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CONCISE REPORT

Protein Co-Isolated With Human Tissue Factor Impairs Recovery of Activity

By Steven D. Carson, Sherman E. Ross, and Robert A. Gramzinski

Preparations of human tissue factor isolated by immunoaffinity chromatography contain variable amounts of 47,000 mol wt, 55,000 mol wt, and multimeric tissue factor when analyzed without reduction on polyacrylamide gels in sodium dodecyl sulfate (SDS). When analyzed after reduction, the 47,000 mol wt tissue factor apoprotein and a protein of about 12,000 mol wt are observed. Elution of tissue factor from polyacrylamide gel slices, followed by reassociation with lipids, restored proportionately much greater tissue factor activity with the 47,000-mol wt protein than with the 55,000-mol wt form. Cyanogen bromide cleavage at the single tissue factor methionine revealed that the 12,000-mol wt protein is associated with the carboxyl-terminal peptide derived from the 47,000-mol wt protein. These results reveal that association of the 12,000-mol wt protein with the cytoplasmic domain of tissue factor can modulate its activity in vitro.

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HUMAN TISSUE FACTOR, isolated from brain[1] and placenta[2] using factor VII-affinity chromatography, has been electrophoretically characterized as a 44,000- to 47,000-mol wt protein with variable proportions of an apparent dimer. Tissue factor preparations isolated by immunoaffinity[3] contain the 47,000-mol wt tissue factor protein, as well as variable amounts of a protein of about 55,000 mol wt. The 55,000-mol wt component is composed of the 47,000-mol wt–tissue factor apoprotein and a disulfide-linked protein of about 12,000 mol wt.[4] The origin and role of the 12,000-mol wt protein are not yet known.

MATERIALS AND METHODS

Bovine brain phosphatidylserine (PS) and egg yolk phosphatidylcholine (PC) were from Sigma Chemical Co (St. Louis). Bovine factors VII and X were prepared by established methods.[5-7] The factor VII was provided by Drs Arabinda Guha and Yale Nemerson. Working stocks were stored at −20°C in tris-saline (0.05 mol/L Tris/0.1 mol/L NaCl/0.01% NaN_3, pH 7.6) with 50% glycerol. Tissue factor was purified from human brain and placenta using monoclonal antibody (MoAb) HTF1-7B8.[3] The procedures involved homogenization of the tissue, dehydration with acetone, and extraction with chloroform/methanol (1/1), followed by extraction of the tissue powder with tris-saline/2% Triton X-100.[8] The Triton extract was passed over an Affi-Gel-HTF1-7B8 column, preceded by columns of Sephacryl S-200 and Affi-Gel-RIg9C[9] to preadsorb material that associates nonspecifically with the affinity matrix. Four samples were prepared with some procedural variations and are referred to as 1 to 4, as numbered in Fig 1. Tissue homogenizations were all done in the presence of 1 mmol/L dithiothreitol (DTT). The homogenate for preparation 1 was obtained from Dr D. Hagerman (University of Colorado School of Medicine) following homogenization of a fresh placenta in potassium salts/1 mmol/L EDTA/0.1 mmol/L androstenedione/0.4 mmol/L phenylmethylsulfonylchloride. The other three homogenizations were done in the presence of 5 mmol/L EDTA/1 mmol/L phenylmethylsulfonylfluoride (PMSF) and either 20 mmol/L benzamidine (preparations 2 and 3) or 10 mmol/L benzamidine (preparation 4). Tissue factor was extracted from the tissue powders with 2% Triton X-100 in tris-saline (preparation 1), containing 10 mmol/L benzamidine (preparation 2), 10 mmol/L benzamidine and 5 mmol/L EDTA (preparation 3), or 10 mmol/L benzamidine, 5 mmol/L EDTA, 1 mmol/L PMSF, and 1 mmol/L DTT (preparation 4). After the 2% Triton X-100 extract was applied, the Affi-Gel-HTF1-7B8 column was sequentially washed with 0.1% Triton X-100 (preparation 1), and 0.1% Triton X-100 with 5 mmol/L EDTA (preparation 2), 2% Triton X-100 followed by 0.1% Triton X-100/5 mmol/L EDTA (preparation 3), or 2% Triton X-100/5 mmol/L EDTA, followed by 0.1% Triton X-100 (preparation 4), all solutions made in tris-saline. After washing, the column was eluted with 4 mol/L guanidinium chloride in tris-saline/0.1% Triton X-100. The eluted tissue factor was dialyzed against tris-saline/0.1% Triton X-100, concentrated on an Amicon YM-30 membrane to about 3 mL, and precipitated with 4 vol cold acetone. Tissue factor was redissolved in tris-saline/0.1% Triton X-100 to give 0.4 to 0.9 mg/mL.

