Induction of Tissue Factor in the Arterial Wall During Recurrent Thrombus Formation

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Objective—Tissue factor (TF) is normally expressed at low levels in the media of blood vessels, but it is readily induced after vessel injury. It is not known whether vascular damage per se or thrombus formation is responsible for this phenomenon.

Methods and Results—Cyclic flow variations (CFVs), attributable to recurrent thrombus formation, were induced in stenotic rabbit carotid arteries with endothelial injury. CFVs were observed for 30 minutes and 2, 4, and 8 hours in different groups of animals. Another group of rabbits pretreated with hirudin before inducing arterial damage to inhibit thrombus formation was observed for 8 hours. Arterial sections were immunostained for TF. Undamaged arteries served as controls. In additional rabbits, in situ hybridization experiments were performed. No TF expression was observed in the media of control vessels, whereas a progressive increase in TF mRNA and protein expression was observed in carotid arteries as CFVs progressed. No increase in TF expression was observed in animals pretreated with hirudin. In vitro experiments demonstrated that TF mRNA is induced in smooth muscle cells stimulated with activated platelets as well as with some platelet-derived mediators.

Conclusions—This phenomenon may contribute to sustain intravascular thrombus formation after the initial thrombogenic stimulus. (Arterioscler Thromb Vasc Biol. 2003;23:1684-1689.)

Key Words: tissue factor • intravascular thrombosis • platelets • platelet-derived mediators

Tissue factor (TF), a transmembrane glycoprotein, plays a pivotal role in the activation of the coagulation cascade. TF binds circulating factor VII (FVII) with high affinity, resulting in formation of a complex that activates factors IX and X, ultimately leading to generation of thrombin and thrombus formation.1–3

Several studies indicate that TF-dependent activation of the coagulation cascade plays an important role in the pathophysiology of intravascular thrombus formation.3–10 Because the formation of the complex TF-FVII represents the initial event in the activation of the coagulation cascade, TF expression in the arterial wall is tightly regulated. For instance, endothelial cells, being in contact with circulating blood, normally do not express TF in significant amounts, whereas the activity of this protein increases significantly from the media to the adventitia.4,7,11–15 The end point of this tight regulation of TF expression in the vascular wall is the avoidance of unwanted intravascular activation of the coagulation, together with a prompt hemostasis in the event of loss of integrity of the vascular wall.

TF expression within the vascular wall is not steady but rather can be modulated by a variety of substances. Phorbol esters,16,17 interleukin 1,18 α-thrombin,19 endotoxin,17,20 tumor necrosis factor,21 and oxygen free radicals22 all induce TF expression, whereas prostacyclin and its analogues result in inhibition of TF expression.23,24 TF can also be rapidly induced in rat aortas after balloon angioplasty,25 but whether vessel injury per se or intravascular thrombus formation26 caused by the vascular injury that follows angioplasty is responsible for this induction is still unknown.

Therefore, the aims of the present study were to investigate (1) whether TF is upregulated in the arterial wall during ongoing thrombosis, (2) which cellular populations are involved in this phenomenon, and (3) whether activated platelets and some platelet-derived mediators are involved in this TF induction.

Methods

Rabbit Model of Thrombosis

The rabbit model of recurrent intravascular thrombus formation used in the present study has been described in detail elsewhere27,28 and represents a modification of the canine model originally described by Folts and colleagues.29,30 This experimental preparation is based on...
carotid arterial damage with a superimposed external stenosis resulting in cyclic flow variations (CFVs), a typical pattern of flow attributable to recurrent cycles of thrombus formation and dislodgement.27

Experimental Protocol

Immunohistochemical Experiments
Animals were divided into 2 groups. In the first group, CFVs were observed for 30 minutes (group Ia) and 2 (group Ib), 4 (group Ic), and 8 hours (group Id, n=6 in each group). The second group of animals was included to differentiate the effects of the injury per se from those of thrombus formation. It is known, in fact, that vascular injury may upregulate TF in rat aortas.23 Group II rabbits (n=6) were treated 10 minutes before performing the damage with a bolus of hirudin (1 mg/kg IV) to prevent CFVs. Arterial damage was performed, and an external stenosis was created exactly as described for the animals in group I; however, because of hirudin administration, CFVs did not develop in any of the 6 rabbits. Animals were followed for 8 hours and then euthanized. Contralateral carotid arteries, not subjected to damage and, therefore, not developing CFVs, served as negative controls. For additional information, please see the online supplement at http://atvb.ahajournals.org.

