Thrombin and Vascular Development
A Sticky Subject

Martin Moser, Cam Patterson

Abstract—Formation of the vasculature is an essential step in embryogenesis. It was observed decades ago that the vasculature and the intravascular blood compartment, which uses the former as a means of transportation, develop in a close spatial and temporal relationship. In this review, we discuss the role of the blood coagulation system as a tool to coordinate angiogenesis. Several mouse models lacking coagulation factors result in impaired thrombin generation and display a phenotype of disturbed cardiovascular development. Similar phenotypes are observed in mouse models of impaired thrombin binding to its cellular receptor, protease-activated receptor-1, or of disrupted signaling via G proteins. Most interestingly, the available data provide evidence that thrombin signaling in vascular development cannot be explained by a model based only on the classic extrinsic and intrinsic coagulation pathways. Because angiogenesis in adults follows the same signaling patterns as angiogenesis in embryos, it is important to learn about these pathways, hoping that they may serve as therapeutic targets in cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2003;23:922-930.)

Key Words: thrombin ■ angiogenesis ■ vascular development ■ thrombosis ■ hemostasis

The formation of the cardiovascular system, together with the circulating blood compartment, is a critical step in embryonic development. More than 80 years ago, it was observed that both blood vessels and blood develop in a tight temporal and spatial relationship. A close interaction between the 2 partners is essential for normal embryonic development. In recent years, it has become more and more evident that the blood coagulation system (and especially its common final agonist, thrombin) represents a major regulatory tool in vascular development. Indeed, the endothelial cell surface, which is the point of contact between the fluid blood compartment and the vessel wall, is the site where the coagulation cascade conducts its activity, especially when the endothelium is injured or denuded. From this perspective, it would make sense that the coagulation cascade is a participant not only in blood clotting but also in other interactions between the circulating blood and blood vessels. In this review, we examine the evidence that supports a role for the coagulation system in general, and thrombin in particular, in normal angiogenesis. The term angiogenesis will be used for both the process of blood vessel sprouting from preexisting vasculature as it occurs in adult organisms as well as for the vascular events in the embryo occurring midway through gestation. At this stage, the vasculature has been partially assembled and is undergoing remodeling to complete the circulation.

The Coagulation Cascade
The traditional view of the coagulation cascade focuses on its role in hemostasis. Events that result in vascular damage trigger the plasma coagulation cascade, which in turn leads to the activation of prothrombin and subsequently, to the cleav-
age of fibrinogen and the formation of a fibrin clot (Figure A). The extrinsic pathway depends on tissue factor (TF) and activated factor VII (FVIIa), which form a complex that activates factor X (FX). FX can also be activated by a catalytic complex formed by the intrinsic pathway. This complex is composed of the serine protease FIXa and its cofactor FVIIIa assembled on an appropriate phospholipid surface. Once FX has been activated, it assembles, together with the nonenzymatic cofactor FVa, into a macromolecular catalytic prothrombinase complex. This complex also assembles on procoagulant phospholipid surfaces, such as activated platelets or inflammatory cells, adhering to the site of vascular damage. The prothrombinase complex finally cleaves prothrombin to thrombin. Thrombin, as the common final enzyme of the coagulation cascade, can act on circulating fibrinogen to convert it to fibrin or alternatively, interact with cell surface receptors and induce intracellular pathways.

**How Does Thrombin Affect Angiogenesis?**

The classic components of the coagulation cascade are well established; however, the mechanisms by which these same molecules participate in developmental events within the vascular system is not intuitive. A clearer picture of how individual coagulation factors contribute to proper vascular development has developed during the past decade by analysis of in vitro experiments and even more convincingly, by loss-of-function mutations in mice. There are several steps involved in obtaining a fully functional blood vessel by angiogenesis: extracellular matrix surrounding the preexisting vessel must be degraded to allow endothelial cells to proliferate and sprout. Endothelial cell permeability as well as endothelial cell adhesion to extracellular matrix and surrounding cells needs to be modified. Finally, endothelial cells must be stimulated to support proliferation and migration.

Studies in the chorioallantoic membrane model of angiogenesis have demonstrated that thrombin is capable of inducing angiogenesis in a fibrin-independent manner. More detailed in vitro studies have aimed to investigate thrombin’s role in the individual steps involved in angiogenesis. Thrombin has been shown to elicit direct and indirect stimulation of metalloproteinases, which are capable of degrading extracellular matrix. Additionally, vascular permeability and endothelial cell migration and proliferation are increased after thrombin stimulation. These effects—necessary for angiogenesis—are independent of thrombin’s fibrinogen-cleaving activity and are conducted through thrombin receptors and intracellular signaling pathways.

