Plasma Vitronectin Polymorphism in Normal Subjects and Patients With Disseminated Intravascular Coagulation

By Maureen G. Conlan, Bianca R. Tomasini, Renee L. Schultz, and Deane F. Mosher

VITRONECTIN, or serum spreading factor, is a major cell adhesion protein of plasma. Other adhesive glycoproteins in the circulation include fibrinogen, fibronectin, and von Willebrand factor (vWF). Although these proteins have similar functions and have an Arg-Gly-Asp cell recognition sequence, they are structurally and immunologically distinct. VITRONECTIN was recently demonstrated to be identical to S-protein, the inhibitor of formation of the membrane lytic complex of complement. VITRONECTIN may regulate blood coagulation by inhibiting the rapid inactivation of thrombin by antithrombin III in the presence of heparin. A trimolecular complex is formed among vitronectin, thrombin, and antithrombin in serum and when the purified proteins are mixed together. The plasma concentration of vitronectin is 200 to 400 \( \mu g/mL \), and significant amounts are present in urine and amniotic fluid. VITRONECTIN has also been found in the extracellular matrix of tissues.

Without reduction vitronectin migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a broad band of molecular weight (mol wt) 75,000. With reduction two major bands are seen of mol wt 75,000 and 65,000. The presence of the 65,000-mol wt band is due to cleavage near the carboxyl terminus. The two fragments of mol wt 65,000 and 10,000 are held together by a disulfide bridge. VITRONECTIN has been cloned independently from two different human liver cDNA expression libraries and has been detected by immunobassay in the culture medium of human HepG2 cells but not in media of cell lines and strains derived from a variety of nonhepatic human tissues. These observations suggest that the liver is the major source of plasma vitronectin.

Vitronectin may be important in certain disease states. For this reason the investigators have surveyed a variety of patient plasmas by a quantitative enzyme-linked immunosorbent assay (ELISA) and have used the immunoblot technique to study the various forms of vitronectin present in the plasmas. The investigators concentrated on patients with evidence of disseminated intravascular coagulation because they were interested in whether they could find evidence for fragmentation of vitronectin or disulfide-bonded complex formation between vitronectin and thrombin-antithrombin.

**MATERIALS AND METHODS**

**Purification of vitronectin.** Vitronectin was purified from freshly frozen citrated human plasma (obtained from Badger Red Cross, Madison, WI) according to the method of Dahlbäck and Podack.

**Antibodies.** Mouse monoclonal antihuman vitronectin was used in the form of spent media from hybridoma cells obtained from Dr Ed Hayman and colleagues (La Jolla Cancer Research Foundation, La Jolla, CA). Mouse monoclonal antihuman S-protein was obtained from Boehninger Mannheim, Indianapolis. Alkaline phosphatase-conjugated rabbit antimouse IgG was from Sigma Chemical Co, St Louis. Rabbit antisera to human plasminogen, alpha-1-antitrypsin, antithrombin III, prothrombin, alpha-2-antiplasmin, and alpha-2-antiplasmin-plasmin complex neoantigen and goat anti-serum to human neutrophil elastase were from Calbiochem. Peroxi-
dase-conjugated goat or sheep secondary antibodies were obtained from Cappel Laboratories, Westchester, PA.

**Plasma samples.** Blood samples from nine healthy laboratory volunteers were obtained by venipuncture and put into polypropylene tubes containing 1/10 volume of 3.8% sodium citrate or 3.8% sodium citrate plus protease inhibitors (4 mmol/L EDTA, 200 U/mL aprotinin [Boehringer Mannheim], 8 mmol/L epsilon-aminocaproic acid [Sigma], and 20 µmol/L D-phenylalaninyl-L-prolyl-L-arginine chloromethyl ketone [Calbiochem]). The plasma was immediately spun down and frozen at −70°C. Repeat samples were obtained 1 week later from five of the nine volunteers and were processed into citrated plasma as above or into serum after the samples were allowed to clot for 30 minutes at 37°C in glass tubes.

