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The Transglutaminase in Vascular Cells and Tissues Could Provide an Alternate Pathway for Fibrin Stabilization

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A thrombin-independent transglutaminase (TG) has been identified in vascular cells and tissues from human, rabbit, rat, porcine, and bovine sources. The vascular TG had several properties that were similar but not identical to guinea pig liver TG. Both enzymes had similar chromatographic and electrophoretic properties, preferentially cross-linked the α-chains of fibrinogen, and reacted with polyclonal and monoclonal anti-guinea-pig liver TG antibodies. However, the TG from adult bovine aortic endothelial (ABAE) cells exhibited a novel Ca^{2+}/Mg^{2+} dependence for enzymatic activity that was distinct from that of purified guinea pig liver TG. The mol wt of the vascular TG (79 ± 3 kd) determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was slightly lower than the purified guinea pig liver TG (85 ± 9 kd). The TG antigen was detected by immunohistochemical techniques in association with the endothelial and smooth muscle cells of arteries, veins, venules, and capillaries. The TG antigen also codistributed with the fibronectin antigen along the hepatic sinusoids. The ABAE cell TG cross-linked α_2-plasmin inhibitor to fibrinogen and caused the modified fibrinogen to be 40-fold more resistant to plasminolysis. A thrombin-independent TG in vascular cells of blood vessels could provide an alternate pathway to inhibit fibrinolysis and promote fibrin stabilization.
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TRANSGLUTAMINASES (TGs) (R-glutaminyl-peptide: amine-γ-glutamyltransferase, E.C. 2.3.2.13) catalyze the formation of covalent bonds between peptide-bound glutamyl residue and either a lysyl group in a protein or a primary amino group in polyamines. Although TG activity has been detected in several tissues and cells,[1,2] the physiologic function of the cell-associated TG is poorly defined.[1] The plasma TG, factor XIIIa, has been traditionally regarded as the enzyme that catalyzes the final steps in the formation of a stable fibrin clot. The important factor XIIIa-mediated reactions that stabilize fibrin include cross-linking of fibrin monomers to each other[3] as well as cross-linking of fibronectin[4] and α_2-plasmin inhibitor[5] to fibrin.

Laki et al[6] first reported that TG activity was increased in the sclerotic portion of aortas from cholesterol-fed rabbits as compared with the healthy-appearing segments. However, the biochemical and immunologic characterization of TG activity was not established. Shainoff and Page[7] reported that insoluble fibrinogen was present in atherosclerotic human aortas and proposed that a thrombin-independent TG could have led to fibrinogen accumulation in the wall of the blood vessel. These observations prompted us to pursue the characterization of the TG activity in vascular cells and tissues.

We have found that cultured adult bovine aortic endothelial (ABAE) cells and vascular smooth muscle (VSM) cells contain a thrombin-independent TG that is biochemically and immunologically similar but not identical to the guinea pig liver TG. The TG antigen was localized to endothelial and smooth muscle cells in human arterial and venous tissues and along the sinusoids in the liver. Vascular TG cross-linked fibrinogen to itself and also cross-linked α_2-plasmin inhibitor to fibrinogen. The α_2-plasmin inhibitor cross-linked fibrinogen was 40-fold more resistant to plasminolysis than was native fibrinogen. The vascular TG could provide an alternate pathway toward fibrin stabilization and inhibit fibrinolysis.

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MATERIALS AND METHODS

Tissue culture dishes were purchased from Falcon Plastics, Oxnard, CA. Tissue culture medium and trypsin were obtained from GIBCO (Grand Island, NY), and calf serum and fetal calf serum (FCS) were from Hyclone Laboratories (Logan, UT). [^{3}H]-Putrescine, Aquasol 2, and Protosil were from New England Nuclear (Boston). Hammerstein casein was purchased from United States Biological Laboratories (Cleveland). BioRad protein assay kit, nitrocellulose membranes, and Enzymobead reagent were from BioRad, Richmond, CA. Diethylaminoethyl (DEAE)-Sephacel was obtained from Sigma, St Louis. Human α-thrombin was supplied by Dr John Fenton, II, New York State Department of Health, Albany, NY. Sephadex G-25M, PD-10 columns were from Pharmacia Fine Chemicals, Piscataway, NJ. Factor XIII-free fibrinogen was prepared as previously described.[8] Rabbit aortic and human aortic and venous tissues were generously provided by Dr Per Otto Hagen, Department of Surgery, Duke University. Pig aorta was purchased from the local slaughterhouse. Affinity-purified anti-guinea-pig liver TG antibody[9] was a generous gift from Dr Peter Davies, Department Pharmacology, University of Texas, Houston. Monoclonal antibody (CUB-7401) against guinea pig liver TG[10] was kindly supplied by Dr Paul Birckbichler, Samuel Roberts Noble Foundation, Ardmore, OK. Goat anti-mouse horseradish peroxidase and goat anti-rabbit horseradish peroxidase conjugates were from BioRad. Anti-factor XIII (A-subunit) antisera from rabbits, rabbit antifibrinogen antisera, and rabbit anti-human fibronectin antisera

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were purchased from Calbiochem, La Jolla, CA. Purified human α_2-plasmin inhibitor was purchased from American Diagnostica, Greenwich, CT. Rabbit antisera to human α_2-plasmin inhibitor was obtained from American Diagnostica, Greenwich, CT.

