Tissue Factor Is Rapidly Induced in Arterial Smooth Muscle after Balloon Injury

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Abstract

Tissue factor (TF) is a major activator of the coagulation cascade and may play a role in initiating thrombosis after intravascular injury. To investigate whether medial vascular smooth muscle provides a source of TF following arterial injury, the induction of TF mRNA and protein was studied in balloon-injured rat aorta. After full length aortic injury, aortas were harvested at various times and the media and adventitia separated using collagenase digestion and microscopic dissection. In uninjured aortic media, TF mRNA was undetectable by RNA blot hybridization. 2 h after balloon injury TF mRNA levels increased markedly. Return to near baseline levels occurred at 24 h. In situ hybridization with a ^{35}S-labeled antisense rat TF cRNA probe detected TF mRNA in the adventitia but not in the media or endothelium of uninjured aorta. 2 h after balloon dilatation, a marked induction of TF mRNA was observed in the adventitia and media. Using a functional clotting assay, TF procoagulant activity was detected at low levels in uninjured rat aortic media and rose by ≈ 10-fold 2 h after balloon dilatation. Return to baseline occurred within 4 d. These data demonstrate that vascular injury rapidly induces active TF in arterial smooth muscle, providing a procoagulant that may result in thrombus initiation or propagation. (*J. Clin. Invest.* 1993. 91:2253–2259.) Key words: vascular smooth muscle • angioplasty • thrombosis • gene expression • vascular injury

Introduction

Tissue factor (TF)[1] is a membrane-bound glycoprotein that is the primary cellular initiator of the clotting cascade (1). TF initiates coagulation by binding its high affinity ligands, circulating Factors VII and VIIa. The resulting bimolecular complex activates Factors IX and X to IXa and Xa, leading ultimately to clot formation (1, 2). The distribution of TF in the arterial wall is not uniform. Immunohistochemical analysis and in situ hybridization demonstrate that TF antigen and mRNA are detectable at high levels in the adventitia of a variety of normal human arteries, including coronary, internal mammary, and aorta (3, 4). In contrast, TF mRNA and antigen are undetectable in normal vascular endothelium and are variably present in the media. In the media of muscular arteries such as coronary and internal mammary, TF mRNA has been demonstrated at low levels; TF antigen has been either undetectable or found at low levels (3, 4). Because it is expressed predominantly in the adventitia, TF is functionally sequestered from circulating blood. The development of intravascular thrombosis may therefore involve the induction of TF in the intimal or medial layers of the vessel wall.

The induction of TF in culture has been demonstrated in a number of cell types. In endothelial cells, TF mRNA or procoagulant activity is induced by agonists such as phorbol esters (5), tumor necrosis factor (6), endotoxin (7, 8), interleukin 1 (9) and α-thrombin (10). In monocytes, TF mRNA and procoagulant activity are stimulated by a variety of mediators of inflammation and antigen-specific cellular immune responses (11). In fibroblasts, TF has been found to be a member of the class of "immediate early" genes induced by serum and growth factors, including PDGF, fibroblast growth factor (FGF), and transforming growth factor β (12, 13). We have recently reported that in cultured vascular smooth muscle cells, TF mRNA is rapidly and markedly induced by a variety of agents implicated in the arterial response to injury, including growth factors, vasoactive agonists, and α-thrombin (14).

Balloon dilatation of the arterial wall provides a model to study the events associated with vessel injury (15), as well as a means to relieve atherosclerotic stenoses (16). Balloon dilatation of the normal pig carotid artery results in the formation of a mural thrombus (17) that is felt to participate in the development of intimal hyperplasia and restenosis by providing growth factors that stimulate smooth muscle migration and proliferation (18, 19). In humans, thrombosis after balloon angioplasty has been demonstrated by a variety of techniques, including angiography (20), angioscopy (21), and histologic examination of coronary atherectomy specimens (22). Given its role as an initiator of clotting, TF may participate in the thrombotic complications associated with vessel injury.

This study tests the hypothesis that vessel injury results in the induction of TF in arterial vascular smooth muscle. We present data indicating that TF mRNA and activity are rapidly induced in vivo in the media of balloon-injured rat aorta. The induction of TF in medial smooth muscle suggests that these cells may play a role in the generation or propagation of thrombus.

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Received for publication 13 November 1992 and in revised from 8 February 1992.

1. *Abbreviations used in this paper:* CS, calf serum; FGF, fibroblast growth factor; PTCA, percutaneous transluminal coronary angioplasty; TF, tissue factor.

