THROMBIN AND PROTEASE-ACTIVATED RECEPTORS IN CANCER

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Results from clinical studies indicate a strong association between thromboembolism and solid malignancy. Being central to blood coagulation and displaying a number of cellular postclotting activities, the serine protease thrombin has been localized within or adjacent to malignant tissues possibly associated with fibrin(oid) deposits. Thrombin proteolytically activates its cellular receptors including the prototypic protease-activated receptor 1 which is expressed by various tumor cells. Current molecular and cellular studies provide mounting evidence that the activation of protease-activated receptor(s) is the main mechanism whereby thrombin exerts its modulating effects on the malignant phenotype including tumor growth, local progression, and distant metastasis. A detailed understanding of the molecular interplay between thrombin, thrombin receptor(s) and cancer biology may be helpful to develop new therapeutic approaches consisting of the suppression of thrombin receptor(s) in malignancy.


INTRODUCTION

Cancer represents a spectrum of diseases sharing common pathobiological principles including active proliferative state due to growth signal autonomy and insensitivity to growth-blocking signals, resistance to apoptosis, escape from finite replicative potential, angiogenic ability, and tissue invasion and metastasis (1). The key processes making tumor cells survive, grow aggressively and metastasize are highly complex mechanisms making its full explanation at the cellular and molecular level a challenging task. The type of tumor, different stages of its progression, and participation and regulation of a large number of gene products need to be incorporated into a steadily evolving overall picture of malignancy. In this review, we focus on the multiple implications of the serine protease thrombin and its cellular receptors as related to tumor growth, local progression, and distant metastasis.

THROMBIN, HEMOSTASIS, AND CANCER

The trypsin-like serine protease thrombin (EC 3.4.21.5) is generated by the proteolytic cleavage of its proenzyme prothrombin by coagulation factor Xa. Physiologically, thrombin generation is restricted to sites of vascular injury. The clotting activities of thrombin are central to hemostasis and include the conversion of soluble fibrinogen to insoluble fibrin occurring at even only 5-10 nM thrombin concentration (2,3). In addition, increasing attention is being paid to what is collectively referred to as thrombin's cellular or postclotting effects. These effects include proinflammatory action with stimulation of transvascular leukocyte migration, regulation of microvascular permeability with formation of interendothelial gaps, and induction of mitogenesis in fibroblasts, smooth muscle cells, and malignant cells, to name but a few (4-8).

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An area of growing interest is the association between thrombin and malignancy. Both clinical and laboratory evidence have shown that thrombin is implicated in the growth, progression, and metastasis of cancer. It has long been known from clinical practice that patients with solid malignancies suffer from an excess thromboembolic rate (9,10). Venous thromboembolism is usually considered as a paraneoplastic syndrome complicating the natural course of cancer (11). Equally or perhaps more important, thromboembolism might actively contribute to the progression and spread of cancer. Clinical research has provided abundant evidence of a thrombophilic and/or prothrombotic state in patients suffering from a variety of malignancies including lung, colorectal, ovarian, pancreas, breast, stomach, and prostate cancer (10,12-16). For example, abnormal coagulation profiles consisting of increased levels of coagulation activation/fibrinolysis markers (prothrombin fragment 1+2, thrombin-antithrombin complex, D-dimer) were repeatedly reported in patients with prostate cancer (16-18).

A multitude of factors, both genetic and acquired, contribute to the pathogenesis of venous thromboembolism in cancer patients (19). Host- and tumor-derived mechanisms are equally involved (Table 1).

### Table 1. Pathogenic factors contributing to cancer-associated venous thromboembolism

- Generation of tissue factor and cancer procoagulants
- Synthesis of cytokines and growth factors
- Induction of platelet and endothelial cell activation
- Diminished fibrinolysis
- Decrease in natural anticoagulants
- Abnormal protein metabolism (dysproteinemia)
- Inflammation and acute phase response
- Hemodynamic changes
- Surgery, chemotherapy, hormone therapy

**LOCAL GENERATION OF THROMBIN IN TUMOR TISSUES**

The modulation of a given tumor phenotype by thrombin requires the presence of enzymatically active thrombin within or adjacent to malignant tissue. Several different methodologies have been used to approach this particularly important issue. Using immunohistochemistry, the presence of prothrombin fragment 1+2, which is released from prothrombin during its proteolytic conversion to thrombin has been demonstrated in pancreatic, laryngeal, and prostatic cancer indicating local thrombin generation (20,21). Furthermore, prothrombin was detected in pancreatic cancer cells and their stromal compartment. In addition, tumor sections stained positive for tissue factor and coagulation factors VII, X, VIII, IX and XII (22). Taken together, these data indicate that in pancreatic carcinoma extravascular blood coagulation might proceed leading to the local generation of thrombin.