In Situ Hybridization
To verify whether TF induction evidenced by immunohistochemistry was the consequence of exposure of preformed, encrypted TF11,12 or required TF gene transcription and de novo protein synthesis, in situ mRNA hybridization experiments were performed in additional groups of animals. For these experiments, animals were divided into 2 groups. In the first group, CFVs were observed for 30 minutes (group IIIa) and 2 (group IIIb), 4 (group IIIc), and 8 hours (group IIId, n=4 for each group). Group IV animals (n=4) included rabbits with arterial damage and external stenosis pretreated with hirudin, as previously described. For additional information, please see the online supplement.

TF Activity
To verify whether TF induction evidenced by immunohistochemistry was accompanied by TF protein expression, TF procoagulant activity was measured in arterial sections obtained from animals with CFVs. Also in this case, animals were divided into 2 groups. In the first group, CFVs were observed for 30 minutes (group Va) and 2 (group Vb), 4 (group Vc), and 8 hours (group Vd, n=4 for each group). Group VI animals (n=4) included rabbits with arterial damage and external stenosis pretreated with hirudin, as previously described. For additional information, please see http://atvb.ahajournals.org.

Detection of Cellular Populations Responsible for TF Expression
To characterize the cellular populations involved in TF upregulation, serial adjacent sections of carotid arteries from animals in group Id (8 hours of CFVs) were subjected to immunohistochemical staining specific for neutrophils using an antibody anti-CD 15 (Dako, Glostrup, Denmark), monocytes using an antibody anti-CD 68 (Dako), and smooth muscle cells (SMCs) using an anti-SMC α-actin antibody (Sigma Chemicals, St Louis, Mo).

Activated Platelets and TF Protein and mRNA Expression in SMCs In Vitro
To test the hypothesis that platelet-derived mediators are responsible for thrombus-induced TF expression in SMCs, additional in vitro experiments were performed. Rabbit aorta SMCs were harvested and cultivated as previously described.31 For additional information, please see the online supplement.

Statistical Analysis
Data are expressed as mean±SD. A one-way ANOVA was used for multiple comparisons among groups. If an F value was significant, differences for individual groups were tested with Student’s t test for unpaired observations with Bonferroni’s correction. P<0.05 was considered significant.

Results

Baseline TF Expression
Consistent with previous studies,4,25 no significant TF expression was observed in the media and in the intima of control, undamaged vessels, whereas a slight positivity was found only in focal spots in the adventitia (Figure 1A). No monocytes or neutrophils were detected in control vessels, whereas an intense positivity for α-actin was observed in the media, as expected (data not shown).

Time Course of TF Expression During CFVs
Figure 1 illustrates immunohistochemical staining for TF in arterial sections obtained from animals subjected to different time periods of CFVs. After 30 minutes of CFVs, a slight positivity for TF was observed in the media (Figure 1B), averaging 0.47±0.44 of the semiquantitative score (Figure 2), although this increase did not reach statistical significance. TF expression increased progressively to 1.3±0.45, 1.8±0.55, and 2.3±0.60 at 2, 4, and 8 hours, respectively (Figure 2). At 8 hours, the media was intensively and diffusely positive for TF, indicating that intravascular thrombus formation is associated with a significant TF upregulation in the arterial wall (Figures 1C through 1E). Interestingly, arterial sections obtained from animals pretreated with hiru-
din, subjected to the same degree of vascular damage but not developing CFVs, showed only a slight positivity for TF (Figure 1F) that was not statistically different from that observed in control, undamaged vessels (Figure 2). These data strongly suggest that thrombus formation plays a prominent role in inducing TF upregulation in the arterial wall rather than the damage of the vessel.

Characterization of the Cellular Population Expressing TF
To determine the cellular population responsible for the observed expression of TF during recurrent thrombosis, adjacent sections were stained for TF and either neutrophils, monocytes, or SMCs. These sections revealed that TF localized mostly with SMCs, indicating that SMCs were predominantly responsible for TF upregulation, although at later time points, ie, 8 hours, some degree of TF-expressing cells infiltrating the arterial wall could be observed, particularly in the luminal side of the vessels (Figure 3).