J. Clin. Invest.

0021-9738/93/05/2253/07 $2.00
Volume 91, May 1993, 2253–2259

Methods

Balloon injury and isolation of rat aortas. Balloon injury of the aorta was performed in adult male Sprague-Dawley rats (300–350 g) under general anesthesia (intraperitoneal ketamine 150 mg/kg). A 2 French balloon embolectomy catheter (American Edwards Laboratories, Santa Ana, CA) was introduced via the femoral artery and advanced to the level of the aortic arch. The balloon was then inflated and the catheter withdrawn along the full length of the thoraco-abdominal aorta. The inflated balloon has a diameter of 4 mm, which is approximately twice the diameter of the rat aorta. The balloon was deflated and the procedure repeated three times. Animals were killed at varying times and the aortas removed. Aortas were incubated at 37°C for 30 min in HBSS (Gibco Laboratories, Grand Island, NY) supplemented with 350 U/ml of collagenase (Worthington Biochemical Corp., Freehold, NJ). After collagenase treatment, separation of the adventitia from the media was performed under a dissecting microscope. This technique is identical to that used for the isolation of cultured vascular smooth muscle cells and results in a cell population that is > 95% vascular smooth muscle (23). A portion of the aortic media to be used for RNA blot hybridization was then frozen in liquid nitrogen. The remainder of the media and the adventitia were then immersed in TBS (0.1 M NaCl, 0.05 M Tris, pH 7.5) and stored separately at −80°C for analysis of TF activity. For light microscopy and in situ hybridization, portions of non-collagenase treated aorta were fixed in freshly prepared 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) at 4°C overnight. After fixation samples were immersed in 10% sucrose/isotonic PBS for 3 h at 4° and then embedded in OCT (Miles Scientific, West Haven, CT) blocks and stored at −80°C. Using a cryostat, 8-μm sections were thaw-mounted onto polylysine-coated slides and immediately frozen at −80°C.

Cell culture. Vascular smooth muscle cells were isolated from the thoracic aortas of 200–300-g male Sprague-Dawley rats by enzymatic dissociation (23). Cells were grown in DME supplemented with 10% heat-inactivated calf serum (CS), 100 U/ml penicillin, and 100 μg/ml streptomycin, and then serially passaged before reaching confluence. Cells were incubated in DME with 0.4% CS for 48–72 h to produce quiescence. Under these conditions incorporation of [^{3}H]thymidine into DNA is < 15% of that seen with 10% CS (24). Experiments with cultured smooth muscle were performed six times, using cells from subculture passages 5–10.

Isolation of the rat TF cDNA. A cDNA library was constructed in λZAPII (Stratagene Corp., La Jolla, CA) from mRNA isolated from PDGF-stimulated rat aortic vascular smooth muscle cells. Screening of this library with the mouse TF cDNA probe (12) resulted in the isolation of a 1,800-bp clone which contained the entire coding region and 3′ untranslated region and part of the 5′ untranslated region of the rat TF. The derived amino acid sequence of the coding region had 82% homology with that of the mouse (Rosenfield, C.-L., Y., Nemerson, and M. B. Taubman, manuscript in preparation).

RNA preparation and blot hybridization. Total RNA was extracted from rat aorta and cultured rat aortic smooth muscle cells by the guanidinium isothiocyanate/cesium chloride procedure (25). Agarose gel electrophoresis, transfer to nitrocellulose, and hybridization to ^{32}P-labeled cDNA were performed as previously described (26). Prehybridization and hybridization were done at 42°C. Final washes for all blots were in 0.5× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7) and 0.1% SDS at 65°C for 1 h. The full-length TF insert was labeled by random oligomer priming to a specific activity of > 10^8 cpm/mg and used at 2×10^6 cpm/ml. RNA from rat aorta was harvested from three animals for each time point studied: control (uninjured), and 2, 4, 8, 24 h, 3 d, and 7 d after balloon injury.

In situ hybridization. In situ hybridization was carried out using the methods of Wilcox et al. (3). Before hybridization, frozen sections were pretreated sequentially with paraformaldehyde and proteinase K (1 μg/ml) and prehybridized for 2 h. Hybridizations were performed using 7.5×10^5 cpm of ^{35}S-labeled rat TF cRNA. After hybridization, sections were washed with 2× SSC, treated with RNAse A (20 μg/ml, 30 min at 37°C), and then washed in 2× SSC and 50% formamide at 54°C for 2 h. The tissues were then dehydrated by immersion in a graded alcohol series containing 0.3 M ammonium acetate. Sections were dried, coated with NTB2 nuclear emulsion (Kodak, Rochester, NY), and exposed in the dark at 4°C for 2 wk. After development, the sections were counterstained with hematoxylin. To confirm probe specificity, RNA blots derived from rat aortic smooth muscle cells were hybridized with the antisense probe under identical conditions to those used for in situ hybridization, and yielded a single band of 2.2 kb, corresponding to the rat TF mRNA. Hybridizations were performed on control (uninjured) aortas and on aortas 2 and 24 h after balloon injury. Three animals were examined at each time point. Six sections were examined per animal.

To generate specific TF cRNA probes, a 687-bp fragment from the 5′ end of the rat TF cDNA (including 63 nucleotides of the 5′ untranslated region and sequence corresponding to amino acids 1–208) was subcloned into the EcoRI site of pGEM-3Z (Promega Corp., Madison, WI). ^{35}S-labeled sense and antisense cRNAs were transcribed with [^{35}S]CTP (New England Nuclear, Boston, MA) using Sp6 and T7 polymerases, respectively. The specific activity of the probes was ≈ 200 Ci/mmol.

Immunohistochemistry. Immunohistochemistry was performed on sections from uninjured and injured aortas using an anti-von Willebrand factor antibody (Dako Corp., Carpinteria, CA) to confirm that balloon dilatation resulted in endothelial removal. Before incubation with the primary antibody, sections were treated with pepsin (1 mg in 1 ml 0.01 M HCl) at room temperature for 5 min. The primary antibody was detected with the avidin–biotin complex (Vector Laboratories, Burlingame, CA) immunoperoxidase technique using diaminobenzidine (Kodak) as the chromogen (27).

Analysis of tissue factor activity. Control and balloon-injured rat aortic media and adventitia were separately immersed in TBS and then sonicated at 20 kHz for 20 s (model W375; Heat Systems-Ultrasonics Inc., Farmingdale, NY). 50-μl aliquots were assayed in duplicate for TF activity using modifications of a standard two-stage procedure (28, 29). Each aliquot was incubated at 37°C for 1 min with 50 μl of rat Factor VII–X concentrate and 50 μl of a 5-mM $CaCl_2$, 12.5-μM phosphatidyl serine/phosphatidyl choline (30/70; wt/wt) in TBS. Concentrates of Factors X, VII, and VII_a were prepared from mouse serum (Pel-Freez Biologicals, Rogers, AR) as previously described (14). 50 μl of human plasma was then added at 37°C and the clotting time was recorded. To generate a standard TF activity curve, a mouse brain homogenate was prepared as follows: 1.5 g of stripped mouse brain (Pel-Freez) was suspended in 15 ml isotonic saline, disrupted by sonication, centrifuged at 1,000 $g \times 15$ min, and the supernatant aliquoted and stored at −80°C. The protein concentration of the homogenate was determined using Bradford reagent (protein assay; Bio-Rad Laboratories, Richmond, CA) with BSA as a standard. When assayed as above, 1–30 μg of homogenate generated a linear response. The amount of protein required to produce a clotting time of 25 s was arbitrarily assigned a value of 100 activity units. TF activity in tissue samples was expressed in activity units per milligram of protein.

Results

Induction of TF mRNA in balloon-injured aorta. To examine the induction of TF in vessel injury, dilatation of rat aortas was performed as described (see Methods). This injury consistently resulted in intimal hyperplasia (Fig. 1 *B*). To investigate the induction of TF mRNA in aortic media, the adventitia was removed using enzymatic digestion and microscopic dissection. The removal of endothelium in injured vessels was confirmed by the absence of staining using anti-von Willebrand factor antibody. Endothelium was detected with this antibody in control, uninjured rat aorta and in reendothelialized aortas examined 3 mo after injury (data not shown). As shown in Fig.

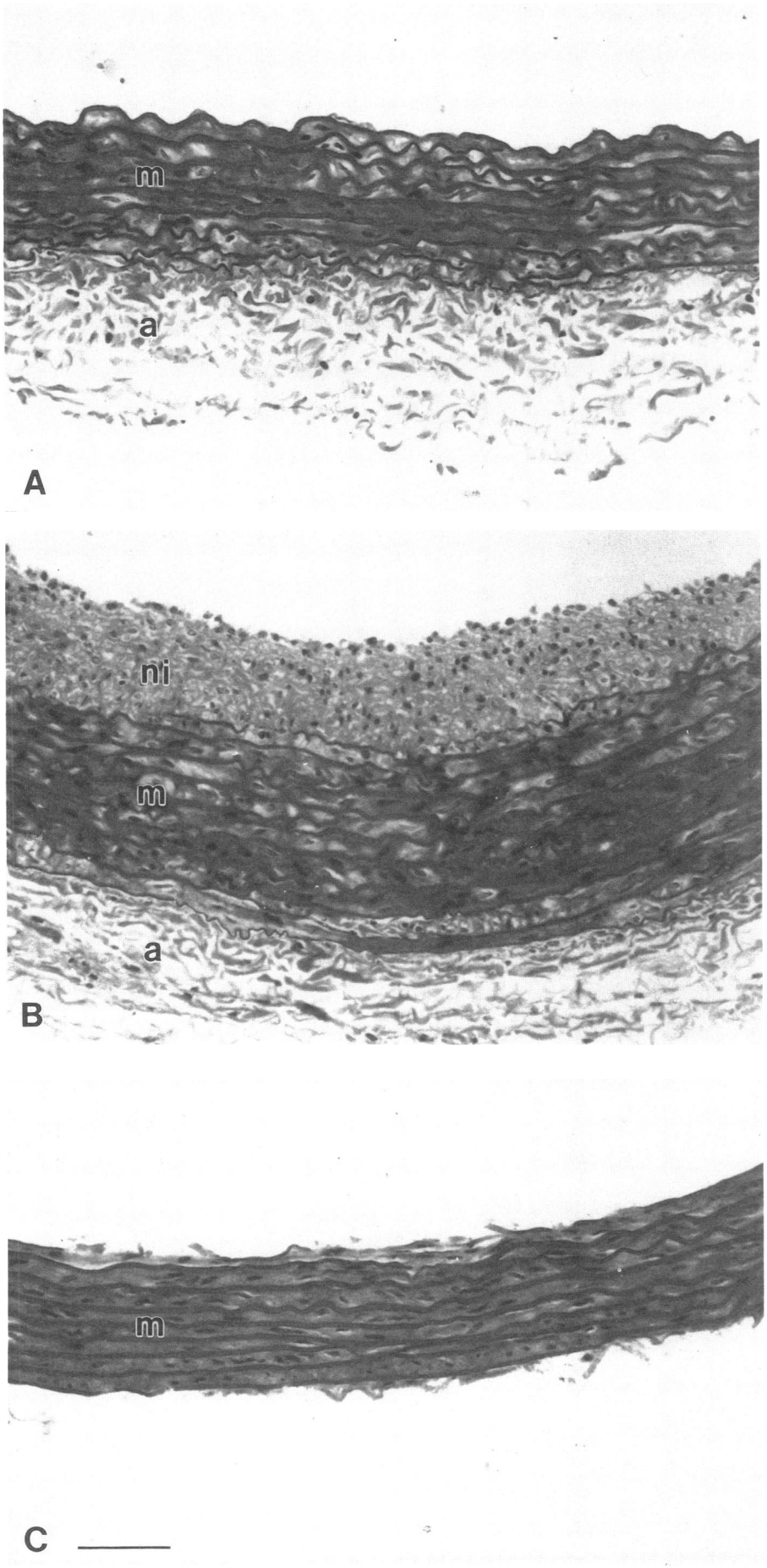

Figure 1. Hematoxylin and eosin stain of rat aortic tissue. The luminal surface is found on the top of each photograph (*ni,* neointima; *m,* media; a, adventitia). (*A*) Rat aorta 2 h after balloon injury. (*B*) Rat aorta 12 wk after balloon injury, demonstrating intimal hyperplasia (n = 4 animals). (*C*) Medial preparation of rat aorta 2 h after balloon injury. The adventitia has been removed with collagenase digestion and microscopic dissection, yielding an essentially pure medial preparation. Removal of endothelium was documented by the absence of staining with anti-Factor VIII (Willebrand factor) antibody (not shown). Scale bar (*C*): 50 μm.

1 *C* balloon injury and adventitial removal resulted in an essentially pure medial preparation.

TF mRNA levels were undetectable in uninjured (time 0) rat aortic media analyzed by RNA blot hybridization (Fig. 2 *A*). After balloon injury TF mRNA levels were markedly increased at 2 h and returned to near baseline levels by 24 h. No TF mRNA was seen at 3 or 7 d after injury.

