

Tumor Angiogenesis: Thrombin and Metalloproteinases in Focus

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INTRODUCTION

The role of thrombin (activated factor II) in blood coagulation has been characterized in detail, from the cleavage of fibrinogen to the multiple effects on platelets and endothelial cells, under both normal and pathological conditions. However, relatively little is known about the effects of thrombin on endothelial cells during the process of tumor angiogenesis. This will be discussed in the present report, together with the relationship between thrombin and metalloproteinases that are essential in tumor angiogenesis: membrane-type 1 metalloproteinase-1 (MT1-MMP), activating progelatinase-A to gelatinase-A (or MMP-2). These are examples of a group of 20 metalloproteinases so far studied which are involved in tumor angiogenesis and matrix invasion, causing disruption of the vascular basement membrane. Thrombin might be involved with these proteases by either direct or indirect activation, as it activates progelatinase-A independent of MT1-MMP. The picture is far from clear: is the latter an alternative or a redundant pathway for MMP-2 activation?

PROTEASES IN THE EARLY STEPS OF TUMOR ANGIOGENESIS

Solid tumors often feature a defective vascular organization leading the neoplastic tissue to hypoxia (Brown and

Giaccia, 1998; Guillemin and Krasnow, 1997; Blancher and Harris, 1998). Hypoxia, in turn, stimulates the first steps of tumor angiogenesis; hypoxia also blocks the degradation of a transcription factor that controls a number of cell functions, with the final result of allowing neoplastic cell survival at low oxygen tension. This factor, called HIF-1 (hypoxia inducible factor-1), is a dimer composed by two subunits termed α and β . HIF-1 is constitutively synthesized by the cell under normal conditions: it contains an oxygen-dependent degradation domain that is sensitive to ubiquitin-proteasome degradation. Thus, at normal oxygen tension, HIF-1 is continuously degraded and thereby kept inactive (Srinivas *et al.*, 1998). When oxygen tension is reduced, as in solid tumor hypoxia, HIF-1 degradation no longer occurs, and its dimeric form enters the nucleus. There HIF-1, which is a gene transactivator, induces the expression of a number of genes, including those encoding for the tumor suppressor p53, erythropoietin, regulators of glycolysis, cyclooxygenase-2, and inducible nitric oxide synthase. Furthermore, HIF-1 induces the overexpression of the gene coding for the main angiogenic vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) (Blancher and Harris, 1998; Chiarugi *et al.*, 1999). VEGF binds two specific receptors on the endothelial cell surface, termed FLT-1 and KDR. These are platelet-derived growth factor (PDGF)-like transmembrane receptors with intrinsic tyrosine kinase activity (for review see Korpelainen and Alitalo, 1998; Dvorak *et al.*, 1995). Binding of VEGF to these receptors evokes multiple

effects on endothelial cells, including stimulation of DNA synthesis and proliferation, expression of the so-called “tissue factor,” activation of thrombin through the clotting alternative pathway, increase of vascular permeability, and protein extravasation.

Extravasated fibrinogen, when cleaved by thrombin, forms a barrier of fibrin; accordingly, it was observed several years ago that the extracellular matrix of neoplastic tissue is abnormally rich in fibrin. However, this barrier must be remodeled for hypoxia-induced tumor angiogenesis to proceed. It seems that VEGF and thrombin stimulate endothelial cell movement and rearrangement so that the cells form tunnel-like structures inside the perivascular matrix. The formation of these tunnel-like structures, resembling rudimentary capillaries with a fibrin scaffold, is called tubulogenesis (Pepper *et al.*, 1996; Werb, 1997). Obviously, endothelial cells must also overcome the vascular basement membrane in order to enter the extracellular matrix; most often, the basement membrane is hydrolyzed by tumor-associated metalloproteinases, which are mainly represented by gelatinase-A and gelatinase-B (MMP-9). Therefore, tubulogenesis occurs concomitantly with the partial destruction of basement membrane. Accordingly, metalloproteinases of the endothelial cell membrane, such as MT1-MMP and gelatinase-A, are expressed very early in the process of tumor angiogenesis and appear essential in angiogenesis. Consistent with this hypothesis, metalloproteinase inhibitors so far gave the best results in clinical trials as anti-angiogenic compounds for cancer treatment. The fumagillol derivative TNP-470 was shown to be particularly effective (Kudelka *et al.*, 1998).

Beside VEGF, other factors are critical during the first steps of tumor angiogenesis, such as the inducible forms of nitric oxide synthase and of cyclooxygenase-2, both under the control of the *trans*-acting transcriptional factor termed NF κ B (Xie *et al.*, 1994). These enzymes are to be considered VEGF cofactors in the process of tumor angiogenesis. Consistently, when their functions are blocked by specific inhibitors, the entire process of neoangiogenesis is also impaired (Tsujii *et al.*, 1998).

