New Tricks for Old Dogs
Nonthrombotic Effects of Thrombin in Vessel Wall Biology

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Abstract—Thrombin is a serine protease that potently activates platelets and catalyzes the conversion of fibrinogen to fibrin. Thrombin also exerts direct effects on vascular cells, such as smooth muscle cells, via interactions with members of the protease-activated receptor family. Evidence in several animal models implicates thrombin-mediated signaling events in the response to injury that typifies vascular lesion formation in atherosclerosis and restenosis. In this review, we examine the activation of protease-activated receptors by thrombin, the downstream signaling events mediated by these receptors, and the physiological role of thrombin in vascular cells and vascular disease. (Circ Res. 2001;88:987-997.)

Key Words: atherosclerosis • thrombin • vascular biology

Conceptually, our understanding of the interface between the thrombosis and hemostasis systems, on one hand, and vessel wall biology, on the other hand, is undergoing a gradual evolution. In its earliest formulation, the interface between these two processes was the inert barrier provided by the vascular endothelium; disruption of this barrier was considered the major stimulus to activation of the coagulation cascade. A level of complexity was added by the discovery that platelet proteins (such as platelet-derived growth factor) and hemostatic factors (such as thrombin) have potent activities on vascular biology independently of any coagulation effects. Conversely, we now know that vascular endothelial and smooth muscle cells (SMCs) produce proteins like tissue factor that modulate the coagulation cascade in the absence of endothelial disruption. This situation becomes even more complex when the effects of coagulation factors on cardiovascular development are considered; the recent demonstration that factor V–deficient mice, which cannot generate thrombin, die in utero of vascular defects in the absence of hemorrhage shows how closely these two systems are interrelated.1 Not only is the interface between coagulation and vessel wall biology not as simple as it was once thought, the boundaries between these two systems can now hardly be defined at all. It is not surprising, then, that the central molecule in blood coagulation, thrombin, also has profound effects on virtually every aspect of vascular wall biology, including regulation of vessel tone; SMC proliferation, differentiation, and migration; vascular development; atherogenesis; and angiogenesis (Figure 1). The purpose of this review is to take thrombin out of the narrow context of its role in the coagulation cascade and in platelet function (these aspects of thrombin function have been reviewed elsewhere2,3) and to focus on the roles played by thrombin and its vascular receptors in SMC biology and vascular disease. Although to date pharmacologic inhibition of thrombin has yielded mixed results in the treatment and prevention of atherogenesis, a...
more precise understanding of how thrombin mediates its effects in the vasculature may well lead to more focused therapeutic approaches.

Thrombin Activation and Inhibition

Thrombin is produced predominantly on the surface of circulating platelets as a result of proteolytic activation of the 72-kDa zymogen prothrombin, which is constitutively synthesized in the liver and released into the circulation. Therefore, activation of thrombin can occur at a distance from its place of secretion, and, in particular, activation occurs at sites enriched for enzymes that facilitate the conversion of prothrombin to thrombin. In the circulation, factor Xa and its cofactor Va activate thrombin, and the activation of Xa is controlled by tissue factor, a membrane protein that is expressed at sites of vascular injury. Activated thrombin participates in clot formation via its serine protease activity, which cleaves fibrinogen to fibrin. Thrombosis is also facilitated by the ability of thrombin to activate platelets directly.

Knowledge of the structural determinants of the enzymatic activity of thrombin is important for understanding the mechanisms by which thrombin activity may be regulated. Because thrombin plays such a vital role in hemostasis, it comes as no surprise that its activity can be regulated by a variety of endogenous and exogenous factors and that thrombin is a prime target for pharmacologic intervention, as described in detail later in this review. The activity of thrombin can be regulated indirectly via factors that modulate the activation of thrombin (such as activated protein C, which degrades factors V and VIII and therefore inactivates the coagulation cascade). However, direct inhibitors of thrombin, such as the endogenous proteins antithrombin III and heparin cofactor II and the exogenous inhibitors hirudin and bivalirudin (Hirulog), can also target the activity of this enzyme with specificity. Direct inhibitors of thrombin take advantage of the unique structural elements of the activated thrombin molecule, and the activity of direct inhibitors can best be explained on the basis of structural features that determine their effects.

