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Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque

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ABSTRACT Tissue factor (TF)-producing cells were identified in normal human vessels and atherosclerotic plaques by in situ hybridization and immunohistochemistry using a specific riboprobe for TF mRNA and a polyclonal antibody directed against human TF protein. TF mRNA and protein were absent from endothelial cells lining normal internal mammary artery and saphenous vein samples. In normal vessels TF was found to be synthesized in scattered cells present in the tunica media as well as fibroblast-like adventitial cells surrounding vessels. Atherosclerotic plaques contained many cells synthesizing TF mRNA and protein. Macrophages present as foam cells and monocytes adjacent to the cholesterol clefts contained TF mRNA and protein, as did mesenchymal-appearing intimal cells. Significant TF protein staining was found deposited in the extracellular matrix surrounding mRNA-positive cells adjacent to the cholesterol clefts and within the necrotic cores. These results suggest that deposition of TF protein in the matrix of the necrotic core of the atherosclerotic plaque may contribute to the hyperthrombotic state of human atherosclerotic vessels.

Tissue factor (TF) is a membrane-bound glycoprotein that functions in the extrinsic pathway of blood coagulation by acting as a cofactor for factor VII (1, 2). TF binds to coagulation factor VII, and the resulting factor VIIa–TF complex acts as a catalyst for the conversion of factors X to Xa and IX to IXa, leading to the formation of thrombin (3, 4). Thus, TF facilitates both intrinsic and extrinsic pathways of coagulation and is a key protein in the activation of the coagulation cascade.

TF activity has been isolated from a number of different tissues and has been cloned from adipose (5), fibroblast (6), and placental (7, 8) cDNA libraries. A 2.3-kilobase band corresponding to human TF mRNA has been identified by Northern blots using RNA from adipose tissue, small intestine, placenta, kidney (5), and brain (8). Cultured endothelial cells produce low levels of TF mRNA (5). TF procoagulant activity in endothelial cells is enhanced by the addition of endotoxin (9), thrombin (10), phorbol esters (9, 11), interleukin 1, or tumor necrosis factor (12, 13) to the culture medium. TF mRNA has also been identified in the monocyte cell line U937 (8) and TF activity has been identified in normal monocytes after activation with endotoxin or phorbol esters (14, 15).

There is no information concerning the cellular distribution of TF-producing cells within the tissues from which it has been isolated. It has been postulated that TF is not exposed to blood elements but must be associated with the vasculature where it could act quickly in response to vascular damage. With cDNA probes and antibodies specific for the TF protein, it is now possible to do more precise cellular localizations using in situ hybridization and immunohistochemistry. We report here the localization of TF-producing cells in the normal vessel wall and atherosclerotic plaques.

METHODS

Tissue Preparation. Normal human saphenous veins and internal mammary arteries were obtained during coronary bypass surgery. Human atherosclerotic plaques were obtained from patients undergoing carotid endarterectomy surgery. The tissue samples were removed and immersed in freshly prepared 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7.4). The tissues were fixed at 4°C for 3 hr to overnight and then immersed in 15% (wt/vol) sucrose/iso- tonic phosphate-buffered saline for 2–4 hr at 4°C to act as a cryoprotectant. The tissues were then embedded in optimal cutting temperature compound (O.C.T., Miles Scientific) blocks and stored at −70°C. Finally, the tissues were sectioned at 10 μm thickness by using a cryostat, thaw-mounted onto polylysine-coated microscope slides, immediately re- frozen, and stored at −70°C with desiccant.

In Situ Hybridization. In situ hybridizations were carried out as described (16, 17). Prior to hybridization the sections were pretreated sequentially with paraformaldehyde (10 min) and with proteinase K at 1 μg/ml (10 min) and prehybridized for 1–2 hr in 100 μl of prehybridization buffer [50% (vol/vol) formamide/0.3 M NaCl/20 mM Tris-HCl, pH 8.0]/5 mM EDTA/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin/10% (wt/vol) dextran sulfate/10 mM dithiothreitol]. The hybridizations were started by adding 600,000 cpm of the 35S-labeled riboprobe in a small amount of prehybridization buffer. After hybridization the sections were washed with 2× SSC (two 10-min washes; 1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0), treated with RNase (20 μg/ml for 30 min at room temperature), washed in 2× SSC (two 10-min washes), and washed at high stringency in 0.1× SSC at 52°C for 2 hr. All SSC solutions up to this point of the procedure contained 10 mM 2-mercaptopethanol and 1 mM EDTA to help prevent nonspecific binding of the probe. The tissue was then washed in 0.5× SSC without 2-mercaptoethanol (two 10-min washes) and dehydrated by immersion in a graded alcohol series containing 0.3 M NH4Ac. The sections were dried, coated with NTB2 nuclear emulsion (Kodak), and exposed in the dark at 4°C for 4–8 weeks. After development, the sections were counterstained with hematoxylin and eosin.