The plasminogen activator–urokinase pathway also appears to be involved in tumor angiogenesis (Carmeliet *et al.*, 1998; Fibbi *et al.*, 1998). Whatever its role, it should be noted that knockout mice for the plasminogen gene are still able to manifest normal angiogenesis (Hiraoka *et al.*, 1998), suggesting that the plasminogen activator–urokinase pathway represents redundant control over angiogenesis or, alternatively, that the pathway controls angiogenesis independently of plasminogen, acting on other substrates.

Forming tunnels necessary for neoangiogenesis is not a simple task for endothelial cells. They must move deeply

inside the fibrin-rich extracellular matrix and pave their way far from the vessel where they are originating. To do so, they produce and activate fibrinolytic enzymes in a polar manner. Thus, areas of fibrinolysis appear only at one pole of the involved endothelial cell and tubulogenesis can proceed in a definite direction without randomly digesting the tumor extracellular matrix (Pepper *et al.*, 1996; Werb, 1997). Neoplastic tissue remains, indeed, rich in fibrin, which represents a provisional scaffold for vessel neof ormation. Accordingly, tumor capillaries are coated with a fibrin layer that will be further remodeled by fibroblasts, resulting in the formation of a complete perivascular matrix that includes new basal membranes. However, these “new” capillaries, at first, are not fully functional and appear to be much more permeable than their normal counterparts, as the stimulation of rapid growth and elongation of these neof ormed vessels by VEGF and thrombin occurs more rapidly than the formation of working basement membranes (Dvorak *et al.*, 1995).

Two metalloproteinases expressed by endothelial cells are essential for this process, namely, MT1-MMP and gelatinase-A (also called MMP-2). Gelatinase-A is produced from progelatinase-A on the endothelial cell surface and the mechanism responsible for its activation involves the activity of the transmembrane MT1-MMP (Zucker *et al.*, 1995; Strongin *et al.*, 1995). Normally, a metalloproteinase inhibitor called TIMP-2 binds to the endothelial cell membrane and forms stoichiometric complexes with MT1-MMP, thus preventing its activation. Progelatinase-A (a 73-kDa molecule) also binds to the complex, thus forming a trimer; this determines, on the basis of stoichiometric relationship within the complex, a limited proteolysis of progelatinase-A, resulting in the formation of a zinc-binding active 62-kDa molecule (Zucker *et al.*, 1998). The same authors reported that treatment of endothelial cells with thrombin increases the expression of MT1-MMP, further stressing the tight connections between thrombin, MT1-MMP, and progelatinase-A in activating the endothelial cell-directed proteolysis of tumor extracellular matrix and tubulogenesis. Furthermore, thrombin-induced activation of progelatinase-A, although indirect, provides a link between blood coagulation and extracellular matrix degradation. This is particularly true at the level of the vascular basement membrane, where gelatinase-A is also active on collagen IV and laminin, i.e., the main components of the membrane itself. Along the same line, it was also hypothesized that thrombin might directly associate progelatinase-A complex activation at the level of the endothelial cell surface.

THROMBIN AND METALLOPROTEINASES COOPERATE IN TUMOR ANGIOGENESIS

The extrinsic pathway of coagulation starts with the activation of factor VII by the tissue factor; this leads to factor X activation and a complex is formed at the level of the cell membrane, including activated factor V, which is responsible for the conversion of prothrombin to active thrombin. Active thrombin has a fundamental role in blood coagulation; its activity is counterbalanced by the specific endogenous inhibitor antithrombin-III, whose action on thrombin is potentiated by heparin. Interestingly, antithrombin-III is proteolytically destroyed by stromelysin (another important metalloproteinase) that becomes active when the angiogenesis cascade is triggered (Furie and Furie, 1995).

It has been recognized for many years that thrombin, in addition to its role in blood coagulation, exerts other actions. Thrombin, indeed, beside being a serine protease, also works as a full title agonist for receptors located on the surface of many cell types. In endothelial cells, just as in platelets, it binds to and cleaves a seven-membrane-spanning domain receptor. This receptor couples to a GTP-binding protein and, when activated, triggers an intracellular signaling cascade via the formation of second messengers, such as diacylglycerol and inositol phosphates. This cascade leads to multiple effects: the most notable are DNA synthesis and mitosis, cell retraction with rupture of integrin bridges between cells, abnormal vascular permeability, and further production of tissue factor that, in turn, reinforces the effect of VEGF. Thus, because of these effects on endothelial cells, thrombin can also be considered a direct angiogenetic factor. In fact, this role was easily demonstrated. *In vitro* thrombin stimulates endothelial cell migration and favors the rearrangement and alignment of endothelial cells in Matrigel (an artificial substrate made of collagen and laminin). *In vivo*, thrombin induces angiogenesis in chicken chorionallantoic membrane *via* a mechanism independent of its action on blood coagulation (Haralabopoulos *et al.*, 1997).