Fortunately, the structure of thrombin, in isolation and in association with cofactors, has been extensively and elegantly studied. Thrombin assumes a globular football shape, with a deep cleft that contains the active site catalytic residues of the enzyme. Negatively charged residues that participate in substrate recognition surround the active site. Two additional positively charged patches interact with factors that modulate the activity of thrombin. One such region, the fibrinogen-recognition exosite, sits at the base of the active site cleft and mediates interactions between thrombin and the substrates fibrinogen and protease-activated receptor 1. The endogenous thrombin inhibitor heparin cofactor II docks with this site in a heparin- or dermatan sulfate-dependent manner that allows heparin cofactor II to interfere with the enzymatic activity of thrombin. The exogenous thrombin inhibitor hirudin also binds to the fibrinogen-recognition exosite and the active site to inhibit thrombin function and is used clinically as a potent antithrombin. A second positively charged patch, to the opposite side of the active site cleft from the fibrinogen-recognition exosite, is the heparin-binding site that allows heparin to tether antithrombin III to thrombin. Antithrombin III, in turn, interferes with the negatively charged residues around the active site that facilitate substrate recognition, thus preventing their cleavage by thrombin.

Vascular Thrombin Receptors

Initial observations that thrombin exerts direct effects on platelets, endothelial cells, and SMCs indicated that receptor
mediated events must exist to initiate signaling responses to thrombin. The discovery of the first thrombin receptor (now referred to as protease-activated receptor-1, or PAR1) and the recognition that this receptor was activated by the proteolytic cleavage of its extracellular domain provided a new paradigm for receptor activation. The protease-activated receptors are a family of G protein–coupled receptors that contain protease recognition sequences in their extreme amino-terminal extracellular sequences. Proteolytic cleavage at these sites releases a small peptide and unmasks a new amino-terminal domain that can activate the receptor through intramolecular interactions. Proof of such a mechanism is demonstrated by the fact that synthetic peptides analogous to these unmasked sequences (the tethered ligands) can activate these receptors with an efficiency equivalent to that of the proteases themselves. For example, the synthetic peptide SFLLRN, which is exposed by thrombin-mediated cleavage of the extracellular domain of PAR1, efficiently activates signaling responses by this receptor in thrombin-sensitive cell types.

Both the inability of the PAR1-activating peptide SFLLRN to completely recapitulate thrombin’s effects in other cell types and the likelihood that other receptors of this type existed led to the search for other protease-activated receptors. Presently, 4 members of the protease-activated receptor family have been identified (Table 1). Of these, PAR1, PAR3, and PAR4 can be cleaved and activated by thrombin, although thrombin-mediated cleavage of PAR4 occurs at higher concentrations of thrombin than are required for activation of PAR1 and PAR3, raising the possibility that activation of this receptor may occur in an atypical fashion, as described below. PAR2, in contrast, is not activated directly by thrombin but can be activated under experimental conditions by trypsin and factor VIIa. The existence of multiple thrombin receptors indicates that the cellular responses to thrombin may be determined at least in part by the presence of different combinations of thrombin receptors on different cell types.

**Thrombin Signaling**

The classic activation sequence of thrombin receptors (ie, proteolytic cleavage to unmask a tethered ligand) is best illustrated for PAR1, a thrombin receptor that is activated at low thrombin concentrations. Surprisingly, a pathway for receptor activation that involves intermolecular interactions also exists. When other thrombin receptors are absent, PAR4 requires high concentrations of thrombin for direct activation, presumably because of the lack of high-affinity thrombin interaction sites in the extracellular domain of this receptor. Activation of PAR4 by low thrombin concentrations occurs only when PAR3 is also present. It seems that PAR4 activation under these circumstances is initiated via binding of thrombin first to the PAR3 exosite. The thrombin-bound PAR3 complex then serves as a cofactor that allows trans-cleavage of the PAR4 exosite to unmask the tethered ligand of PAR4 (Figure 2). Trans-activation of PAR2 by PAR1 may also occur, and PAR2 activation may be required for some aspects of thrombin signaling, even though it is not itself a thrombin receptor. The advantages to be gained by such a complicated activation series are not yet explained, although such events probably contribute to the diversity of thrombin-mediated responses and allow for cell type–specific activa-

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**Table 1. Protease-Activated Receptors Identified in Vascular Tissue**

<table>
<thead>
<tr>
<th>Protein Agonists</th>
<th>TRAP</th>
<th>Chromosome</th>
<th>Expression by Vascular Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR1 Thrombin</td>
<td>SFLLRN</td>
<td>5q13</td>
<td>Human atheroma; balloon-injured rat carotid artery; SMCs, endothelial cells, and fibroblasts in culture</td>
</tr>
<tr>
<td>PAR2 Trypsin, tryptase, factor VIIa + tissue factor, factor Xa, thrombin-cleaved PAR-1</td>
<td>SLIGKV (human); SFLLRN (human); SLIGRL (rat, mouse, bovine)</td>
<td>5q13</td>
<td>Balloon-injured rat carotid artery; normal human arteries; human aortic, endothelial, and coronary SMCs in culture</td>
</tr>
<tr>
<td>PAR3 Thrombin</td>
<td>TFRGAP</td>
<td>5q13</td>
<td>Human endothelial cells in culture</td>
</tr>
<tr>
<td>PAR4 Thrombin, cathepsin G</td>
<td>AYPGKF (human); GYPGQV (human); GYPGKF (murine)</td>
<td>19p12</td>
<td>Rat aorta</td>
</tr>
</tbody>
</table>

TRAP indicates thrombin receptor–activating peptide.