Plasma from patients with evidence of disseminated intravascular coagulation (fibrinogen/fibrin degradation products >32 µg/mL) complicating various disease states were from a sample bank of the Special Coagulation Laboratory of the University of Wisconsin Hospital and Clinics. The samples had been collected between 1980 and 1987 in 1/10 volume 3.8% sodium citrate and stored in aliquots at −70°C after completion of diagnostic coagulation studies. Normal plasma samples were collected and treated in a similar manner. The normal plasma pool was from 12 donors with normal screening coagulation studies. Frozen samples for family studies had been obtained with informed consent by Dr Ed Azen, Departments of Medicine and Medical Genetics, University of Wisconsin, and had been made available to us after completion of his studies.

**Immunoblotting.** Plasma was diluted 1:200 for reduced samples and 1:50 for nonreduced samples in 10 mmol/L Tris, 150 mmol/L sodium chloride, pH 7.4 (Tris-buffered saline). Serum was used at a dilution of 1:50. N-ethyl maleimide (NEM, Pierce Chemical Co., Rockford, IL) was added to all the nonreduced samples at a final concentration of 3 mmol/L to minimize thiol-disulfide exchange of the denatured proteins. Samples were mixed with 1/4 volume of reducing (10% glycerol, 10% SDS, and 10% beta-mercaptoethanol in deionized distilled water) or nonreducing (same but without beta-mercaptoethanol) denaturant and boiled for three minutes immediately prior to electrophoresis. Samples were separated by SDS-PAGE on 8% gels and electroblotted onto nitrocellulose filters (Schleicher and Schuell, Keene, NH). One lane of each blot containing the molecular wt (mol wt) markers were stained for protein with 0.1% naphthol blue black in 45% methanol and 10% acetic acid. The remainder of the blot was soaked in Tris-buffered saline containing 3% bovine serum albumin (BSA) for one hour at 37°C, rinsed in Tris-buffered saline, and incubated overnight in Tris-buffered saline containing 1% normal goat serum and 1% to 10% appropriate rabbit antisera or 10% spent medium from mouse antivitronectin hybridoma cells. Blots were then rinsed in Tris-buffered saline and incubated for one hour at room temperature in Tris-buffered saline containing 1% normal goat serum and 1% peroxidase-conjugated goat antirabbit or antimouse IgG. Blots were rinsed again in Tris-buffered saline and incubated in 4-chloro-1-naphthol and hydrogen peroxide substrate solution (Kirkegaard and Perry, Gaithersburg, MD). The lowest amount of vitronectin that could be adequately visualized on the immunoblot was 5 ng/lane. Mol wts of immunostained bands were estimated from plots of the distance migrated by the standards relative to the front (Rf) vs log mol wts of the standards. Standards included fibrinogen subunits, 200,000; phosphorylase, 93,000; bovine albumin, 68,000; ovalbumin, 43,000; and chymotrypsin, 24,500. Some blots were scanned in the reflectance mode on a Transidyne RFT densitometer.

**ELISA.** An indirect or competition ELISA was used to quantify plasma vitronectin. The antibody to vitronectin used in the assay was mouse monoclonal anti-S-protein. This particular monoclonal was chosen because it does not preferentially recognize vitronectin that is complexed to thrombin-antithrombin. Microtiter flat-bottom, 96-well plates were coated with vitronectin by incubating with 200 µL/well of 2 µg/mL vitronectin in 0.1% BSA in Tris-buffered saline for two hours. Plasma samples to be assayed were diluted in Tris-buffered saline, and 250 µL were incubated for 30 minutes with 250 µL of mouse anti-S-protein antibody diluted 1:1,000 to 1:4,000 in 0.5% BSA in Tris-buffered saline. Duplicate wells received 200 µL of plasma sample-antibody mixture and were incubated for 45 minutes. Plates were washed four times with 0.05% Tween 20 in Tris-buffered saline and incubated with 200 µL/well of a 1:800 dilution of alkaline phosphatase-conjugated rabbit antimouse IgG in Tween 20/Tris-buffered saline. Plates were washed and incubated at 37°C with p-nitrophenol phosphate (1 mg/mL in Tris-buffered saline, pH 9) until maximum A405 nm was 0.7. A minimum of three twofold dilutions of each plasma sample were analyzed in duplicate. Individual plasma values were expressed as a percent of pooled normal human plasma and were estimated from a plot of A405 nm vs log dilution of pooled plasma. Compared with wells receiving no vitronectin, color development was inhibited approximately 50% by pooled plasma at a dilution of 1:200. For reasons that are not understood, dilutions of a mixture of purified vitronectin, approximately 400 µg/mL, and 3% albumin yielded a steeper inhibition curve than pooled normal plasma. Therefore the authors cannot express their results as µg/mL vitronectin standard. The coefficient of variation of the assay was 9%. In all instances a visual estimate of amount of vitronectin antigen detected by immunoblotting agreed with the ELISA done on the same sample.