Fig. 2 *B* shows the accumulation of TF mRNA in cultured rat aortic vascular smooth muscle cells in response to serum as a comparison between these cells and the intact aorta. As previously reported (14) TF mRNA was undetectable in quiescent cultured smooth muscle. Stimulation of cells with 10% CS induced a marked rise in TF mRNA levels, which peaked between 60 and 120 min, and returned to baseline by 8 h. Thus, the pattern of induction of TF mRNA in whole aorta is similar to that observed in cultured cells. This suggests that the study of TF mRNA regulation in cultured smooth muscle cells may provide insights into the regulation of TF in vivo.

To investigate the distribution of TF mRNA in normal and injured vessels, aortic sections were hybridized with a ^{35}S-labeled TF cRNA probe. As shown in Fig. 2 *C* a faint signal was seen in uninjured aorta (time 0). 2 h after balloon injury, an intense signal was observed around the entire circumference of the vessel wall, demonstrating the diffuse pattern of TF mRNA induction.

Higher power photomicrographs of aortic sections hybridized to the TF cRNA probes are shown in Fig. 3. In uninjured aortas no hybridization was detected in the intima and media. In contrast, hybridization was seen in the adventitia (Fig. 3 *A*). 2 h after balloon dilatation intense hybridization was found in the media of all preparations (Fig. 3 *B*). Adventitial hybridization was also increased at this time. Hybridization was not detected in the media at 24 h (not shown).

Induction of TF protein in aortic tissue. TF activity was measured at various times after injury to establish that the rise in TF mRNA in aortic tissue was accompanied by the synthesis of active TF protein. Assays were performed on the media and the adventitia after separation using collagenase digestion and microscopic dissection. Low levels of TF activity were measured in uninjured (time 0) rat aortic media (Fig. 4). 2 h after balloon injury there was a $\approx$ 10-fold increase in TF activity. TF activity remained elevated for 24 h and then returned to baseline by 96 h. In contrast to the aortic media, TF activity in the adventitia was high in uninjured aorta ($\approx$ 10-fold higher than that found in injured media) and was unchanged following injury.

Discussion

Platelet deposition and thrombosis are commonly seen in animal models of vascular injury (30) and in the human coronary artery following transluminal balloon angioplasty (31). These processes have been implicated in the development of acute closure that can occur after angioplasty and in the generation of a neointima that can result in late restenosis (22). The data described above demonstrate that TF, a major initiator of the coagulation cascade, is rapidly inducible in vivo. Elevation of TF mRNA levels occurs both in the media and adventitia of balloon-injured rat aorta; in the media, this accumulation of TF mRNA is temporally associated with an increase in TF activity. In adventitia, TF activity remains constant, despite the increase in TF mRNA levels. One explanation for this discrepancy is that the posttranscriptional regulation of TF mRNA differs between adventitial fibroblasts and medial vascular smooth muscle cells. Alternatively, incorporation of TF within the plasma membrane may be limited by the number of available membrane sites and may already be saturated in uninjured adventitial fibroblasts. Because TF activity is markedly

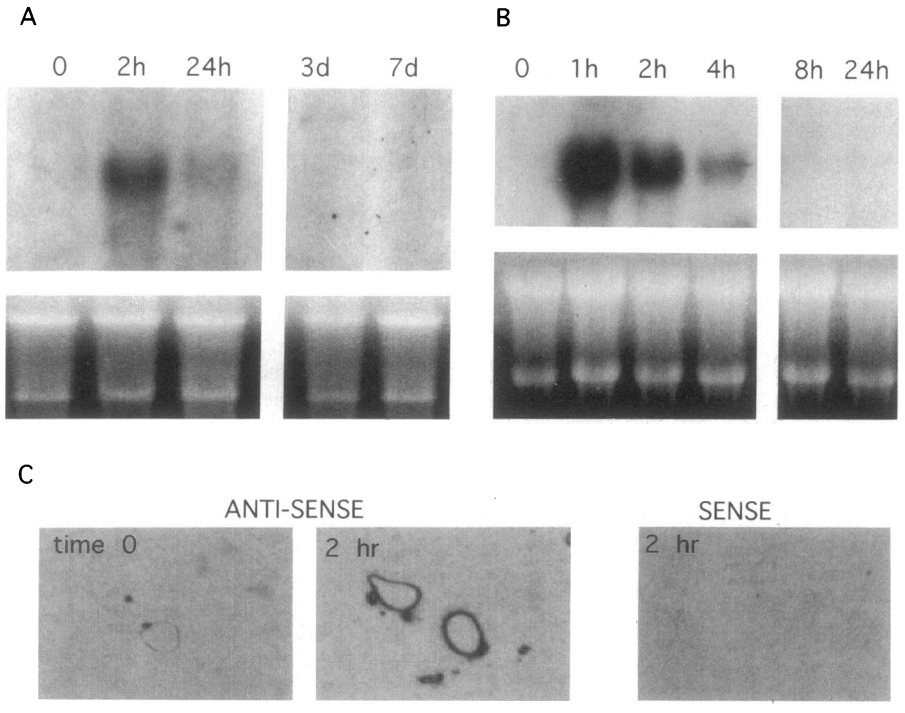

Figure 2. Analysis of TF mRNA in rat aorta and cultured vascular smooth muscle. (*A*) RNA blot hybridization: aortic media. Lanes contain 10 μg of total RNA from the media of normal rat aorta (time 0) or from the media of aortas harvested at various time points (*h*) after injury. Each time point was derived from an individual rat and the results were reproducible in three sets of animals. The levels of TF mRNA at 4 and 8 h (not shown) were similar to those seen at 24 h. (*B*) RNA blot hybridization: cultured vascular smooth muscle (subculture passage 8). Lanes contain 10 μg of total RNA from quiescent cultured rat aortic vascular smooth muscle cells (incubated in DME with 0.4% CS for 48 h; time 0) that were treated for the times indicated with 10% CS. Blots were hybridized to the full-length rat TF cDNA and washed at 0.1× SSC, 65°C. The hybridizing band corresponds to an $\approx$ 2-kb TF species. Ethidium bromide staining of the 18S and 28S ribosomal RNA shown below each blot demonstrates that lanes were equally loaded. (*C*) Autoradiography of sections derived from uninjured (time 0) and balloon-injured (*2 hr*) rat aorta hybridized in situ to an antisense TF ^{35}S-labeled RNA probe. Hybridization of injured rat aorta (*2 hr*) with a sense TF ^{35}S-labeled RNA probe served as a negative control.

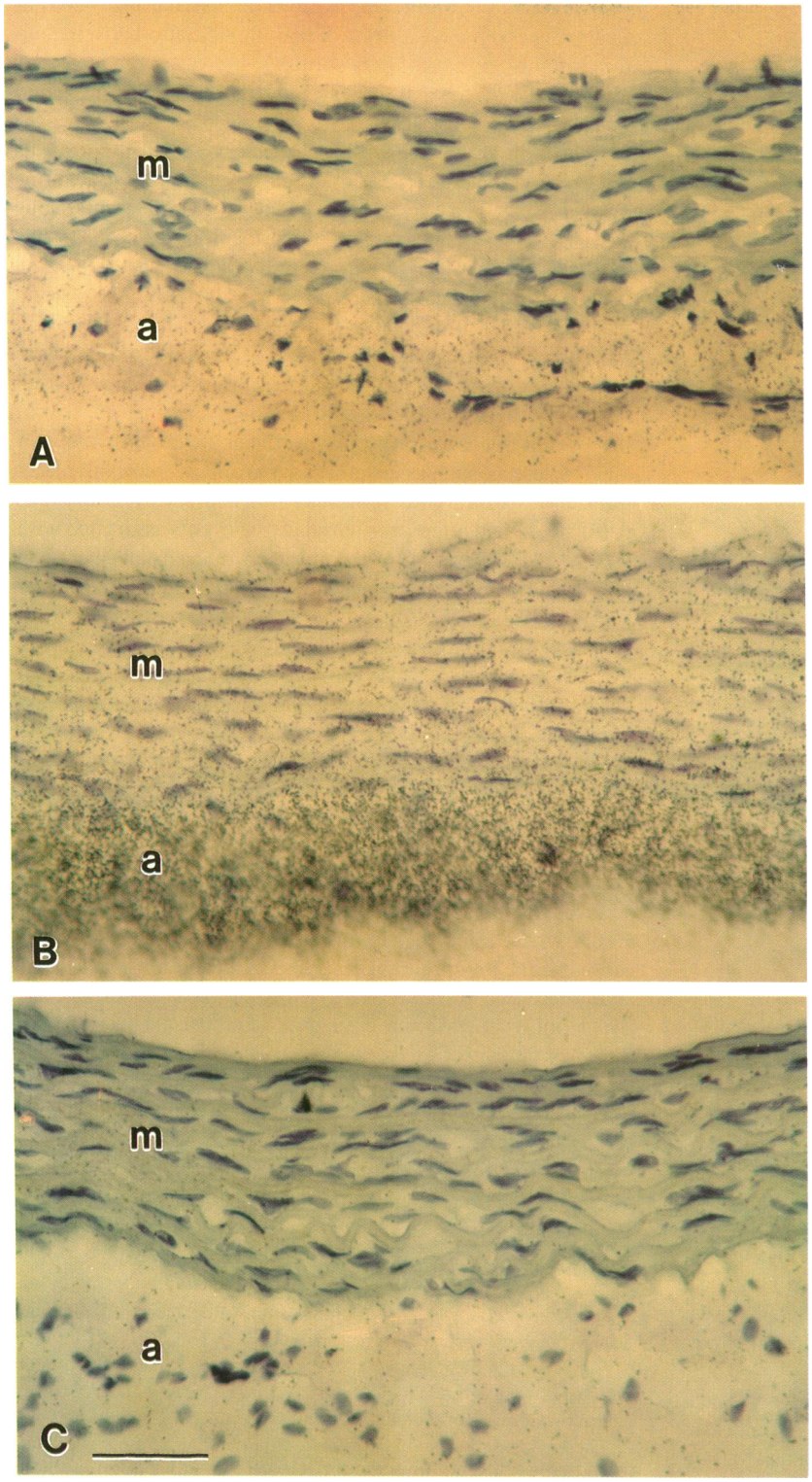

Figure 3. In situ hybridization of rat aorta. The luminal surface is found at the top of each photograph (*m*, media; a, adventitia). Photographs are counterstained with hematoxylin. (*A*) Normal, uninjured rat aorta hybridized with a ^{35}S-labeled TF antisense RNA probe. (*B*) Rat aorta 2 h after injury hybridized with the same probe. (*C*) Rat aorta 2 h after injury hybridized with a ^{35}S-labeled TF sense RNA probe. (n=3 sets of animals; 18 sections per time point). Scale bar (*C*): 50 μm.

diminished in the absence of phospholipids, such as those associated with the cell membrane (32), the inability to incorporate additional TF protein in the plasma membrane would likely prevent a measurable rise in TF activity, even in the presence of increased TF synthesis.

The demonstration that vascular injury induces TF expression in the media may be particularly relevant to percutaneous transluminal coronary angioplasty (PTCA). Balloon injury results in endothelial denudation and disruption of the internal elastic lamina, thereby exposing circulating blood to medial smooth muscle cells (30). Expression of TF on the surface of these cells could provide a site for circulating Factor VII to bind and activate the clotting cascade. In the rat aortic media, TF activity persisted at high levels for at least 24 h. This raises the possibility that TF expression induced by balloon injury may contribute to the early thrombotic complications of PTCA (33). By providing growth factors (18), thrombus formation may also participate in the development of intimal hyperplasia (17, 18), the pathologic hallmark of late restenosis after PTCA.

TF expression in arterial media suggests an additional role

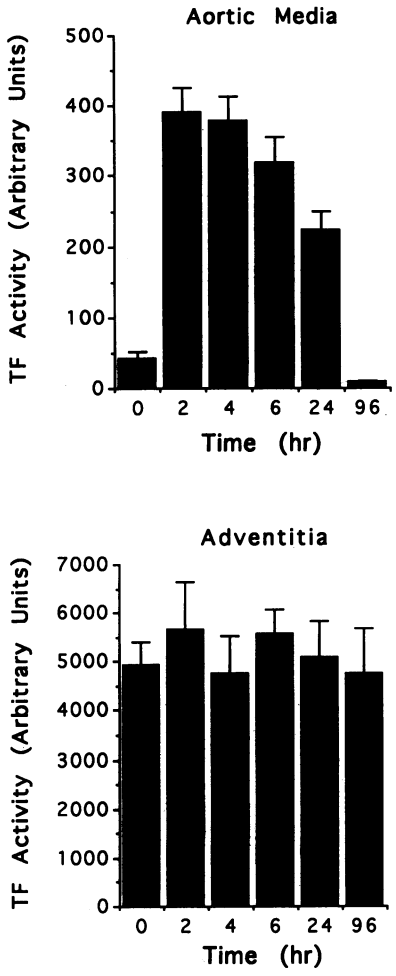

Figure 4. TF Activity in rat aortic media and adventitia. Bars represent the level of TF activity in uninjured (time 0) and balloon-injured rat aortic tissue harvested at various time points (*hr*) after injury. Each bar represents the mean±SEM of duplicate measurements performed in six rats. Units correspond to the amount of Factor Xa generated (described in Methods) and are expressed as total activity per milligram of protein. Note that the scale of the *y* axis for adventitia is one order of magnitude greater than that used for aortic media.

