Role of Protease Activated Receptor 1 and 2 Signaling in Hypoxia-Induced Angiogenesis

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Objective—Tissue factor (TF) initiates coagulation and indirectly triggers thrombin-dependent protease activated receptor (PAR) signaling. The TF–VIIa complex also directly cleaves PAR2 and promotes angiogenesis in vitro in TF cytoplasmic domain-deleted (TFCT) mice. Here we address the effect of PAR1 and PAR2 deficiency on angiogenesis in vivo.

Methods and Results—In hypoxia-driven angiogenesis of oxygen induced retinopathy (OIR), wild-type, PAR1−/−, PAR2−/−, and TFCT mice showed a comparable regression of the superficial vascular plexus during the initial exposure of mice to hyperoxia. However, TFCT mice revascularized areas of central vaso-obliteration significantly faster than wild-type animals. Pharmacological inhibition of the TF–VIIa complex, but not of Xa, and blockade of tyrosine kinase receptor pathways with Gleevec reversed accelerated angiogenesis of TFCT mice to revascularization rates observed in wild-type mice. Genetic deletion of PAR2, but not of PAR1, abolished enhanced revascularization of TFCT mice. PAR1 knock-out animals were indistinguishable from wild-type mice in the model of retinal neoangiogenesis and angiogenesis-dependent subcutaneous tumor growth was unaltered in PAR1- and PAR2-deficient animals.

Conclusion—Loss of the TF cytoplasmic domain results in accelerated hypoxia-induced angiogenesis mediated by TF–VIIa signaling. PAR2 signaling is sufficient for this proangiogenic effect without apparent contributions of mouse host cell PAR1.

Key Words: tissue factor ■ protease activated receptors ■ angiogenesis ■ coagulation ■ thrombin

Tissue factor (TF), a transmembrane glycoprotein expressed by vascular and myeloid cells, is the primary cellular initiator of blood coagulation. TF exerts its biological activities by forming a catalytic enzyme complex with coagulation factor VIIa. The TF–VIIa complex then triggers coagulation by binding and activating factor X, leading to thrombin-dependent fibrin deposition and platelet activation. Thrombin can exhibit pleiotropic effects which are typically mediated through G protein–coupled protease activated receptor (PAR) 1 signaling.1 The TF–VIIa complex also signals directly by cleaving PAR2, but not PAR1.2,3 However, the relative contributions of direct TF signaling, downstream coagulation activation, and indirect protease signaling to the underlying pathogenesis of cancer progression and angiogenesis,3 are incompletely defined in vivo.

TF deficiency results in vascular failure during development and the proangiogenic phenotype of TF cytoplasmic domain deleted (TFCT) mice provided direct evidence that TF is involved in angiogenesis.5 Whether direct TF–VIIa signaling is the only relevant pathway to drive angiogenesis in vivo has not been established. Indeed, thrombin-dependent PAR1 signaling stimulates angiogenesis in certain angiogenesis models in vivo,6–8 and the partial embryonic lethality of PAR1 knock-out mice attributable to vascular failure resembles the lethal phenotype of TF deficient animals.9,10 However, the role of PAR1 in postnatal angiogenesis has not been directly addressed in PAR1 knock-out animals.

Thrombin and/or TF-signaling pathways are also involved in tumor angiogenesis,5,11 but production of proangiogenic factors by tumor cells may mask the specific signaling roles of TF and PARs present on host cells that participate in the angiogenic response. We therefore focused on the oxygen induced retinopathy (OIR) model12 that specifically evaluates the regeneration of a capillary network in the retina driven by hypoxia. In this model, we show that the proangiogenic phenotype of TFCT mice is dependent on the activity of the TF–VIIa complex that acts synergistically with tyrosine kinase receptor signaling pathways. TF-dependent angiogenesis required PAR2, but not PAR1, suggesting that direct TF signaling pathways predominate in TF driven angiogenesis under hypoxia.

Methods

Materials and Mouse Strains

Nematode anticoagulant protein (NAP) c213 was kindly provided by Dr Sriram Krishnaswamy (Children’s Hospital, Philadelphia, Pa),
the Xa inhibitor NAP5 was obtained from Dr George Vlasuk (Corvas, San Diego, Calif), and control antibody CVAM from Dr Mark Anderson (Centocor, Radnor, Pa). TF<sup>CT</sup> mice, which lack the 18 C-terminal residues of the TF cytoplasmic domain, 14 PAR-1<sup>−/−</sup>, and PAR-2<sup>−/−</sup> mice were backcrossed for at least 9 generations into the C57BL/6 genetic background. TF<sup>CT</sup>/PAR-1<sup>−/−</sup> and TF<sup>CT</sup>/PAR-2<sup>−/−</sup> mice were generated by interbreeding after backcrossing was complete.

**Oxygen Induced Retinopathy (OIR) Model**

All animal work was done according to the ARVO statement for the use of animals in ophthalmic and vision research under approved protocols of the TSRI IACUC. OIR was induced by exposing pups to 75% oxygen at postnatal day 7 (P7) for 5 days and returned to room air at P12 to induce relative hypoxia in the avascular areas of the retina. 12 Animals were euthanized at P12 to score the degree of hyperoxia-induced vascular regression and at P17 to score regeneration under relative hypoxia on retinal preparations. 16

Retina flat mounts were fixed with 4% PFA for 1 hour, stained with fluorescence conjugated isoelectric Griffonia simplicifolia (Molecular Probes, 1:150 dilution). For immunohistochemistry, retinas were blocked in 20% fetal bovine and 20% normal goat serum and stained with anti-CD11b (clone M170; BD Pharmingen) and 1:200 of anti-NG2 (Chemicon) overnight, followed by appropriate fluorescence-conjugated secondary antibodies. Whole mount retinas were imaged using confocal microscopy (BioRad Radiance 2100) with a 4× objective lens. 16 By focusing just above the inner limiting membrane of the retina, the prelaminar neovascular tufts were readily distinguished from the underlying superficial vascular plexus. Overlapping images were assembled into a whole retina montages using Photoshop 6.0 (Adobe). Areas of vascular obliteration and neovascular tufts were quantified from these montages.

**NAPc2, NAP5, and Imatinib Treatment**

Immediately after return to normoxia at day P12, mice were treated with intraperitoneal injections of NAPc2 (1 mg/kg) or as controls with either buffer or nonreactive antibody. Dosing was once daily until sacrifice at day P17. NAP5 was injected subcutaneously twice daily at a dose of 1 mg/kg. Imatinib (Gleevec; Novartis) was dissolved in distilled water, pH 4.7, to 50 mg/ml and administered orally (20 mg/kg) once daily.

**Syngeneic Tumor Growth Models**

Growth of syngeneic tumors was characterized in C57BL/6 PAR1<sup>−/−</sup> or PAR2<sup>−/−</sup> mice versus C57BL/6 wild-type controls. The following numbers of tumor cells resuspended in PBS were injected: murine melanoma B16F10 (1.25×10<sup>5</sup> cells/mouse), highly metastatic Lewis lung carcinoma-derivative D121met (5×10<sup>5</sup> cells/mouse), and fibrosarcoma T241 (2×10<sup>5</sup> cells/mouse). Tumor growth was monitored by measuring tumor volumes with calipers. Mice were euthanized after exponential growth phase kinetics had been established.

**Statistics**

Two-way analysis of variance (ANOVA) complemented by the Bonferroni post hoc test was used for pairwise comparisons between test groups. Differences were considered significant when P<0.05.

**Results**

**Deletion of TF Cytoplasmic Domain Accelerates Hypoxic Revascularization**

TF<sup>CT</sup> mice show accelerated developmental retinal angiogenesis. We first analyzed whether regression of vessels under hypoxic conditions in the OIR model is comparable between TF<sup>CT</sup> and wild-type mice. After exposure to 75% oxygen between P7 and P12, retinas were evaluated at P12. Typical for this model, large areas of the central vascular network were obliterated with only a few major vessels remaining centrally in both wild-type and TF<sup>CT</sup> mice (supplementary Figure I, available online at http://atvb.ahajournals.org). Quantitative analysis of obliterated areas confirmed that the retinal vasculature in TF<sup>CT</sup> mice is affected comparably to wild-type mice. In addition, no differences in lectin positive cells (including microglia) were observed in the avascular area between the two strains (supplemental Figure ID). Taken together, these data suggest that deletion of the TF cytoplasmic domain has no observable effect on vascular regression and remodeling in the retina.

On return to normoxia, the now relatively avascular and hypoxic central retina stimulates neovascularization. Regression of the vasculature of wild-type and TF<sup>CT</sup> retinas was quantified in the OIR model 5 days after returning the mice to normoxia. In TF<sup>CT</sup> mice, the superficial vascular plexus formed more quickly than that of wild-type mice, as demonstrated by significantly smaller areas of vascular obliteration in TF<sup>CT</sup> mice relative to wild-type at P17 (supplemental Figure IE). On return to normoxia, the strong hypoxic stimulus in the eye also drives abnormal misdirected sprouting of blood vessels into the vitreous at the interface between the centrally obliterated and peripherally perfused retina. Periretal neovascularization reaches its maximum in wild-type mice 5 days after returning to normoxia. No significant differences in the areas of preretinal neovascular tufts were found between TF<sup>CT</sup> mice and wild-type mice, indicating that more rapid revascularization of the obliterated areas was achieved without increased pathological sprouting into the vitreous.

We addressed whether the more rapid developmental angiogenesis observed in TF<sup>CT</sup> mice may have influenced the regeneration of the superficial retinal vascular plexus in the OIR model. Wild-type or TF<sup>CT</sup> mice were analyzed at an earlier age and exposed to 5 days of hyperoxia starting at P5, followed by 5 days of normoxia and sacrifice at P15. The extent of vascular obliteration was similar in mice exposed to hyperoxia from day P5 to 10 versus P7 to 12 (data not shown). TF<sup>CT</sup> mice show ≈2 day advanced developmental angiogenesis. If advanced development caused accelerated revascularization in TF<sup>CT</sup> mice, one would expect that wild-type mice at P17 show similar regeneration rates as TF<sup>CT</sup> mice at P15. However, the obliterated area after 5 days of relative hypoxia was smaller in mice that were exposed to hyperoxia starting at day P5 relative to day P7 (supplemental Figure IE), indicating faster revascularization of younger mice with a less developed retinal vasculature. Nevertheless, TF<sup>CT</sup> mice showed significantly enhanced revascularization relative to wild-type when pups of the same ages were compared. These data provide evidence that the enhanced regeneration under relative hypoxia is unrelated to the accelerated developmental angiogenesis of TF<sup>CT</sup> mice.

**Inhibition of TF–FVIIa Signaling by NAPc2 Prevents Enhanced Angiogenesis in TF<sup>CT</sup> Mice**

The TF cytoplasmic domain regulates integrin activation, 17 and effects on neovascularization may have occurred independent of TF–VIIa dependent PAR signaling. We tested whether accelerated angiogenesis in TF<sup>CT</sup> mice was, indeed, dependent on activity of the TF–VIIa complex. On return to normoxia, wild-type and TF<sup>CT</sup> mice were treated by daily...
tuft formation after NAPc2 treatment was unchanged in either wild-type or TF\textsuperscript{SCT} mice. Thus, the TF–VIIa complex is necessary for accelerated vascular regeneration.

Because NAPc2 also inhibits coagulation activation, we further used a specific inhibitor of Xa, NAP5. NAP5 has anticoagulant potency comparable to NAPc2,\cite{18} a finding that was confirmed by comparison of the two inhibitors in an endotoxin challenge model (data not shown). Unlike NAPc2 administration, treatment of TF\textsuperscript{SCT} mice with NAP5 did not change the rate of revascularization under relative hypoxia (Figure 1). NAP5 also did not influence revascularization of wild-type mice or preretinal revascularization. These data provide evidence that inhibition of the TF–VIIa complex, but not of downstream coagulation activation, is required to block the proangiogenic phenotype of TF\textsuperscript{SCT} mice.