**Correlation with clinical and laboratory information.** The clinical diagnoses and treatments of the patients were noted, along with autopsy results when available. A database was formed based on measurements of serum albumin, transaminases, bilirubin, alkaline phosphatase, prothrombin time, activated partial thromboplastin time, fibrinogen, fibrin degradation products, antithrombin III, and alpha-2-antiplasmin when these tests were done on the same day that the plasma was sent for special coagulation studies. For those patients classified as having liver failure, the diagnosis was made at the time of hospitalization by attending physicians based on appropriate diagnostic information (eg, enzyme levels compatible with fulminant hepatitis or tomographic evidence of extensive hepatic metastases).

**RESULTS**

**ELISA results and clinical correlations.** Of the patients with evidence of disseminated intravascular coagulation, striking alterations of plasma vitronectin levels were found in patients with liver failure not due to metastases in whom the average value was 42% of normal (Fig 1). Vitronectin levels of patients with metastatic cancer and acute leukemia did not differ significantly from normal with average values of 88% and 86%, respectively. Patients with liver failure due to extensive metastases to the liver, in contrast, had an average level of 57% of normal. Some patients with disseminated intravascular coagulation and no evidence of liver disease had low vitronectin levels. The four lowest levels were found in patients with retained products of conception, Felty's syndrome complicated by myelofibrosis and sepsis, renal failure due to atheromatous emboli, and renal failure due to diabetes.

Correlations were found between plasma vitronectin and both antithrombin III and fibrinogen levels (Figs 2 and 3). When antithrombin III was decreased below normal, vitronectin was similarly low (r = 0.64, Fig 2). The correlation

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were excluded from the analysis (Fig 3). In all patients from 0.71) when patients with evidence of extensive fibrinolysis between vitronectin and fibrinogen levels was greatest (r

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and 65,000 and a minor band of mol wt 45,000 (Fig 4). The

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alkaline phosphatase, on alpha-2-antiplasmin.

between vitronectin and albumin, bilirubin, transaminases,

oking time was prolonged. No significant correlation was observed

whom the vitronectin level was below 40%, the prothrombin

cantly different from normals

cient

correlation

percentage of pooled normal human plasma (NHP). The horizontal

lines indicate the mean (M) and SD. The liver failure, metastatic
cancer and liver failure, and miscellaneous groups were all signifi-
cantly different from normals (P < .01) when analyzed by Student’s t test.

Immunoblotting results. Immunoblots of reduced normal human plasma stained with mouse antivitronectin dem-

strated the presence of two major bands of mol wt 75,000

and 65,000 and a minor band of mol wt 45,000 (Fig 4). The

nonreduced samples revealed a major band of mol wt 75,000

and two minor bands of mol wt 140,000 and 125,000 (Fig 5). The

major band migrated as a closely spaced doublet when

NEM-treated plasma or serum was analyzed (Fig 5) and as a

single broad band when purified vitronectin or plasma not

treated with NEM was analyzed (not shown). Thus it is

probable that the doublets in Fig 4B do not represent

resolution of a heterogeneity of nonreduced vitronectin but

rather artificial “creasing” of the broad vitronectin band

by a comigrating protein from NEM-treated plasma. When

primary antibody was not added, no staining was observed

(not shown), indicating that none of the minor bands repres-

ents crossreactivity of the secondary antibody with human

IgG.

Variation among normal individuals was observed in the relative intensities of staining of the 75,000- and 65,000-mol

wt bands of reduced vitronectin. Three patterns appeared to

be present: primarily the 75,000-mol wt band, primarily the

65,000-mol wt band, and a roughly equal mixture of both

bands (Fig 4A). The variation was independent of gender

and plasma vitronectin concentration. When repeat plasma

samples were obtained a week later, identical banding pat-

terns were observed (Fig 4, A and C). Identical banding

patterns were found in plasma collected with and without

protease inhibitors (Fig 4, A and B) and in plasma and serum

(Fig 4, C and D).

Blots of plasma were probed with a number of antisera to
determine if complex formation between vitronectin and

another protein could account for the 140,000- and 125,000-

mol wt bands stained by antivitronectin in normal nonre-

duced plasma. No staining of the bands was observed with

antisera to antithrombin III, alpha-1-antitrypsin, prothrom-

bin, alpha-2-antiplasmin, alpha-2-antiplasmin-plasmin com-

plex neoantigen, or human neutrophil elastase (not shown).

Nonreduced serum showed more bands of high mol wt

than plasma (Fig 5, A and B). The bands of mol wt 160,000

and higher represent disulfide-bonded vitronectin-thrombin-

antithrombin III complexes.28 The composition of the

110,000-mol wt band was not further investigated.

<table>
<thead>
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<th>M</th>
<th>S.D.</th>
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Fig 4. Immunoblots with mouse antivitronectin of reduced normal human plasma and serum. Lanes a through h in panels A and B are reduced plasma samples from nine individuals drawn into sodium citrate without (A) or with (B) protease inhibitors. Five of the same individuals (c, d, e, g, and h) were rebled a week later and analyzed in panels C and D. Panel C contains samples of citrated plasma. Panel D contains samples of serum. Numbers are mol wt (in thousands) and point to bands mentioned in the text. Samples d, e, and g represent type 1-1, 1-2, and 2-2 banding, respectively. Reflectance densitometry scanning indicated that 67% of the staining in sample d is in the 75,000-mol wt band as compared with 42% in sample e and 16% in sample g.

Plasma samples from 68 patients with evidence of disseminated intravascular coagulation complicating various disease states were studied using the immunoblot technique. The intensities of staining on the immunoblots were compatible with ELISA results. The minor 45,000-mol wt band of reduced samples was observed sporadically. The minor 140,000- and 125,000-mol wt bands were observed in most but not all nonreduced samples and were especially prominent, along with a minor 130,000-mol wt band, in plasma of patients with acute promyelocytic leukemia. None of the nonreduced samples contained the 160,000- and greater mol wt bands found in serum. Plasma samples of patients with acute promyelocytic leukemia were also immunoblotted using monoclonal anti-S-protein. This antibody recognizes an epitope distinct from that recognized by the mouse antivitronectin, in particular an elastase cleavage product of vitronectin that is not detected by the monoclonal antivitronectin antibody on immunoblots (unpublished observation). No additional bands of low mol wt were identified with the anti-S-protein antibody, indicating that despite active fibrinolysis (and possible elastase release from the leukemia cells) there was no degradation of vitronectin.

There was the same marked variation in relative intensities of staining of the 75,000- and 65,000-mol wt bands of the reduced patient samples, as was seen in normal plasmas. There was no association of banding pattern with disease state, and the proportions of the three patterns in evaluable patient immunoblots were not different from normals when analyzed by the chi-square test (Table 1). When normals and patients were grouped together, 18% had mostly the 75,000-mol wt subunit, 22% had mostly the 65,000-mol wt subunit, and 59% had both (Table 1). This distribution was compatible with a Hardy-Weinberg equilibrium of two alleles present in the Wisconsin population at roughly equal frequency (Table 1). The inheritance of vitronectin types in 11 white families with 38 offspring is shown in Table 2. The data are consistent with the interpretation of autosomal codominant inheritance.