At the molecular level, at least 2 major families of proteins are involved in angiogenesis. First, vascular endothelial growth factor-A (VEGF-A) and its close relatives VEGF-B, -C, -D, and -E and placental growth factor regulate angiogenesis by binding to the endothelial VEGF receptors flt-1, flk-1/KDR, and flt-4. This molecular pathway can induce all necessary steps involved in angiogenesis. Thrombin stimulates the transcription of these molecules, most likely through the activation of transcription factor HIF1α, which is involved in the cellular response to hypoxia. A second protein family involved in angiogenesis consists of the angiopoietins (Ang-1 and Ang-2) and their receptors Tie1 and Tie2. Binding of Ang-1 to Tie2 helps to maintain vascular integrity and is involved in vessel maturation. However, Ang-2 antagonizes the interaction of Ang-1 and Tie2, thus resulting in disturbed vessel maturation and enhanced sprouting. Ang-2 is predominantly expressed in areas undergoing vascular remodeling. Interestingly, in vitro stimulation of endothelial cells by thrombin results in upregulation of Ang-2. Another critical molecule involved in angiogenesis is the integrin αv/β3. This adhesion molecule is expressed in activated endothelial cells (eg, in tumor vasculature) and mediates signals involved in vascular remodeling. Accordingly, in vitro stimulation of endothelial cells by thrombin upregulates levels of αv/β3 mRNA, which in turn supports the formation of new vasculature. Taken together, thrombin has proangiogenic activity in various in vitro assays involving several pathways known to be crucial in angiogenesis.

If the coagulation cascade and/or its final effector thrombin do play a role in vascular development and angiogenesis in vivo, then impaired thrombin generation should result in altered vessel formation in the embryo proper as well as in the yolk sac. In several notable models, this has proven to be the case (Table). We will consider this evidence in detail in the following section.
Loss of Extrinsic-Pathway Enzymes

Tissue Factor

Under physiological conditions, TF, the vessel wall–bound initiator of the extrinsic pathway, is a transmembrane protein that is restricted to subendothelial layers of the vessel wall. In situations where blood coagulation is needed rapidly, such as after disruption of vascular integrity or capillary leakage, and also in circumstances of endothelial cell activation (such as in inflammation, septic shock, or within tumor vasculature), TF becomes exposed to the vessel lumen. Subsequently, TF acts as an obligate cofactor for the activation of FX by FVII. Intrinsic FVIII No

Extrinsic TF, the vessel wall–bound initiator of the extrinsic pathway, is a transmembrane protein that is restricted to subendothelial layers of the vessel wall. In situations where blood coagulation is needed rapidly, such as after disruption of vascular integrity or capillary leakage, and also in circumstances of endothelial cell activation (such as in inflammation, septic shock, or within tumor vasculature), TF becomes exposed to the vessel lumen. Subsequently, TF acts as an obligate cofactor for the activation of FX by FVII.

Three independent groups \(^{21-23}\) have reported mouse models of TF deficiency. The common finding of these 3 groups is that, depending on the genetic background, 80% to 100% of TF \(^{-/-}\) embryos die around day 10.5 of embryonic development (ED 10.5). Embryos of this age are pale and show evidence of massive hemorrhaging from embryonic and extraembryonic vessels. The affected embryos lack the large vitelline vessels and have dysmorphic yolk sac capillaries that fuse to form a disordered plexus. Residual blood flow is detected within the embryo proper (which is generally less affected) but not in the yolk sac vasculature. Taken together, these findings are consistent with an underlying defect in vascular development in TF \(^{-/-}\) embryos. Further evaluation of transgenic models that express different deletion mutants of human TF in TF \(^{-/-}\) mice have led to the conclusion that only the extracellular domain, with its FVII binding capacity, is necessary for embryonic survival. \(^{24}\) A deletion mutant of the cytoplasmic domain of TF can still rescue embryonic lethality, whereas expression of mutants with dysfunctional extracellular domains that are unable to bind FVII cannot overcome lethality in TF \(^{-/-}\) embryos.

Taken together, these studies indicate that TF is necessary for proper embryonic development. Lack of TF and presumably, the subsequent lack of active thrombin results in characteristic vascular defects. Its FVII binding capacity is essential for TF’s function in embryonic angiogenesis, whereas induction of intracellular signals by the cytoplasmic domain apparently does not play an important role. In other words, these studies indicate that without initiation of the extrinsic pathway of blood coagulation, vascular development and subsequently embryonic survival are disturbed.