A different approach using hirudin as a specific label localises thrombin to tumor cells of different origin, including small cell carcinoma of the lung, renal cancer and melanoma (23). Lung adenocarcinoma and squamous cell carcinoma are positive only in their stromal compartment. However, colon cancer sections stain negatively, lending support to the notion that the generation of thrombin by either tumor cells or tumor stroma is not a unique feature of all malignancies.

Thrombin converts soluble fibrinogen to insoluble fibrin with the formation of fibrin-rich clots retaining proteolytically active thrombin at their surface (24). This observation is highly relevant since fibrin(oid) deposits are frequently detected within and adjacent to malignant tissue of different origin and localization (25-28). Traditionally, fibrin(ogen) deposition within the tumor stroma is thought to originate from exudation of plasma fibrinogen with subsequent deposition (29). However, recent evidence indicates that certain cancer cells synthesize and secrete fibrinogen polypeptide chains suggesting endogenous synthesis and deposition within the tumor (30). The molecular composition of the tumor (neo)matrix is complex. It is best described as a transitional fibrin-containing gel with ongoing remodeling and changing organization (31). The functional impact of extravascular fibrin turnover in cancer remains to be elucidated.

In vitro experiments have shown that fibrin clot-associated thrombin can be released from the clot surface for a prolonged period of time and its ability to induce cell proliferation is fully retained (32). Due to an excess amount of antithrombin(s), thrombin activity is only short-lived in the blood circulation. However, it is tempting to speculate that enzymatically active thrombin can be steadily released from fibrin(oid) deposits within neoplastic tissue to exert its function without being inhibited by antithrombin(s). Therefore, cancer-associated fibrin clots could represent "reservoirs" of bioactive thrombin which can be released "on demand" (33).
THROMBIN RECEPTORS AND THROMBIN-BINDING SITES

The interplay between malignancy and hemostasis including its central protease thrombin has long been recognized although the underlying molecular mechanisms were poorly understood. Recently, the identification and a steadily increasing molecular understanding of PARs provide mechanistic insight at the molecular level.

The PAR family and their unique mechanism of proteolytic activation

G protein-coupled, seven-transmembrane segment receptors (GPCRs or 7TMRs) constitute a large superfamily of proteins with exceptional ligand diversity which transmit signals from the extracellular environment to the cytoplasm predominantly by recruiting and activating heterotrimeric G proteins. In accordance with the traditional understanding of ligand-receptor interactions, binding of an extracellular ligand to its specific GPCR causes a conformational change and activation of the receptor followed by the generation of a series of signaling events (34). However, it was only recently that a different and unique activation mechanism of GPCR has been identified. The activation of this type of GPCR does not only depend on ligand binding to, but also on the cleavage of the receptor molecule by employing the proteolytic activity of the protease ligand. Accordingly, this family of GPCRs has been designated protease-activated receptors. Following the molecular cloning of the prototypic functional thrombin receptor (PAR-1) from a human megakaryoblastic cell line (35), a four-member family comprising PAR-1, -2, -3 and -4 has emerged (36). Only PAR-2 is known to be activated by trypsin and additional proteases with trypsin-like specificity (37) while the other three receptors are proteolytically activated by thrombin. PAR-1 has been recently suggested to be cleaved and activated by coagulation factor Xa without the participation of thrombin in human cervical carcinoma cells (38). Although the coagulation factors VIIa and Xa activate PAR-2 (39), it is not considered a thrombin receptor and will not further be covered in this review. PAR-3 and PAR-4 are the most recently identified members of the PAR family (40,41).