Tissue culture techniques. ABAE cells and bovine vascular smooth muscle (VSM) cells were cultured by previously described methods on 10-cm plastic culture plates.[11,12] ABAE and VSM cells were used between passages 2 and 15. Confluent ABAE cells reached a density of 900 cells/mm^2. ABAE cells were characterized by the presence of Weibel-Palade bodies[13] and factor VIII-related antigen.[14] VSM cells were characterized by their typical morphology and absence of factor VIII-related antigen. ABAE and VSM cells were cultured with fibroblast growth factor purified from bovine brain.[15] Porcine aortic endothelial cells were cultured by previously described methods in tissue culture flasks and used during the third passage.[14] Rabbit vascular (thoracic aorta) smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

Cell harvesting. Cell monolayers were dissociated by exposure (2 to 3 minutes at 22°C) to trypsin. When cells were rounded (monitored by inverted-phase contrast microscope), the trypsin solution was removed and 5 mL medium supplemented with 10% serum was added to inhibit trypsin. Cells were washed three times with 20 mmol/L Tris-HCl, 130 mmol/L NaCl, pH 7.4 (TBS). After the third wash, the cells from each dish were resuspended in 0.1 to 0.2 mL of 50 mmol/L Tris-HCl, 25% glycerol, pH 8.5. Phenylmethyl sulfonyl fluoride (PMSF) (0.2 mmol/L) was added immediately prior to sonication to limit proteolysis. In a few experiments, cells were removed from tissue culture dishes by scraping with either a rubber policeman or a cell scraper after the monolayers were washed twice with media.

TG assay. Washed and resuspended cells were sonicated three times at 4°C for 15 seconds at 40% output using a model W-220 sonicator, Heat Systems-Ultrasonics, Farmingdale, NY. The protein concentration of the sonicated cells was determined using the BioRad Protein Assay. Cell sonicates (10 to 100 μg) were assayed for TG activity in presence of either 5 mmol/L $CaCl_2$ or 10 mmol/L EDTA, as described previously.[16]

Agarose gel electrophoresis. Sonicates of cultured ABAE cells were incubated with DEAE-Sephacel in TBS and washed. The protein bound to the DEAE was eluted with 0.4 mol/L NaCl. The eluate was subjected to electrophoresis in gels cast from *N,N'*dimethylcasein (0.3%) and agarose (1%) in 75 mmol/L barbital buffer containing 1 mmol/L EDTA.[17] After electrophoresis, the gels were stained for TG activity using monodansylcadaverine.[17]

Immunoblotting of ABAE cell TG, guinea pig liver TG, factor XIII, fibrinogen and α_2-plasmin inhibitor, antigens after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The ABAE cell sonicates or guinea pig liver TG were taken up in a solution containing 1% sodium dodecylsulfate (SDS), 1.5 mol/L urea, 2.5 mmol/L EDTA, 30 mmol/L Tris, 5% 2-mercaptoethanol, and 0.001% bromophenol blue, and were boiled for 5 minutes. The material was then fractionated by slab-gel electrophoresis in an 8% discontinuous polyacrylamide gel (SDS-PAGE) using a 3% stacking gel.[18] The proteins were then transferred to nitrocellulose membrane.[19] The membrane was then incubated with bovine serum albumin (BSA) (3%) in TBS and later reacted with a monoclonal antibody to guinea pig liver TG (CUB-7401) or control unreactive antibody (CUB-11) at 1:1,000 dilution for 8 to 12 hours at 4°C with gentle shaking. The membrane was washed with TBS and then reacted with goat anti-mouse horseradish peroxidase conjugate (1:2,000 dilution) 6 hours at 4°C. In other experiments, the membrane was reacted with affinity-purified anti-guinea-pig liver TG antibody followed by reaction with rabbit anti-goat horseradish peroxidase conjugate, in the manner described above. A similar procedure was used to detect factor XIII, α_2-plasmin inhibitor and fibrinogen antigens. The membrane was reacted with either anti-factor XIII antisera (1:2,000 dilution) anti-α_2-plasmin inhibitor antisera (1:1,000 dilution), or antifibrinogen antisera (1:1,000), followed by reaction with goat anti-rabbit horseradish peroxidase conjugate (1:2,000 dilution). After being washed, the membranes were stained with a color reagent from BioRad.

Purification of TG. Plasma factor XIII was purified as previously described[8] and stored at −70°C. Guinea pig liver TG was purified by DEAE column chromatography.[20]

HPLC gel filtration and DEAE column chromatography of guinea pig liver TG and ABAE TG. Confluent ABAE sonicates and purified guinea pig liver TG preparations were centrifuged at 150,000 g for 60 minutes in a Beckman air-driven ultracentrifuge. The supernatant containing at least 90% of the total enzyme activity was injected into an HPLC gel filtration column (BioSil TSK-125, BioRad) and eluted with 50 mmol/L Tris-HCl pH 8.5. Five-hundred-microliter fractions were collected at a flow rate of 1.0 mL/min. The fractions were then assayed for TG activity.

Confluent ABAE cell TG was partially purified by adsorption to a column of DEAE-Sephacyl. The sonicate was applied to a 15-mL column of DEAE-Sephacyl previously equilibrated with 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 200 μmol/L PMSF, pH 8.5. The column was then washed with 200 mL of the same buffer, and the enzyme was eluted using a linear (0 to 1 mol/L) sodium chloride gradient in the same buffer. Two-milliliter fractions were collected at a flow rate of 30 mL/hour. The column was operated at 4°C. The fractions were then checked for TG activity. Purified guinea pig liver TG was also applied to the same column under similar conditions and its elution was monitored similarly.

Identification of fibrinogen cross-linking pattern. ABAE cell sonicate (12.5 μg) was incubated with purified fibrinogen (60 μg) in the presence of 25 mmol/L DTT, 1.0 mmol/L calcium chloride at 22°C for 60 minutes. The reaction was stopped as described in the preceding section. SDS-PAGE was performed in an 8% polyacrylamide gel. The gel was stained with Coomassie blue and then destained. Purified fibrinogen was electrophoresed under similar conditions and served as markers for α-, β-, and γ-chains. High- and low-mol-wt protein mixtures (BioRad) were electrophoresed simultaneously.

Identification of TG activity in vascular tissues. Human saphenous vein (672 mg) was cut into 2-mm pieces and homogenized five times in 3.0 mL of 0.1 mol/L Tris-HCl, pH 8.5, using a Tissumizer (Tekmar, Cincinnati) at 80% output for 30 seconds. The homogenate was sonicated three times for 15 seconds at 60% output using a cell sonicator. The sonicated material was centrifuged for 15 minutes at 10,000 g, and the supernatant was filtered through a Millex-HA 0.45 μmol/L filtration unit. All procedures were performed at 4°C. The final extract, which had a protein concentration of 3.72 mg/mL, was kept at 4°C until assayed. The enzyme was assayed within 2 hours of homogenization. All other tissues were homogenized and sonicated as described above.

Immunohistochemistry of TG and fibronectin antigens in human tissues. Immunoperoxidase testing was performed by the standard "ABC" method as previously described[21] using frozen sections of various human and animal tissues. When liver was used, endogenous biotin binding was first blocked using excess avidin as described.[21] Sections were stained with diaminobenzidine and 0.03% H_2O_2 and counterstained with hematoxylin. As controls, isotype-specific monoclonal antibodies or culture supernate from the nonreactive fusion partner were used in place of the monoclonal anti-TG antibody supernate.

The distribution of TG in human liver was compared with that of fibronectin. In these experiments, frozen sections were cut as above and, after preincubation with normal goat serum, were stained with

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rabbit antifibronectin antisera at a dilution of 1:2000, followed by biotinylated goat anti-rabbit IgG, and then avidin and biotinylated horseradish peroxidase. Normal rabbit serum and/or mouse monoclonal antibodies were also substituted as controls. Tissues for these studies came from the frozen bank in the immunopathology laboratory at Duke University Medical Center.

Enzyme-linked immunosorbent assay for tissue TG. Enzyme-linked immunosorbent assay (ELISA) for guinea pig liver TG was conducted as described by Birckbichler et al.[10]

Effect of TG-catalyzed cross-linking of α_2-plasmin inhibitor to fibrinogen on plasminolysis. Fibrinogen (32 μg) was incubated with confluent ABAE cell sonicate (5 μg cell protein) and α_2-plasmin inhibitor (4 μg) in a total volume of 50 μL of 0.02 mol/L Tris-HCl, pH 7.4, containing 130 mmol/L NaCl and 5 mmol/L calcium chloride, for 60 minutes at 37 °C. In some incubations, 10 mmol/L dithiothreitol was also present. Reactions were also performed in the presence of 5 mmol/L EDTA. The reactions were stopped by 1% SDS, 1.5 mol/L urea, 2.5 mmol/L EDTA, 30 mmol/L Tris, 5% 2-mercaptoethanol, and 0.001% bromphenol blue, and boiled for 5 minutes. Samples were separated by SDS-PAGE on a 6% to 15% linear polyacrylamide gradient and subjected to immunoblotting as described previously.

ABAE cell sonicate (10 μg) was incubated with fibrinogen (100 μg) and purified α_2-plasmin inhibitor (10 μg) in a total volume of 50 μL of 0.02 mol/L Tris-HCl, 0.13 mol/L NaCl pH 7.4, 5 mmol/L $CaCl_2$ for 30 minutes at 37 °C. Similar reactions were performed either in the presence of 5 mmol/L EDTA or by omitting one of the components from the assay. Fibrinogen was precipitated by protamine sulfate (2 mg/mL), and washed by centrifugation in 0.1 mol/L Tris-HCl, pH 7.4, containing protamine sulfate 2 mg/mL. Fibrinogen was resuspended in 0.02 mol/L Tris-HCl, 0.13 mol/L NaCl, pH 7.4, at a concentration of 0.4 mg/mL and incubated with plasmin (0.005 to 0.16 CU/mL) for 10 minutes at 37°C. Samples were solubilized in 1% SDS, 1.5 mol/L urea, 2.5 mmol/L EDTA 30 Tris, pH 6.8, and electrophoresed on a 4% to 15% gradient SDS-polyacrylamide gel. Gel was stained with Coomassie blue and dried, and the degradation of the fibrinogen was quantitated by scanning densitometry. The absorbance of the fibrinogen bands in the presence of plasmin was divided by the absorbance of fibrinogen in the absence of plasmin and multiplied by 100 to yield the percentage degraded.