Recently, a provocative report by Nguyen *et al.* (1999), claims that thrombin rapidly and efficiently activates gelatinase-A in human microvascular endothelial cells, *via* a mechanism independent of MT1-MPP. Although this finding is very relevant, it is not clear whether this effect of thrombin represents the main operating pattern in angiogenesis or just an alternative pathway cooperating with the well-known MT1-MMP/progelatinase-A activation (Sato *et al.*, 1999; Zucker *et al.*, 1995; Strongin *et al.*, 1995; Hiraoka *et al.*, 1998). Whatever the case, the direct activation of progelatinase-A, which is essential for the destruction of basement

membrane, by thrombin is a fact that deserves further investigation. If confirmed under other experimental conditions, this mechanism provides a direct link between thrombin (i.e., between coagulation) and early phases of angiogenesis, namely, induction of endothelial cell proliferation, extravasation of fibrinogen and formation of fibrin, and destruction of basement membrane by gelatinase-A, with the consequent migration of stimulated endothelial cells through the extracellular matrix.

The level of complexity of this field is very high as each player (i.e., each protease in this case) appears to perform more than one role. A recent article by Hiraoka *et al.* (1998) demonstrates that MT1-MMP, the most studied membrane-bound metalloproteinase, is also able to degrade cross-linked fibrin gels and is directly responsible for the invasive, as well as the tubulogenetic, behavior of tumor-associated endothelial cells. That MT1-MMP is relevant in angiogenesis can also be inferred by the fact that it is the only protease which is expressed at the surface of endothelial cells as a

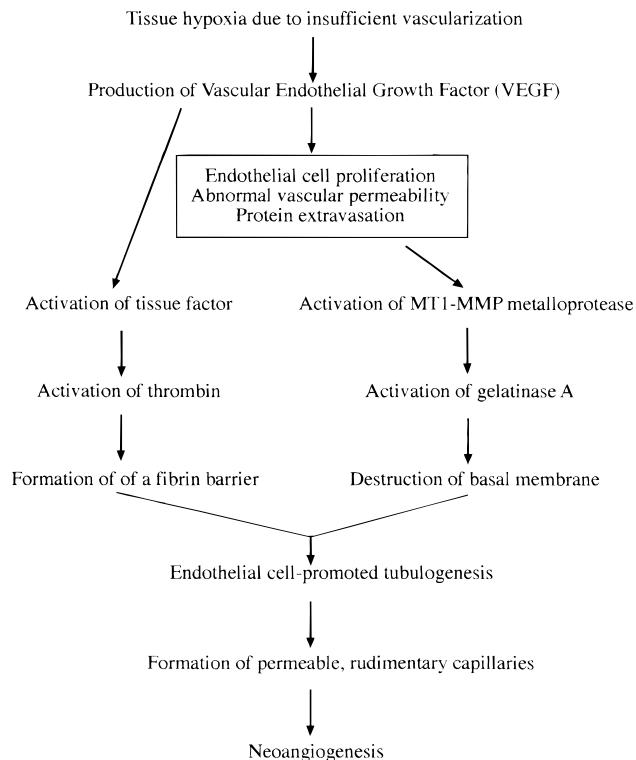


FIG. 1. Summary of the cooperative roles of thrombin and metalloproteinases in the early steps of tumor angiogenesis and the tight interconnections between blood coagulation and formation of new vessels.

zymogen, but as an active 63-kDa enzyme; the posttranslational activation of the enzyme is in fact performed by intracellular convertases of the trans-Golgi, belonging to the group of furin (Sato *et al.*, 1996). Which factors are responsible for induction of the expression of MT1-MMP remains to be determined, but there is no doubt that this is at the moment one of the most interesting aspects of the control of tumor angiogenesis. (Westermarck and Kahari, 1999).

In conclusion, we would like to stress the cooperative roles of thrombin and metalloproteinases in the early steps of tumor angiogenesis and the tight interconnections between blood coagulation and formation of new vessels phenomena summarized in the schema of Fig. 1.

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REFERENCES

- Blancher, C., and Harris, A. L. (1998). The molecular basis of the hypoxia response pathway: Tumor hypoxia as a therapy target. *Cancer Metastasis Rev.* **17**, 187–194.
- Brown, J. M., and Giaccia, A. J. (1998). The unique physiology of solid tumors: Opportunities (and problems) for cancer therapy. *Cancer Res.* **58**, 1408–1416.
- Carmeliet, P., Moons, L., Dewerchin, M., Rosenberg, S., Herbert, J. M., Lupu, F., and Collen, D. (1998). Receptor-independent role of urokinase-type plasminogen activator in pericellular plasmin and matrix metalloproteinase proteolysis during vascular wound healing in mice. *J. Cell Biol.* **140**, 233–245.
- Chiarugi, V., Magnelli, L., