Extrahepatic Synthesis of Factor VII in Human Atherosclerotic Vessels

Josiah N. Wilcox, Sumiko Noguchi, Juan Casanova

Objective—Coagulation is initiated by the interaction of tissue factor (TF) with plasma coagulation factors VII (FVII) and X (FX). TF is highly expressed in atherosclerotic lesions, but little is known about the synthesis of FX or FVII outside of the liver. Previous studies suggested that macrophages synthesize FVII. We therefore hypothesized that macrophages within atherosclerotic lesions may produce FVII, leading to partial activation of the coagulation cascade.

Methods and Results—Immunohistochemistry was performed using antibodies against FVII, FX, and TF on normal and atherosclerotic vessels. In atherosclerotic lesions, FVII immunostaining was colocalized with TF in macrophages and spindle-shaped smooth muscle cells. FVII mRNA was also detected in these cells using in situ hybridization, suggesting the local synthesis of FVII in atherosclerosis. Reverse transcriptase–polymerase chain reaction confirmed the presence of FVII mRNA in normal and atherosclerotic vessels as well as smooth muscle cells, fibroblasts, and keratinocytes in vitro.

Conclusions—The localization of FVII synthesis outside the liver may be indicative of other cellular functions for this coagulation protein. The observed coexpression of TF and FVII may contribute to autocrine signaling via thrombin-independent mechanisms and may represent a novel mechanism contributing to growth in the setting of vascular disease.

Key Words: tissue factor ▪ factor VII ▪ factor X ▪ thrombosis ▪ atherosclerosis

The coagulation cascade is initiated by the interaction of tissue factor (TF) with plasma coagulation factors VII (FVII) and X (FX), which promotes the generation of thrombin. TF also activates factor IX (FIX) to IXa and is a key protein in the activation of both the intrinsic and extrinsic pathways of coagulation. In normal arteries, TF is found in the adventitia, where it is sequestered away from the circulating coagulation factors until rupture of the vessel. In the setting of atherosclerosis, TF mRNA and protein have been identified in the fibrous cap and necrotic core of the plaque, where they are involved in the initiation of coagulation on plaque rupture.

In general, FVII and FX are synthesized only in the liver and are found circulating in the blood. Very little is known about the synthesis of FVII or FX outside of the liver. Previous studies have shown that isolated human peripheral blood monocytes stimulated with lipopolysaccharide express FVII activity on their surface. Human alveolar macrophages may display FVII activity and contain FVII mRNA. Together, these data suggest that monocyte/macrophages or lymphocytes may be sites of FVII synthesis. We therefore hypothesized that macrophages or other cells within atherosclerotic lesions may produce FVII, which could contribute to thrombosis associated with plaque rupture.

In the present series of experiments, we have examined the localization of FVII in normal and atherosclerotic vessels using immunohistochemistry, in situ hybridization, and reverse transcriptase–polymerase chain reaction (RT-PCR). These studies demonstrate that FVII is locally synthesized by macrophages and smooth muscle cells (SMCs) in the setting of atherosclerosis. Additional studies on cultured human SMCs, fibroblasts, and keratinocytes confirm that these cells contain FVII mRNA. The finding of FVII synthesis outside the liver may be indicative of other cellular functions for this coagulation protein.

Methods

Tissue Preparation

Protocols for obtaining tissue samples for all studies were approved by the Emory University Hospital Human Investigation Committee. Human aorta samples representative of different stages of atherosclerosis were obtained from transplant donors. The stages of atherosclerotic development were based on the degree of intimal lesion formation, the presence of inflammatory cells, and regions of necrosis, as previously described. Normal aorta segments (n = 3) had almost no intimal development and no inflammatory cells as defined by CD68 immunohistochemistry; early atherosclerotic aortas (n = 4) had only minor intimal development with scattered macrophage staining just under the luminal surface; and advanced atherosclerotic aortas (n = 4) had a thickened intima, numerous CD68-positive macrophages in the intima and media, and regions of necrosis and cholesterol deposits. Advanced carotid atherosclerotic plaques were collected from patients undergoing carotid endarterec-
tomy (n=6). Normal human internal mammary artery specimens (IMA) (n=8) were obtained as excess tissue from coronary artery bypass surgery. Archived samples of normal baboon brachial arteries that had been collected under prior Emory University Institutional Animal Use and Care Committee approved studies were examined for FVII staining (n=3). The baboon vessels were perfusion fixed using 4% paraformaldehyde in 0.1 mol/L sodium phosphate (pH 7.4). Tissue samples were collected, immediately immersed in 4% paraformaldehyde at 4°C for 3 to 4 hours, cryoprotected in 15% sucrose/isotonic phosphate-buffered saline overnight at 4°C, embedded in optimal cutting temperature compound (Miles Laboratories), frozen in liquid nitrogen, and stored at −70°C until sectioning. Six-μm cryosections were cut, thaw-mounted onto Superfrost/Plus slides (Fisher Scientific), refrozen, and stored at −70°C with desiccant until immunohistochemistry or in situ hybridization.

