REVIEW ARTICLE

Thrombin's central role in angiogenesis and pathophysiological processes

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ABSTRACT. A plethora of endogenous modulators of angiogenesis have been identified and their roles in the molecular and cellular events that mediate and regulate angiogenesis have been proposed. In this review, we summarize the recent findings on the role of thrombin/thrombosis on angiogenesis and other related pathophysiological processes. The mechanisms by which thrombin itself and its receptor PAR1 orchestrate many cellular events through interaction with a variety of other factors and cell types are discussed. These new data point to the complexity of the regulatory processes involved in the angiogenic cascade, which may be tissue specific, and dependent upon the pathology involved. The understanding of these events may provide targets for therapeutic intervention in disease states where angiogenesis is disturbed.

Keywords: thrombin, proteinase-activated receptor 1, angiogenesis, endothelial cells, apoptosis, coagulation cascade

Thrombin is a serine protease that is generated in the blood from its inactive precursor prothrombin. Thrombin plays two important, opposing functions [1]. It acts as a procoagulant factor when it converts fibrinogen into an insoluble fibrin clot that anchors platelets to the site of the lesion, and initiates the processes involved in wound repair. This action is reinforced and amplified by: the activation of transglutaminase factor XIII that covalently stabilizes the fibrin clot, the inhibition of fibrinolysis *via* activation of tissue factor pathway inhibitor (TAFI), and the proteolytic activation of factors V, VIII and XI. Thrombin also acts as an anticoagulant through activation of protein C. This function takes place *in vivo* through binding of thrombin to thrombomodulin, an endothelial membrane receptor. Upon binding, the ability of thrombin to cleave fibrinogen is suppressed, but the specificity of the enzyme toward zymogen protein C is markedly enhanced. This reaction is further potentiated by the presence of a specific endothelial cell protein C receptor. Activated protein C (APC) cleaves and inactivates factors Va and VIIIa, two essential cofactors of coagulation factors Xa and IXa that are required for thrombin generation. By this mechanism, APC down-regulates both the amplification and progression of the coagulation cascade [2]. In addition, thrombin is irreversibly inhibited at the active site by the serine protease inhibitor antithrombin with the assistance of heparin [3, 4], and by the thrombin-specific, heparin cofactor II [5].

In addition to its central role in the coagulation cascade, thrombin has long been known to trigger important cellular effects. The main mechanism responsible for these cellular actions of thrombin is mediated by the proteinase-activated receptors (PARs), a novel family of G-protein-coupled receptors [6]. PARs utilize an intriguing mechanism to convert an extracellular proteolytic cleavage event into a transmembrane signal. These receptors carry their own ligands, which remain silent until thrombin and other proteases cleave at a specific site within the extracellular N-terminus exposing a new N-terminal-tethered ligand domain that binds and activates the cleaved receptor [6]. PAR1, the first member of this family to be cloned and the first receptor for which this unique mechanism of activation was described [7], has been shown to respond with very high affinity to thrombin. However, there is evidence that other proteases, such as plasmin [8], factor Xa [9], APC [10] as well as matrix metalloproteinase-1 (MMP-1) [11], can activate this receptor under certain conditions, and induce downstream signals. Besides PAR1, PAR4 also has been found to be cleaved preferentially by thrombin unmasking a unique N-terminal-tethered ligand sequence [12]. PAR1 is responsible for platelet activation in humans at low thrombin concentrations, and its action is reinforced by PAR4 at higher enzyme concentrations [6].

Furthermore, a variety of "non-receptor" targets have also been proposed for thrombin. However, the ability of thrombin to affect cell signaling through PARs-independent mechanisms is an issue that can often be overlooked. For instance, disruption of extracellular matrix-integrin signaling by cleaving either the integrins or the matrix with which they interact, would, in principle, alter cell behavior. In this regard, the ability of thrombin to

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activate endothelial cell-derived metalloproteinases [13], which in turn may remodel the ECM, could lead to PAR-independent signaling. Thrombin can also yield from within its sequence, chemotactic-mitogenic peptides released by proteolytic processing [14, 15]. These thrombin-derived peptides can cause effects by interacting with cell surface receptors that are not PARs [16]. On the other hand, thrombin, at concentrations generated in the circulation in certain settings, can cleave other substrates to release biologically active peptides. In an inflammatory setting that results in fibrin deposition, this substrate can yield proteolytic cleavage products with biological properties [17]. In particular, the action of thrombin on fibrin (ogen) can yield a 14-amino acid chemotactic peptide sequence, termed human fibrinopeptide B, from the N-terminus of the β-chains [18, 19].

INTERRELATION BETWEEN THE BLOOD COAGULATION SYSTEM AND ANGIOGENESIS

Activation of clotting, vascular thrombosis and deposition of extracellular fibrin are common, early steps in many physiopathological processes. Coagulation and the haemostatic plug provide the basic stimulus for initiation of the angiogenic response induced by inflammation, tissue injury and wound healing [20-22]. Similarly, components of the coagulation system contribute to cancer biology [23].

Tissue factor (TF) has been recognized to play an important role in stimulating angiogenesis [24]. TF is aberrantly expressed in many tumour cell types and increased TF expression in tumours is associated with increased angiogenesis and higher tumour grades [25-28]. TF-induced angiogenesis may be due to the up-regulation of vascular endothelial growth factor (VEGF) and down-regulation of thrombospondin [29, 30]. TF was also demonstrated to mediate angiogenesis through activation of its cytoplasmic domain. Phosphorylation of the TF-cytoplasmic domain results in cell migration and PARs signaling [31]. The activation of PAR1 and PAR2 by either the TF/FVIIa complex or the TF/FVIIa/FXa complex led to an acceleration of angiogenesis [32]. The information currently available on the multiple effects of the TF pathway on tumour pathophysiology and angiogenesis provide the basis for considering TF as a target for anti-tumour and anti-angiogenic therapy. On the other hand, tissue factor pathway inhibitor (TFPI), the naturally occurring TF inhibitor, has been shown to exhibit anti-tumour effects *in vitro* and *in vivo* [33]. In addition, TFPI inhibits angiogenesis in the chick embryo system and significantly reduces melanoma, colon and lung carcinoma-induced angiogenesis [34]. Furthermore, TFPI-2, a structural homologue of TFPI, which inhibits the TF/FVIIa complex, has been shown to play a part in the maintenance of the stability of the tumour environment and inhibits invasiveness and growth of neoplasms, as well as formation of metastases [35]. TFPI-2 has also been shown to induce apoptosis and inhibit angiogenesis in experimental models [36, 37]. Of interest, TFPI-2 in endothelial cells is up-regulated by VEGF and suppresses proliferation of endothelial cells [38]. This may represent a mechanism for negative-feedback regulation of VEGF activity.

In addition, APC and protein C inhibitor (PCI) have been demonstrated to essentially contribute, not only to the regulation of haemostasis but also to cell inflammation, proliferation, apoptosis, tumour biology and angiogenesis [39]. Regarding angiogenesis, it was recently reported that APC increases proliferation of vascular endothelial cells and angiogenesis by APC receptor-mediated activation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase and endothelial nitric oxide synthase (eNOS) pathways [40]. Consistently, PCI was shown to inhibit the growth and metastatic potential of breast cancer cells and angiogenesis, *in vivo* and *in vitro*, through a mechanism independent of its protease inhibitory activity [41].

A variety of endogenous angiogenesis inhibitors have been described that are derived from the proteolytic processing of parent proteins with distinct actions [42]. In particular, the generation of anti-angiogenic forms of antithrombin [43] and prothrombin kringle-2 [44], provides additional evidence of a more general process in which components of the clotting system play major roles in the regulation of angiogenesis [45]. Cleavage of the carboxyl-terminal loop of antithrombin induces a conformational change in the molecule, and the cleaved conformation has potent anti-angiogenic and anti-tumour activity in mouse models [46]. In this regard, prothrombin kringle-2 domain also exhibits anti-endothelial cell proliferative activity [44]. Furthermore, recombinant human prothrombin kringle-1 and 2 have potent anti-angiogenic activities in a chick embryo angiogenesis model, and inhibit Lewis lung carcinoma tumour growth and metastasis in mice [47].