RESULTS

Enzymatic properties of vascular TG. A thrombin-independent, calcium- and thiol-dependent TG activity was detected in sonicates of ABAE and VSM cells by incorporation of [^{3}H]-putrescine into dimethylcasein. The ABAE and VSM TG activity was maximal between pH 8.5 and 9.0. The TG assay was sensitive and detected 40 ng of either purified guinea pig TG, human factor XIIIa, or the TG activity in 1 to 10 μg of ABAE or VSM cell protein. Incubating sonicates of ABAE and VSM cells with 1 mmol/L *N*-ethylmaleimide or 1 mmol/L iodoacetic acid inhibited 98% of the TG activity. TG activity was not detected in the culture media before or after ABAE cells were cultured for 10 days.

Immunoblotting using polyclonal anti-factor XIII a-chain antisera did not detect the factor XIII antigen in 200 μg of confluent ABAE cell protein. This immunoblotting technique was capable of detecting 50 ng of purified human or bovine factor XIII. Thrombin, which is necessary for the activation of plasma and platelet factor XIII, did not modify the TG activity of ABAE cell sonicates. In contrast to factor XIIIa, which initially catalyzes γ-chain cross-linking of fibrinogen, the vascular TG selectively cross-linked the α-chains of fibrinogen and not the β- or γ-chains (Fig 1).

More than 90% of the TG activity remained in the cytoplasmic fraction of confluent ABAE cells after centrifugation for 60 minutes at 150,000 g. The presence of calcium chloride (5 mmol/L) or EDTA (5 mmol/L) had no effect on the distribution of the TG from disrupted ABAE cells after centrifugation. To determine whether there was latent TG activity in the particulate or soluble fraction of ABAE cell sonicates, each fraction was incubated with 0.5% NP-40 or 0.5% Triton X-100 (Sigma, St Louis) to solubilize the TG. The nonionic detergent treatment did not change the TG activity in either fraction.

Presence of TG in vascular tissues and cells. TG activity was also detected in homogenates from both human and several other mammalian blood vessels (Table 1). Thrombin was not required for the expression of the TG activity from any of the cells or tissues. The TG activity was only detected in fresh sonicates of tissues and cells and was destroyed by freezing at −70 °C unless glycerol (25% vol/vol) was present during freezing. TG activity was also detected in a variety of other cultured vascular cells, including human umbilical vein endothelium (Table 1). Aortic endothelial and smooth muscle cells could be the major source of TG activity in bovine aortic tissues since they had a higher specific activity than the original tissue. The specific activity of confluent ABAE cells remained constant for up to 15 cell passages. However, the specific activity of nonconfluent cells was sevenfold lower (data not shown). Results for all the cells described in Table 1 were from postconfluent cells.

Immunologic characterization of vascular TG. Immunologic similarity between ABAE cell TG and guinea pig liver TG was demonstrated by immunoblotting techniques

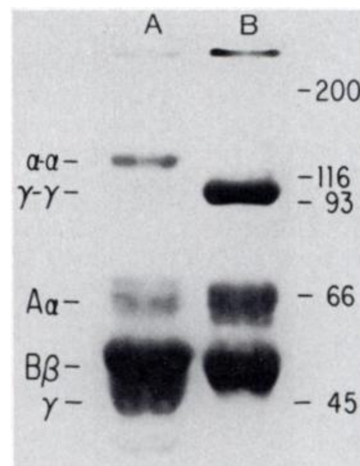

Fig 1. Pattern of fibrinogen cross-linking by confluent ABAE cell sonicate (lane A) and purified platelet factor XIIIa (lane B). The mobility of the mol wt standards are shown × 10^{-3} on the right margin. Purified fibrinogen was incubated with either ABAE sonicate or purified platelet factor XIIIa at 22°C for 1 hour. The incubation mixture was separated by SDS-PAGE as described in the text. Location of the Aα-, Bβ-, and γ-chains of fibrinogen as well as the γγ- and αα dimers are shown on the left margin.

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Table 1. Distribution of TG Activity in Cells and Tissues

Tissue/Cell	Specific Activity*
Tissues	
Human abdominal aorta	0.80
Porcine abdominal aorta	2.64
Rabbit abdominal aorta	2.15
Rabbit inferior vena cava	4.10
Bovine abdominal aorta	1.96
Cells	
Porcine aortic endothelial cells	2.24
Porcine VSM	1.82
Rabbit VSM cells	1.30
Adult ABAE	122.00
Bovine VSM	36.40

Tissues and cells were homogenized, sonicated and assayed for enzyme activity as described in the Materials and Methods section.

*Specific activity is defined as nanomoles of putrescine incorporated into casein by 1 mg of cell protein in 1 hour at 37°C.

using the culture supernatant from a hybridoma cell line producing monoclonal anti-guinea-pig liver transglutaminase antibody (CUB-7401).[10] The confluent ABAE cell extract revealed a major 79 ± 3-kd band that migrated slightly ahead of the guinea pig liver TG (lane 1; Fig 2). Purified guinea pig liver TG migrated as a single 85 ± 9-kd protein under these conditions (lane 2, Fig 2). Tissue culture media before culturing ABAE cells did not have any detectable TG antigen. However, the culture supernatant did have a very small amount of TG antigen that migrated as a higher mol wt antigen ~150 kd (data not shown). The TG antigen in rabbit VSM, porcine aortic endothelial cells, and an extract of porcine aortic tissue also migrated as an 80-kd antigen. Occasionally, lower mol wt TG antigens were detected in the sonicates of ABAE cells. Inclusion of protease inhibitors in the cell lysis buffer did not completely reduce the appearance of these lower mol wt antigens. The confluent ABAE sonicates did not react with the culture supernatant from a nonreactive hybridoma cell line (CUB-11). The monoclonal antibody (CUB-7401) did not cross-react with 5 μg of either factor XIII, fibrinogen, or fibronectin. The ELISA assay detected 0.3 ng TG antigen in 100 ng cell protein.