Thrombin recognizes and binds to the aminoterminal exodomain of PAR-1 specifically cleaving the peptide bond located between the residues Arg-41 and Ser-42. This cleavage results in a new aminoterminal ("tethered ligand") starting with the amino acid residues Ser-Phe-Leu-Leu-Arg-Asn (SFLLRN). This newly formed tethered ligand binds into the transmembrane domain of PAR-1 thus eliciting signalling events (Fig. 1). The activated PAR-1 initially internalizes via clathrin-coated pits and is then sorted away from recycling pathways and delivered to lysosomes for degradation (42). A pool of intracellular intact PAR-1 "reserve" can quickly repopulate the cell surface without the requirement of new receptor synthesis (43). Similar activation mechanisms have been described for all members of the PAR family. Several synthesized thrombin receptor activation peptides (TRAPs) comprising the corresponding tethered ligand amino acid residues are powerful experimental tools to mimic the thrombin-mediated activation of protease-activated receptors. Being the prototypic member of the PAR family, PAR-1 is believed

![Figure 1. Proteolytic mechanism of protease-activated receptor 1 (PAR-1) activation. Thrombin binds to and cleaves the extracellular receptor aminoterminus between residues Arg-41 and Ser-42 thus unmasking a new aminoterminus. The resulting shorter N-terminal segment (tethered ligand) docks intramolecularly within the transmembrane receptor body to generate intracellular signalling.](image)

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to mediate most if not all of the cellular effects exerted by thrombin, while PAR-3 may function as a tethering protein for thrombin. PAR-4 is rather insensitive to thrombin demonstrating low affinity (44).

**Expression profile of thrombin receptors**

Physiologically, PAR-1 is expressed by different human tissues including blood and blood vessel cells (45), and in the central nervous system (46). PAR-3 is detectable in mouse platelets, rat brain capillary endothelial cells and astrocytes, and human airway smooth muscle cells (47-49). Notably, human platelets do not express PAR-3 (36). Both PAR-1 and PAR-4 are expressed in human platelets, and either of them independently mediates thrombin signalling, PAR-1 at low while PAR-4 at high thrombin concentrations (50). It has been suggested that PAR-4 may act as a “backup” mechanism in human platelets in the absence of PAR-1 functionality. The recent development of PAR knockout mice has provided a valid approach to comprehensively elucidate the physiological and pathological importance of the different members of the PAR family (51).

The prototypic thrombin receptor PAR-1 has recently been detected in most established malignant cell lines and in a number of cancer tissues obtained from different primary origin (Table 2). It seems tempting to speculate on an association between the expression level of thrombin receptors and the tumor phenotype including differentiation and the propensity to form metastasis. For example, PAR-1 is expressed in oral squamous cell carcinomas with PAR-1 protein levels being lower in non-metastatic compared to metastatic cells (52). PAR-1 protein was not detected in the epithelia of normal pancreatic tissue whereas immunofluorescence staining and Western blot studies of pancreatic cancer cell lines revealed a correlation between PAR-1 expression intensity and the grade of differentiation. The level of mRNA\(^{PAR-1}\) is lower in normal pancreas compared to pancreatic cancer tissue. The levels of mRNA\(^{PAR-1}\) differ up to 25-fold between different pancreatic cancer cell lines (53). Compared to normal prostate tissue or soft tissue metastasis-derived prostate cancer cells, prostate cancer cells derived from bone metastases express increased amounts of PAR-1 indicating an association between PAR-1 expression and bone metastases (54,55).

These findings support a hypothetical relationship between the level of thrombin receptor PAR-1 expression, carcinogenesis, and cancer progression.

The progression of a given tumor requires bidirectional communication with its microenvironment. PAR-1 expression has been demonstrated in stromal cells surrounding malignant tissue. Up-regulated PAR-1 expression is detected in cancer stromal fibroblasts compared to normal or benign counterparts (56) suggesting that the aberrant PAR-1 expression in and/or around tumor tissues contributes to abnormal cellular communication in malignancy.

Considerably less work has been performed to investigate the expression profile of the additional thrombin receptors PAR-3 and PAR-4 in cancer cells and tissues. However, mounting evidence suggests the presence of multiple PAR species in human malignant cells. Human astrocytoma cells express PAR-1 and PAR-4 (57). In primary human renal carcinoma cells, co-expression and cellular surface clustering of PAR-1 and PAR-3 was detected (58) pointing to receptor interactions. Similarly, a complex PAR expression profile was reported for breast cancer cells (59). Highly invasive breast cancer cells MDA-MB-231 express very high levels of functional PAR-1, PAR-4, and trypsin receptor PAR-2, while minimally invasive MCF7 cells have only trace amounts of PAR-1 and low levels of PAR-4 and PAR-2. These cell lines should be helpful to study in detail the relative contribution of the individual thrombin receptors to cellular effects exerted by thrombin. It was also demonstrated that the expression profile of thrombin receptors in a given tumor varies with the histopathological type. Table 2 summarizes the expression profile of established cell lines and primary cancer tissues except for blood-related cells (45). It should be noted that only SIT1 melanoma cells were shown not to express PAR-1. Moreover, this cell line is unique in not expressing any of the thrombin receptor species identified so far (60). Partly controversial data have been obtained for PAR expression in several cell lines including MCF-7 and MDA-MB-231 breast cancer cells (59,61-63), and LnCAP prostate cancer cells (64,65).