A cDNA probe specific to human TF subcloned in the SP64 plasmid (Promega Biotec), provided by Karen Fisher and Richard Lown (Genentech), was labeled by transcription (18) using 35S-labeled UTP (specific activity, 1200 Ci/mmol; 1 Ci = 37 GBq; Amersham). This was a 1.3-kilobase probe and included the entire coding sequence for human TF extending...
FIG. 1. (Legend appears at the bottom of the opposite page).
from nucleotide 1 in the 5' flanking region to a HindIII site at nucleotide 1347 in the 3' untranslated region (5). The final specific activity of this probe was 300 Ci/mmol.

Immunohistochemistry. TF antibody RD010 was prepared by immunizing rabbits with recombinant human TF protein (19). The IgG fraction of the serum was purified by affinity chromatography on a recombinant human TF-Sepharose column. This antibody was shown to react with a single band at 42 kDa on a Western blot, neutralize TF activity, and immunoprecipitate TF protein (20).

Immunohistochemistry was performed according to the manufacturer's direction using the Vectastain ABC alkaline phosphatase system (Vector). The final reaction product was stained with the alkaline phosphatase substrate kit 1 to give a final stain that appeared red.

The affinity-purified TF antibody RD010 was used at a concentration of 4.4 μg/ml. An IgG fraction of the preimmune serum was used as a control for the TF immunohistochemistry at the same IgG concentration as RD010. This was prepared by passing the preimmune serum over a protein A-Sepharose column. Additional antibodies specific for human macrophages (ref. 21; HAM56, gift of Allen Gown, University of Washington), smooth muscle cells (ref. 22; HHF35, gift of Allen Gown), or human endothelial cells (ref. 23; anti-Ulex lectin, Vector) were used on some serial sections to aid in cell identification.

RESULTS

TF Localization in Normal Vessels. Normal human saphenous vein and internal mammary artery specimens (nine sections for each tissue, three sections from each of three individuals) were examined for the presence of TF. Endothelial cells were negative for TF mRNA and protein in all vascular tissues examined. Many scattered cells in the tunica media of the saphenous vein contained TF mRNA as determined by in situ cRNA hybridization (Fig. 1A). Immunohistochemical staining of these cells, however, was very weak but appeared to be cell-associated and correlated well with the in situ results (Fig. 1B). The strongest labeling was seen over the adventitia where adventitial fibroblasts showed intense TF protein staining (Fig. 1B) and mRNA hybridization. The internal mammary artery specimens also showed positive cRNA hybridization and intense protein labeling over the adventitial fibroblasts. However, few medial cells contained TF mRNA, as indicated by the in situ hybridization, and no protein could be detected in the media by immunohistochemistry. Additional normal arteries obtained from hearts discarded from recipients of transplants were screened for TF mRNA by in situ hybridization (three sections each from the following tissues: aorta, n = 2; left anterior descending coronary artery, n = 1; right coronary artery, n = 2; left circumflex artery, n = 1). These arterial samples came from idiopathic dilated cardiomyopathy hearts removed at the time of cardiac transplantation. These hearts typically have no significant atherosclerotic disease, and this was confirmed in our samples. All of these vessels showed positive adventitial cells but at most a single medial cell containing TF mRNA.

In general, more cells were found to be positive in the media of the saphenous vein by in situ hybridization than could be detected by immunohistochemical staining. Visual comparison of the hybridization signal over the adventitial fibroblasts and that of positive cells in the media suggested that these cell types do not differ greatly in their content of TF mRNA. However, a similar comparison of the immunohistochemical staining intensity over these cells suggests that the adventitial fibroblasts contain much more TF protein than do the medial cells. This could suggest that there is some alteration in TF translation or secretion in the medial cells resulting in the reduced protein content. Alternatively, antibody RD010 may react differently with the TF in the medial or adventitial cells due to some change in the TF protein itself.

The morphology of TF-positive cells in the media of the saphenous vein differed from that of typical smooth muscle cells. The cytoplasm of the TF-positive cells stained poorly with eosin and did not display a typical fusiform-shaped cytoplasm but rather appeared more cuboidal in shape, with small dense nuclei. Cells with this morphology do not stain with smooth muscle α-actin antibodies (HHF35; data not shown) and must be considered undefined, since we lack a positive immunohistochemical marker for these cells.