Chromatographic and electrophoretic characterization of vascular TG. High-pressure liquid chromatography (HPLC) gel filtration, DEAE column chromatography, and casein-agarose electrophoresis were used to ascertain whether there was a single functional form of TG in ABAE cells. TG activity in confluent ABAE cells eluted as a single peak from the HPLC gel filtration column with the same retention time (RT = 7 minutes) as purified guinea pig liver TG. More than 90% of the ABAE TG activity adsorbed to and eluted from a DEAE-Sephacyl column as a single peak at a NaCl concentration (0.35 to 0.38 mol/L), similar to purified guinea pig liver TG.

Identification of TG activity after electrophoretic separation in nondenaturing casein-agarose gels has been used by others to characterize the TG from various tissues.[17] ABAE cell TG partially purified by DEAE-Sephacel chromatography and purified guinea pig liver TG migrated in casein-agarose gels as a single band with an Rf of 0.6 relative to bromphenol blue dye. In contrast, purified factor XIIIa migrated more slowly, with an Rf of 0.1.

Effect of divalent cations on TG activity. We found a unique effect of calcium and magnesium ions on the TG in ABAE cells (Fig 3). A very narrow range of calcium ion

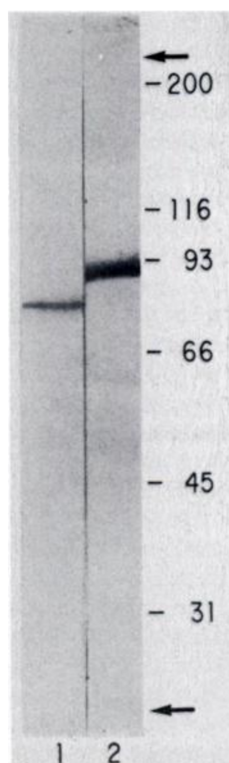

Fig 2. Detection of tissue TG antigen in confluent ABAE cell sonicates by SDS-PAGE and immunoblotting. Confluent ABAE cell sonicate (200 μg) from passage 8 (lane 1) and purified guinea pig liver TG (2 μg) (lane 2) were subjected to SDS-PAGE and Western blotting as described in the text. Monoclonal antibody (CUB-7401) was used to locate the TG antigen on the nitrocellulose membrane. Mobility of the mol-wt standards are shown × 10^{-3} on the right margin. Top arrow, origin; bottom arrow, distance of bromphenol blue dye migration.

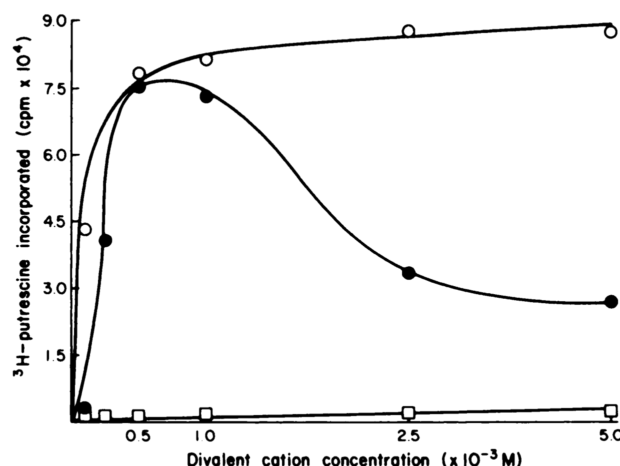

Fig 3. Effect of $CaCl_2$ and $MgCl_2$ concentration on ABAE cell TG activity. Fifty micrograms of protein from sonicates of ABAE cells was incubated with increasing concentrations of $CaCl_2$ (●----●), $MgCl_2$ (□----□) or $MgCl_2$ in presence of 0.1 mmol/L $CaCl_2$ (O—O). The amount of [^{3}H]^2-putrescine incorporated into dimethylcasein was determined after 30 minutes of incubation at 37°C. EDTA (1 mmol/L) was added to each reaction mixture to chelate unbound divalent cations. The $CaCl_2$ and $MgCl_2$ concentrations added to the reaction mixtures were 1.0 mmol/L higher than the desired final concentration.

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concentration was required for optimum TG activity; and when this range was exceeded, a reduction in TG activity was observed. When magnesium ions were substituted for calcium ions, TG activity was not detected. However, in the presence of suboptimal concentrations of $CaCl_2$ (0.1 mmol/L), $MgCl_2$ promoted ABAE cell TG activity (Fig 3). This was consistently observed in five experiments, using both confluent ABAE and VSM cells. The Mg^{2+}-dependent increase in TG activity was also studied using partially purified ABAE cell TG obtained from ABAE cells following HPLC gel filtration. Magnesium chloride (1 mmol/L) and 0.5 mmol/L $CaCl_2$ increased TG activity to 64% of the level achieved by 1 mmol/L calcium chloride. Such an effect was not observed with purified guinea pig liver TG.

Identification and localization of TG antigen in human tissues. When human skin was analyzed by immunohistochemistry, the TG antigen was confined to the blood vessels in the dermis. A section of two blood vessels from the skin showed intense staining along the endothelial portion of a medium-sized vein (Fig 4, arrow). In the vessel of a medium-sized artery, the TG antigen localized mainly to the smooth muscle cell layer (Fig 4). A medium-sized artery showed positive staining with anti-TG monoclonal antibody along the endothelial surface. Staining was also observed along the surface of smooth muscle cells when a cross-section of the vessel was studied (data not shown). Human and rabbit aorta also stained for TG antigens in the endothelium, VSM cells, and interstitial connective tissue of the intima and media (data not shown).

Although TG was purified from guinea pig liver >10 years ago, the location of this enzyme in the liver was never established. We have detected TG antigen lining the sinusoids of human liver (Fig 5) as well as rat and guinea pig livers (data not shown). There was no detectable staining of hepatocytes, and the nonreactive antibody (CUB-11) produced no immunoreactivity (Fig 5). When liver tissues were stained with antifibronectin antibody, a reaction occurred similar to the pattern observed with the TG, suggesting that

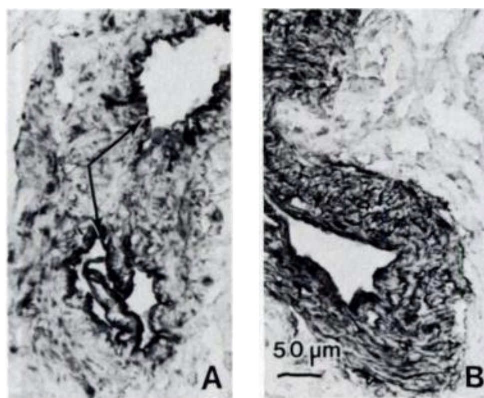

Fig 4. Frozen section of two blood vessels from human skin stained with anti-TG. (A) A medium-sized vein shows intense staining along the endothelial portion of the vessel (arrow) with less staining in the wall. In contrast, a medium-sized artery (B) shows localization mainly within the smooth muscle layer.

both proteins were distributed in the same tissue compartment (Fig 5).

Effect of ABAE cell lysates on plasmin degradation of fibrinogen. The ABAE cell TG cross-linked purified α_2-plasmin inhibitor to fibrinogen (Fig 6A and B). The high-mol-wt complexes (Fig 6A) were not observed when α_2-plasmin inhibitor or $CaCl_2$ was omitted from the reaction or when the reaction was conducted with 5 mmol/L EDTA. Fibrinogen–α_2-plasmin inhibitor complexes with mol wt of 132, 195, 260, and >260 kd were identified using either antifibrinogen or anti–α_2-plasmin inhibitor antibody. Because these complexes reacted with both antibodies and since the α-chain (67 kd) is the preferred cross-linking site in fibrinogen for the ABAE TG (Fig 1), we believe these complexes to be α_2-plasmin inhibitor cross-linked to the α-chain monomers or α-chain polymers of fibrinogen. The complexes of mol wt $\geq$ 260 kd represent α_2-plasmin inhibitor cross-linked to α-chain polymers. The exact stoichiometry of α_2-plasmin inhibitor cross-linked to fibrinogen cannot be determined from these results.

ABAE cell sonicates also made fibrinogen resistant to plasmin degradation. In the absence of either TG, calcium ions, or α_2-plasmin inhibitor, plasmin (0.005 CU/mL) degraded 50% of the fibrinogen (Fig 6B). After α_2-plasmin inhibitor was cross-linked to fibrinogen by ABAE cell TG, the plasmin concentration to degrade 50% of the fibrinogen was increased 40-fold (0.20 CU/mL) (Fig 6B). The formation of the cross-linked complexes between fibrinogen and α_2-plasmin inhibitor and the resistance of modified fibrinogen to plasmin degradation were unaffected by dithiothreitol in the cross-linking reaction (data not shown).

DISCUSSION

Although previous investigators have detected TG activity in homogenates of rabbit aorta,[6] the TG was neither biochemically characterized nor immunohistochemically localized. Cultured ABAE and VSM cells contain a thrombin-independent TG with biochemical and immunologic properties similar but not identical to guinea pig liver TG. This protein was biochemically and immunologically distinct from factor XIIIa.[22] Both the vascular and guinea pig liver TG enzymes preferred uncharged polyamines (pH optimum 8.5 to 9.0), required a free sulfhydryl group for activity, reacted with polyclonal and monoclonal antibodies specific for guinea pig liver TG, were predominantly cytoplasmic enzymes, selectively cross-linked the α-chains of fibrinogen, and eluted from DEAE columns under similar conditions. Both enzymes were also inhibited in a noncompetitive manner by GTP.[23,24]

We observed a novel Ca^{2+}/Mg^{2+} effect on TG activity in confluent ABAE cells. With suboptimal concentrations (0.1 mmol/L) of calcium ions, an Mg^{+2}-dependent increase in TG activity occurred. To our knowledge, this is the first observation of this type of Ca^{2+}/Mg^{2+} effect on TG activity. The Ca^{2+}/Mg^{2+} effect was not observed with purified guinea pig liver TG, demonstrating that this was not an artifact of our assay system. Since the Ca^{+2}/Mg^{+2} effect was less prominent when the partially purified ABAE TG was stud-