Interestingly, thrombin, the final common effector of the coagulation cascade, has also been found to have important roles in angiogenesis [48]. The angiogenesis-promoting effect of thrombin was first demonstrated in the chick chorioallontoic membrane (CAM) system [49]. Thrombin also promoted the formation of blood vessels in matrigel plug injected subcutaneously into mice [50]. In these *in vivo* systems, it was shown that the angiogenic action of thrombin is dose-dependent and requires that the catalytic site of thrombin be functional, since the D-Phe-Pro-Arg-chloromethylketone-thrombin (PPACK-thrombin, chemically inactivated analog of thrombin at the active site) is without effect and competes with thrombin for its angiogenic action. An analog of thrombin (γ-thrombin), which is catalytically active but lacks the anion-binding exosite for binding fibrinogen and therefore cannot form fibrin, is also active in promoting angiogenesis. In addition, thrombin receptor-activating peptide SFLLRN, which acts as an agonist peptide for activating PAR1, is also effective in activating angiogenesis. These findings led us to conclude that the angiogenic action of thrombin can be receptor-mediated and independent of fibrin formation, and can therefore be modulated without interfering with blood coagulation.

In line with this conclusion, data obtained by analysis of animal models (knockout mice) with impaired coagulation factors, suggests that thrombin is the critical protein

involved in vascular development and that this activity is independent of its coagulant action, depending mostly on signaling *via* the thrombin receptors [51]. Lack of thrombin generation (as seen in TF-/-, FX-/-, FV-/-, FI-/- mice), results in severe vascular defects in embryonic development. Notably, similar phenotypes occur in models of impaired thrombin binding to its PAR receptor (PAR1-/- mice) or in a model lacking the corresponding G-protein in endothelial cells (Gα13-/- mice). However, in mice lacking circulating platelets (NF-E2 -/- mice), embryonic development is not altered and embryonic bleeding is not reported [52]. Similarly, mice lacking the fibrinogen alpha chain, which is required for effective thrombus formation, do not display a vascular phenotype nor do they bleed during embryonic development [53]. Thus, both down-stream events of thrombin activation (*i.e.* platelet activation and fibrinogen cleavage), do not seem to be important in controlling embryonic vascular development.

THROMBIN-MEDIATED ANGIOGENESIS: INVOLVEMENT OF PAR1 ACTIVATION

Thrombin, through PAR1 signaling, stimulates endothelial cells and regulates the release, expression and activation of the majority of angiogenesis mediators. Thrombin-induced angiogenesis in a chick CAM system is associated with up-regulation of VEGF as well as angiopoietin-2 (Ang-2) [54]. In line with this, thrombin up-regulates VEGF [55] and Ang-2 [56] in endothelial cells. Another important effect of thrombin is the potentiation of the mitogenic activity of VEGF on endothelial cells [57]. When endothelial cells are pre-incubated with thrombin and subsequently exposed to VEGF, the mitogenic activity is increased by more than 100% over that expected from the additive effects of thrombin and VEGF alone. This synergistic effect of thrombin with VEGF can be explained by the finding that thrombin significantly increases mRNA levels and functional receptor protein for the VEGFR-2. Thus, the up-regulation of the VEGF receptor by thrombin sensitizes endothelial cells to the action of VEGF for the activation of angiogenesis. In this context, it was recently demonstrated that thrombin markedly up-regulated growth-regulated oncogene-α in endothelial cells, and that this chemokine, in turn, mediates the thrombin-induced increase of vascular regulatory growth factors (VEGF, Ang-2) and receptors (VEGFR-2) [58]. Furthermore, different studies have reported that thrombin up-regulates the hypoxia-inducible factor 1 alpha (HIF-1α) under non-hypoxic conditions, by a reactive oxygen species (ROS)-dependent mechanism both in endothelial cells [59] and vascular smooth muscle cells [60, 61]. Thrombin has also been shown to activate the proliferation of endothelial cells by acting directly as a mitogenic factor [62]. This effect of thrombin involves the phosphorylation of extracellular signal-regulated protein kinase 1/2 (Erk1/2, MAPK) and is mediated by EGF receptor transactivation through MMP-dependent release of heparin-binding EGF [63]. Also, neuron-derived orphan receptor-1, a nuclear receptor, has been shown to mediate thrombin-induced endothelial cell mitogenesis and migration [64].

It has also been shown that thrombin alters endothelial cell function *via* PAR1 signaling by decreasing endothelial cell ability to adhere to extracellular matrix proteins [65]. This action of thrombin, together with its ability to activate the MMP-2 in a PAR1-independent manner [66, 67], may be of great importance during the initial stages of angiogenesis, when endothelial cells must detach from their anchorage sites on the vessel wall, degrade the surrounding basement membrane, migrate to distal sites, proliferate, and form the lumen of new vessels. It may also be important in this respect that thrombin increases the levels of the mRNA and protein of β3 integrin subunit in endothelial cells (68). As a result, endothelial cells exposed to thrombin have an increased ability to interact with proteins of the extracellular matrix such as vitronectin and fibronectin. Integrin αvβ3, on the surface of endothelial cells, recognizes the RGD sequence present in proteins of the extracellular matrix. Interaction of the RGD sequence with endothelial cell αvβ3 integrin, regulates the attachment, migration, growth and apoptosis of these cells.

As mentioned previously, the proteolytic cleavage of the N-terminal region of human PAR1 by thrombin, at the R_{41}/S_{42} bond, results in the release of a 41-amino acid peptide. Besides PAR1 activation, it was recently demonstrated that this peptide could also exert biological actions [69]. The name of "parstatin" has been coined for this peptide. Parstatin suppressed both basic angiogenesis and that stimulated by bFGF and VEGF in the chick CAM model and in the rat aortic ring model of angiogenesis. Parstatin also inhibited *in vitro* endothelial cell migration and capillary-like network formation in the Matrigel and fibrin angiogenesis models. Treatment of endothelial cells with parstatin resulted in inhibition of cell growth by inhibition of the phosphorylation of ERK1/2 in a specific and reversible fashion, and by promoting cell cycle arrest and apoptosis, through a mechanism involving the activation of caspases. The molecular mechanism by which parstatin could exert its effects remains unknown. However, parstatin acts as a cell-penetrating peptide, exerting its biological effects intracellularly. It has been shown that the parstatin activity is dependent on its N-terminal hydrophobic domain within residues 1 to 23. Therefore, these data suggest that, similarly to the role of thrombin in haemostasis, where it can be both prothrombotic and antithrombotic, the role of thrombin in angiogenesis can be proangiogenic and antiangiogenic. Parstatin, the cryptic peptide generated by thrombin, may represent an important negative regulator of angiogenesis, with possible therapeutic applications.

On the other hand, it was recently reported that parstatin is an effective agent for cardioprotection during ischaemia and reperfusion of the rat myocardium [70]. It was also shown that parstatin causes vasodilation in isolated rat coronary arterioles. Both the cardioprotective and vasodilatory properties of parstatin were dependant on NOS and K_{ATP} channels. In particular, these data implicate the up-regulation of endothelial derived nitric oxide synthase and increases in bioavailable NO asimportant mechanisms behind parstatin's cardioprotective and vasodilatory effects. Collectively, these studies in rat hearts and coronary vessels, strongly support the concept that parstatin has a protective role during ischaemia-reperfusion by protecting endothelial function.

In addition to modulating the preexisting endothelial cells, thrombin may also impact repair mechanisms and angiogenesis by affecting bone marrow-derived progenitor cells. It was recently shown that human EPCs, as well as CD34+ cells, expressed the thrombin receptor PAR1 on their surface, at levels similar to those found on mature endothelial cells [71]. Thrombin, through PAR1, acts as a potent inducer of bone marrow-derived cell proliferation, migration, and differentiation into endothelial cells [72], by means of an angiopoietin-dependent mechanism [73]. Furthermore, thrombin inhibits apoptosis and causes proliferation of vascular progenitor cells, expressing markers for both activated endothelial cells and vascular smooth muscle cells, suggesting a significant role for thrombin in regenerative repair by circulating progenitor cells [74].

Apart from its effect in endothelial cells, thrombin exerts a wide range of effects on platelets, which contribute to the control of many functions, including angiogenesis. Vessel wall injury or thrombus formation stimulates platelet to adhere to subendothelial matrix and to undergo activation by thrombin, leading to aggregation and degranulation. Platelets stimulate endothelial cell proliferation and tube formation *in vitro* and induce angiogenesis *in vivo* [75, 76]. The absence of platelets inhibits the early stages of angiogenesis and contributes to a decreased number of new vessels *in vivo* [77, 78]. It should be emphasized that platelet progenitor cells (megakaryocytes) synthesise and secrete VEGF, whereas mature platelets transport and, upon activation by thrombin, release this growth factor [79-81]. Moreover, platelet α-granules are a source of a plethora of other pro-angiogenic factors, including VEGF-C [81], basic fibroblast growth factor (bFGF) [82] and platelet-derived growth factor (PDGF) [83]. On the other hand, apart from being pro-angiogenic, platelet α-granules are also a source of inhibitors of angiogenesis, such as thrombospondin [84], Ang-1 [85] and endostatin [86]. It is of interest that thrombin, which influences platelet activity through platelet PAR1 and PAR4 receptors, triggers VEGF secretion *via* PAR1 activation whereas thrombin activation of PAR4 leads to the release of endostatin [86]. Activated platelets are also a source of microvesicles circulating in the bloodstream [87], which have been shown to induce angiogenesis both *in vitro* and *in vivo* [88, 89].

Furthermore, thrombin is clearly accepted as a principal physiological regulator of inflammation, which is an early key process for ischaemia-induced angiogenesis [90]. In this regard, many studies have shown that thrombin and its receptors exist not only in the vascular wall and cells, but also in immune-privileged tissues and cells, which play important proinflammatory roles. Indeed, PAR1 is expressed by many immune cells [91], including macrophages, monocytes, lymphocytes and mast cells, and PAR4 appears to play a key role in thrombin-regulated leukocyte rolling and adherence [92]. Consequently, considerable accumulated data documents the ability of thrombin to trigger many of the responses associated with inflammation, including endothelial cell activation (P-selectin display, increased adhesion of leukocytes and platelets), along with increased vascular permeability [93], mast cell degranulation [94], chemotaxis of neutrophils and their increased adhesion to the endothelium [95, 96], and to the induction of cytokine release from epithelial and vascular smooth muscle [97] and endothelial cells [98, 99]. Also, thrombin acting through PAR1, has been shown to play an essential role in the generation of monocyte chemoattractant protein, which is a key molecule for the recruitment of monocytes and macrophages [100].

THROMBIN-MEDIATED ANGIOGENESIS: INVOLVEMENT OF PAR1-INDEPENDENT MECHANISMS

During wound healing, inflammation or malignant tumour growth, the plasma protein fibrinogen leaks into the extravascular tissue, binds to specific receptors on inflammatory and tumour cells and is cleaved by thrombin generated in the local microenvironment [21, 101]. Several reports provide evidence that this fibrin network has a supportive role for endothelial cell adhesion. The fibrin matrix also appears to be an excellent substrate for the invasion of endothelial cells and subsequent formation of new capillary-like structures [102]. Fibrin bridges cell-matrix interactions essential for physiological and pathological events, which are accomplished through exposure of cryptic sites in the molecule that facilitate adhesion to cell-surface receptors [103]. For example, binding of endothelial cells to fibrin *via* the adhesion molecule vascular endothelial cadherin may be necessary for capillary tube formation [104]. Endothelial cells express different adhesion molecules on their surface based on the extracellular matrices they encounter. Fibrin matrix provokes an angiogenic response by up-regulating the expression of αvβ3 receptors that facilitate endothelial invasion and capillary tube formation [105, 106]. The αvβ3 integrins provide survival signals to endothelial cells during their interaction with fibrin.