TF Localization in Atherosclerotic Plaques. Human atherosclerotic plaques obtained from carotid endarterectomy surgery were examined for TF mRNA and protein using the above techniques. Extensive mRNA hybridization was seen in several regions of atherosclerotic plaque. Positive cells were found scattered throughout the fibrous cap (Fig. 1C), the base and shoulder region of the plaque, as well as in the necrotic core adjacent to the cholesterol clefts (Fig. 1F). Six plaques were screened and cells showing TF mRNA hybridization were seen in all of them. The normal media underlying the endarterectomy specimens did not contain any TF protein or mRNA-positive cells.

The necrotic cores of the plaques were characterized by extensive TF protein localization in the extracellular matrix particularly surrounding some cholesterol clefts (Fig. 1E and G). Additional protein staining was seen in the macrophage-rich foam cell regions of many of the atherosclerotic plaques examined (Fig. 1D). Such foam cell-rich regions often lay underneath the fibrous cap and adjacent to the necrotic cores.

Fig. 1. Localization of TF in the normal human saphenous vein (A and B) and in human carotid endarterectomy specimens (C-G). (A) In situ hybridization using a specific 35S-labeled TF cRNA probe indicated that there were scattered TF-producing cells in the tunica media and adventitia. (B) Cells containing TF protein were detected by immunocytochemistry using antibody RD010 and the Vectastain alkaline phosphatase method (positive cells stain red). Scattered cells in the tunica media were lightly stained by antibody RD010, whereas strong immunohistochemical staining was always seen in the adherent adventitial fibroblasts. Endothelial cells lining the lumen of the normal vessel were always negative for TF protein and mRNA. L, lumen; M, media; A, adventitia. (C) Localization of TF in the human atherosclerotic plaque by in situ hybridization. Carotid endarterectomy specimens were hybridized to an 35S-labeled TF riboprobe and revealed many cells producing TF in the fibrous cap of the atherosclerotic plaque. (D) Localization of tissue factor protein in macrophage foam cells of the atherosclerotic plaque by immunohistochemistry with antibody RD010. (E and F) Co-localization of TF protein and mRNA in the same cells of the plaque. (E) Immunohistochemistry with TF antibody RD010 indicated strong staining of the necrotic core region of the plaque particularly in areas adjacent to the cholesterol clefts. (F) In situ hybridization of a section immediately adjacent to the one shown in E indicated that cells containing TF mRNA were found adjacent to the cholesterol clefts, suggesting local synthesis of the TF protein detected in this region. Not all of the protein detected here was cell associated. Arrows point to two cholesterol clefts followed on the serial sections. G and H) Identification of cells producing TF in the necrotic core of the atherosclerotic plaque. Serial sections stained with the TF antibody RD010 (G) (same section as shown in E) or with a monoclonal antibody (HAM-56) to human macrophages (H) were compared and indicated that the TF-protein-producing cells in the necrotic core may be macrophages. Additional adjacent sections in the series were screened with markers specific for smooth muscle (HHF-35), endothelial cells (Ulex europaeus lectin binding), or T cells (anti-Leu4), all of which were negative in this region but labeled the appropriate cells elsewhere in the section. Arrows point to two cholesterol clefts followed on the serial sections (E-H). (Magnifications are as follows: for A, ×760; for B, ×615; for C, G, and H, ×310; for D, ×1230; for E, ×75; for F, ×760.)
Finally, as with the normal vessels, no TF mRNA or protein was detected in either the endothelium lining the vascular surface or the small vessels within the plaques.

It was possible to confirm that the protein staining and the *in situ* hybridization labeled the same regions on serial sections (see Fig. 1 E–G). In a few instances we could identify the same cell on two adjacent serial sections and show that this cell was positive with both the TF antibody and the *in situ* mRNA hybridization. This observation served as a control for both the immunohistochemistry and hybridization analysis. Additional controls were done on serial sections in every experiment. The *in situ* hybridizations were controlled by hybridization of serial sections with platelet-derived growth factor A chain or platelet-derived growth factor receptor-specific riboprobes (17). Different patterns of hybridization were seen with these probes compared to TF. TF immunohistochemistry was always controlled by incubation of serial sections with preimmune serum that failed to label any cells at all.

All of the carotid endarterectomy specimens examined (n = 16) showed positive TF protein staining in some region of the plaque. Prominent staining of regions surrounding the cholesterol clefts in the necrotic core was a feature observed in five out of sixteen plaques in which this feature was present. The others showed variable staining in monocytes, macrophage foam cells, or mesenchymal-appearing intimal cells (17). A femoral plaque obtained by endarterectomy was examined and showed TF protein staining consistent with that seen in the carotid plaques. The mRNA hybridizations were in agreement with the immunohistochemistry and all six carotid plaques examined by *in situ* hybridization had positive intimal cells.