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**Figure 2.** Thrombin receptor activation mechanisms: direct activation versus trans-activation. Top, Traditional pathway of thrombin receptors by thrombin, in which soluble thrombin directly cleaves the extracellular domain of a receptor (in this case, PAR1) to unmask a receptor-activating tethered ligand. Bottom, Example of receptor trans-activation, in which soluble thrombin in low concentrations interacts with the first receptor (in this case, PAR3) and the thrombin-receptor complex itself serves as an enzyme to cleave the second receptor, PAR4.
tion sequences to occur. In addition, as described below, the recruitment of multiple receptor types by thrombin extends beyond the G protein–coupled receptor family.

Ongoing work from several laboratories indicates that signaling downstream from the thrombin receptors is surprisingly complex. The events that occur consequent to receptor binding and G protein activation involve broadly dissimilar, parallel signaling pathways that frequently intersect and even activate signaling via other, nonthrombin-activated receptors (Figure 1). For these reasons, assigning specific effects of thrombin to each pathway has been a challenge. Several of these pathways have been characterized in a variety of cell types and are described briefly in this review. More recently, generation of reactive oxygen species (ROS) as signaling intermediates and crosstalk between thrombin and other receptor families have been implicated in events closely linked to vascular dysfunction. These recently elucidated thrombin-activated signaling events are therefore described in greater detail.

G Protein–Coupled Receptor Signaling
Although each of the protease-activated receptors that respond to thrombin undoubtedly mediate different thrombin responses, most of what is known about thrombin signaling downstream of the receptors themselves has derived from studies of PAR1. PAR1 couples with at least 3 G protein families, G12/13, Gq, and Gi, to elicit diverse downstream signaling events.21–23 The well-characterized pathways activated via these G protein–dependent events include Rho activation by Gi3 that causes cytoskeletal changes affecting vascular cell migration.24,25 Gq-dependent signaling activates phospholipase C26 which in turn leads to mitogen-activated protein kinase (MAPK) phosphorylation and receptor tyrosine kinase trans-activation, both necessary events in thrombin-mediated proliferation. Gβγ interactions activate phosphoinositide 3-kinase, which promotes Ca2+ release that is required for SMC growth in response to thrombin.27 It is clear that diverse cellular responses to thrombin depend on this rich signaling network and that ample opportunity exists for integration of signaling events elicited by thrombin and by other activation pathways to modulate the cellular phenotype.

Thrombin and ROS
ROS induce activation of growth-related signaling pathways in a manner similar to that of endogenous growth factors, indicating that ROS may lie downstream of some growth factors in the course of intracellular signaling.28 Growth factors known to play a role in atherogenesis, such as platelet-derived growth factor29 and angiotensin II,30 generate intracellular ROS, and ROS in turn can elicit specific growth responses in SMCs.31 It is therefore quite interesting that ROS production by a unique SMC oxidase is also required for mitogenic signaling events elicited by thrombin.32 This oxidase is similar but not identical to the neutrophil NAD(P)H oxidase. SMCs express p22phox, a membrane-associated component of the oxidase, and p47phox, a cytoplasmic component that translocates to the activated membrane–anchored oxidase on stimulation.32,33 Characterization of the NAD(P)H oxidase in SMCs has indicated that in these cells, the function of the catalytic membrane–associated gp91phox subunit is likely replaced by a homologue (a member of the nox family) that has functional and structural similarity to the neutrophil oxidase component gp91phox.34 A recent review by Griendling et al35 presents a more complete discussion of the components of the vascular NAD(P)H oxidase.

