as growth factors activated by action of plasmin, laminin fragments, and acute phase proteins in plasma of cancer patients. Consequently, we investigated whether plasma samples displaying these three characteristic features would mediate protection against apoptosis in an assay in vitro.

**Table I**

Occurrence of protein alterations correlating with protective effect against apoptosis

<table>
<thead>
<tr>
<th></th>
<th>Fibrinogen α-chain dimer</th>
<th>Laminin α-chain fragment</th>
<th>Antitrypsin fragment</th>
<th>Elevated acute phase proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1/16 (6%)</td>
<td>4/16 (25%)</td>
<td>7/16 (44%)</td>
<td>2/16 (13%)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0/8 (0%)</td>
<td>3/8 (38%)</td>
<td>8/8 (100%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>Prostate CA</td>
<td>14/24 (58%)</td>
<td>19/24 (79%)</td>
<td>24/24 (100%)</td>
<td>17/24 (71%)</td>
</tr>
<tr>
<td>Lung CA</td>
<td>4/8</td>
<td>8/8</td>
<td>8/8</td>
<td>5/8</td>
</tr>
<tr>
<td>Breast CA</td>
<td>6/8</td>
<td>8/8</td>
<td>8/8</td>
<td>5/8</td>
</tr>
</tbody>
</table>

**Fig. 1.** Characteristic alterations in the plasma protein composition of cancer patients. Silver-stained 2D polyacrylamide gels (13%) of plasma derived from (A) a healthy donor and (B) a prostate cancer patient, serving as representative examples, are shown. Hexagons, proteins displaying decreased intensities in various cancer patients; circles, proteins displaying increased intensities in various cancer patients.
Anti-apoptotic Plasma Proteins in Cancer Patients

TABLE II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>M_{obs}</th>
<th>M_{th}</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa Da</td>
<td></td>
<td></td>
</tr>
<tr>
<td>804–818</td>
<td>1659.9</td>
<td>1659.677</td>
</tr>
<tr>
<td>881–893</td>
<td>1661.9</td>
<td>1661.690</td>
</tr>
<tr>
<td>1595–1609</td>
<td>1661.9</td>
<td>1661.820</td>
</tr>
<tr>
<td>1012–1023</td>
<td>1725.2</td>
<td>1725.663</td>
</tr>
<tr>
<td>1405–1423</td>
<td>1889.6</td>
<td>1888.936</td>
</tr>
<tr>
<td>1358–1375</td>
<td>2134.7</td>
<td>2134.041</td>
</tr>
<tr>
<td>1024–1041</td>
<td>2295.8</td>
<td>2296.067</td>
</tr>
<tr>
<td>1005–1023</td>
<td>2414.1</td>
<td>2413.992</td>
</tr>
<tr>
<td>845–865</td>
<td>2472.7</td>
<td>2472.118</td>
</tr>
<tr>
<td>1330–1354</td>
<td>2612.4</td>
<td>2613.348</td>
</tr>
<tr>
<td>1049–1076</td>
<td>3152.3</td>
<td>3151.546</td>
</tr>
</tbody>
</table>

Fig. 2. Confirmation of laminin γ-chain fragment in plasma of cancer patients. Silver-stained 2D gels of immunoprecipitates from plasma derived from a healthy control (left) and plasma from a prostate cancer patient using an anti-laminin γ-chain antibody (right) are shown. Spots corresponding to the laminin γ-chain, as well as immunoprecipitated IgG light chains cross-reacting with the Sepharose beads (as determined by control experiments), are indicated.

of patients suffering from systemic inflammation. Interestingly, supplementation of medium with plasma from healthy human individuals resulted in significantly higher apoptotic indices as compared with supplementation with FCS (Fig. 3). Heat-denaturation treatment of plasma samples abrogated the inhibitory effect (not shown), identifying proteins as causal factors. However, we could not find significant inhibitory effects by application of single acute phase proteins such as α-1-acidic glycoprotein, antichymotrypsin, haptoglobin, and α-1-antitrypsin, as well as combinations thereof (added at concentrations observed in plasma of cancer patients; not shown). In contrast to antibody- and drug-induced apoptosis, UV light-induced apoptosis was found unaffected by supplemented plasma (Fig. 3). This observation suggested that anti-apoptotic plasma constituents would act directly on the cell surface, potentially by interference with death receptor activation.