The fibrin matrix also provides storage of pro-angiogenic growth factors, such as bFGF, VEGF and insulin-like growth factor-1. Within the fibrin, sequestered growth factors are protected from proteolytic degradation [107]. Degradation of the matrix by proteolytic enzymes, generated during invasion by endothelial and/or tumour cells, releases sequestered growth factors, which bind to cognate receptors on the invading cells, promoting cell proliferation and migration for tumour angiogenesis [108, 109]. Moreover, fibrin E-fragment, which is produced by proteolytic cleavage of fibrin, has been shown to stimulate angiogenesis in the chick chorioallontoic membrane assay [110].

Similarly, it is known that thrombin is also trapped within fibrin matrix and is protected from inactivation by its circulating inhibitors. Binding of thrombin to the fibrin or subendothelial extracellular matrix leaves the majority of the molecule functional and available for cellular interaction [111]. Indeed, thrombin has been proposed as a novel ligand of αvβ3 and α5β1 integrins [68, 112]. When endothelial cells are cultured on thrombin-coated surfaces, the interaction between thrombin and cellular integrins facilitates their attachment and migration, and protects them from apoptosis. These effects of thrombin are independent of its catalytic action and PAR1 activation, and involve the

single RGD (Arg-187, Gly-188, Asp-189) sequence within the thrombin molecule [113]. DIP-thrombin, an active site,chemically-inhibited analogue, or the catalytically inactive thrombin mutant S195A, which replaces the active site serine with alanine, are equally effective in promoting cell attachment and migration. The crystal structure of thrombin shows that most of the RGD sequence is buried and not available for interactions with integrins. However, when thrombin is immobilized, it can assume a non-canonical conformation, exposing the RGD sequence to the solvent and allowing functioning as an epitope, which is recognized by specific integrins that mediate cellular signaling without the involvement of the catalytic activity of the enzyme [112].

The aforementioned effects of thrombin most likely contribute to the initiation of angiogenesis, providing a plausible explanation for the angiogenesis *par excellence* occurring within thrombi in several pathophysiological conditions. For example, a very common clinical observation is that after thrombosis in a large vein, the thrombus is recanalized by new vessels that can be seen with angiography. Interestingly, recent data provide evidence that thrombin bound to a fibrin clot confers angiogenic and haemostatic properties to endothelial progenitor cells, which have been shown to be involved in recanalizing venous thrombi [114].

THROMBIN PROTECTS ENDOTHELIAL CELLS FROM APOPTOSIS

A growing body of evidence has accumulated showing that thrombin is pro- or anti-apoptotic in several cell types, including epithelial and neuronal cells, fibroblasts and tumour cells [115]. In these cells, activation of PAR1 has been shown to induce or inhibit apoptosis, depending on the thrombin concentration or that of PAR1 agonist peptides. In contrast to these observations in other cell types, it was found recently that thrombin protects endothelial cells from apoptosis *via* a mechanism in which its catalytic active site and PAR1 activation have limited contribution [63]. This protective effect of thrombin may be of importance for the migrating endothelial cells during angiogenesis. A further demonstration of the distinct mechanism of thrombin-induced cell survival was obtained from experiments with DIP-thrombin, a chemically inactivated thrombin analogue at the active site. DIP-thrombin mimics the anti-apoptotic effect in endothelial cells almost to the same extent as thrombin itself. In addition, it was shown that αvβ3 and α5β1 integrins play an essential role in the activation of cell survival by thrombin. When echistatin, which is a very potent antagonist of β3- and β1-integrin families, or neutralizing monoclonal antibodies against αvβ3 and α5β1, are combined with thrombin, its protective effect is almost abolished. Collectively, these findings suggest that thrombin inhibits apoptosis in endothelial cells by at least two mechanisms: a minor contribution is mediated by PAR1 activation and a major contribution by interaction with αvβ3 and α5β1integrins in which the catalytic site of thrombin is not necessary.