Functional studies indicate that both p22phox and nox1 are necessary for ROS generation in SMCs,33,34 and recent data from our laboratories indicate that p47phox is also required for oxidase activity in SMCs (P. Barrylane, C. Patterson, M.S. Runge, unpublished data, 2001). The p47phox component of the oxidase becomes phosphorylated and is recruited to the cell membrane after thrombin stimulation, and this same oxidase component is upregulated after vascular injury in the rat carotid injury model, suggesting that the oxidase in general, and p47phox in particular, plays a central role in signaling events leading to vascular lesion formation. Of interest, angiotensin II activates the SMC oxidase to generate intracellular ROS and initiate cell growth,33 indicating that this oxidase may mediate a central pathway that is activated in vascular pathologies. However, the precise mechanisms that lead from thrombin-mediated receptor activation to oxidase assembly and ROS generation remain obscure. In neutrophils, several different signaling pathways have been implicated in p47phox phosphorylation and receptor activation, but the proximal kinases that activate the ROS-producing capacity of this oxidase continue to be obscure.36 It remains to be determined whether the pathways for thrombin-mediated oxidase activation will be different or similar to the pathways that activate the neutrophil NAD(P)H oxidase.

Although ROS generation via the SMC NAD(P)H oxidase plays a necessary role in thrombin-induced effects in SMCs, the precise signaling events mediated by these ROS are still unclear. It seems that ROS are relatively proximal events in the thrombin-signaling pathway insofar as thrombin-induced MAPK activity and Janus kinase/signal transducers and activators of transcription signaling are ROS-dependent.37 Intriguingly, recent data indicate that ROS may feedback directly on G proteins themselves that then activate signaling via MAPKs and other pathways.38 Clearly, a great deal more needs to be determined about the role of ROS in thrombin-mediated signaling and, by extension, in atherogenesis and other thrombin-associated phenomena. Ascertainment of the role of ROS in these events is not a trivial point, because antioxidant therapies have been tested in several formulations for their effectiveness in modulating the progression of vascular lesions, albeit with little success.39 The gap in our understanding of the role of ROS in thrombin-mediated signaling certainly contributes to this oxidative paradox but offers the potential for development of new therapeutic interventions based on growing knowledge of these events.

Crosstalk Between Thrombin-Mediated Signaling and Other Receptor Types
Trans-activation of one protease-activated receptor by another, as described above, provides one means of crosstalk between receptors, albeit within the same family. G protein–coupled receptors, and in particular the protease-activated receptors, also interact heterotypically with other receptor
families to elicit appropriate signaling responses. The first well-characterized example of such an interaction was described between PAR1 and the epidermal growth factor (EGF) receptor.40 Thrombin was found to elicit activation of the EGF receptor, and thrombin signaling could be inhibited efficiently by a dominant-negative EGF receptor. Surprisingly, this effect seemed to be independent of EGF itself, which led to the initial belief that this receptor trans-activation was ligand-independent. However, these receptor interactions are in fact much more complicated, and subsequent studies have shown that EGF receptor activation by G protein–coupled receptors is dependent not on EGF but on another ligand that interacts with the EGF receptor, heparin-binding EGF-like growth factor (HB-EGF).41,42 HB-EGF is synthesized as an inactive, membrane-bound precursor. Protease-activated receptor activation by thrombin induces metalloproteinase activity that sheds the active ectodomain of HB-EGF, which then can activate the EGF receptor. Such interactions provide yet another unexpected twist to the thrombin-signaling story, although some issues, such as the nature of the metalloproteinase activity that is stimulated by this system, remain to be clarified.

Receptor tyrosine kinases are not the only heterotypic receptors that interact functionally with the thrombin receptors in vascular SMCs. Interactions between platelet integrins and protease-activated receptors have been well-characterized, and more recent studies indicate that β3-containing integrins communicate with thrombin receptors and that integrin signaling is necessary for maximal thrombin-mediated proliferative events in SMCs.43 The precise contributions of direct interactions between protease-activated receptors and integrins or of intracellular interactions and ligand-dependent integrin signaling through molecules such as thrombospondin remain to be determined. However, the functional importance of these interactions is likely to be significant, insofar as integrins are required for phenotypic responses in SMCs, such as adhesive and migratory cell-matrix events that are central to the vascular response to injury.44,45

**Thrombin and Vascular Function**

It is now clear that the effects of thrombin extend beyond its central role in coagulation and platelet function. Receptors for thrombin are present on vascular SMCs and endothelial cells,46 and thrombin stimulation of the endothelium has effects that are prothrombotic47 and proinflammatory.48 Thrombin is mitogenic and chemotactic for inflammatory cells,49,50 such as lymphocytes and macrophages, which may be present in the vessel wall in pathologic conditions. Thrombin is a potent stimulus for endothelium-dependent vasodilatation mediated by activation of nitric oxide production.51 Thrombin also modulates endothelial cell growth responses. Thrombin is among the most potent stimuli for nonhypoxic vascular endothelial growth factor (VEGF) expression52 and also upregulates VEGF receptors in endothelial cells.53 These latter effects may explain, in part, the effects of thrombin on angiogenesis and vascular development, as described below.

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**TABLE 2. Some Secondary Growth Factors and Signaling Molecules Implicated in Thrombin-Induced Proliferation of Vascular SMCs**

<table>
<thead>
<tr>
<th>Secondary Growth Factor</th>
<th>Signaling Molecules</th>
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<tbody>
<tr>
<td>Autocrine production of PDGF-AB</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>Autocrine production of basic fibroblast growth factor</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>Autocrine production of epiregulin</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>HBEGF-mediated trans-activation of EGF receptor</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>Flavin-containing oxidases</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>Insulin-like growth factor-1 receptor</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>RhoA</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>pp60c-src and p21ras</td>
<td>G protein–coupled receptor kinase 2</td>
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<tr>
<td>β1 integrins</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>Nuclear factor-κB</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
<tr>
<td>G protein–coupled receptor kinase 2</td>
<td>G protein–coupled receptor kinase 2</td>
</tr>
</tbody>
</table>

**Thrombin and Vascular SMCs**

Although modulation of endothelial cell function by thrombin might be easily understandable, in consideration of the role of thrombin in maintaining hemostasis, a role for thrombin in SMC biology indicates that its effects are not limited to coagulation events and are more diverse than originally thought. Therefore, the importance of initial observations that thrombin stimulated SMC proliferation cannot be overestimated.54

Although thrombin-induced proliferation is perhaps the best-described cellular response of SMCs, the cellular program elicited by thrombin in this cell type is relatively complex (Table 2). Thrombin elicits an inflammatory response that includes secretion of interleukin-6 and monocyte chemotactic protein-155 and stimulates SMCs to synthesize collagen56 that may contribute to extracellular matrix accumulation. Smooth muscle migration is also induced in response to thrombin stimulation.57 The inflammatory, fibrotic, and chemotactic responses to thrombin may be major contributors to the deleterious response to injury that culminates in obstructive vascular lesions. Heat shock proteins such as Hsp70, Hsp90, and Hsp27 are also activated and upregulated in SMCs by thrombin,58,59 and these proteins may contribute to the atherogenic response, because expression of these proteins is particularly concentrated in SMCs surrounding the necrotic core of atherosclerotic plaques.60

Finally, an interaction between thrombin and smooth muscle that deserves mention is the activation of thrombin that occurs in SMCs undergoing cell death via the apoptotic pathway.61 Phosphatidyserine that is exposed at the membrane of cells undergoing apoptosis facilitates the assembly of a prothrombinase complex that accelerates the generation of thrombin. Apoptosis of SMCs can occur after vascular injury62 and in response to changes in extracellular matrix composition63,64 and may exacerbate the wound-healing response.65 The generation of active thrombin by these apoptotic SMCs may serve as a positive feedback loop to accelerate the atherogenic process and as such may provide one explanation for the uncontrolled proliferation of SMCs after vascular injury.
Thrombin and Vascular Lesion Formation

There has been speculation for some time that thrombin participates in vascular lesion formation. Indeed, the thrombotic effects of thrombin alone would implicate this protease in vascular lesion formation in the setting of vascular injury and atherosclerosis. Recent studies indicate that thrombin does in fact increase the response to injury in these settings; however, these effects seem to be mediated primarily via direct effects of thrombin on vascular arterial cells rather than indirectly through its hemostatic effects.

Concentrations of thrombin are increased at the arterial surface after denuding injury and remain elevated for up to 10 days. Both activated thrombin and its precursor, prothrombin, can be detected within the neointima of human atherosclerotic lesions. Effects of this thrombin on adjacent cells are suggested not only by its presence in activated form but also by upregulation of its cognate receptor PAR1. PAR1 expression is upregulated within 6 hours of injury to the rat carotid artery and remains so during the course of neointima formation. Increased expression of PAR1 has also been observed in atherosclerotic plaques from human arteries but not in normal arteries. Thus, thrombin and its receptors are in the right place at the right time to contribute to the vasculoproliferative response to injury.

A critical question raised by the presence of active thrombin within the arterial walls is how this thrombin is regulated. The major endogenous inhibitor of thrombin in the intravascular compartment is antithrombin III, yet little antithrombin III is present within vascular lesions and no antithrombin III/thrombin complexes are detectable. It has been suggested that plasminogen activator inhibitor 1 (PAI-1) can interact with activated thrombin to inhibit thrombin-mediated activity and that vitronectin is a cofactor in this complex. This complex may be converted either to an inactive thrombin/PAI-1 complex or scavenged by the LDL receptor–related protein present on SMCs and macrophages. Indeed, active thrombin, PAI-1, and vitronectin can be found colocalized within human atherosclerotic lesions, suggesting that this is a physiologically relevant mechanism for regulation of thrombin activity within vascular lesions. The existence of a regulatory mechanism involving inhibition of active thrombin by PAI-1 suggests that decreased PAI-1 expression or activity within arteries should enhance the amount of free thrombin available to stimulate vascular cells, with deleterious consequences in the context of lesion formation. Such a scenario is supported by observations that mice lacking PAI-1 have greater neointimal formation after injury than do wild-type mice, indicating an inhibitory role for PAI-1 in lesion formation, perhaps via its ability to sequester active thrombin.

