Mechanism of thrombin-induced angiogenesis

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Abstract

Clinical, laboratory, histopathological and pharmacological evidence support the notion that a systemic activation of blood coagulation is often present in cancer patients. Additionally, thrombin was shown to promote tumour progression and metastasis in animals, and epidemiological studies suggest an increased risk of cancer diagnosis after primary thromboembolism. We have proposed that the aforementioned results may be related to our finding that thrombin is a potent activator of angiogenesis. This is a thrombin receptor-mediated event (the receptor is referred to as protease-activate receptor) and is independent of fibrin formation. Many cellular effects of thrombin on endothelial cells can contribute to the angiogenic action of thrombin. (i) Exposure of endothelial cells to thrombin cause a time- and dose-dependent decrease in the attachment of these cells to basement membrane components, with a concomitant increase in matrix metalloproteinase 2 activation. (ii) Thrombin upregulates the expression of integrin $\alpha_\beta_3$, the marker of the angiogenic phenotype of endothelial cells. (iii) Thrombin has chemotactic and aptoptactic effects on endothelial cells and upregulates the expression of the vascular endothelial growth factor (VEGF) receptors (KDR and Flt1). Thus, thrombin synergizes with the key angiogenic factor VEGF in endothelial cell proliferation. Furthermore, thrombin enhances the secretion of VEGF and matrix metalloproteinase 9 of PC3 prostate cancer cells. These results can explain the angiogenic and tumour-promoting effect of thrombin and provide the basis for development of thrombin receptor mimetics or antagonists for therapeutic application.

Introduction

Trousseau was the first to observe frequent blood coagulation in cancer patients [1]. Since then many investigators have provided evidence supporting the view that systemic activation of the blood clotting cascade is evident in patients with cancer [2]. For example, high levels of circulating fibrinopeptides are present in patients with cancer, which

Key words: integrin, thrombosis, tumour.

Abbreviations used: CAM, chick chorioallantoic membrane; cRGD, cyclic Arg-Gly-Asp-o-Phe-Nme-Val; PPACK, o-Phe-Pro-Arg-chloromethylketone; DIP, di-isopropylfluorophosphate; TRAP, thrombin receptor activating peptide; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

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indicates active intravascular coagulation and fibrinolysis. Additionally, it has been shown that many tumour cell types express the transmembrane protein tissue factor, which, when exposed to circulating factor VII, activates factor X leading to generation of thrombin and the formation of fibrin. Thus, many tumour cells elicit procoagulant activity directly or indirectly through interaction with platelets, leukocytes and endothelial cells. Zacharsky et al. [3] have shown by immunostaining the presence of thrombin in a variety of tumour types.

These findings explain the hypercoagulability observed in cancer, but do not answer the question as to whether thrombin/thrombosis contribute directly to the promotion of tumour growth and metastasis. Indeed, results from epidemiological studies [4] show that after primary thromboembolism the risk of overt cancer diagnosis increases 3–6-fold within 6 months following the thromboembolic episode. These clinical results are in line with animal experiments. When B16 cells are treated with thrombin this increases dramatically their metastatic ability [5].

We have proposed that a plausible explanation for the tumour-promoting effects of thrombin could be related to our finding that thrombin is a potent angiogenic factor [6,7], since angiogenesis is considered an essential requirement for tumour growth and metastasis [8].

**Activation of angiogenesis by thrombin**

The angiogenic action of thrombin was demonstrated in the chick chorioallantoic membrane (CAM) system [6] and in the in vivo Matrigel system [7]. In the CAM system it was shown that the angiogenic action of thrombin is dose-dependent and requires the functional catalytic site of thrombin. γ-Thrombin, which is catalytically active, but lacks the anion-binding exosite and cannot form fibrin, is equally as active as α-thrombin in activating angiogenesis in the CAM. In addition, the agonist peptide thrombin receptor activating peptide (TRAP) to the thrombin receptor is also effective in activating angiogenesis. The fact that γ-thrombin and TRAP mimic the angiogenic action of thrombin, but cannot form fibrin, led us to conclude that the angiogenic action of thrombin is receptor-mediated and independent of blood coagulation (fibrin formation). This implies that this new action of thrombin can be modulated without interfering with the blood coagulation cascade. Corrosion casting experiments in the CAM system show that the vascular density of the small capillaries and the plexus are affected the most in the presence of thrombin [9].