Tissue factor activity was determined using a continuous chromogenic assay[8,10] following relipidation with PS/PC (30/70).[11] Polyacrylamide gel electrophoresis (PAGE) was conducted in sodium dodecyl sulfate (SDS)[12] using prestained standards (Diversified Biotech, Newton Center, MA). Gels stained with Coomassie blue R250 were scanned with a Helena Quick Scan densitometer using a 525-nm filter. Western blots[13] were conducted using tissue factor electrophoretically transferred to nitrocellulose (in 0.01 mol/L Tris HCl pH 8.1). The transfers were blocked with bovine serum albumin (BSA, 50 mg/mL in tris-saline), then incubated with HTF1-7B8 conjugated to horseradish peroxidase.[14] Immunoreactive proteins were detected with H_2O_2 and diaminobenzidine.

Tissue factor activity was recovered from unstained polyacrylamide gels run with nonreduced samples.[15-17] Gel slices (1 mm) were placed in 100 μL of tris-saline/0.1% Triton X-100 at 4 °C overnight. One-microliter aliquots were combined with 10 μL PS/PC (2.5 mg/mL) in 0.25% sodium deoxycholate, diluted to 60 μL with tris-saline/1 mg/mL bovine serum albumin, then made 5 mmol/L in $CdCl_2$ by addition of 5.5 μL of a 50-mmol/L solution.[11]

Prior to cleavage with CNBr,[18] tissue factor was modified with N-ethylmaleimide.[19] To 42 μL of tissue factor, 5 μL of 10 mmol/L N-ethylmaleimide in water was added at 25 °C. After 15 minutes an additional 5 μL of reagent was added. After a total of 30 minutes reaction time, the modified tissue factor was divided into two aliquots one of which was retained as a pre-CNBr sample. Tissue factor in the second aliquot was acetone-precipitated and redissolved in 50 μL of 70% formic acid. One sample (25 μL) was taken for

From the Department of Pathology, University of Colorado Health Sciences Center, Denver, CO.

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Address reprint requests to Steven D. Carson, PhD, Department of Pathology, B216, University of Colorado Health Sciences Center, 4200 E Ninth Ave, Denver CO 80262.

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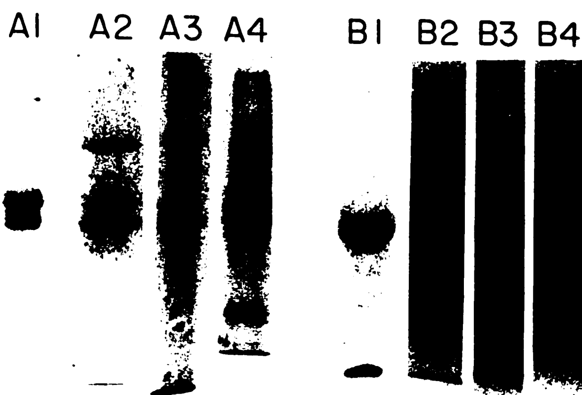

Fig 1. Polyacrylamide gel analyses of tissue factor preparations, before (A) and after reduction (B). The samples were from placenta (1 and 4) or brain (2 and 3). Sample 1 was analyzed on a 10% to 20% polyacrylamide gradient, and samples 2, 3, and 4 were analyzed on 10% to 15% polyacrylamide gradients.

acid-treatment control, and an additional 25 μL of 70% formic acid was added. The remaining 25 μL was made 15 mg/mL with CNBr by addition of 25 μL 30 mg/mL CNBr in 70% formic acid. After 12 hours at 25°C in the dark, the samples were dried in a vacuum centrifuge (Savant), redissolved in 50 μL water, and redried. The three aliquots were redissolved in water and Laemmli sample solvent,[12] which contained no reducing agent.

RESULTS

As expected from previous work,[1-4] all of the tissue factor preparations contained a principal component with electrophoretically estimated mol wt near 47,000 (Fig 1). This 47,000-mol wt protein is the predominant component in reduced samples (Fig 1B), accompanied by lesser amounts of a protein of about 12,000-mol wt (apparent in Fig 1B1) and occasionally by traces of residual dimer. An as yet unidentified contaminant of 25,000 to 30,000 mol wt was observed in sample 4. When analyzed without reduction, variable proportions of proteins larger than 47,000-mol wt are apparent. The presumably dimeric form of tissue factor $(TF)_2$ has been observed in all reports to date,[1-3,8,20] but few reports mention the 55,000-mol wt protein.[4,8] This 55,000-mol wt protein, which varies in amount among preparations, is comprised of the 47,000-mol wt tissue factor protein associated with a protein of 12,000-mol wt by disulfide bonds (TF + 12K), in agreement with other reports (G. Broze, personal communication).[4,8] As is evident from Fig 1, the 55,000-mol wt protein would not have been observed when samples were examined only after reduction, and the 12,000-mol wt protein is not evident in the gels with lower polyacrylamide concentrations (Fig 1B2-B4).

The results summarized in Fig 2 demonstrate that the 55,000-mol wt protein and the multimeric proteins are derived from the tissue factor apoprotein. As shown in the Western blot probed with HTF1-7B8 (Fig 2D), each of the proteins stained with Coomassie blue R250 (Fig 2C) were immunologically reactive as tissue factor. When tissue factor was eluted from gel slices, reconstituted with PS/PC, and assayed, the 47,000-mol wt protein contained most of the

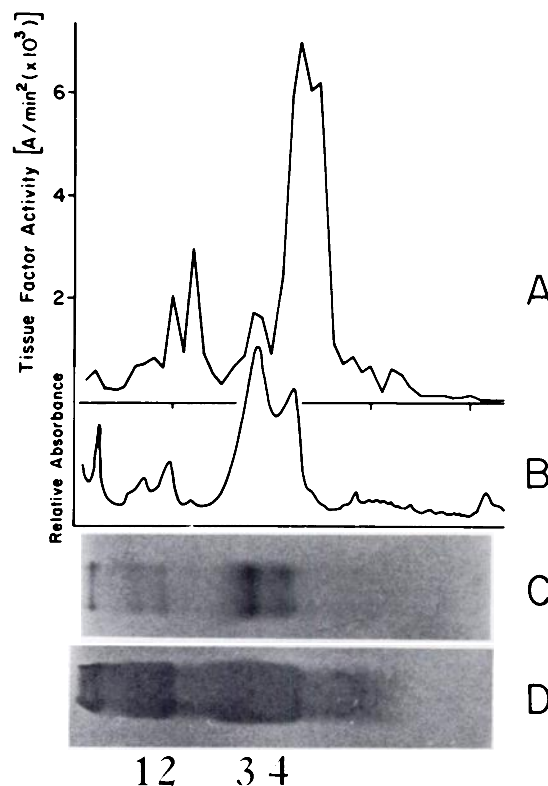

Fig 2. Electrophoretic characterization of nonreduced placental tissue factor. The stained 10% polyacrylamide gel (C) was densitometrically scanned (B). Unstained lanes were transferred to nitrocellulose and blotted with antibody HTF1-7B8 (D) or sliced, eluted, reconstituted, and assayed for active tissue factor (A). The gel top is to the left, and calculated molecular weights of the bands are (1) 112,000, (2) 93,000, (3) 55,000, and (4) 47,000. After reduction (Fig 1B1) only bands near 47,000 mol wt and 12,000 mol wt are observed, of which the 47,000-mol wt band is reactive with HTF1-7B8.[8]

recoverable tissue factor activity (Fig 2A). Summation of the tissue factor activity recovered from all of the gel slices accounted for 100% of the tissue factor applied to the gel, of which approximately 8% was contained in the 55,000-mol wt (TF + 12K) peak and 9% in the multimer peaks.

Tissue factor contains five cysteinyl residues of which four are amino-terminal and one carboxyl-terminal with respect to a membrane-spanning domain.[18] This distribution of cysteinyl residues is maintained with respect to the single methionine present in tissue factor,[18] and cleavage with CNBr separates the 47,000-mol wt protein into 41,000-mol wt amino-terminal and 6,000-mol wt carboxyl-terminal peptides (CNBr41K and CNBr6K). Analysis of CNBr-generated peptides, in the absence of disulfide bond reduction (Fig 3), revealed peptides of 41,000-mol wt and 18,000-mol wt. These peptides are expected if both 55,000-mol wt and 47,000-mol wt proteins yield identical amino-terminal CNBr peptides, while the 18,000-mol wt peptide is derived from the 6,000-mol wt carboxyl-terminal peptide associated with the 12,000-mol wt protein. These results demonstrate that the 12,000-mol wt protein associates with the carboxyl-terminal cysteine of tissue factor.

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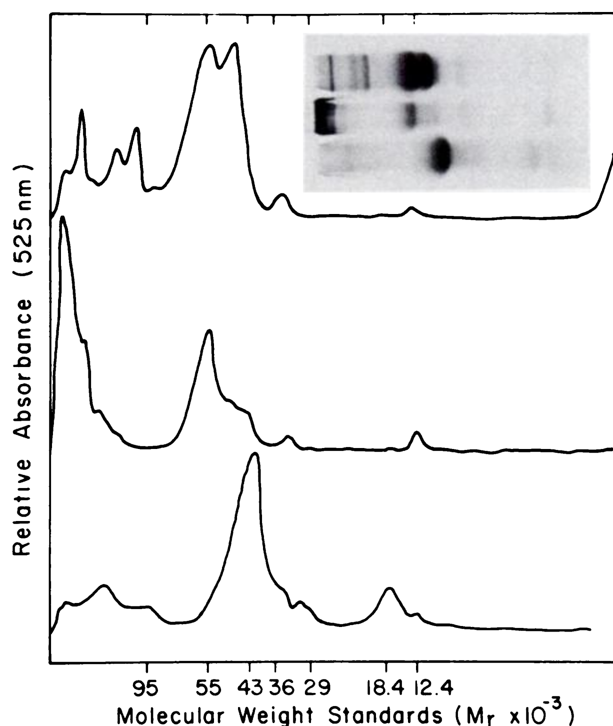

Fig 3. Placental tissue factor was reacted with CNBr and characterized electrophoretically on a 10% to 20% polyacrylamide gradient in the presence of SDS. The stained gel (inset) was scanned at 525 nm. The densitometric profiles and stained lanes correspond to (top) pre-CNBr sample, (center) acid treatment only, and (bottom) CNBr digest. The gel top is to the left.

DISCUSSION

There is broad agreement that human tissue factor is a single polypeptide with electrophoretically estimated mol wt near 47,000,[1-4] the gene has been mapped to the short arm of human chromosome 1,[21] and the cDNA sequence encodes only the 47,000-mol wt protein (the sequence actually predicts a nonglycosylated mol wt of 29,593 but accounts for the entire amino acid sequence of the 47,000-mol wt protein).[18] The present experiments have revealed that the 47,000-mol wt protein can be actively reconstituted with phospholipids, whereas relatively little activity can be reconstituted using the electrophoretically isolated 55,000-mol wt protein. This finding indicates that association of the 12,000-mol wt protein with the tissue factor apoprotein interferes with recovery of its procoagulant activity, and it is clearly not required for its activity. Considered with the genetic information,[18,22] these results further suggest that the 12,000-mol wt protein is not a "light chain" required for tissue factor expression but is capable of modulating its activity in vitro.

The amino acid sequence of human tissue factor[18] reveals that the molecule contains a large extracellular (glycosylated) amino-terminal domain and a small cytoplasmic carboxyl-terminal domain separated by a single membrane-spanning hydrophobic sequence. The cysteine with which the 12,000-mol wt protein associates is cytoplasmically disposed, whereas the factor VII-binding site is presumably in the extracellular domain. Presumably the reducible homodimer $(TF)_2$ is formed via a disulfide bond involving this cysteinyl residue on two tissue-factor molecules. If only a single cysteine is available on the 12,000-mol wt protein and this residue is involved in association with tissue factor, this heterodimer (TF + 12K) should not be able to form higher multimers, and the observed bands other than $(TF)_2$ are inexplicable. If, however, an additional cysteine is present on the 12,000-mol wt protein, composition of the higher mol wt bands can be predicted. Two bands are evident in Fig 2C (bands 1 and 2) corresponding to about 112,000-mol wt and 93,000-mol wt. An additional peak at slightly smaller molecular weight that corresponds to a peak of activity in Fig 2A is faintly apparent in Fig 2B. One interpretation of this pattern of proteins and tissue factor activity suggests that band 1, band 2, and the third peak might represent $(TF + 12K)_2$, (TF + 12K + TF), and $(TF)_2$, respectively. The experiment shown in Fig 3 used tissue factor and (TF + 12K), in which free sulfhydryls were modified by reaction with N-ethylmaleimide. When this preparation of tissue factor was digested with CNBr without prior modification of sulfhydryls, an additional band was observed near 34,000-mol wt (not shown). CNBr will promote the formation of mixed disulfides.[22] Considering the above model, the 34,000-mol wt species could be $(CNBr6K + 12K)_2$.

It remains unclear why the appearance of the (TF + 12K) species is so unpredictable among preparations. At present there is no evidence as to whether this species is an artifact that appears during isolation of tissue factor or whether it actually exists in an intact cell. If the association is artifactual, it is intriguing that the association is specific for the 12,000-mol wt protein and that other proteins with free sulfhydryls are not found in association with tissue factor. While the origin of the 12,000-mol wt–tissue factor complex remains debatable, the nature of this association and its effect on recoverable tissue factor activity have clearly been demonstrated in this study. Modulation of tissue factor activity by modification of its cytoplasmic domain is a possible mechanism by which cells could mediate the initiation of coagulation. Insofar as very little is known with regard to tissue factor expression and inhibition in vivo, the effects of this 12,000-mol wt protein observed in vitro are relevant to formulating and testing models of tissue factor regulation.

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NOTE ADDED IN PROOF

The 25,000 to 30,000-mol wt protein in preparation 4 has been identified as the light chain of the monoclonal antibody HTF1-7B8. It has been eliminated by omitting DTT from the 2% Triton extraction.

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