**Additional thrombin-binding sites**

In addition to PARs, other thrombin-binding sites including thrombomodulin and glycoprotein Ib\(a\) (GPIb\(a\)) are variably expressed by malignancies of different cellular origin (76,77).

Thrombomodulin (TM) is a thrombin-binding glycoprotein expressed on the luminal vascular endothelial cell surface. It is a potent negative coagulation regulator acting as a molecular switch by converting thrombin from a procoagulant protease to an anticoagulant (78). In addition, TM precludes thrombin from activating PAR-1 (79). Expression of TM seems to be restricted to certain types of malignancy including transitional cell (76) and squamous cell carcinoma (80), ovarian (81) and pancreatic cancer (82). Clinical studies have indicated that the expression of TM in lung squamous cell carcinoma is inversely correlated with malignancy with a decrease of TM expression being associated with metastasis and poor prognosis (83,84). Potential mechanisms underlying a less aggressive tumor phenotype in TM-expressing cancer include the abrogation of thrombin procoagulant activities, reduction of pericellular thrombin concentration by its TM-mediated internalization, and inhibition of PAR-1 activation.
### Table 2. Expression profile of thrombin receptors (PAR-1, PAR-3 and PAR-4) by malignant cells*

<table>
<thead>
<tr>
<th>Established cell cultures</th>
<th>Protease-activated receptor</th>
<th>Assay</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>DU145 (prostate carcinoma)</td>
<td>+</td>
<td>RT-PCR, FCM</td>
<td>63, 65</td>
</tr>
<tr>
<td>LnCAP (prostate carcinoma)</td>
<td>+</td>
<td>RT-PCR, FCM</td>
<td>65</td>
</tr>
<tr>
<td>A172 (glioblastoma)</td>
<td>+</td>
<td>RT-PCR</td>
<td>65</td>
</tr>
<tr>
<td>SNB-19 (glioblastoma)</td>
<td>+</td>
<td>RT-PCR, ICC</td>
<td>57</td>
</tr>
<tr>
<td>MZ4met (melanoma)</td>
<td>+</td>
<td>RT-PCR, Southern, Western</td>
<td>68</td>
</tr>
<tr>
<td>5K-Mel-28 (melanoma)</td>
<td>+</td>
<td>RT-PCR</td>
<td>68</td>
</tr>
<tr>
<td>5i1 (melanoma)</td>
<td>-</td>
<td>Northern</td>
<td>60</td>
</tr>
<tr>
<td>A549 (lung carcinoma)</td>
<td>+</td>
<td>RT-PCR, ICC</td>
<td>69</td>
</tr>
<tr>
<td>O22 (laryngeal carcinoma)</td>
<td>+</td>
<td>Western</td>
<td>52</td>
</tr>
<tr>
<td>Hep-2g (laryngeal carcinoma)</td>
<td>+</td>
<td>ICC</td>
<td>70</td>
</tr>
<tr>
<td>MIA PaCa-2 (pancreatic carcinoma)</td>
<td>+</td>
<td>ICC</td>
<td>71</td>
</tr>
<tr>
<td>MDA-MB-436 (breast carcinoma)</td>
<td>-</td>
<td>FCM</td>
<td>62</td>
</tr>
<tr>
<td>MDA-MB-439 (breast ductal carcinoma)</td>
<td>+</td>
<td>Northern, ICC, Western</td>
<td>61</td>
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<tr>
<td>MDA-MB-231 (breast adenocarcinoma)</td>
<td>+/-</td>
<td>FCM, Northern, ICC, Western</td>
<td>59, 61, 63, 62</td>
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<tr>
<td>MCF7 (breast adenocarcinoma)</td>
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<td>59, 61, 63, 62</td>
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<td>HeLa (cervical carcinoma)</td>
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<td>RT-PCR, Southern, Western</td>
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<tr>
<td>Tu686, 686LN (oral squamous cell carcinoma)</td>
<td>+</td>
<td>Western</td>
<td>52</td>
</tr>
<tr>
<td>KB (oral epidermoid cancer**)</td>
<td>+</td>
<td>RT-PCR</td>
<td>72</td>
</tr>
<tr>
<td>Clone A (colon adenocarcinoma)</td>
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<td>Nested RT-PCR, Southern, Sequencing, Western</td>
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<tr>
<td>HCT-8, SW-48, HT-29 (colon carcinoma)</td>
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<td>RT-PCR, Southern, Western</td>
<td>68</td>
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</table>