**TF–VIIa Synergizes With Growth Factor Signaling in Hypoxic Angiogenesis**

In the in vitro aortic ring assay, TF–VIIa driven angiogenesis required the presence of platelet-derived growth factor-BB (PDGF-BB), indicating crosstalk with tyrosine kinase receptor-mediated signaling.\cite{5} Imatinib (Gleevec) is a potent tyrosine kinase domain inhibitor of the PDGF\(\beta\) receptor and related kinases, such as c-kit and bcr-abl and inhibits PDGF-dependent signaling in the mouse.\cite{19} We treated wild-type and TF\textsuperscript{SCT} mice after return to normoxia in the OIR model with Gleevec. Gleevec selectively blocked revascularization in TF\textsuperscript{SCT}, but not in wild-type mice, without influencing preretinal neovascularization (Figure 2A and 2B). These data show that TF–VIIa signaling synergizes with tyrosine kinase receptor signaling to enhance angiogenesis in vivo.

PDGF signaling is important for the recruitment and retention of pericytes with blood vessels. We addressed the question of whether Gleevec-induced inhibition of angiogenesis in TF\textsuperscript{SCT} mice reduced pericyte recruitment to angiogenic vessels. No differences were observed in the extent of nascent blood vessel coverage by NG2-positive pericytes of vehicle-versus Gleevec-treated TF\textsuperscript{SCT} or wild-type mice (Figure 2C). Thus, our results suggest that the synergistic effect of tyrosine kinase receptor with TF–VIIa signaling in angiogenesis is unrelated to pericyte recruitment to regenerating blood vessels.

**Accelerated Revascularization in TF\textsuperscript{SCT} Mice Is Dependent on PAR-2, but not PAR-1 Signaling**

Although NAPc2 and NAP5 have similar potency to block coagulation, thrombin generation may not have been completely suppressed. PAR1 is the major thrombin receptor on mouse endothelial cells and interstitial cells, such as fibroblasts,\cite{20} but not on platelets. Analysis of PAR1\textsuperscript{−/−} mice therefore provides an additional line of evidence for the role of thrombin signaling in hypoxia-induced angiogenesis. PAR1\textsuperscript{−/−}, PAR2\textsuperscript{−/−}, TF\textsuperscript{SCT}/PAR1\textsuperscript{−/−}, and TF\textsuperscript{SCT}/PAR2\textsuperscript{−/−} were compared with wild-type mice in the OIR model. Regression of the superficial vascular plexus under hypoxia was similar in all genotypes analyzed at day P12. The area of vascular obliteration observed in PAR1\textsuperscript{−/−} or PAR2\textsuperscript{−/−} mice after 5
days of relative hypoxia at P17 was similar to wild-type mice. Neovascular tuft formation in PAR1−/− and PAR2−/− mice was also identical to that observed in wild-type mice (Figure 3). These data demonstrate that PAR1 signaling downstream
of TF is not contributing to angiogenesis, consistent with the finding that coagulation inhibitors did not suppress neovascularization in wild-type mice.

In contrast to the TFCT mice, the area of vascular obliteration in TFCT/PAR2−/− was comparable to wild-type mice after 5 days of relative hypoxia at P17 (Figure 3). This demonstrates that accelerated regrowth of the retinal vascular network under hypoxia in TFCT mice is dependent on PAR2 signaling. Specificity for PAR2 is further documented by the finding that TF−/− mice were indistinguishable from TFCT mice with regard to vascular regeneration and tuft formation. Thus, PAR1 signaling does not appear to enhance or diminish angiogenesis in the TF-dependent angiogenic response of TFCT mice in vivo.