Inhibition of DISC Formation in the Presence of Patient-derived Plasma—To test the latter hypothesis, we investigated whether DISC assembly would be affected in the presence of plasma samples with anti-apoptotic properties. During Fas-mediated apoptotic signaling the CD95 receptor recruits FADD and subsequently caspase-8 upon multimerization (16), i.e. the DISC is formed. Jurkat cells were suspended in medium supplemented with 1% plasma and treated with anti-Fas antibody 30 min thereafter. The DISC was isolated by immunoprecipitation of the activated CD95 receptor, as described under “Experimental Procedures.” Indeed, less FADD and caspase-8 were reproducibly found recruited in the presence of plasma of patients suffering from neoplastic disease (Fig. 4). However, consistent with the data from apoptotic cell counting, this property was not specific for
cancer patients, because a similar, but again less pronounced, effect was observed with plasma derived from patients suffering from inflammatory diseases (Fig. 4). In addition, as shown in Fig. 4, the unbound protein fractions (lysates) displayed higher levels of FADD and caspase-8 compared with controls, confirming that the recruitment to the receptor was impeded. In conclusion, some of the anti-apoptotic features of selected plasma could be attributed to interference with DISC formation, an essential step in apoptotic signaling by the CD95 death receptor in many cell types.

**DISCUSSION**

In this study we demonstrate that characteristic alterations of the plasma protein composition occur in patients with neoplastic diseases. These alterations were strongly suggestive for the generation of anti-apoptotic capability.

Elevated plasma levels of fibrinogen γ-chain dimer (Fig. 1), as observed specifically in plasma of cancer patients, are caused by transglutaminase activity and have been described as indicative for tumor-associated fibrin deposition and fibrinolysis (7). Fibrinolysis is mediated mainly via the plasma protease plasmin (8), which exerts pleiotropic effects (24, 25). Generally, plasma proteases, like metalloproteinases and plasmin, are capable of activating growth factors by cleavage of their inert proforms, a process essential during morphogenesis and chronic disease, e.g. chronic wounds (26, 27). Growth factors further mediate proliferative and survival signals to cells (28–30). Indeed, deregulation of hemostasis resulting in long lasting plasmin activation has been described to occur during neoplastic disease, which might account for the present observation (26). Extensive proteolytic activity of plasmin (31) may also be involved in the generation of proteolytic products of antitrypsin and laminin γ-chain (Fig. 1). Although low levels of degradation products of antitrypsin were also detected in several healthy controls (Table I), the amount detected in plasma of many cancer patients by far exceeded that of any controls (Fig. 1 provides a representative example). The same notion holds true in the case of the laminin fragments. Laminin is a basement constituent (32) that is degraded during invasive neoplasia preferentially by plasmin and plasma metalloproteinases (33–35). In addition, laminin has been described as constituent of blood platelets (36). Detectable plasma levels of laminin fragments may potentially indicate basement membrane destruction accompanying invasive disease as described for other laminin fragments detected in urine of cancer patients (37). However, one has to bear in mind that activation of plasmin and metalloproteinases is not restricted to neoplastic disease but may well occur during inflammatory diseases (38), restricting the potential use of their reaction products as diagnostic markers for neoplastic disease. Indeed, neoplastic disease is often accompanied by chronic local inflammation (39), which might result in latent protease activities and accumulation of proteolytic products in plasma. Laminin degradation facilitates tumor cell metastasis (40), and the resulting degradation products have also been described to mediate strong proliferative and survival activity (9).

Evidence for an apparently high proteolytic activity by proteases such as plasmin in plasma of cancer patients was observed in exactly those samples displaying highly elevated levels of protease inhibitors, represented by many acute phase proteins (Fig. 1). Indeed, the acute phase response observed in a vast majority of cancer patients might represent an adaptive response to the present proteolytic activity. Acute phase proteins are synthesized by the liver, and their synthesis rate is increased during inflammatory disease (41). During tumor growth these proteins have also been described to accumulate in peripheral blood (42).

Acute phase proteins themselves have been described to interfere with apoptosis induction. In the case of two protein family members, α1-acid glycoprotein and α1-antitrypsin, a direct anti-apoptotic mode of action has been demonstrated on tumor necrosis factor-induced apoptosis of hepatocytes (12). With respect to Fas-induced apoptosis we found some, but no statistically significant inhibition, by applying purified acute phase proteins such as α1-acidic glycoprotein, antichymotrypsin, α1-antitrypsin, and haptoglobin. However, we can-
not rule out a much stronger contribution of these proteins to the observed effects in vivo, as they may become modified during either inflammatory and/or malignant diseases, giving them properties potentially quite different from the commercially available proteins isolated from healthy donors (43).

Inhibition of the apoptotic pathway is a key strategy of various tumor cells to escape cell death, which may eliminate most initiated cells in a normal organism (1, 44). Indeed, apoptosis inhibition by extracellular factors has also been described in the case of soluble Fas (45), elevated extracellular K⁺ ions (5), and lysosphingolipids (6). Plasma signifying the above-described characteristic proteome alterations substantially inhibited Fas- or staurosporine-induced apoptosis in Jurkat cells in contrast to plasma derived from healthy individuals (Fig. 2). Jurkat cells served as a reference model, because they rapidly undergo apoptosis in a highly reproducible manner, and this model system has already been investigated extensively in our laboratory (18, 46). In addition, mechanisms of Fas-induced cell death have been described well (47). In search of a potential mechanism of action of plasma protein-mediated apoptosis inhibition, we observed that supplementation of plasma samples, featuring the respective characteristics, strongly inhibited the assembly of the CD95-DISC, hence indicating that interference occurs at very early stages in the apoptotic pathway. As apoptosis induction by the formation of a DISC is similar in many cell types, the obtained data with respect to the mechanism of interference with cell death induction may be of general validity.

We demonstrated that the generation of anti-apoptotic features of plasma is reflected by unique and characteristic alterations of the plasma proteome. Although we identified several candidate anti-apoptotic proteins in plasma of cancer patients, we cannot rule out the additional contribution of other factors. For example, soluble Fas described as elevated in plasma of cancer patients (45) could interfere with DISC assembly. However, we can rule out soluble Fas as a major contributor to interference with DISC assembly, as in our experimental settings the utilized anti-Fas antibody concentration exceeded the highest reported plasma-soluble Fas concentrations from cancer patients by several hundredfold. Our data rather suggest that a common set of multiple factors potentially act in a synergistic manner resulting in significant apoptosis inhibition. As a result, the described distinct proteome alterations proved to be highly indicative for apoptosis inhibition. Further research will be required to identify other factors, as well as the contribution of each single factor to the observed effects.

Acknowledgment—We thank Josef Gotzmann for critically reading the manuscript.

* This work was supported by Herzfelder-Stiftung, Vienna, Austria. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed: The Smurfit Inst. of Genetics, Trinity College, Dublin 2, Ireland. Tel.: 353-1-6081089; Fax: 353-1-6798558; E-mail: Christopher.Gerner@univie.ac.at.

REFERENCES

3. Harris, C. C. (1996) p53 tumor suppressor gene: from the basic research laboratory to the clinic—an abridged historical perspective. Carcinogenesis 17, 1187–1198
20. Trauth, B. C., Kla, C., Peters, A. M., Matzku, S., Muller, P., Falk, W.