<table>
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<th>Primary culture or tissue</th>
<th>Assay</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Non-breast carcinoma tissue specimens (gastric, lung, thyroid, ovarian)</td>
<td>+</td>
<td>IHC, ISH</td>
</tr>
<tr>
<td>Primary glioblastoma cells</td>
<td>+</td>
<td>ICC</td>
</tr>
<tr>
<td>Breast carcinoma tissue specimens</td>
<td>+</td>
<td>ISH</td>
</tr>
<tr>
<td>Oral squamous cell carcinoma</td>
<td>+</td>
<td>IHC, RT-PCR</td>
</tr>
<tr>
<td>Primary renal cell carcinoma</td>
<td>+</td>
<td>RT-PCR, ICC, EM</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma</td>
<td>+</td>
<td>RT-PCR, Immunofluorescence, Western</td>
</tr>
</tbody>
</table>

*Reports were only considered if direct proof of PAR expression was obtained at either the mRNA or protein level. Publications providing indirect evidence for the expression of PAR (e.g. by recording cellular phenomena after treatment with thrombin and/or TRAPs) were not included.

**The identity of KB cells as oral epidermoid cancer cells has recently been questioned (75).**

**Abbreviations:**

- RT-PCR: Reverse transcription-polymerase chain reaction
- ICC: Immunocytochemistry
- FCM: Flow cytometry
- IHC: Immunohistochemistry
- ISH: In situ hybridization
- EM: Electron microscopy
- ND: Not done

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The relative importance of TM expression as related to the cellular effects of thrombin has not been conclusively established. However, it should be noted that in A549 lung cancer cells TM constitutes about 90% of all thrombin binding sites (85). In our laboratory, we demonstrated that TM is not expressed in metastasis-derived human prostatic cancer cell lines DU145 and LnCAP (unpublished data) which is consistent with the concept that reduced or absent TM expression seems favourable for thrombin-mediated PAR-1 activation with a plethora of downstream effects contributing to tumor growth and progression.

GPIbα forms part of the glycoprotein Ib-IX-V complex primarily expressed on the platelet surface (86). GPIbα contains several ligand-binding domains for von Willebrand factor (vWF), Mac-1, P-selectin, coagulation factor XII, and thrombin. Significant insight has been obtained into thrombin binding to platelet GPIbα suggesting that GPIbα may act as a cofactor for PAR-1 activation by thrombin (87). However, the effects of binding of thrombin to tumor cell surface GPIbα are largely unknown mainly due to the long-held belief that GPIbα expression is restricted to cells of megakaryocytic lineage (88). Only few studies convincingly ruled out the expression of GPIbα by the malignant cells under consideration (89). Although formal proof of GPIbα/thrombin interaction in malignant cells is lacking, indirect evidence has been provided. GPIbα has been detected in human breast cancer cell lines and primary breast tumor tissue samples. In addition, it may play an important role in tumor-induced platelet aggregation and in the hematogenous spread of breast cancer cells (90,91). The functional importance of GPIbα expression by malignant cells and its molecular interplay with thrombin and/or PAR-1 remains to be studied.

THROMBIN, PAR, AND TUMOR BIOLOGY

At the present time, it seems premature to conclude that cellular effects evoked by the exposure of malignant cells to thrombin are exclusively mediated by the proteolytic activation of thrombin receptor(s). However, their significant contribution to tumor growth, local progression, and distant metastasis is being gradually established.

Tumor progression: cell proliferation

The mitogenic effect of thrombin on smooth muscle cells, fibroblasts, and endothelial cells has long been appreciated (4,5,92). Thrombin treatment of pigment epithelial cells activates S-phase reentry in vitro (93). In interferon-γ-differentiated growth-arrested U937 promonocytic cells, the proliferation-stimulating effect of thrombin was demonstrated by downregulation of p21^{wdc} and upregulation of cyclin D1 expression (94).

In addition, thrombin displays mitogenic activity towards a variety of malignancies including astrocytoma, laryngeal, colon and prostate cancer (6,65,70,95). Although the molecular mechanisms involved in thrombin-mediated tumor cell mitogenesis are not yet understood in full detail, it elicits signalling events through coupling with multiple G proteins and complex signalling pathways including Ras protein, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) cascade(s). For example, thrombin treatment of 1321N1 astrocytoma cells lead to polyphosphoinositide hydrolysis, Ca^{2+} mobilization, and DNA synthesis. Either microinjection of a dominant interfering mutant of human Ras or an inhibitory antibody against Ras into these cells inhibited DNA synthesis after thrombin treatment (6). The mitogenic activities of thrombin and PAR-1 activation peptide on human epidermoid carcinoma cells can be abolished by the PKC inhibitor bisindolylmaleimide (70). The MAPK cascade forms a critical part of the signalling mechanisms in eukaryotic cell regulation (96). Specific inhibitors for MAPK kinase or MEK suppress thrombin/PAR-1-induced cell proliferation in human colon cancer cells (95) and prostate cancer cells (Liu et al, in preparation).

Recent studies observed dose-dependent dual effects of thrombin on the proliferation kinetics of certain types of tumor cells in vitro. At low concentrations, thrombin leads to increased cell proliferation while high concentrations of thrombin cause impaired tumor cell growth and/or apoptosis (97,98). Strikingly, the opposite pattern was also observed in rat C6 glioma cells with inhibition of proliferation at lower thrombin concentrations (99). Potential mechanisms underlying the different cellular proliferation kinetics in a variety of cell lines include the impact of thrombin on cell cycle control and apoptosis (98). Thrombin-induced changes in cell cycle control and apoptosis were associated with p53-independent, STAT-1-dependent up-regulation of p21^{wdc} and caspases (63). However, inconsistent expression patterns of p53, p21 and bcl-2 in thrombin-induced apoptosis have been described (98). Preliminary evidence suggests the involvement of a variety of signaling mechanisms in the regulation of apoptosis by thrombin in malignant cells (100).

The molecular mechanisms of thrombin-induced mitogenesis and cell cycle control in malignant cells remain to be fully elucidated. Of particular importance will be the molecular dissection of apoptosis-inducing pathways triggered by thrombin at higher concentrations.

Tumor progression: angiogenesis

A solid tumor can not grow beyond a critical limit of 1-2 mm in diameter without gaining access to the host circulatory system that supplies the tumor with nutrients and waste-disposal capacity. Once the primary tumor gains access to the blood-supplying system, growth at the primary site is no longer limited. Tumor-associated angiogenesis is the process of generation of new blood vessels that connect the tumor...

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with the host blood vessel system. Although angiogenesis
physiologically takes place in a limited number of occasions,
such as embryogenesis, wound healing and the proliferative
phase of the female reproductive cycle, tumor cells can initi-
ate (neo)vascularization by secretion of an array of proangio-
genic factors. Thrombin has been reported to play a critical
role in triggering and sustaining tumor-associated angiogen-
esis (101-103).

Vascular endothelial growth factor (VEGF) is a potent
proangiogenic cytokine directly acting on endothelial cells to
promote new vessel formation. Up-regulation of VEGF ex-
pression and secretion by tumor cells and surrounding en-
dotheial cells is a key aspect of tumor-associated angiogenesis
(104). VEGF expression is more pronounced in human pro-
state cancer compared to normal prostate tissue (105). We ob-
erved that DU145 and LnCAP prostate cancer cells secrete
increased amounts of VEGF in comparison to non-tumori-
genic SV40-immortalized prostate epithelial cells PNT1A
(unpublished data) supporting the concept that VEGF lev-
els are related to malignancy and metastasis. Both thrombin
and PAR-1 activation peptide stimulate VEGF expression
and secretion from cancer cells and vascular smooth muscle
cells with a dose- and time-dependent pattern (106,107).
The mechanism of increased expression of VEGF seems to
involve stabilization of the mRNA. The increase of VEGF
mRNA is inhibited by wortmannin (a PI3Kinase inhibitor)
and H7 (a broad spectrum serine/threonine kinase inhibitor)
demonstrating that PI3Kinase and serine/threonine kinase
pathways are involved (106). Thrombin-induced VEGF re-
lease from vascular smooth muscle cells is significantly re-
duced by applying neutralizing antibodies against platelet-
derived growth factor (PDGF), transforming growth factor
(TGF)-β, and basic fibroblast growth factor (bFGF) showing
its dependence on the endogenous formation of a variety of
growth factors (107). On the other hand, incubation of hu-
moral umbilical vein endothelial cells (HUVEC) with VEGF
accelerates tissue factor generation and the conversion of in-
active prothrombin to enzymatically active thrombin (108)
suggesting the functionality of a feedback system in throb-
min/VEGF-stimulated tumor neoangiogenesis. In addition,
overexpression of VEGF receptors (Flt-1 and KDR) was ob-
served in tumor blood vessel cells compared to normal
gastrointestinal hyperplasia (110).

Interleukin-8 (IL-8) is another inducer of tumor neoan-
giogenesis (111,112). IL-8 directly increases endothelial cell
proliferation, survival, matrix metalloproteinases (MMP)
expression, and tumor-associated angiogenesis (113). Thrombin-mediated activation of PAR-1 causes the release
of IL-8 from human respiratory epithelial cells (69). How-
ever, an indirect mode of action of thrombin on IL-8 expression
in tumor tissue has also been demonstrated (114). Thrombin-
catalyzed fibrin formation has been detected in oral squamous
cell carcinoma in vivo. Although the application of thrombin
had no effect on IL-8 expression in these cells, incubation
with fibrin caused a dose- and time-dependent stimulation of
IL-8 expression. In contrast, a similar stimulation of IL-8 by
fibrin was not observed in non-malignant oral cells.

An important family of proteins involved in vascular de-
velopment and stability has been identified and designated
angiopoietins (115). It has been reported that thrombin en-
hances the expression and secretion of angiopoietin-2 from
HUVEC. Hirudin pretreatment inhibits this cellular re-
response showing thrombin specificity (116). There are still
other metastasis-associated molecules which are regulated
by thrombin. Chicken chemotactic and angiogenic factor
cCAF) being the product of the 9E3/CEF4 gene, is a proan-
giogenic protein. cCAF is highly expressed in Rous sarcoma
virus-induced tumor where thrombin acts as the most potent
natural stimulator of 9E3/CEF4 gene (117). Platelet-activat-
ing factor (PAF) is a phospholipid mediator of inflammation
and is correlated with microvessel density. PAF has been de-
tected in breast cancer tissue and thrombin stimulates PAF
production in human breast cancer cell lines. Animal experi-
ments demonstrated that PAF receptor antagonists abolished
the formation of new tumor vessels in SCID mice injected with
MDA-MB231 breast cancer cells (118).

It has long been established that a number of growth fac-
tors participate in the regulation of tumor-associated an-
giogenesis. However, the individual functional importance
of any of these factors related to the progression of a given
malignancy remains unknown. Within the tumor microenvi-
ronment, thrombin-mediated activation of PAR-1 expressed
by tumor cells and/or surrounding stromal cells might play a
critical role in triggering and sustaining new vessel forma-
tion. Thrombin and the proangiogenic VEGF may emerge a
positive feedback system to promote tumor-associated an-
giogenesis: VEGF induces local thrombin generation within
tumor tissues, and thrombin enhances VEGF secretion thus
further contributing to the vascularization of malignancy.

**Tumor cell adhesion, invasion, and metastasis**

Tumor cell adhesion to platelets, vascular endothelium, and
extracellular matrix (ECM) is critical to tumor invasion and
metastasis. Thrombin regulates the expression of various ad-
hesion molecules in tumor cells and modulates their adhesion
to endothelial cells, platelets, and ECM components includ-
ing fibronectin, laminin, collagen and vWF (52,73,119,120).
Thrombin enhances the adhesion of melanoma cells transfec-
ted with full-length PAR-1 sense cDNA to fibronectin
compared to mock-transfected cells demonstrating the im-
portance of PAR-1 in thrombin-induced cell adhesion (89).

Thrombin/PAR-1-mediated cell adhesion involves the
up-regulation of a variety of adhesion molecules including integrin $\alpha_v\beta_3$, integrin $\alpha IIb-IIIa$ receptor, ICAM-1, VCAM-1, and P-selectin (119,121,122). Both thrombin and PAR-1 activation peptide induce the expression of ICAM-1 and VCAM-1 in endothelial cells (121). Thrombin-induced tumor cell adhesion to platelets is abolished by inhibitors of platelet integrin $\alpha IIb-IIIa$ receptor occupancy and by polyclonal antibodies directed against the integrin $\alpha IIb-IIIa$ ligands fibronectin and vWF (119). Monoclonal antibodies against the integrin $\alpha IIb-IIIa$ and P-selectin also inhibit the effect of TRAP-activated platelets on enhancing tumor cell adhesion to endothelial cells (123).