Heparin cofactor II is an additional factor that may regulate the activity of thrombin in vascular lesions. Heparin cofactor II binds to and inactivates thrombin and can potently inhibit thrombus formation in models of thrombosis. As mentioned above, heparin cofactor II inhibits thrombin by directly interacting with the fibrinogen-recognition exosite of thrombin, and the activity of heparin cofactor II is increased by interactions with dermatan sulfate–containing proteoglycans of the extracellular matrix. Interestingly, proteoglycans derived from atherosclerotic vessels, such as biglycan and decorin, are impaired in their ability to activate heparin cofactor II, compared with proteoglycans derived from normal arteries. Taken together, these results indicate an additional means by which thrombin activity is regulated within the vasculature and suggest that alterations in dermatan sulfate–containing molecules may accelerate atherosclerotic lesion formation by attenuating the inactivation of thrombin.

The functional role of thrombin in lesion formation has been addressed elegantly in several different models, and hirudin, a potent thrombin inhibitor, has been a potent tool in this regard. Hirudin administration in the periprocedural period reduces neointimal formation after injury in minipig and rabbit models. This effect may be attributable to prolonged inhibition of thrombin activity within arteries by hirudin after short-term administration. Interestingly, studies performed in rats demonstrate that prolonged (but not short-term) administration of hirudin is effective in inhibiting the vasculoproliferative response. Likewise, administration of hirudin via adenosine gene therapy, which produces hirudin over a period of days, is effective in blocking lesion formation in the rat carotid injury model. Taken together, these studies demonstrate the importance of thrombin generation in lesion formation but also emphasize the significance of the time course of thrombin inhibition and possibility of species-specific responses to thrombin inhibition in the vasculoproliferative process.

Targeting the receptor PAR1 using inhibitory antibodies has been a second approach to inhibiting thrombin activity experimentally. The feasibility of this approach has been indicated by the inhibition of thrombus formation in Green monkeys and by blocking neointimal formation after balloon injury in the rat. Because rodent platelets (in contrast with those in primates) do not express PAR1 and are activated by thrombin in a PAR1-independent manner, these experiments provide presumptive evidence that the effects of thrombin on vascular lesion formation are attributable in significant part to direct effects of thrombin on vascular cells. More precise studies of the role of thrombin signaling via the PAR1 receptor have been performed using genetically modified mice that lack the PAR1 receptor. These experiments generally confirm the preceding studies but also suggest that the role of this pathway may be more complex than previously indicated. Neointimal and medial areas are decreased in PAR1-deficient mice after denuding injury, yet in these experiments luminal diameters also decrease, suggesting a component of adverse remodeling that may indicate changes in extracellular matrix components in the PAR1-deficient mice. These studies, taken together with the species-specific effects of thrombin inhibitors, demonstrate that the complexity of this system needs to be taken into account in the translation of this approach to human studies (Table 3). In addition, doses of hirudin used in the human trials have been far lower than those used in animal studies, owing to the risks of bleeding associated with the use of higher doses in humans. More promising approaches for sustained, local, high-concentration delivery, including the use of impregnated stents, may be a useful means to reap the benefits of...
Thrombin and Angiogenesis

Thrombosis has long been associated with angiogenesis-associated diseases such as cancer, and thrombin itself can be detected in a variety of tumor types. However, an appreciation that thrombin generation may directly promote angiogenesis has only emerged recently. Angiogenesis is stimulated by thrombin in both in vivo and in vitro model systems. Given the preclinical data, it is disappointing that clinical studies using the direct thrombin inhibitors hirudin and bivalirudin have failed to show efficacy in inhibition of restenosis after angioplasty in humans. However, the realization provided from animal studies that the consequences of thrombin inhibition on lesion formation may depend on the duration of thrombin inhibition and on other factors. In the human studies reported so far, the duration of thrombin inhibition has been relatively short and the doses used were far lower than those used in animal studies. Consideration that thrombin inhibition in these trials was either too brief or incomplete raises the possibility that locally delivered hirudin (or other thrombin inhibitors), perhaps delivered via impregnated stents, may be a more reasonable approach to inhibit thrombin effectively, locally, and for prolonged periods.