In Situ Hybridization
To determine whether TF protein expression was accompanied by de novo TF mRNA transcription, in situ hybridization experiments were performed. These experiments showed a pattern of TF mRNA expression similar to that of TF protein expression evidenced by immunohistochemistry. In particular, in arterial sections incubated with the antisense probe, a progressive increase in TF mRNA expression was observed, starting at 30 minutes of CFVs and peaking at 8 hours (Figure 4). Minimal amounts of TF mRNA, again mainly localized in the adventitia, were detected in undamaged vessels; no detectable signal was observed with the sense probe (negative controls). Carotid arteries obtained from animals pretreated with hirudin showed minimal induction of TF mRNA (Figure 4), indicating that the positivity for TF mRNA was specific.

TF Activity in Carotid Arteries With and Without CFVs
TF procoagulant activity increased significantly in the media isolated from vessels with CFVs compared with the media obtained from control arteries, starting at 2 hours and peaking at 8 hours. Data are summarized in online Table I, available at http://atvb.ahajournals.org.

Activated Platelets and TF mRNA and Protein Expression in SMCs In Vitro
TF mRNA was barely detectable in quiescent SMCs. Stimulation with collagen-activated platelets caused a time-dependent increase in TF mRNA levels compared with unstimulated SMCs, peaking at 2 hours. TF mRNA levels progressively decreased thereafter, returning to baseline values at 24 hours. SMCs stimulated with collagen alone (Figure 5) or with nonactivated platelets (data not shown) did not show any increase in TF mRNA expression. TF protein, evaluated as procoagulant activity, showed a similar pattern of expression, although with a time shift of approximately 2 hours (Figure 5).

TF Expression in SMCs Stimulated With Platelet-Derived Mediators
To determine the relative contribution of different platelet-derived mediators on TF expression in SMCs, TF procoagulant activity was measured at different time points after stimulation with increasing concentrations of ADP, U46619, 5-HT, PAF, platelet-derived growth factor, and TRAP. All agonists except ADP induced a time- and dose-dependent increase in TF expression, the most potent being platelet-derived growth factor and TRAP. Of note is the observation of a synergistic effect when all agonists were added together at subthreshold concentration (online Table 2, available at http://atvb.ahajournals.org).

Discussion
The main findings of the present study are as follows: (1) TF mRNA, protein, and procoagulant activity are readily upregulated in the arterial wall during ongoing recurrent thrombus
TF is the main initiator of the extrinsic coagulation pathway. The tight binding between TF and FVII, leading to the formation of a complex able to activate factors IX and X, is the first step of this pathway.1–4 As a consequence, TF expression in the arterial wall is finely regulated. TF is normally sequestered from circulating blood, because endothelial cells do not express this protein in significant amounts,4 whereas its activity increases from the media to the adventitia.7,11–15 Therefore, when atherosclerotic plaque ruptures, preformed TF present in the subendothelium is suddenly exposed to flowing blood, contributing to formation of a thrombus and to the sudden onset of acute coronary syndromes.5–9

Apart from the preformed protein, induction of TF in the arterial wall might also represent an important mechanism regulating thrombus formation. In fact, TF can be induced by a variety of stimuli, including endotoxin,17,20 phorbol esters,16,17 interleukin-1,18 α-thrombin,19 tumor necrosis factor,21 and oxygen free radicals.22 Interestingly, previously published studies have already described induction of TF in the arterial wall after vessel injury using immunohistochemistry and either in situ hybridization or RNA blot analysis.25,34–36 Furthermore, a recent study showed not only that TF is rapidly induced in rabbit aortas after balloon injury but also that TF activity in the developing neointima is maintained at high levels for at least 8 weeks after the injury.37 However, data from those studies do not allow one to dissect out the relative contribution of the injury per se from that of thrombus formation, because mural thrombi almost invariably form after balloon injury. The present study was designed to clarify this issue, and our data indeed demonstrate that TF in the arterial wall is primarily induced as a consequence of platelet activation, thrombin generation, and thrombus formation rather than vessel injury per se.