Invasion is the passage of tumor cells through the basement membranes and their migration into the surrounding tissues, and represents a critical step in the cascade of local cancer progression and distant metastasis (124). Basement membranes are comprised of multiple components including collagens, laminins, and proteoglycans. They form tight barriers between the different tissues but may become permeable during tissue development, repair, inflammation, and tumor invasion/metastasis. The process of metastasis of a given tumor initially requires cellular detachment from the primary tumor location and degradation of the basement membrane and interstitial connective tissue. At a later stage, enhanced adhesion to stromal components is a prerequisite to form metastases at distant sites.

One well-characterized example of extracellular proteases known to be involved in invasion is the urokinase-type plasminogen activator (uPA) which converts plasminogen to plasmin. Plasmin is a broad-spectrum protease that degrades extracellular matrix components. In PC-3 prostate cancer cells, thrombin generates a dose- and time-dependent increase in uPA expression and secretion. This effect is mediated by the proteolytic activation of thrombin receptor as PAR-1 activation peptide also causes increased uPA synthesis (125). Moreover, thrombin and TRAP activate matrix metalloproteinase 2 which plays a key role in the degradation of the extracellular matrix during tumor invasion (52). In addition, thrombin enhances the heparanase-induced degradation of heparan sulfate proteoglycans in tumor stroma (126,127).

Experimental evidence suggests that the levels of thrombin and its receptor are implicated in tumor invasion and metastasis (128). Metastatic human breast carcinoma cell lines and tissues express high levels of PAR-1. The introduction of PAR-1 antisense cDNA significantly inhibits the invasive potential of metastatic cells in vitro (61). Animal experiments have shown that both preinjection of thrombin or PAR-1 activation peptide into mice and pretreatment of tumor cells with thrombin significantly enhance pulmonary metastasis. It was also noted that a five-fold increase of metastasis occurred when using tumor cells transfected with PAR-1 cDNA compared to mock-transfected cells (119). In addition, inhibition studies demonstrated that blocking of the coagulation cascade at the level of thrombin diminishes hematogenous metastasis of the human melanoma cell line M24met in SCID mice confirming the importance of thrombin during tumor metastasis (67).

Tumor cell-induced platelet aggregation is considered an important step in hematogenous metastasis. Microvesicles from the cell-free supernatant of U87MG human glioblastoma cells result in platelet aggregation and coagulation with a thrombin-dependent pattern (129). A panel of human pancreatic cancer cell lines induces platelet aggregation, which can be inhibited by the addition of hirudin making the involvement of thrombin likely (130).

**WE ARE (ALMOST) THERE: CONCLUSIONS AND OPEN QUESTIONS**

Thrombin can be generated in malignant tissues with the capability of exerting a multitude of modulating effects on tumor cells and their stromal components. It is beyond any doubt that the prototypic thrombin receptor PAR-1 expressed by cancer cells mediates most if not all of the cellular effects of thrombin. Being involved in a given tumor phenotype including growth, angiogenesis, adhesion, invasion and metastasis, thrombin and its cellular receptor(s) significantly contribute to overall tumor biology (Fig. 2). However, due to the complexity and diversity of cancer, the molecular mechanisms of the pathogenesis of malignancy and the particular impact of the coagulation protease thrombin have not yet been worked out in detail even for a small portion of human cancers. Therefore, ongoing and future basic, translational and clinical research is expected to answer a number of open questions.

First and foremost, more and convincing *in situ* data need to prove the concept that malignant tissues harbor the molecular machinery to generate amounts of proteases (in particular thrombin) known from *in vitro* studies to have an impact on malignant cells via PAR activation. Once in place, PAR activation by thrombin generates a cleaved peptide (TR1-41) whose functional implications are largely unknown. Considering tumor biology, we need to understand if these peptides are a major culprit or just innocent bystanders.

Another set of unanswered questions relates to the molecular interplay between the major thrombin receptor PAR-1 and other thrombin receptors and binding sites including PAR-3, PAR-4, thrombomodulin, and GPIb$\alpha$. Approaches allowing to manipulate gene expression such as transgenic, antisense, and gene targeting tools including tissue-specific and inducible gene-knockout, and small interfering RNA (siRNA) techniques are expected to provide molecular insight (131,132).

Finally, newly designed PAR agonists and antagonists will be tested to selectively modulate tumor growth and metas-
tasis (44,133,134). New approaches are continuously being used with the attempt to target the cellular thrombin-PAR interface. A detailed understanding of the association between thrombin receptor and cancer may give rise to new therapeutic approaches consisting of the suppression of thrombin receptor(s) in cancer.

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