Factor VII

FVII is the most important soluble ligand for TF. The complex of TF and FVII activates the FVII substrate FX. As TF gene disruption results in high embryonic lethality, the report of Rosen and coworkers \(^{25}\) that FVII \(^{-/-}\) mice develop to term normally is somewhat surprising. Vascular defects, as detected in TF \(^{-/-}\), mice are not present in FVII \(^{-/-}\) embryos, but the latter neonates die from severe bleeding within the first days after birth. This means that embryonic FVII is necessary for survival after birth but obviously not for embryonic development. FVII’s major upstream interaction partner, TF, is necessary for normal angiogenesis, whereas embryonically expressed FVII as TF’s most important ligand is not.

How Can the Discrepancy Between the Phenotypes of TF \(^{-/-}\) and FVII \(^{-/-}\) Mice Be Explained?

First, maternal FVII may cross the placenta and replace embryonic FVII in FVII \(^{-/-}\) embryos. In intracardiac blood samples from FVII \(^{-/-}\) embryos, the FVII procoagulant activity is <0.05% of adult levels. \(^{26}\) In addition, injection of supraphysiological amounts of recombinant human FVII into pregnant mice results in <0.1% of maternal recombinant human FVII levels in the embryo. These data indicate that FVII is unlikely to cross the placental barrier in major amounts, although transfer of small, and perhaps physiologically relevant, amounts of FVII cannot be excluded. Interestingly, it has been shown that small amounts of other coagulation factors (TF, FV) are able to rescue the phenotypes of their respective null mice. \(^{24,26}\) Consequently, it is still possible that amounts of FVII that are inaccessible to current detection technology still have sufficient potency to maintain thrombin signaling and proper angiogenesis.

### Mouse Models for Thrombin Signaling Defects

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Mutation</th>
<th>Embryonic Lethality</th>
<th>ED Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrinsic</td>
<td>Tissue factor</td>
<td>80%–100%</td>
<td>8.5–10.5</td>
<td>Bleeding and vascular defects in embryonic and extraembryonic tissues</td>
</tr>
<tr>
<td>Extrinsic</td>
<td>FVII</td>
<td>No</td>
<td>(\ldots)</td>
<td>Neonatal bleeding; death within 24 days</td>
</tr>
<tr>
<td>Common</td>
<td>FX</td>
<td>(\sim)30%</td>
<td>11.5–12.5</td>
<td>Bleeding, no vascular defects detected, high rate of resorption</td>
</tr>
<tr>
<td>Common</td>
<td>FV</td>
<td>(\sim)50%</td>
<td>9.5–10.5</td>
<td>Bleeding and vascular defects in yolk sac</td>
</tr>
<tr>
<td>Common</td>
<td>FII</td>
<td>(\sim)50%</td>
<td>10.5</td>
<td>Severe bleeding, vascular defects reported in one of two studies</td>
</tr>
<tr>
<td>Intrinsic</td>
<td>F IX</td>
<td>No</td>
<td>(\ldots)</td>
<td>Neonatal spontaneous bleeding</td>
</tr>
<tr>
<td>Intrinsic</td>
<td>FVIII</td>
<td>No</td>
<td>(\ldots)</td>
<td>Prolonged bleeding after injury</td>
</tr>
<tr>
<td>Receptor</td>
<td>TM</td>
<td>Complete</td>
<td>(\sim)8.5</td>
<td>Developmental retardation, no red blood cells</td>
</tr>
<tr>
<td>Receptor</td>
<td>PAR 1</td>
<td>(\sim)50%</td>
<td>(\sim)9.5</td>
<td>(Pericardial) bleeding, small vascular defects</td>
</tr>
<tr>
<td>Signaling</td>
<td>G(_{\alpha})13</td>
<td>Complete</td>
<td>(\sim)10.5</td>
<td>Severe vascular defect in yolk sac and embryo proper</td>
</tr>
<tr>
<td>Combined</td>
<td>FV and PAR-1</td>
<td>(\sim)96%</td>
<td>(\sim)12.5</td>
<td>Severe bleeding in embryonic and extra-embryonic tissues</td>
</tr>
</tbody>
</table>
Second, although FVII is the main TF ligand, other proteins may still compensate for the loss of FVII. The recombinant nematode protein rNAPc2 specifically inhibits the TF/FVIIa complex in an FXa-dependent manner. If FVII were the only ligand of TF, then in vitro culturing of wild-type embryos in the presence of rNAPc2 should abolish FXI-induced thrombin generation and should subsequently result in a vascular phenotype similar to that seen in TF −/− mice. There are several possibilities why this was not the case in the reported studies.25 First, embryos were cultured in vitro in the presence of rNAPc2 from ED 8.5 to ED 10.5. This time frame may not be early enough for rNAPc2 to interfere with FVII activity or angiogenesis. Second, rNAPc2 may have been inactive because of the lack of sufficient amounts of FXa. Finally, proteins other than FVII might have bypassed the blockade of FVII activity and might still have resulted in activation of the common coagulation pathway. These experiments are at least consistent with the possibility that FVII is not the only TF ligand and that other proteins might compensate for FVII loss or inactivation. Thus, although the differences in phenotypes of the FVII- and TF-null mice are not entirely resolved, because maternal transfer or bypass pathways cannot be excluded, a model consistent with an essential role for extrinsic pathway-dependent thrombin generation can be derived from these studies.

Loss of Common-Pathway Enzymes

Factors V and X

FX is the first protein of the common pathway of the coagulation cascade. It can be activated by either the extrinsic or intrinsic pathways and in turn activates prothrombin by using nonenzymatic FV as a cofactor. Approximately one third of FX −/− mouse embryos die between ED 11.5 and ED 12.5.27 In studies examining these mice, only a few embryos could be examined histologically because of a high rate of resorption of affected embryos. Bleeding was evident in some of the embryos that could be evaluated, but a vascular defect could not be directly determined. Therefore, to the limits of detection in this model, the FX −/− genotype causes embryonic lethality and bleeding as the most obvious cause, although developmental defects of the vasculature cannot be excluded. Because FX and FV act together, it is of particular interest to compare FV −/− and FX −/− mice.28 In contrast to the molecular couple TF and FVII, in which the inactivation of each molecule had very different effects on embryonic development, deletion of FV has consequences similar to those observed in FX −/− mice. The FV −/− genotype results in 50% embryonic lethality at ED 9.5 to 10.5. The surviving embryos continue to develop to term, but neonates die within 2 hours of massive hemorrhage. Additionally, and in contrast to FX −/− mice, a vascular phenotype with abnormalities in the yolk sac vasculature, as well as a reduced number of blood islands, is evident in FV −/− mice. Thus, the results obtained from both FX −/− and FV −/− mice would be consistent with the hypothesis that reduced thrombin generation results in defective angiogenesis.

To address questions about temporal and spatial effects of FV expression, FV was expressed under the control of a liver-specific promoter in FV −/− mice. Even undetectably low FV protein activity (<0.1% of normal wild-type levels) was able to rescue neonatal lethality but under the conditions of this study did not overcome midembryonic lethality,26 possibly because transgene expression was insufficient at the appropriate time in embryonic development. The promoter used to drive FV expression in these studies was only active after ED 9.5, but in wild-type whole embryos, RNA transcripts of FV have been detected by reverse transcription–polymerase chain reaction as early as ED 7.5. Consequently, FV expressed in this manner might have been absent from the embryo at the crucial time in vascular formation, which occurs earlier than ED 9.5. Alternatively, transgene expression might not have taken place in the appropriate cell type to rescue vascular defects. Under physiological circumstances, the major portion of circulating FV is being secreted by hepatic cells, but it is not entirely clear whether low amounts of FV are also expressed by endothelial and vascular smooth muscle cells.29–31 Because FV is expressed under the control of a liver-specific promoter in this study, it is not known whether FV that might be produced by vascular cells has special developmental relevance. If a transmembrane anchoring of FV to vascular cells is necessary, a lack of FV expression in these cells might account for the observed vascular defects. In any event, FX −/− or FV −/− genotypes result in a similar midembryonic lethality of up to 50%. Interestingly, FV −/− embryos can be rescued by transgenic expression of extremely low amounts of FV, indicating that the amount of coagulation factors needed for embryonic survival and proper angiogenesis are much lower than the amounts needed for sufficient coagulation.26

Factor II

The assembly of the prothrombinase complex results in cleavage of prothrombin to active thrombin. Two independent studies of FII gene disruption in mice have been reported. In both, embryonic lethality was ≈50% at ED 10.5, independent of the genetic background. The results of 1 group indicate that most mice that survived this critical stage developed to term without any detectable developmental failures.32 After delivery, neonates died during the first few days of severe hemorrhage. The investigators could not detect an abnormal vascular phenotype, either in embryos that died at ED 10.5 or in surviving pups. A second report indicated that most embryos surviving beyond ED 10.5 developed to ED 14.5, but almost all embryos had died by ED 18.5.33 In this study, embryos that survived the first critical step at ED 10.5 had characteristic defects in yolk sac vasculature. The rare neonates were extremely pale and died of hemorrhage on the first postnatal day. The description of the yolk sac vascular defect (enlarged capillaries and capillary fusion into a venous plexus) is consistent with a primary vascular defect.

The common feature of these 2 reports is that a loss of embryonic FII results in incomplete embryonic lethality. As vascular defects are only observed in 1 study, it might be assumed that the different genetic backgrounds of the mouse models have a major influence (Sun et al32, 50:50, 129/Black Swiss; Xue et al33, C57BL/6J). In the model that showed vascular defects, late embryonic lethality was also higher,
which may be indicating that FIIa deficiency has a stronger effect on the C57BL/6J background, and this could be compensated to a lesser extent than when similar mice are on a mixed genetic background.

The common pathway of the coagulation cascade is essential for thrombin generation. Mouse models of disrupted common-pathway coagulation factors result in midembryonic lethality of \( \approx 50\% \), notably in the case of FX and in 1 study of FII, which presented a clear vascular phenotype. These data support the hypothesis that thrombin as the final common effector of the coagulation cascade is important in embryonic angiogenesis and survival. It is noteworthy that lethality does not exceed \( \approx 50\% \) in many of these models, which indicates that other molecules or pathways can at least partially compensate for the loss of common-pathway factors during vascular development.

Loss of Intrinsic-Pathway Enzymes
In contrast to the effects of loss of extrinsic-pathway enzymes, loss-of-function mutations of intrinsic-pathway enzymes would not necessarily be expected to have a critical influence on vascular development, because small amounts of thrombin can still be produced without the involvement of the intrinsic pathway. Activated FIX cleaves its substrate FVIII, and mice lacking FIX have a phenotype similar to human hemophilia B. Mice surviving to adulthood exhibit a spontaneous bleeding tendency but have no evident vascular defects during development. Disruption of the FVIII gene in mice results in a phenotype similar to but milder than that of human hemophilia A (possibly owing to inherent differences between the mouse and human FVIII proteins). For both FVIII and FIX deficiency, vascular defects are reported neither in humans nor in mice. Because thrombin generation is not entirely dependent on the intrinsic pathway, the lack of vascular defects in these models does not necessarily discount a central role for thrombin in embryonic angiogenesis.

Considerations About Impaired Thrombin Generation
As expected, disruption of the reported genes encoding plasma coagulation cascade proteins results in a neonatal bleeding disorder. Pups generally die shortly after birth of severe bleeding, indicating that sufficient plasma coagulation is especially required for survival after birth. However, in some cases, bleeding has also been reported during embryonic development, especially in mutants with accompanying vascular abnormalities. This raises an ontogenetic question about the relation between vascular developmental abnormalities and bleeding in these mice.

Does the Defective Plasma Coagulation Cascade Also Account for Embryonic Bleeding?
Insights into this question have come from analysis of mouse models with defective blood clotting caused by factors downstream of thrombin activation. Mice without the transcription factor NF-E2 lack circulating platelets. These mice die of hemorrhage, indicating that their clotting system is indeed defective. Surprisingly, embryonic development is not altered in these mice, and embryonic bleeding is also not reported. Consequently, insufficient platelet activation as a result of low thrombin levels would not necessarily explain embryonic bleeding in coagulation factor–deficient mice. Along the same lines, Su and coworkers reported that mice lacking the fibrinogen \( \alpha \)-chain (which is required for effective thrombus formation) do not suffer from disturbances in embryonic development or from embryonic bleeding. Thus, both downstream events of thrombin activation, platelet activation and fibrinogen cleavage, do not seem to be important in controlling embryonic blood loss.

If ‘Classic Blood Clotting Capacity’ Is Not Necessary to Prevent Embryonic Bleeding, Why Do Embryos Lacking Coagulation Factors Bleed?
Because platelets and fibrinogen are not necessary to prevent bleeding in embryos, it is even more likely that the vascular component of hemostasis is playing an essential role under these circumstances. This assumption is consistent with the vascular defects observed in TF \(-/-\) and FII \(-/-\) mice. The phenotypes of these mice indicate that bleeding might have occurred as a result of physical disintegration of the endothelial cell layer, allowing for blood cell extravasation. Abnormal vascular patterning is frequently found in the yolk sac as well as in the embryo itself in these models and is often accompanied by extravasated red blood cells. Primary vascular defects, either followed by incorrect organ development or even more likely by fatal blood loss, seem to be the underlying cause for embryonic lethality in these mutants. The fact that vascular defects result in bleeding and embryonic death is a common response to vascular perturbations and has also been observed in Tie1 \(-/-\) and Tie2 \(-/-\) mice. It is possible that each of the coagulation factors (TF, FX, FV, FII, and maybe FVII) has a distinct influence on vascular development. However, because the common final step of the coagulation cascade is thrombin activation, the most parsimonious assumption is that thrombin itself has a direct effect on vascular development.