**Immunohistochemistry**

Immunohistochemistry was performed with specific monoclonal antibodies for FVII (Novo Nordisk, Denmark, FVII-F7A2B17, 1/460 dilution), TF (Genentech Inc, RD010, 1/450 dilution), and a polyclonal antibody for FX (DAKO Patts, 1/800 dilution) using the Vectastain ABC alkaline phosphatase system (Vector Labs). The secondary antibodies were biotinylated horse anti-mouse IgG (1:400, Vector Labs) for FVII or goat anti-rabbit IgG (1/200, Fisher Scientific) for TF and FX. The final reaction product was stained with the alkaline phosphatase substrate kit I (Vector Labs) to give a red end product, and tissues were counterstained with hematoxylin. Serial sections treated with secondary antibodies only or with nonimmune IgG did not show any staining. For cell identification, serial sections were stained with markers for endothelial cells (Ulex lectin; Vector Laboratories, 1/200 dilution), α-smooth muscle actin staining on the serial sections suggested that the spindle-shaped cells were either fibroblasts or myofibroblasts. FX protein was consistently found in SMCs or myofibroblasts.3,4 Many vessels displayed significant FVII staining of monocytes and inflammatory cells in the adventitia (Figure 1). Serial sections stained with the TF antibody failed to find any corresponding TF in these cells in the same regions. We hypothesize that FVII may be deposited or induced in these tissues during the surgical procedure to isolate the vessels due to mechanical injury or thrombosis. In support of this hypothesis, perfusion-fixed baboon vessels typically show no medial FVII staining at all (Figure 1) and rarely have significant numbers of inflammatory cells in the adventitia. FX staining was not detected in any normal vessels (not shown).

**Localization of FVII, FX, and TF in Human Normal Aorta by Immunohistochemistry**

Normal human aortas (n=3) and IMA (n=8) showed variable weak staining for FVII in the endothelium, medial wall, and inflammatory cells in the adventitial surrounding these vessels (Figure 1). Some vessels showed very little staining at all, whereas others, particularly the smaller IMAs, consistently showed stronger staining of the medial wall. FVII staining in these regions was typically not associated with TF (Figure 1). Consistent with previous studies, TF was not detected in the endothelial or smooth muscle layers of the normal vessels but was localized primarily in adventitial fibroblasts.3,4 Many vessels displayed significant FVII staining of monocytes and inflammatory cells in the adventitia (Figure 1). Serial sections stained with the TF antibody failed to find any corresponding TF in these cells in the same regions. We hypothesize that FVII may be deposited or induced in these tissues during the surgical procedure to isolate the vessels due to mechanical injury or thrombosis. In support of this hypothesis, perfusion-fixed baboon vessels typically show no medial FVII staining at all (Figure 1) and rarely have significant numbers of inflammatory cells in the adventitia. FX staining was not detected in any normal vessels (not shown).

**Localization of FVII, FX, and TF by Immunohistochemistry in Atherosclerotic Vessels**

Human aortas with early (n=4) and advanced (n=4) atherosclerotic lesions as well as carotid arteries with advanced atherosclerotic lesions (n=6) were examined by immunohistochemistry for the distribution of FVII, FX, and TF. FVII and TF immunostaining were found colocalized throughout atherosclerotic development from the earliest fatty streaks to the most advanced lesions. In early intimal thickening, TF and FVII were colocalized with CD68-positive foamy macrophages, whereas FX staining was not detected in these vessels (Figure 2). In advanced lesions, FVII protein staining was found in macrophages overlying the necrotic core (Figure 3) and in spindle-shaped SMCs in the fibrous cap (Figure 4). A comparison of the distribution of FVII and α-smooth muscle actin staining on the serial sections suggested that the spindle-shaped cells were either SMCs or myofibroblasts. FX protein was consistently found distributed throughout the matrix of the necrotic core and in the regions adjacent to the cholesterol clefts in areas that also contained immunoreactive TF (Figure 3). In some advanced carotid lesions, TF, FX, and FVII were all colocalized in the inflammatory zone overlying the necrotic core (online Figure II, available at http://atvb.ahajournals.org).
Localization of FVII mRNA in Human Normal and Atherosclerotic Aorta by In Situ Hybridization

To confirm that FVII immunostaining was attributable to local synthesis of FVII in the vessel wall, in situ hybridization was performed using a 35S-labeled human FVII antisense riboprobe (Figure 5). FVII mRNA was found in inflammatory and mesenchymal-appearing cells in the intima of early and advanced atherosclerotic aorta. The mRNA localization by in situ hybridization colocalized with FVII protein staining. FVII mRNA was not found in medial SMCs of normal or atherosclerotic vessels but could often be found in the adventitia in mesenchymal-appearing cells surrounding the vasa vasorum. Control in situ hybridizations were performed using either a 35S-labeled human FVII sense riboprobe or a riboprobe directed against VWF mRNA, which either showed no hybridization or localization to endothelial cells, respectively (not shown).

RT-PCR Studies

To confirm the presence of FVII mRNA in vascular tissues, we also examined snap-frozen normal human IMAs and carotid endarterectomy samples as well as cultured cells for the presence of FVII mRNA by RT-PCR (Figure 6). These results confirmed the presence of FVII mRNA in both normal and atherosclerotic vessels. FVII mRNA was also detected in human aortic SMC as well as dermal fibroblasts and keratinocytes in vitro. Together these results suggest that many different types of cells make FVII outside the liver.

Discussion

TF is a highly thrombogenic protein. In normal vessels, TF is typically found in the adventitia sequestered away from the blood and the circulating coagulation proteins. Thus the coagulation cascade is only activated when a blood vessel breaks and the circulating factors come into contact with TF localized in the adventitia. Previous studies have shown that TF is upregulated in the setting of atherosclerosis and may be involved in thrombus formation associated with plaque rupture. Additional studies have demonstrated that TF is upregulated at sites of vascular injury after angioplasty and may play a role in the proliferative process associated with restenosis, possibly attributable to local thrombin generation.
in the intima. Very little information exists, however, about the distribution or synthesis of other coagulation factors outside of the liver, specifically FX and FVII, which are required for TF activity. Previous work concluded that FVII is a liver-specific gene. However, there are several reports indicating that macrophages or monocytes may produce FVII. Mouse peritoneal macrophages in culture make several vitamin K-dependent coagulation factors. Human alveolar macrophages in vitro make FVII protein, and these cells also display procoagulant activities in FVII-depleted serum, which is blocked by FVII antibodies. Human peripheral blood

Figure 2. Localization of FVII, TF, and FX protein in early atherosclerotic lesions of human aorta. Immunohistochemistry was performed to localize FVII (A), TF (B), and FX (C) in human aortas showing early fatty streak development. FVII and TF staining were found in early intimal thickening containing numerous CD68-positive inflammatory cells (D). Comparison of FVII and TF staining with CD68 staining confirmed that FVII and TF protein were localized in macrophages in the early atherosclerotic intima. FX staining was not found in the intima of early lesions (C). Arrows indicate border of internal elastic lamina. Magnification ×50.

Figure 3. Localization of FVII, TF, and FX protein in foam cell regions of advanced carotid atherosclerotic plaques. FVII (A), FX (B), and TF (C) were localized in advanced atherosclerotic plaques obtained from carotid endarterectomy surgery by immunohistochemistry. FVII protein staining was found in the luminal endothelial cells and macrophage foam cells overlaying the necrotic core (nc). FX staining was not cell-associated but was found distributed throughout the necrotic core separate from FVII. TF was colocalized with FVII in foamy macrophages just beneath the luminal surface and in the necrotic core colocalized with FX. The presence of a continuous endothelial layer and macrophages in this lesion was confirmed by staining of serial sections with Ulex lectin or CD68, respectively (not shown). Magnification ×125.
monocytes produce a proteolytically active form of FVII when stimulated with endotoxin. Macrophages can be induced to synthesize TF on stimulation with inflammatory cytokines and produce TF in human atherosclerotic plaques. We therefore began this study with the hypothesis that macrophages in the atherosclerotic lesion might make both FVII and TF, thus increasing the prothrombotic potential of the lesions. Our results confirmed our initial hypothesis but indicate that the expression of FVII is much more widespread than initially thought.

The present series of experiments provides evidence that FVII is synthesized outside of the liver and is found in a variety of cells in normal and atherosclerotic vessels. Normal vessels showed only weak staining for FVII, possibly associated with some tissue injury during surgical isolation. No FVII staining was found in perfusion-fixed baboon vessels. In early and advanced atherosclerotic lesions, however, there were extensive deposits of FVII, mostly found in SMCs and macrophage-rich regions colocalized with TF. Foam cells and macrophages in the necrotic core adjacent to the cholesterol clefts and foamy macrophages in early intimal thickenings all showed strong cytoplasmic staining with FVII antibodies. Although it is possible that FVII protein staining found in normal and atherosclerotic vessels originated from the blood, the finding of FVII mRNA by both in situ hybridization and RT-PCR suggests that these tissues are sites of FVII synthesis.

These studies demonstrated that FX was found in macrophage-rich regions in the necrotic core of advanced atherosclerotic vessels, where it was colocalized with FVII and TF. FX staining was primarily localized in the extracellular matrix of these regions and was not cell associated. It has been generally thought that FX is synthesized primarily in the liver, but other organs, such as kidney, spleen, and intestine, have been shown to produce small amounts of FX. FX has been described localized on the cell surface of lymphocytes within germinal centers of human lymph nodes. Future studies will need to be performed to determine if FX mRNA may be found outside the liver or in atherosclerotic lesions.