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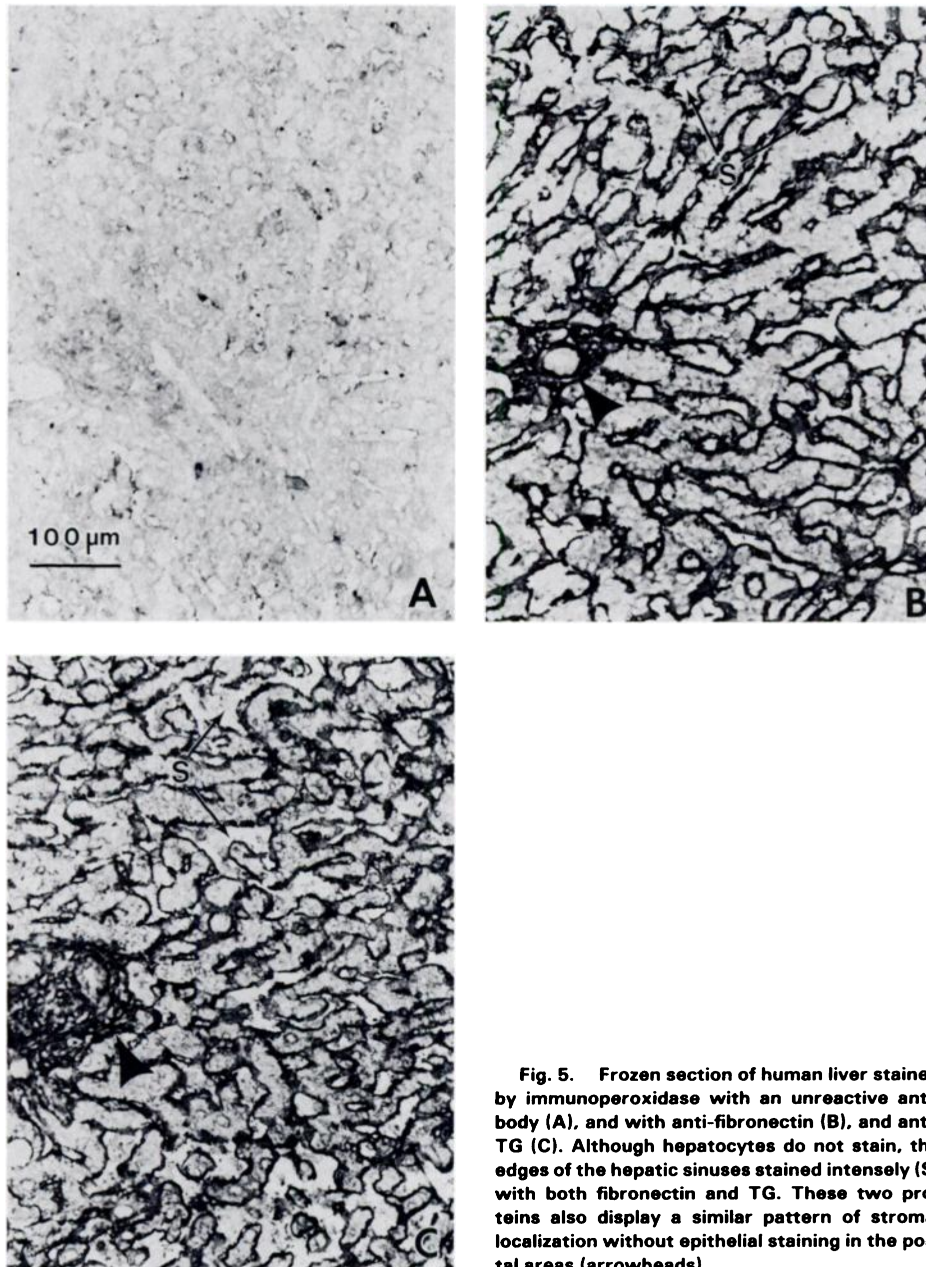

Fig. 5. Frozen section of human liver stained by immunoperoxidase with an unreactive antibody (A), and with anti-fibronectin (B), and anti-TG (C). Although hepatocytes do not stain, the edges of the hepatic sinuses stained intensely (S) with both fibronectin and TG. These two proteins also display a similar pattern of stromal localization without epithelial staining in the portal areas (arrowheads).

ied, Mg^{+2} ions may be modulating ABAE cell TG activity by directly binding to the TG or binding through an intracellular cofactor. A delicate balance between intracellular Ca^{2+}/Mg^{2+} in the milieu of the TG may be a mechanism for regulating the intracellular activity of this enzyme. Mg^{+2} ions have been reported as capable of partly replacing the Ca^{2+} requirement of partially purified liver TG.[28]

The mol wt of the ABAE TG as determined by SDS-PAGE was ~5 kd lower than the guinea pig liver TG. The two proteins may be synthesized similarly but processed differently within the cells. Variability in the mol wt of the vascular TG could be due to intracellular proteases. Lacking amino acid sequences, we conclude that the two enzymes are similar but not identical.