Why Are Vascular Defects Not Reported in All Mouse Models Lacking Thrombin Activity?
Mice lacking FVII, FV, and (in 1 study) FII have no reported vascular abnormalities. However, it cannot be assumed that this refutes the notion of a central role for thrombin in vascular development. Depending on the technique used to examine the vascular system, it is sometimes technically very difficult to assess small, microscopic endothelial discontinuities. Also, embryos that are halved in development at a certain time are being resorbed quickly and are not always accessible for further examination. It is therefore possible and indeed likely that embryonic lethality in FV and FII is connected to vascular defects in development, even though they cannot be easily visualized.

Why Are Some Models Characterized by Embryonic Lethality While Others Are Not?
Assuming that thrombin is necessary for proper angiogenesis, it might seem surprising that the mouse models for FVII, FVIII, and FIX do not exhibit embryonic lethality. For the proteins of the intrinsic pathway (FVIII and FIX), this might
be because small amounts of thrombin can be generated even in the absence of the intrinsic pathway. These small amounts might be sufficient to maintain the thrombin signaling that is required for angiogenesis. Other coagulation factors have been shown to rescue coagulation in their respective null mouse models, even in amounts that are not detectable by serum activity assays. This phenomenon might also explain why FVIII- or FIX-null mice develop normally during embryogenesis. The case is different for FVII. The extrinsic pathway is required for thrombin generation, so FVII−/− mice should not be able to produce even small amounts of thrombin. In this case, it is more likely that maternal transfer of trace amounts of FVII is responsible for their survival to term. Potentially, even small amounts of FVII are sufficient to support basal thrombin signaling and prevent vascular defects. Such a phenomenon might also occur for other soluble coagulation factors (FX, FIX, and FII).

Clearly, the data obtained by analysis of models deficient in thrombin generation suggest that thrombin is critical for angiogenesis, but enough questions remain to preclude definitive statements regarding the role for thrombin in this process and how thrombin’s action on cardiovascular development occurs. However, knowing that thrombin does not influence vascular development by downstream events of the coagulation cascade, such as fibrinogen cleavage or platelet activation, it may be assumed that thrombin binding to its cellular receptors would be the important event in this process.

**Thrombin Receptors**

There are at least 2 different types of thrombin receptors, thrombomodulin (TM) and the protease-activated receptors (PARs). PARs convert an extracellular protease cleavage event into an intracellular signal by using a unique mechanism. The N-terminal extracellular domain can be cleaved by thrombin to unmask a cryptic N-terminus (“tethered ligand”), which serves as an activation signal for the receptor. PARs are G protein–coupled receptors. They interact with the Gq13, Gq, and Gi families of G proteins and hence, influence a broad range of intracellular pathways. Four different PARs are present in mice and humans, and of these, PAR1, PAR3, and PAR4 are activated by thrombin, whereas PAR2 is activated by trypsin and tryptase but not by thrombin.

TM is a transmembrane molecule that is structurally unrelated to PARs.39 By binding to TM, thrombin loses its procoagulant activity, and instead, the natural anticoagulant protein C is activated,40 creating a negative-feedback loop that results in cleavage of active FV and FVIII and consequently, less procoagulant activity. Disruption of the TM gene in mice results in embryonic lethality at 2 different stages. A first block occurs before significant expression of thrombin and earlier than establishment of a functional cardiovascular system, indicating a thrombin-independent function of TM. These mutant embryos fail to develop endocardium and lack circulating blood cells.41 By replacement of TM deletion mutants, its function in embryonic development might be assigned to the extracellular domain of TM.42 Selective expression of full-length TM in the extraembryonic tissues of TM−/− embryos overcomes the early block in embryogenesis. These embryos develop normally in midgestation without signs of vascular defects but then die of consumptive coagulopathy between ED 12.5 and 16.5.43 These data suggest that TM plays an important role in placental development and/or in the interaction between maternal and embryonic tissues, but a direct link to angiogenesis is not obvious from these studies.