Several studies have suggested a correlation between lipids and the coagulation factors TF, FX, and FVII. Macrophages express TF on stimulation by plasma lipoproteins, modified LDL, or oxidized LDL in vitro. Oxidized LDL has also been demonstrated to stimulate endothelial production of TF in vitro. In the setting of atherosclerosis, TF can be detected in foam cells present in the early fatty streak and is also found in advanced lesions in macrophages and foam cells in regions adjacent to cholesterol clefts. These observations suggest a role for oxidized lipids in the induction of TF expression in macrophages. Levels of FVII and FX in plasma are positively related to plasma cholesterol and triglyceride concentrations. Plasma levels of HDL-cholesterol and LDL-cholesterol are correlated with FVII and FX activity in the plasma, and chylomicron and VLDL have a

Figure 4. Localization of FVII in SMCs in the fibrous cap of advanced human atherosclerotic lesions. Human carotid endarterectomy specimens were stained with antibodies against FVII (A), TF (B), α-smooth muscle actin (C), or CD68 (D). FVII protein was consistently localized in spindle-shaped SMCs (A) in the fibrous cap. Note that the region where the FVII-positive cells are located is rich in α-actin-positive SMCs (C) but devoid of CD68-positive macrophages (D). Magnification ×125.
strong binding affinity for FVII. Tissue factor pathway inhibitor circulates in the blood in association with plasma lipoproteins, and tissue factor pathway inhibitor levels are also correlated with plasma lipid levels as well. The colocalization of FVII and FX with TF in macrophages in the atherosclerotic plaques in the present study may suggest that lipids may play a role in the activation of the TF-dependent coagulation pathway in these vessels.

Figure 5. The distribution of FVII mRNA in human atherosclerotic aorta. In situ hybridization was performed on human aortas with early atherosclerotic lesions using 35S-labeled human FVII riboprobe. FVII mRNA was localized in inflammatory cells and mesenchymal-appearing cells in the intima of early atherosclerotic aorta (A). FVII mRNA was also found in the adventitia localized to adventitial fibroblasts and cells associated with the vasa vasorum (B). FVII protein, (C) and mRNA (D) were colocalized in the intima of early atherosclerotic lesions in the aorta by immunohistochemistry and in situ hybridization. Magnification, A and B, ×80; C and D, ×50.

Figure 6. RT-PCR demonstrating the presence of FVII mRNA in normal and atherosclerotic vessels and non-liver cells in vitro. Total RNA was isolated from snap-frozen human liver (LIV), internal mammary artery (IMA), advanced carotid atherosclerotic plaques obtained by carotid endarterectomy (TEA), and human keratinocytes (KER), SMCs, and fibroblasts (FIB) maintained in vitro and analyzed by RT-PCR, as described in Methods. A predicted 325-bp band corresponding to FVII was detected in extracts from normal and atherosclerotic vessels, confirming the localization of FVII mRNAs in these tissues. FVII mRNA was also detected in SMCs, fibroblasts, and keratinocytes. The FVII band in the vessels and cells was sequenced and confirmed to be identical to human FVII mRNA.
cascade. Recent work suggests that the interaction of FVII with TF may directly activate a thrombin-independent signaling cascade, resulting in an increase in cytosolic calcium and upregulation of egr-1 and several other growth-related genes. The colocalization of TF and FVII in SMCs and macrophages in atherosclerotic lesions raises the possibility of autocrine or paracrine signaling, which might affect cell proliferation within the lesions.

In conclusion, these studies indicate that FVII and FX are found in the setting of atherosclerosis in association with TF, suggesting the possibility of a partial activation of the coagulation cascade within the intima of the developing plaque, which may increase the thrombotic potential of the lesions. Alternatively, autocrine production of TF and FVII may stimulate a thrombin-independent signaling pathway, resulting in expression of several growth-associated genes. The novel finding of widespread synthesis of FVII in extrahepatic tissues raises the possibility of alternative cellular functions for this coagulation factor.

Acknowledgments

This work was supported by NIH grants HL49743 and HL48667 (to Dr Wilcox). The authors wish to thank Hongzhi Xu for expert technical assistance and Mirella Esban of Novo Nordisk for useful discussions and providing the FVII antibody and the Western blot in online Figure I.

References


Extrahepatic Synthesis of Factor VII in Human Atherosclerotic Vessels
Josiah N. Wilcox, Sumiko Noguchi and Juan Casanova

Arterioscler Thromb Vasc Biol. published online October 24, 2002;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2002/10/24/01.ATV.0000043418.84185.3C.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/