for the vascular smooth muscle cell in the response to vessel injury. It has been established that luminal narrowing after vessel injury results in large part from the mass effect of smooth muscle proliferation, migration, and secretion of extracellular matrix (17, 19). Previously, we have reported that injury of the rabbit aorta induces *JE* and *KC*, two PDGF-inducible genes that encode a monocyte and neutrophil chemotactic factor, respectively (34). We now report that vascular smooth muscle may play an additional role; specifically, as an important mediator of the thrombotic response to injury through the elaboration of TF. Thus, the vascular smooth muscle cell may act as a multifunctional cell involved in all phases of vascular injury, including thrombosis, inflammation (35), and intimal hyperplasia.

TF mRNA and protein can be induced in cultured vascular smooth muscle by a variety of agents that have been implicated in the response to vessel injury and in the pathogenesis of atherosclerosis (14). PDGF may be released by platelets that are known to accumulate at sites of vessel injury (36), as well as by smooth muscle cells and monocytes (18). Basic FGF is also released locally by mechanically traumatized cells (37) and may provide the signal to activate the TF gene. α-Thrombin is generated at the site of injury by the activation of the clotting cascade (38). Angiotensin II has been implicated in the response to vascular injury by the recent reports that rats treated with angiotensin converting enzyme inhibitors developed lesser degrees of intimal hyperplasia after arterial dilatation (39, 40). The ability of these agonists to induce TF mRNA in tissue culture raises the possibility that one or more of them may mediate the induction of TF in the vasculature. Alternatively, the mechanical effects of balloon dilatation (stretch, alteration in shear stress) may stimulate increases in TF mRNA levels and protein activity through a non agonist-mediated mechanism.

The induction of TF within the vessel wall has implications not only in the pathogenesis of the early and late complications associated with PTCA, but also in the process of spontaneous atherogenesis, for which a response to injury hypothesis has been advanced (41). Numerous lines of evidence point to the importance of thrombosis in the generation of atherosclerotic plaque (42). Immunocytochemical studies using monoclonal antibodies have detected fibrin, fibrinogen, and fibrin degradation products in the intima, neointima, and deeper medial layers of atherosclerotic vessels (43, 44). Thrombosis within the arterial tree also contributes to the acute complications of atherosclerosis, such as unstable angina and myocardial infarction (45). We chose a model in which a severe injury was performed in an attempt to simulate the events associated with PTCA. The generation of thrombus in myocardial infarction and unstable angina is often the result of plaque fissuring or rupture (46), events which are likely to be associated with lesser degrees of injury. Thus, it will be important to establish whether more subtle forms of injury such as endothelial denudation without arterial stretch are also able to induce TF expression.

This study investigated the procoagulant activity of a normal artery after a single balloon injury. Earlier studies have demonstrated that a second injury to a previously ballooned artery that has developed a neointima results in increased fibrin formation and platelet-fibrin microthrombi on the aortic surface (47, 48). This observation may be relevant to clinical PTCA where injury occurs in an already diseased artery. Because it is the predominant cell type in the neointima, vascular smooth muscle may play a role in the increased thrombogenicity of injured neointima. Our data demonstrate that the induced expression of TF is one mechanism through which vascular smooth muscle may participate in the thrombotic response to balloon injury. To investigate the hypercoagulability of injured neointima, it will therefore be important to determine whether neointimal smooth muscle cells have an enhanced expression of TF at baseline and/or in response to injury.

Acknowledgments

We gratefully acknowledge the contributions of Dr. Claire-Lise Rosenfield to the work reported in this manuscript.

This research was supported in part by National Institutes of Health Grants HL-43302 and HL-19019. J. Marmur is a fellow of the Medical Research Council of Canada, Ottawa, Canada. M. Mendlowitz received support from the Louis and Harold Price Foundation, Inc. This is publication No. 123 from the Brookdale Center for Molecular Biology of the Mount Sinai Medical Center.

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