**DISCUSSION**

The purpose of these experiments was to localize sites of TF biosynthesis in both the normal and atherosclerotic vessel. In the normal vessel, we have shown by immunohistochemistry and *in situ* hybridization that TF is synthesized by scattered cells in the tunica media and fibroblasts in the adventitia surrounding the vessels. We did not find any evidence of TF mRNA or protein localization in endothelial cells of any vessel studied. Previous cell culture work has suggested that induction of TF synthesis by endothelial cells represents a major procoagulant mechanism by which endothelial cells participate in homeostasis (24). However, cultured fibroblasts (24–26) and vascular smooth muscle cells (25) have been shown to produce TF at much higher levels. It is not clear if induction of endothelial TF biosynthesis is a normal *in vivo* mechanism by which the endothelium modifies homeostasis or represents the response of the endothelium to infection and endotoxin stimulation.

Atherosclerotic plaques were examined and found to have many more TF mRNA-containing cells and stronger TF protein staining than the media of normal saphenous veins, internal mammary artery, or regions of normal media underlying the plaque. TF mRNA was found in mesenchymal-like intimal cells in the atherosclerotic intima as well as in macrophages and cells adjacent to the cholesterol clefts. Immunohistochemistry indicated that there was a considerable amount of TF protein trapped in the extracellular matrix of the necrotic core of the atherosclerotic plaque. This was not cell-associated but was probably synthesized locally since cells adjacent to these regions contained TF mRNA. There is no evidence that TF is a secreted protein (5, 8); however, it is possible that the TF protein found in the necrotic core may be shed from the surfaces of the synthesizing cells and trapped in the surrounding matrix. Alternatively, the TF protein in this region may originate from cells that have died and left TF-rich membranes behind.

The production of TF by macrophages has been demonstrated by other investigators (27, 28). The immunostaining of macrophage foam cells suggests that in these cells TF is intracellular as well as possibly cell surface associated. It is not clear to what extent such stores of TF are macrophage-derived or whether this protein originates from phagocytosis of surrounding necrotic core debris. It is interesting to note that Levy *et al.* (28), have found that certain lipoprotein fractions can induce procoagulant activity originating from mononocytes/macrophages.

Evidence indicates (29) that TF-initiated coagulation is the major *in vivo* coagulant pathway. It is a highly thrombogenic protein and requires only phospholipid and factor VII/VIIa for its activity. The TF-factor VIIa complex directly activates factor IX and X leading to the generation of thrombin. Factor VII is normally present in blood but requires binding to TF for activation of factors IX and X. Since normally there is no *in vivo* coagulation in the absence of vascular damage, it is reasonable to assume that TF is not exposed to blood (7, 29). This is consistent with our findings, since normal vessel endothelial cells in direct contact with the blood do not synthesize or store TF. TF is found in scattered smooth muscle-like cells in the tunica media and adventitial cells adherent to the vessel. Vessel wall rupture into these areas would be required to expose the blood to significant stores of procoagulant TF activity. Zaug (30) has shown that damaged human aorta exhibits factor VII-dependent procoagulant activity, in support of this hypothesis.

Advanced human atherosclerosis is characterized by intimal smooth muscle cell proliferation accompanied by accumulation of fats, inflammatory cells including macrophages, and T cells within the atherosclerotic plaque (24, 31, 32). Commonly, the critical event that converts an asymptomatic atherosclerotic plaque into a symptomatic one is thrombosis (33–35), whereas non-diseased arteries rarely become thrombotic. It has been suggested that plaque rupture is the integral event that precipitates clot formation (36–39). An occlusive mural thrombus accompanies most cases of acute myocardial infarctions (40–43). Plaque rupture or cracking that exposes the necrotic core region to the lumen is usually found to underlie such thrombi in both the coronary (33, 44, 45) and cerebral arteries (46). The source of the thrombogenicity of the plaque has not previously been determined, but it has been suggested that coagulation occurs when blood components come into contact with fats or the collagen matrix within the plaque. Our studies clearly demonstrate that there is significant synthesis of TF in atherosclerotic plaques accompanied by strong staining of TF protein in the necrotic core and in foam cell-rich regions of the plaque. These results suggest that overproduction and/or trapping of TF protein in the atherosclerotic plaque may play a significant role in the thrombosis associated with plaque rupture.

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