In contrast, PARs (the second set of thrombin receptors) do play an important role in angiogenesis. Disruption of the gene for PAR1 results in 50% embryonic lethality.44 At ED 8.5, PAR1−/− mice are still indistinguishable from wild-type littermates, but at ED 9.5, a cohort of null mice are significantly smaller than their siblings, and their hearts do not beat. Hemorrhage, especially in the pericardium, is apparent in more than a third of the PAR1−/− embryos. Delay in development of the embryo occurs in conjunction with abnormalities in placental development. At this time, about half of the PAR1−/− embryos die, while the other half of the embryos “catch up” with their PAR1+/− littermates. A cause of embryonic death has not been determined definitively, but it is noteworthy that failed hemostasis is not present. Platelets of the surviving PAR1−/− mice react normally to thrombin, whereas fibroblasts from the same mice are insensitive to thrombin. These observations indicate that PAR1 is not required for proper platelet responses to thrombin, and PAR3 is now recognized as the receptor responsible for thrombin signaling in mouse platelets.46 Histological analysis of PAR1−/− mice indicates that small openings develop in the wall of the sinus venosus that appear large enough to allow red blood cell passage. Interestingly, transgenic expression of PAR1 under the control of an endothelial cell–specific promoter is able to decrease the embryonic lethality to ∼14%. This suggests that lethality in PAR1−/− embryos is related, in large part, to lack of PAR1 in endothelial cells. PAR1−/− mice suffer from embryonic lethality at about the same time as observed in coagulation factor–deficient mice and also without evidence of a hemostatic disorder. The activity of the plasma coagulation cascade is not altered, which is consistent with the fact that thrombin’s proteolytic activity to cleave fibrinogen is not related to the presence or absence of PAR1. In addition, platelet function is also not altered in PAR1−/− embryos. Taken together, these observations indicate that thrombin must have an influence on embryonic lethality and angiogenesis by binding to its PAR1 receptor.

Because PARs are G protein–coupled receptors, additional insight into thrombin’s role in vascular development might be derived by considering the phenotype of G protein–deficient mice. Go13 is a PAR1-interacting protein that is required for intracellular thrombin signaling. Disruption of the Go13 gene results in 100% embryonic lethality in homozygous mice between ED 9.5 and 10.5.47 Even earlier, at ED 8.5, yolk sacs from null embryos are opaque and roughened. Although endothelial cells are present, they are not organized in a honeycombed array as seen in wild-type embryos. Vascular structures are absent in ED 9.5 yolk sacs from null mice, and blood islands can only occasionally be observed. In the embryo proper, the vasculature is dysmorphic, and defects in the endothelial cell layer result in leakage of blood cells into
Diversity of Thrombin Signaling

Because lethality in the different coagulation factor–deficient models is, for the most part, incomplete, it seems likely that the thrombin signaling pathway is not as straightforward as a simplified model of the coagulation cascade might suggest. For example, Griffin and coworkers tested the simple model that FV mediates conversion of prothrombin to thrombin, which in turn then signals via PAR1, by using genetically modified mice (Figure C). Embryonic lethality for FV−/− mice and PAR1−/− mice are very similar (∼50%). Lethality of FV−/− mice tends to occur slightly later than reported for PAR1−/− mice, although it cannot be excluded that this is due to experimental variability or genetic background differences of the investigated mice. Both mouse models show similar (though not identical) phenotypes, with embryonic and extraembryonic bleeding as well as vascular defects. If the simple model is true, then intercrosses of FV−/− and PAR1−/− mice should produce a phenotype identical to that of either deficiency alone. Surprisingly, however, only 4% of the FV−/−, PAR1−/− embryos survived to term. The dead embryos were pale and severely necrotic, with pooled blood in the pericardial and exocoelomic cavities. This result suggests that the FV and PAR1 pathways might interact but do not overlap completely. Under the assumption that no maternal FV crosses to the embryo, this would mean that PAR1 can still be activated, even in the absence of embryonic FV. This would also imply either that molecules other than FV can convert prothrombin to thrombin, or that thrombin is not the only protease that can activate PAR1. Indeed, Riewald and coworkers have shown that in the absence of thrombin, FX itself has the capacity to cleave PAR1 and to induce the same downstream signals induced by thrombin cleavage of PAR1 (Figure B). This would suggest that, under certain circumstances, prothrombin-dependent activation of PAR1 can be bypassed, which would be consistent with the partial embryonic lethality in FII−/− mice. In the surviving mice, these alternative pathways might be activated to compensate for the loss of prothrombin. It is also possible to interpret the studies with mice that are FV−/−, PAR1−/− to indicate that FV−/− increases death of PAR1−/− mice. This can be explained by a model in which FV has functions in addition to those involved in signaling via PAR1. FV or a factor(s) dependent on FV might act on molecular players in embryonic angiogenesis in addition to PAR1. Of note, fibrinogen has been excluded from playing such a role by intercrossing PAR1−/− and fibrinogen−/− mice.

The search for FV-dependent signaling molecules continues. Strong candidates are the other PAR family members, which might either be involved in angiogenesis under physiological conditions or only compensate for PAR1 deficiency in its absence. Interestingly, it has been demonstrated that PAR2 can be activated by FVII in vascular endothelial cells in an FX- and TF-dependent manner. This effect seems to be independent of thrombin and might account for continued FV-dependent endothelial cell signaling, even in the absence of PAR1 (Figure B). It has also been shown that PAR3 has the same thrombin-interacting sequences as PAR1. Thus, in the absence of PAR1, thrombin might activate PAR3, which then acts as a cofactor for PAR4 in mice to activate similar intracellular signaling events. Whether this mechanism plays a role in angiogenesis is not yet known.