Mechanical Injury

TABLE 3. Animal and Human Studies Examining the Effects of Direct Thrombin Inhibition on Neointimal Formation After Vascular Injury

<table>
<thead>
<tr>
<th>Study</th>
<th>Comparison</th>
<th>Administration</th>
<th>Model</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarembock (1991)</td>
<td>Hirudin vs heparin</td>
<td>IV bolus + 2-hour infusion</td>
<td>BA of atherosclerotic femoral artery in hypercholesterolemic rabbits</td>
<td>Reduction in cross-sectional narrowing by plaque in hirudin-treated group</td>
</tr>
<tr>
<td>Walters (1994)</td>
<td>Hirudin + P-PACK vs placebo</td>
<td>IV bolus of hirudin + 7-day infusion of P-PACK</td>
<td>BA of aorta in rabbits</td>
<td>Reduction in intimal/medial ratio in antithrombin-treated rabbits</td>
</tr>
<tr>
<td>Bittl (1995)</td>
<td>Bivalirudin vs heparin</td>
<td>IV bolus</td>
<td>Human coronary angioplasty</td>
<td>No difference in event-free survival</td>
</tr>
<tr>
<td>Serruys (1995)</td>
<td>Hirudin vs heparin</td>
<td>IV bolus + 24-hour infusion + 2 additional days subcutaneously</td>
<td>Human coronary angioplasty</td>
<td>No difference in event-free survival</td>
</tr>
<tr>
<td>Jang (1995)</td>
<td>Hirudin vs heparin</td>
<td>IV bolus + 3-day infusion</td>
<td>BA of atherosclerotic femoral artery in rabbits</td>
<td>No difference in neointimal area</td>
</tr>
<tr>
<td>Rade (1996)</td>
<td>Hirudin vs β-galactosidase</td>
<td>Locally delivered adenoviral vector</td>
<td>BA of carotid artery in rats</td>
<td>Reduction in neointima in hirudin-treated group</td>
</tr>
<tr>
<td>Sarembock (1996)</td>
<td>Hirulog vs heparin</td>
<td>IV bolus + 2-hour infusion</td>
<td>BA of atherosclerotic femoral artery in rabbits</td>
<td>Reduction in lumen stenosis and intimal plaque area in hirulog-treated group</td>
</tr>
<tr>
<td>Abendschein (1996)</td>
<td>Hirudin vs heparin</td>
<td>IV bolus + 3-hour infusion</td>
<td>BA of carotid artery in hypercholesterolemic minipigs</td>
<td>Reduction in lumen stenosis in hirudin-treated group</td>
</tr>
<tr>
<td>Buchwald (1996)</td>
<td>PEG-hirudin vs heparin</td>
<td>IV bolus + subcutaneous administration for 3 or 14 days</td>
<td>Stenting of coronary artery in pigs</td>
<td>Treatment with PEG-hirudin reduced neointimal area</td>
</tr>
<tr>
<td>Hadoke (1996)</td>
<td>Hirudin vs placebo</td>
<td>IV bolus + subcutaneous administration for 24 hours or 28 days</td>
<td>BA of subclavian artery in hypercholesterolemic rabbits</td>
<td>24-hour treatment with hirudin had no effect; 28-day treatment with hirudin resulted in increased neointimal area and SMC proliferation</td>
</tr>
<tr>
<td>Gerdes (1996)</td>
<td>Hirudin vs placebo</td>
<td>IV bolus + infusion for 2 hours + subcutaneous injection; in rats, IV infusion was continued for 3 or 14 days</td>
<td>BA of normal carotid artery in rats, rabbits, and hypercholesterolemic minipigs and BA of atherosclerotic carotid artery in rabbits</td>
<td>Neointimal area was reduced in hirudin-treated rabbits but not in minipigs; in rats, neointimal area was reduced by 3-day and 14-day infusion of hirudin</td>
</tr>
<tr>
<td>Barry (1997)</td>
<td>Hirudin vs heparin</td>
<td>IV bolus at time of procedure and 24 hours later</td>
<td>BA of femoral artery in rabbits</td>
<td>Reduction in neointimal area in hirudin-treated rabbits</td>
</tr>
<tr>
<td>Thome (1998)</td>
<td>Hirudin vs heparin</td>
<td>IV bolus + 2-hour infusion at time of procedure; IV bolus 24 hours later</td>
<td>BA of atherosclerotic femoral artery in hypercholesterolemic rabbits</td>
<td>Double bolus regimen of hirudin resulted in decreased plaque area</td>
</tr>
<tr>
<td>Gallo (1998)</td>
<td>Hirudin (different regimens)</td>
<td>IV bolus + continuous infusion via minipump</td>
<td>BA of coronary artery in pigs</td>
<td>IV bolus + 2-week infusion decreased neointimal formation by 40%</td>
</tr>
<tr>
<td>Bishop (1999)</td>
<td>Hirudin vs β-galactosidase</td>
<td>Local delivery of adenovirus</td>
<td>BA of atherosclerotic femoral artery in hypercholesterolemic rabbits</td>
<td>Increased neointimal area in hirudin-treated animals attributable to inflammation</td>
</tr>
</tbody>
</table>