The observation that damaged vessels obtained from animals pretreated with hirudin, in which CFVs could not be induced, did not show a significant increase in TF compared with vessels obtained from animals with CFVs obviously points toward a prominent role of thrombin in this phenomenon. However, it should be outlined that it is likely that other platelet-derived chemical mediators also play a role in vivo. This is suggested by 2 lines of evidence. First, pretreatment with hirudin not only eliminated the effects of thrombin but also those of other mediators, because CFVs did not develop. Second, direct stimulation of SMCs in vitro with platelet-derived mediators showed that most of them (with the possible exception of ADP) are able to induce TF. Thus, although probably thrombin occupies a central role in TF formation; (2) a prominent role for this TF upregulation is played by thrombin generated during thrombus formation rather than by the arterial damage per se; (3) TF is predominantly upregulated in arterial smooth muscle cells, with very little contribution of infiltration of the arterial wall by TF-expressing cells, such as monocytes and neutrophils; and (4) platelets and soluble platelet-derived chemical mediators induce TF mRNA in SMCs in vitro, suggesting their important role in inducing TF in the arterial wall in vivo.

**Figure 4.** Time course of TF mRNA expression in vessels with CFVs and subjected to in situ hybridization (magnification ×32 for each panel). A and B, Control, undamaged vessels with no CFVs and stained with sense (A) and antisense probe (B). No staining for TF was detected in the media of sections hybridized with sense probe, whereas a slight positivity was observed in the adventitia of sections hybridized with antisense probe. C, Vessel harvested after 8 hours of CFVs and stained with sense probe. No staining for TF was observed in the media, whereas a slight background staining was present in the adventitia. D, Vessel harvested after 8 hours of CFVs and stained with antisense probe. An intense positivity for TF is detectable in the media and in the intima, which appear almost uniformly stained for TF. E and F, Vessels obtained from animals pretreated with hirudin and stained with sense (E) or antisense (F) probe. No staining for TF was detected in the media and only a weak background signal was evident in the adventitia of sense-hybridized sections, whereas in sections hybridized with antisense probe, there was a more intense staining for TF mRNA mainly localized in the adventitia.

**Figure 5.** Time course of TF mRNA (A and B) and protein expression (C) in SMCs stimulated in vitro with activated platelets. Both TF mRNA and protein were rapidly induced by activated platelets, peaking at approximately 2 and 4 hours, respectively. No induction was observed with nonactivated platelets (data not shown) or with collagen alone.
induction after arterial damage, an important contribution of other mediators is likely to occur.

The demonstration that intravascular thrombus induces TF expression in the media may be particularly relevant to the pathophysiology of acute coronary syndromes as well as of percutaneous interventions. Induction of TF on the surface of SMCs exposed to flowing blood could not only provide a site for additional activation of the coagulation cascade but could also participate in the development of neointimal hyperplasia, as recent preliminary data from our laboratory seem to suggest. It should be emphasized, however, that the results obtained in the experimental model used in the present study might be specific for this model and that it remains to be determined whether the same findings can be observed in models characterized by a more severe vascular injury, such as balloon-induced injury.

Candidates for the observed thrombus-induced TF upregulation are activated platelets and platelet-derived mediators. Data from the present study indicate that soluble mediators secreted from activated platelets rapidly induce TF mRNA and protein in SMCs in culture. In vivo, however, the potential role of other cell types, such as leukocytes, or mediators, such as thrombin, cannot be ruled out. The gene coding for TF belongs to the so-called immediate early genes, a group of sentinel genes that are able to answer immediately to different noxious stimuli by promoting the transcription of sequences encoding for proteins that explicate a protective effect. This is consistent with the data from the present study that supports the existence of a new pathophysiological mechanism originally triggered by intravascular thrombus formation. After the initial thrombogenic stimulus, activated platelets and perhaps other components of the thrombus stimulate SMCs in the arterial wall to synthesize new TF, which may help sustain the thrombotic process and activate cell proliferation and perhaps gene expression. In this pathophysiological scheme, TF may represent a link between thrombosis, inflammation, and atherosclerosis.

In conclusion, the present study suggests the existence of a positive feedback loop for expression of TF in the arterial wall. Preformed TF is important in initiating intraarterial thrombus formation, which, in turn, is able to stimulate the synthesis of new TF, thus sustaining the thrombotic process and perhaps SMC proliferation.

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References


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