Other recent data indicate that the interaction between thrombin and PAR1 is not restricted to direct cleavage of the receptor by the protease. As discussed earlier, thrombin can bind to TM and in turn activate the “endogenous anticoagulant” protein C. After being activated, protein C can use its receptor (endothelial cell protein C receptor) as a cofactor to cleave PAR1 and induce downstream intracellular signaling. This might serve as an example of the complexity of molecular interactions that are involved in thrombin signaling. These extensive interactions and the resulting parallel options for particular pathways are supported by the genetic data, as only TF−/− and Gα13−/− mice undergo complete embryonic lethality due to defects in angiogenesis. For the other coagulation factor–null mouse models, compensating pathways rescue at least a cohort of the embryos.

Summary

Several lines of evidence have demonstrated that thrombin signaling is essential for angiogenesis. Thrombin’s angiogenic activity seems to be mostly independent of its coagulant activity and more dependent on signaling via the thrombin receptors. There is some evidence that not only thrombin but also other molecules (FVII, FX) might be able to activate these receptors under certain conditions and induce downstream signals. In vitro data indicate that thrombin stimulates several critical pathways in angiogenesis, including VEGF, VEGF receptors, angiopoietin-2, and integrin αv/β3. These data support the observations obtained in mouse models, wherein a lack of thrombin generation (TF−/−, FX−/−, FV−/−, FII−/−) results in severe vascular defects in embryonic development. Similar phenotypes occur in models of impaired thrombin binding to its PAR receptor (PAR1−/−) or missing the corresponding G protein (Gα13). It is striking that TF−/− embryos as well as Gα13−/− embryos suffer from complete lethality at midgestation in contrast to other coagulation factor or PAR deficiencies. This phenomenon might indicate that TF is an essential initiator of the extrinsic pathway and that Gα13 is a common component of the signaling pathway downstream of PAR1. In contrast, the loss of other proteins involved in this pathway might be compensated for by other family members or by the induction of different pathway interactions.

The use of coagulation cascade proteins to link the fluid blood component with vessel wall development may be an important means to control the close temporal and spatial connection of these 2 components of the cardiovascular system. It might be that gaps in the developing vasculature are detected by TF exposure to the bloodstream and that
through its subsequent activation of the coagulation cascade, a signal is being created to seal off the leaking vessel by endothelial differentiation and growth. It is noteworthy that although there is an increasing understanding of the signaling mechanisms described in this review, the particular molecules and events that link thrombin signaling to angiogenesis are not yet known. VEGF and its cellular receptors as well as the transforming growth factor-β family of growth factors that are essential for early embryonic patterning and angiogenesis are potential candidates to fill the missing link. Our understanding of the molecular mechanisms involved in signaling required for angiogenesis is far from complete, but by close analysis of these pathways, it might be possible in the future to create molecular tools to manipulate angiogenesis in human disease.

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References

1. Sabin FR. Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of development. Contr Embryol. 1920;36:213–262.
6. Garcia JG, Pavalko FM, Patterson CE. Vascular endothelial cell acti-
8. Wang HS, Li F, Runge MS, Chaikof EL. Endothelial cells exhibit dif-
9. Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through coop-
   erative mechanisms involving the αβ3 integrin, osteopontin, and thrombin. Am J Pathol. 1996;149:293–305.
10. Eriksson U, Alitalo K, Structure, expression and receptor-binding pro-
   perties of novel vascular endothelial growth factors. Curr Top Microbiol
13. Tsopanoglou NE, Maragoudakis ME. On the mechanism of thrombin-
14. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonnier PC, Yancopoulos GD. Iso-
24. Parry GC, Mackman N. Mouse embryogenesis requires the tissue factor
26. Yang TL, Cui J, Taylor JM, Yang A, Gruber SB, Ginsburg D. Rescue of
defective embryonic hemorrhage in factor V deficient mice by low level
ehemorrhage and incomplete block to embryogenesis in mice lacking
30. Cerveny TJ, Fass DN, Mann KG. Synthesis of coagulation factor V by
31. Rodgers GM, Kane WH, Pitas RE. Formation of factor Va by athero-
sclerotic rabbit aorta mediates factor Xa-catalyzed prothrombin activ-

Moser and Patterson

Thrombin and Vascular Development

929
Thrombin and Vascular Development: A Sticky Subject
Martin Moser and Cam Patterson

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