BA indicates balloon angioplasty; IV, intravenous.
systems. Several factors apparently contribute to the angiogenic effects of thrombin. VEGF is a potent angiogenic factor that acts via endothelial cell–specific receptor tyrosine kinases, and thrombin is a potent stimulus for release of VEGF stores within platelets. Thrombin also upregulates expression of the VEGF receptors to potentiate VEGF activity on endothelial cells. VEGF in turn accelerates thrombin generation, suggesting these two factors act as part of a positive feedback loop to accelerate the angiogenic process. The effects of thrombin in the setting of angiogenesis are not limited to its modulation of the VEGF axis, because thrombin also activates matrix metalloproteinases that are required for tissue remodeling in angiogenesis.

An additional and interesting link between thrombin and angiogenesis derives from recent evidence indicating that proteolytic fragments of antithrombin III and prothrombin themselves serve as inhibitors of angiogenesis. Thrombin-cleaved antithrombin III was purified as an antiangiogenic factor derived from small-cell lung cancer cells. Likewise, the kringle-2 domain of prothrombin, which is derived from factor Xa–mediated cleavage of prothrombin, was identified in the serum of lipopolysaccharide-treated rabbits as an antiproliferative factor for vascular endothelial cells. Although the physiological role of these proteolytic fragments remains to be determined, their identification suggests that the thrombin activation system has a more central role in the angiogenic process than has been previously appreciated. In addition, thrombin-mediated angiogenesis seems to be under both positive and negative feedback controls. A great deal more about this angiogenic pathway, including what role thrombin inhibitors such as hirudin will play in therapeutic modulation of angiogenesis, remains to be determined.

Thrombin and Vascular Development

Although initial reports indicating a role for the thrombin pathway in vascular development were unanticipated, such an association is easier to understand given the role of thrombin in angiogenesis, because angiogenesis is a critical component of the vascular development program. Deletion of prothrombin by homologous recombination in mice results in characteristic defects in yolk sac vasculature. Similar defects are observed in factor V–deficient mice, which fail to generate active thrombin. It might be presumed that the developmental effects of thrombin deficiency are attributable to its effects on hemostasis; however, several lines of evidence indicate that this is not entirely the case. First, embryonic hemorrhage was noted in only one of the two reports of prothrombin deficiency; similarly, factor V–deficient embryos are devoid of hemorrhage. Second, deficiency of PAR1 also results in developmental defects (which have not yet been completely characterized), even though this thrombin receptor is not required for platelet activation or fibrin generation in mice. Third (and quite remarkably), mice deficient in either fibrinogen or platelets develop normally, indicating that coagulation per se is not required for the vascular phenotypes observed in the thrombin pathway during development. A direct effect of thrombin on vascular cells is plausible insofar as expression of PAR1 is highest in endothelial cells at early stages of vascular development.

The exact role played by thrombin in vascular development is not yet clear, although it is tempting to speculate that thrombin may be required for local control of VEGF signaling. The developing vascular system is exceptionally sensitive to quantitative changes in VEGF expression, insofar as mice lacking a single VEGF allele die in vitro of a vascular developmental catastrophe and VEGF receptor expression is required for normal endothelial cell development. However, other functions for thrombin on vascular development, instead of or in addition to effects on VEGF signaling, are likely. An important source of information in this regard will be complete descriptions of the developmental phenotypes of mice lacking the different thrombin receptors.

Summary

Thrombin is one of the most well-characterized factors that participates in the vascular response to injury, yet many hypotheses regarding its role in atherogenesis and restenosis have fallen apart on the basis of emerging data indicating direct effects of thrombin on vascular cells, the existence of a novel family of thrombin receptors that are activated in cis and trans via tethered ligands, and trans-activation of receptor tyrosine kinases via metalloproteinase-induced growth factor activation. Despite our expanding knowledge of the role of thrombin in vascular lesion formation in animal models, we still have no effective means to test its role in human disease. Advances in pharmacology and biodelivery or the development of agents that interfere with downstream thrombin-mediated signals may mean that these experiments are on the horizon.

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