DISCUSSION

The two groups that cloned vitronectin did so with expression libraries from liver,6,7 and vitronectin secretion has been shown only for the HepG2 hepatoma cell line.19 The investigators found that plasma vitronectin levels were low in patients with disseminated intravascular coagulation and liver failure from a variety of etiologies. There was a high degree of correlation between plasma vitronectin and antithrombin III or fibrinogen. Antithrombin III and fibrinogen are synthesized by the liver,25,26 and both proteins may be markedly diminished in liver failure.27,28 Low fibrinogen is not specific for liver disease, since it may also be markedly reduced in consumptive disorders such as defibrination syndrome and fibrinolysis.27 Antithrombin III is one of the best predictors of liver failure, although it also may be consumed during disseminated intravascular coagulation.27,28 Kemkes-Matthes and colleagues30 recently found, using an electroim-
The two published vitronectin cDNA sequences determined differences in susceptibility to cleavage of the aminoterminus of vitronectin. The larger band (type 1-1), roughly equal amounts of both bands (type 1-2), and mostly the smaller band (type 2-2) and point to bands mentioned in the text.

Table 1. Distribution of 75,000 and 65,000 Mol Wt Bands in Reduced Plasma

<table>
<thead>
<tr>
<th>Group</th>
<th>Majority 75,000 (1-1)</th>
<th>Both 75,000 and 65,000 (1-2)</th>
<th>Majority 65,000 (2-2)</th>
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</thead>
<tbody>
<tr>
<td>Normals</td>
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<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Patients</td>
<td>12</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>61</td>
<td>23</td>
</tr>
<tr>
<td>proportion</td>
<td>0.18</td>
<td>0.59</td>
<td>0.22</td>
</tr>
<tr>
<td>Expected*</td>
<td>24</td>
<td>51</td>
<td>28</td>
</tr>
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</table>

*Calculated assuming that the frequency of allele 1 is 0.48 and the frequency of allele 2 is 0.52.

Fig 5. Immunoblots with mouse antivitronectin of nonreduced normal human plasma and serum. (A) plasma samples; (B) serum samples. These samples were from the five individuals, lanes c, d, e, g, and h, analyzed in Fig 4. C and D. Numbers represent mol wt (in thousands) and point to bands mentioned in the text.

Minor bands staining for vitronectin were noted in reduced samples of normal plasma at mol wt 45,000 and in nonreduced samples at mol wt 140,000 and 125,000. The 45,000-mol wt band probably represents a cleavage product. There is a precedence for circulating vitronectin cleavage products in somatomedin B, a fragment of mol wt 5,000 derived from the aminoterminus of vitronectin. The larger bands must represent disulfide-bonded complexes of vitronectin or of vitronectin and other protein(s). As described in the introduction, the investigators suspected that patients with defibrination syndrome might have increased vitronectin degradation products. Such bands were not found.

As others have noted, the relative intensities of the 75,000- and 65,000-mol wt bands of vitronectin vary among individuals. The investigators found that for an individual the relative intensities of the two bands were constant over a week's time and were not influenced by the method of blood collection. Individual patterns were of three types: mostly the larger band (type 1-1), roughly equal amounts of both bands (type 1-2), and mostly the smaller band (type 2-2). The distribution of individuals in these categories was approximately 1:2:1 and was compatible with a Hardy-Weinberg equilibrium of two alleles. Family studies are consistent with autosomal codominant inheritance of these two alleles. Therefore it appears that the differences in the ratios of the 75,000- and 65,000-mol wt bands are due to individually determined differences in susceptibility to cleavage of the 75,000-mol wt nascent polypeptide. Such cleavage could take place intracellularly during post-translational processing or after secretion. The difference in susceptibility could be due to an inherited polymorphism of the protease responsible for the cleavage or to an inherited polymorphism of vitronectin. The two published vitronectin cDNA sequences predict a polymorphism at residue 381, which can be methionine or threonine. This polymorphism is very close to the presumptive cleavage site between residues 379 and 380.

Thus the two cDNA clones may represent the two alleles,
with the presence of one residue at position 381 favoring easy cleavage (predominance of the 65,000-mol wt band) and the presence of the other residue not favoring cleavage (predominance of the 75,000-mol wt band). The investigators are currently carrying out sequencing vitronectin of individuals with a predominance of one or the other bands to test this hypothesis. A similar situation, ie, an amino acid substitution influencing a nearby proteolytic cleavage, has recently been shown to account for part of the polymorphism of the PRH1 salivary protein locus.12

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