**Cellular actions of thrombin related to angiogenesis**

Many, but not all, of the cellular actions of thrombin are mediated through activation of the thrombin receptor. This receptor is expressed in many cell types (endothelial, macrophages, platelets, smooth muscle cells and fibroblasts). A variety of G-proteins can be coupled to the thrombin receptor and this determines the nature of the cellular responses to thrombin. The transduction mechanisms involved include phospholipases C and A₂, protein kinase C, mitogen-activated protein kinase, tyrosine kinase and adenylate cyclases. Through these mechanisms thrombin modulates a multitude of cellular effects [10].

We have explored several actions of thrombin on endothelial cells, the key players in the angiogenic cascade, which may contribute directly or indirectly to angiogenesis.

**Endothelial cell adhesion to extracellular matrix components [11]**

Brief exposure of endothelial cells to physiological concentrations of thrombin causes a marked inhibition of their ability to adhere to basement membrane collagen IV or to laminin. When endothelial cells were exposed to 1 IU/ml thrombin, 50% inhibition was observed within 5 min. This effect is reversible, since reincubation of thrombin-treated cells with fresh growth medium restored their ability to attach to extracellular matrix components. This effect of thrombin is specific and depends on activation of the thrombin receptor. Hirudin abolishes this effect of thrombin. The chemically inactivated thrombin [D-Phe-Pro-Arg chloromethylketone (PPACK)-thrombin] is without effect, but it competes with α-thrombin. TRAP mimics the effect of thrombin on cell adhesion. The transduction mechanism involved is via cAMP, since forskolin or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine restores adherence to endothelial cells that have been exposed to thrombin. This cellular action of thrombin on endothelial cell adhesion may represent an important early event in the activation of the normally quiescent endothelial cells in the initiation of the angiogenic cascade. Endothelial cells need to overcome the barrier of
their anchorage to basement membrane components in order to migrate to distal sites, proliferate and form the lumen of the new vessel.

**Activation of gelatinase A**
A key early event in angiogenesis is the local dissolution of the basement membrane of the parent vessel, by the activated endothelial cells, to allow for detachment and migration to distal sites. Thrombin has been shown to activate gelatinase A [matrix metalloproteinase 2 (MMP-2)] [12], which degrades basement membrane collagen type IV. This not only allows for migration of endothelial cells, but also liberates other angiogenic factors that are sequestered in the extracellular matrix.

**Endothelial cell proliferation**
Thrombin (1.5 IU/ml) has been shown to increase DNA synthesis in endothelial cells by 40–90% over that in controls. PPACK-thrombin has no effect and hirudin abolishes the effect of thrombin [13].

**Synergism of thrombin with vascular endothelial growth factor (VEGF) on endothelial cell proliferation [13]**
We have shown that 8–12 h after exposure of endothelial cells to thrombin, the cells are sensitized to the action of VEGF. The mitogenic activity is increased more than 100% over that expected from the additive effects of thrombin and VEGF alone. The thrombin-treated cells responded to VEGF-induced DNA synthesis in a synergistic manner only 8 h after exposure to thrombin. At earlier times (0.5–4 h), the potentiating effect of thrombin in VEGF-induced DNA synthesis was not evident [13]. The effect is dose-dependent and specific, since it is abolished by hirudin, and receptor mediated, since PPACK-thrombin has no effect, while TRAP mimics the effect of thrombin.

**Thrombin upregulates VEGF receptor expression in endothelial cells [13]**
The aforementioned synergistic effect of thrombin with VEGF can be explained by our finding that thrombin increases mRNA levels for the VEGF receptors KDR and Flt-1 by almost 100% over that of controls. The effect is dose-dependent (with a maximum at 1.5 IU/ml thrombin) and is evident after 8–12 h of thrombin treatment; it declines to control levels after 16 h. This is not owing to a general increase in RNA synthesis because reverse transcriptase-PCR with the same preparations did not show any change in fibroblast growth factor receptor-1 mRNA levels in the thrombin-treated cells as compared with the control cells.

This thrombin receptor-mediated upregulation of VEGF receptors is mediated by protein kinase C and mitogen-activated protein kinase signalling pathways. This was shown using specific activators and inhibitors of these transduction mechanisms [13].

**Involvement of integrin αβ₃ in the mechanism of the angiogenic action of thrombin**
Integrin αβ₃ has been identified as a marker of the angiogenic phenotype of endothelial cells in vascular tissue [14]. Antibodies or peptide antagonists of this integrin inhibited angiogenesis induced by basic fibroblast growth factor in the CAM system or the rabbit cornea model [15]. Furthermore, integrin αβ₃ antagonists inhibit tumour-induced angiogenesis by inducing apoptosis in angiogenic blood vessels without effects on mature vessels, which express minimal αβ₃. Brooks et al. [15] have shown that gelatinase A (MMP-2) and αβ₃ integrin are functionally associated on the surface of angiogenic blood vessels. A fragment of MMP-2 from the C-terminal prevents the enzyme binding to αβ₃ and blocks collagenolytic action.

We have several lines of evidence to suggest that αβ₃ integrin is involved in the mechanism of activation of angiogenesis by thrombin.

We have shown that thrombin-treatment of endothelial cells increases αβ₃ mRNA levels and protein synthesis. The increase in β₃ mRNA levels is evident 8–12 h after thrombin treatment; these levels increase by approx. 130% and there is an approx. 70% increase in β₃ protein levels.

Endothelial cells are shown to adhere to immobilized thrombin. The process is shown to be saturable and concentration-dependent. The catalytic site of thrombin is not involved since di-isopropylfluorophosphate (DIP)-thrombin is equally effective. However, when cells are pretreated with an antagonist peptide to αβ₃ [cycled Arg-Gly-Asp-d-Phe-Nme-Val (cRGD)] cell adhesion to thrombin is prevented. An inactive cRGD peptide has no effect indicating the involvement of αβ₃ in the adhesion of endothelial cells to thrombin.

Endothelial cells migrate in a haptotactic Boyden chamber assay through a microporous membrane towards immobilized thrombin in a dose-dependent manner. This migration is also αβ₃-dependent, because it is blocked by the antagonist peptide cRGD (but not the inactive...
cRGD). The same results are obtained with the chemically inactivated DIP-thrombin, and this is in line with the cell adhesion results described above. The involvement of $\alpha_v\beta_3$ in the haptotactic effect of thrombin on endothelial cells is further demonstrated using immobilized vitronectin as an $\alpha_v\beta_3$ ligand. The motility of endothelial cells towards vitronectin is prevented when soluble thrombin or an anti-$\alpha_v\beta_3$ antibody is present. These results may explain the clinical observation that robust angiogenesis occurs within thrombi. Thrombin in the plasma is inactivated rapidly, but within thrombi it is protected and probably acts as a haptotactic factor and activator of angiogenesis.

Endothelial cells in culture undergo apoptosis once serum is removed. We found that endothelial cells have longer survival and decreased levels of apoptosis on plates coated with thrombin. The administration of soluble cRGD peptide or anti-$\alpha_v\beta_3$ antibody resulted in a significant induction of apoptosis in endothelial cells on thrombin-coated plates. These results suggest that thrombin promotes $\alpha_v\beta_3$-dependent endothelial cell survival, which is required in the process of detachment from their anchorage site on the basement membrane and their migration to distal sites during angiogenesis.

As discussed above, thrombin causes an activation of MMP-2; 1.0 IU/ml causes more than 60% activation of the latent form of MMP-2. This process is also $\alpha_v\beta_3$-dependent. When thrombin was used in combination with cRGD, MMP-2 activation was reduced in a dose-dependent fashion.

In the CAM system, 1.0 IU/ml thrombin caused an approx. 100% increase in angiogenesis, as evidenced by the rate of collagenuous protein biosynthesis, which we have shown to be a reliable, sensitive and quantitative index of angiogenesis [16]. The combination of cRGD peptide with thrombin decreased the thrombin promotion of angiogenesis from 100%, to 28%. cRGD alone caused a 30% inhibition of basal angiogenesis in the CAM system as compared with controls.