The involvement of thrombin in endothelial cell survival may provide new insights into the role of thrombin in vascular protection and provides evidence for an essential contribution of thrombin in the establishment and maintenance of vessel wall integrity. Vascular protection may provide an attractive, alternative, mechanistic framework for understanding the impact of thrombin on the cardiovascular system. Thrombin, through its multiplicity of effects on angiogenesis, survival, interaction with other growth factors and many cell types, may have the unique ability to orchestrate the requirements for the development of mature blood vessels. In this regards, thrombin may prove to be useful in the treatment of occlusive and ischaemic cardiovascular diseases. The principle goal of angiogenic therapy is to develop collateral vessels with vascular stability that can provide sufficient blood flow to the ischaemic tissue [116]. Indeed, using a rabbit hindlimb ischaemic model, we have shown that a single intramuscular injection of thrombin can enhance the angiogenic response to ischaemia at the arteriolar level, leading to a significant increase in the regional collateral circulation [117]. These findings are further supported by other reports that have shown that thrombin significantly increases, not only the number of vessels in CAM, but also their diameters and lengths [118]. In addition, the use of anticoagulant drugs after induction of tissue ischaemia hampered a spontaneous angiogenic response in a rodent hindlimb ischaemia model [119]. Accordingly, intramuscular injection of fibrin matrices promoted angiogenesis in a rabbit hindlimb ischaemia model [120].

THROMBIN AND PAR1 AS TARGETS FOR ANTI-ANGIOGENIC THERAPY

The fact that thrombin plays an important role in angiogenesis may suggest a crucial role for thrombin and its receptors in tumour progression and metastasis, as angiogenesis is considered an essential requirement for both of these processes. Indeed, much clinical, histopathological, epidemiological and pharmacological evidence support the notion that the coagulation system contributes to tumour biology [121]. Most tumours cells have constitutively active TF on their surface capable of generating thrombin, which in turn promotes the pericellular deposition of fibrin. Fibrin provides a provisional matrix suitable for attachment and invasion of tumour cells and facilitates endothelial cells to invade the tumour providing the neoplastic mass with the necessary vasculature for growth and metastasis. Thrombin itself has been shown to contribute to a more malignant phenotype by activating platelet-tumour aggregation, tumour adhesion to subendothelial matrix, tumour growth and metastasis [122]. These observations led researchers to use anticoagulants in clinical studies for cancer treatment. Indeed, there is an increasing body of evidence suggesting that adjunctive therapy with anticoagulants may improve prognosis in cancer patients [123]. Both heparins and vitamin K antagonists have been tested. The data from prospective randomized clinical

trials in cancer patients to evaluate the effect of low-molecular-weight heparin on cancer survival are promising and have created new interest in this area [124].

It is likely that thrombin could be acting as a growth-stimulatory signal through activation of PAR. Persistent thrombin signaling through PAR1 acts as an additional tumourigenic event in malignant cells or cells programmed to become malignant, through a combination of various events [125]. PAR1 has been identified as an oncogene [126] and is reported to be highly expressed in tumour cells and in carcinoma biopsy specimens [127, 128]. PAR1 has also long been proposed to be involved in the invasive and metastatic processes of cancers of breast, colon, lung, pancreas, prostate and melanoma [129-134].

In line with these findings, the cell-penetrating pepducin P1pal-7, which acts as potent PAR1 antagonist, significantly blocks tumour growth and angiogenesis of breast cancer xenografts in nude mice [11]. Recently, two newly developed PAR1 antagonists, SCH79797 and RWJ56110, have been evaluated for their effects in the angiogenic cascade [135]. Using the *in vivo* model of the chick chorioallantoic membrane system of angiogenesis, it was shown that SCH79797 and RWJ56110 are very potent anti-angiogenic agents. This inhibitory effect is dose-dependent and is evident both for basic angiogenesis and that stimulated by thrombin. PAR1 antagonists also inhibit capillary-like structure formation by endothelial cells cultured either in medium containing serum or the combination of bFGF and VEGF. Furthermore, the anti-angiogenic effect of PAR1 antagonists is well correlated with their inhibitory effects on endothelial cell growth. These agents not only arrest endothelial cell proliferation and prevent vessel growth, but also induce regression of existing vessels by increasing endothelial cell apoptotic death. It is of interest that the inhibitory effect of PAR1 antagonists was evident only when endothelial cells were in a fast-growing state. Together, these results provide further evidence that thrombin and its receptor, PAR1 are key molecules that mediate angiogenesis, and validate the concept that inhibitors of these targets would be effective anti-angiogenic agents and, as such, have potential therapeutic applications in cancer and other angiogenesis-related diseases.

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