The vascular tissues that contained the TG antigen included human aorta, renal capillaries, and hepatic arteries and veins, as well as arterioles and venules in the skin. Using immunohistochemistry, we found that the TG and fibronectin antigens codistribute along the sinusoids of human liver. At the light microscopic level, it is not possible to determine whether the sinusoidal localization of the TG is the result of its presence in sinusoidal endothelial cells, binding to the extracellular matrix, or its presence in the sinusoidal membranes of hepatocytes. Hepatocytes may also synthesize guinea pig liver TG but not have sufficient intracellular antigen to be detected by the monoclonal antibody. Either hepatic endothelial cells or hepatocytes may synthesize the protein and secrete it into the extracellular matrix where it

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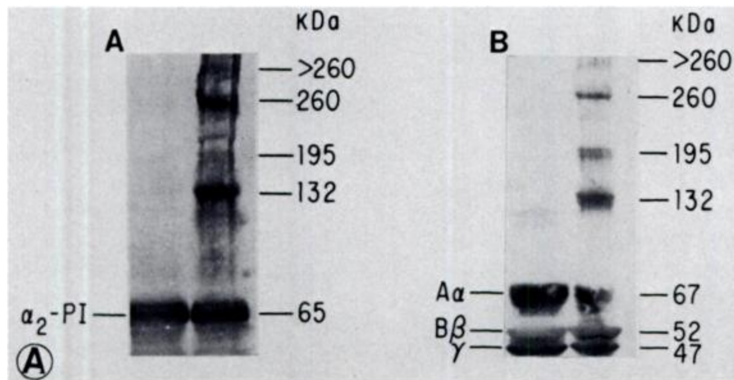

Fig 6A. Cross-linking of α_2-plasmin inhibitor to fibrinogen by ABAE cell sonicates. ABAE cell sonicate was incubated with purified fibrinogen and α_2-plasmin inhibitor in the presence of 5 mmol/L $CaCl_2$ or 5 mmol/L EDTA as described in the Materials and Methods section. Following SDS-PAGE and immunoblotting, the membranes were reacted with either anti-α_2-plasmin inhibitor antisera (A) or anti-fibrinogen antisera (B), followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. Antigen bands were detected using a color reagent from BioRad. α_2-Plasmin inhibitor migrated as a 65-kd protein band. The high-mol-wt complexes formed in the presence of 5 mmol/L $CaCl_2$ (mol wt = ≥132 kd) contain both α_2-plasmin inhibitor antigen and fibrinogen antigen.

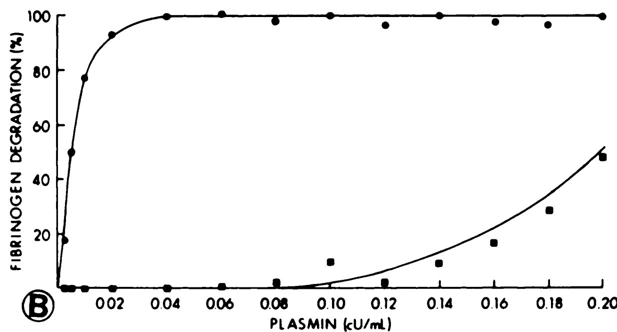

Fig 6B. Effect of ABAE cell lysates on fibrinogen degradation by plasmin. Plasmin was incubated with either fibrinogen alone (●—●) or with fibrinogen cross-linked to α_2-plasmin inhibitor by ABAE cell lysates (■—■) as described in the Materials and Methods section. After plasmin treatment for 20 minutes at 37°C, the reaction mixture was solubilized and subjected to SDS-PAGE. Coomassie blue-stained fibrinogen bands were quantitated by scanning densitometry as described in the text.

may be bound to fibronectin. Fibronectin is a substrate for the plasma TG factor $XIII_a$[4,25] and the tissue TG.[26] The monoclonal antibody used in this study does not cross-react with human factor XIII, fibrinogen, fibronectin, collagen, or bovine actin. Fibronectin is synthesized and secreted into extracellular tissues by vascular cells.[27] A similar mechanism may account for the TG distribution in vascular tissues. The immunohistochemical results represent the first demonstration of the in vivo distribution pattern of the TG antigen in human tissues. Preliminary electronmicroscopic immunohistochemistry studies suggested that the TG antigen was associated with the hepatic sinusoidal cells and not the hepatocytes (D. Bainton and C.S. Greenberg, unpublished observations).

The vascular TG described in this study is distinct from the plasma, TG factor XIIIa. Neither thrombin-dependent TG activity nor factor XIII-related antigen was detected in the cells and tissue preparations used in the present study. Furthermore, the vascular TG (partially purified or crude cell sonicates) did not cross-react with polyclonal rabbit anti-factor XIII antibody. Finally, in contrast to γ-chain cross-linking of fibrinogen by factor XIIIa, the vascular TG preferentially cross-linked the α-chains to form dimers, mol wt = 132,000. Purified guinea pig liver TG also selectively cross-linked the α-chains of fibrinogen. These results demonstrate that the α-chain of fibrinogen is the preferred substrate for the ABAE cell and guinea pig liver TG. The ABAE cell TG also catalyzed the cross-linking of α_2-plasmin inhibitor to the α-chains of fibrinogen. This is the first demonstration that a tissue-derived TG can function to cross-link a_2-plasmin inhibitor to fibrinogen. The modified fibrinogen was nearly 40-fold more resistant to plasminolysis as compared with native fibrinogen. We have obtained similar results using the purified guinea pig liver TG (C.S. Greenberg and K.E. Achyuthan, unpublished observations).

The demonstration of a thrombin-independent TG in vascular cells and tissues that can utilize fibrinogen as a substrate provides a mechanism by which insoluble cross-linked fibrinogen could be deposited in normal and atherosclerotic vascular tissues.[7] In addition, α_2-plasmin inhibitor cross-linked to fibrinogen by the vascular TG might function to protect fibrinogen from plasmin degradation and then lead to accumulation of fibrinogen in the atherosclerotic plaques.[7] The vascular TG could play an important role in fibrinolysis and could also provide an alternate pathway to promote fibrin stabilization following vascular injury.

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