Thrombin has been shown to induce proangiogenic effects in vivo9,21 and PAR1-deficiency leads to partial developmental lethality attributable to vascular failure.9,10 We addressed whether the apparently normal hypoxia-induced angiogenic response in PAR1−/− mice was peculiar to retinal neovascularization or may also occur in other types of angiogenesis in adult mice. Subcutaneous tumor growth is an angiogenesis-dependent process that was shown to be sensitive to thrombin inhibition, although in these models tumor growth inhibition may result from blockade of tumor cell–specific thrombin signaling rather than the host (eg, angiogenic) compartment.

We had previously shown that syngeneic tumors grew more rapid in TFCT mice,5 and we used these models to further address whether PAR1-deficiency in the host contributes to angiogenesis (Figure 4). All 3 tumor models showed similar tumor expansion in wild-type and PAR1−/− mice, excluding that PAR1 on host cells makes an essential contribution to tumor angiogenesis. In addition, we tested tumor growth in PAR2−/− mice using the T241 model. PAR2−/− was reproducibly without effect on the growth and expansion of subcutaneous tumors. Thus, individual PAR1 or PAR2 deficiency had no appreciable effect in postnatal mice on either angiogenesis-dependent tumor growth or hypoxia-driven retinal angiogenesis.

**Discussion**

In this study, we present data that provide new insight into the role of PAR signaling in angiogenesis in vivo. We demonstrate enhanced retinal revascularization of TFCT mice in an in vivo model of hypoxia-driven angiogenesis. Using a specific inhibitor of TF–VIIa, NAPc2,13 we further provide direct evidence that the proangiogenic phenotype of TFCT mice is dependent on the extracellular domain of the TF–VIIa complex. We have previously shown that PDGF-BB cooperates with TF–VIIa to stimulate angiogenesis in vitro.9 We here show in vivo that Gleevec, a kinase domain inhibitor of the PDGFRβ receptor, prevents the enhanced revascularization observed in TFCT mice. Although PDGF-BB signaling is critical for crosstalk between endothelial cells and pericytes, we did not observe any abnormalities in pericyte distribution on nascent or mature retinal vessels in Gleevec-treated animals. Alternatively, TF may synergize with endothelial cell PDGFRβ receptor signaling, recently shown to play a role in early endothelial differentiation.22 Additional studies with more selective tyrosine kinase inhibitors are necessary to exclude that other targets of Gleevec, such as bcr-abl or c-kit, are synergizing with TF. Our data, nevertheless, provide clear evidence for cooperation between TF and growth factor signaling during angiogenesis in vivo.

Enhanced revascularization of the superficial retinal vascular plexus in TFCT mice was dependent on PAR-2 signaling, providing evidence that TF–VIIa targets PAR2 to support angiogenesis in vivo. Our data show that PAR1-deficiency alone or in combination with TF cytoplasmic domain deletion
had no effect on hypoxia-driven ocular angiogenesis. PAR1 is cleaved by Xa, thrombin, or activated protein C (APC), but direct blockade of Xa, and therefore indirect inhibition of downstream coagulation protease generation with the Xa inhibitor NAP5, also had no effect on angiogenesis in TF mice. Thrombin has been shown to promote tumor growth, but the growth of syngeneic tumors was also not reduced in PAR1−/− or PAR2−/− mice. PAR4 does not play a major role in thrombin signaling in cultured endothelial cells, but may compensate, under certain circumstances, for PAR1 deletion. However, the proangiogenic phenotype of TF mice was completely reverted to wild-type levels by PAR2-deficiency, establishing a major and predominant role for PAR2 in angiogenesis.

Developmental angiogenesis can be stimulated by injection of PAR2 agonist peptide, trypsin, or VIIa into the rat eye, further emphasizing the role of PAR2 as a proangiogenic receptor. Both tumor necrosis factor (TNF) and Tie-2 upregulate contribution to the proangiogenic effects of PAR2 agonists. The Tie-2 ligand angiopoietin 2 is known to sensitize the endothelium to TNF stimulation. Because angiopoietin 2 is stored in Weibel-Palade bodies and PAR2 activation induces Weibel-Palade body release, PAR2 may function in an autocrine loop of angiopoietin-2-mediated Tie-2 signaling. However, PAR2-deficient mice showed normal revascularization in the hypoxia-driven OIR model and supported subcutaneous tumor growth normally, indicating that TNF and Tie-2 dependent effects involving PAR2 may play more important roles in angiogenesis associated with inflammation, rather than hypoxia-driven neovascularization.

The minor contribution of PAR1 to angiogenesis observed in this model is unexpected, because thrombin has been shown to stimulate angiogenesis in vivo and endothelial progenitor cell differentiation in vitro. In addition, PAR1 antagonists suppress thrombin-dependent neovascularization. Although it is expected that a PAR1 antagonist suppresses an angiogenic response induced by direct application of thrombin, the present study raises the issue whether thrombin is generated at sufficiently high concentrations in vivo to drive angiogenesis. Alternatively, the effect of thrombin in vivo may involve the activation of platelets that are a rich source of proangiogenic factors and play an important role in neovascularization. Mouse platelets do not depend on PAR1 for activation, and our study raises the question whether thrombin regulates angiogenesis predominantly through platelet activation.

PAR1 signaling on host cells other than platelets has been linked to angiogenesis by several studies that showed angiogenic responses to PAR1 agonist peptides. One possible explanation for the different conclusion from our and previous studies may rest with the use of a nonselective agonist peptide that activates both PAR1 and PAR2. Indeed, chemokinetic effects by thrombin-mediated PAR1 activation are dependent on the crossactivation of PAR2 by thrombin cleaved PAR1. Thus, the demonstrated effects of thrombin or the chosen PAR agonist peptide on endothelial progenitor differentiation may have involved signaling from PAR2, rather than PAR1 alone.

The presented data are consistent with a model in which TF–VIIa signaling can promote angiogenesis independent of thrombin generation in vivo. Two possible mechanisms could support this model. On the one hand, proangiogenic TF may be localized extravascularly and, thus, separated from the bulk of the coagulation factors in the plasma. In tumor cells, we have recently found that hypoxia can induce ectopic, nonhepatic synthesis of VIIa. The implication of this finding is that the TF–VIIa complex can form and may signal independent of extravasation of coagulation factors. Alternatively, the coagulant activity of TF may be disabled. We have recently described a disulfide/thiol exchange mechanism involving protein disulphide isomerase that suppresses TF-dependent coagulation while preserving direct TF–VIIa signaling. Although it remains to be established whether angiogenesis is promoted by TF expressed in endothelial cells or other vascular-associated cells (eg, microglia or pericytes), such a mechanism may turn off the coagulant activity of TF and leave TF–VIIa mediated PAR2 activation as the dominant protease signaling pathway during neovascularization.

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Disclosures

None.

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**Fig. 1: Hypoxia-driven angiogenesis of WT and TF^{ACT} mice.** (A) Schematic presentation of OIR model. At P17 there is significant residual central vasoobliteration. Associated pre-retinal neovascularization, at the interface between perfused and non-perfused retina, is seen as bright spots in retinal flat mounts. (B) Representative images and (C) quantitative analysis of avascular areas of TF^{ACT}, PAR1-/- and PAR2-/- retinas at P12 demonstrate similar vascular regression. Means and standard deviations, n = 11 (wt), 9 (TF^{ACT}), 9 (PAR1-/-) and 6 (PAR2-/-). (D) wild-type and TF^{ACT} retinas were stained for CD11b (green) and isolectin *Griffonia simplicifolia* (red) to visualize microglia at P12. Numbers of microglia were similar in wild-type and TF^{ACT} mice in both the central avascular area and the periphery containing residual vascular plexus. Images were taken using 20X magnification. (E) Quantitative analysis of vascular regeneration and preretinal neovascularization of wild-type and TF^{ACT} mice. TF^{ACT} and WT mice were exposed to 75% oxygen at P5 or P7 for 5 days. Retinas were analyzed 5 days after return to normoxia at P15 and P17, respectively (retinas analyzed: P15, WT n = 10, TF^{ACT} n = 11; P17, WT n = 10, TF^{ACT} n = 11); * denotes p<0.05.