Many of the aforementioned effects of thrombin may contribute to its angiogenic action. The question arises as to which one of the multitude of effects of thrombin on endothelial cells and other cell types is the most important in the activation of angiogenesis and is therefore a suitable target for therapeutic modulation? It is likely that thrombin plays a different role depending on the particular site and the pathology involved. Temporal and spatial factors may play a determining role. It is likely that thrombin plays a key role in orchestrating the events described above in the microenvironment at the site of angiogenesis, where many factors and cell types participate.

**Role of thrombin in angiogenic diseases and therapeutic implications**

Thrombin is present in situations such as wound healing and inflammation and in the placenta, as well as in many tumour types, in which its presence has been shown by immunostaining [3]. Zacharsky et al. [3] have shown the presence of thrombin in rheumatoid synovial fluid, placenta macrophages, and the capillaries of freshly incised skin, but not in unperturbed skin or aged incisions. Bleeding and blood coagulation, and therefore, thrombin generation, are primary events not only in cancer and wound healing, but also in diabetic retinopathy, within the atherosclerotic plaque etc. The presence of thrombin in all these conditions in which angiogenesis is activated suggests that it may play a pivotal role in this cascade.

In this paper we have reviewed our studies on the cellular actions of thrombin on endothelial cells, actions which may be involved in several steps of the angiogenic cascade. Many of these actions are thrombin receptor-mediated events and can be mimicked or antagonized with agents that do not interfere with blood coagulation. This opens up the possibility of using thrombin-mimetics to promote angiogenesis. Such non-thrombogenic analogues of the activated thrombin receptor may have therapeutic applications in wound healing, ischaemic conditions, non-healing ulcers and other conditions where promotion of angiogenesis is desirable. Conversely, antagonists of the activated thrombin receptor, which do not interfere with blood coagulation, may be of potential use for anti-angiogenic therapy in cancer and other angiogenic diseases.

It is of interest, in this respect, that thrombin receptors are preferentially expressed in highly metastatic breast carcinoma cell lines and breast carcinoma biopsy specimens [5]. These receptors can be activated by thrombin or other proteases, which are expressed at high levels in invasive tumour cells.

Activation of thrombin receptors was shown to upregulate the expression of VEGF receptors in endothelial cells. This sensitizes the endothelial cells to the VEGF, the key angiogenic factor and the specific endothelial cell mitogen. This, in connection with overexpression of VEGF by tumour cells after thrombin receptor activation,
may result in mutual activation of tumour and endothelial cells, leading to promotion of angiogenesis and tumour progression.

References

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Structural analysis of the interaction between urokinase-type plasminogen activator and its receptor: a potential target for anti-invasive cancer therapy

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Abstract

The ability to degrade the extracellular matrix by controlled proteolysis is an important property of malignant cancer cells, which enables them to invade the surrounding tissue and to gain access to the circulation by intravasation. One proteolytic system thought to be involved in these processes is the urokinase-mediated plasminogen activation. Expression of a glycolipid-anchored receptor for urokinase-type plasminogen activator (uPA) targets this system to the cell surface. This receptor (uPAR) is composed of three homologous modules belonging to the Ly-6/uPAR/x-neurotoxin protein domain family. Integrity of the three-domain structure of uPAR is required for maintenance of its sub-nanomolar affinity for uPA, but the functional epitope for this interaction is primarily located in uPAR domain I. Using affinity maturation by combinatorial chemistry, we have recently identified a potent 9-mer peptide antagonist of the uPA–uPAR interaction having a high affinity for uPAR (Kd < 1 nM). Photoaffinity labelling suggests that this peptide interacts with a composite binding site in uPAR involving both domains I and III. When tested in a chicken chorioallantoic membrane assay that was developed to quantify intravasation of human cells, this antagonist was able to reduce the intravasation of HEP-3 cancer cells by approx. 60 %.

Introduction

Degradation of the extracellular matrix by cell-surface-associated proteolysis plays an important role in the tissue remodelling events that occur during morphogenesis, angiogenesis, mammary gland involution, wound healing and dissemination of cancer [1]. A number of proteolytic systems are associated with the cell surface; these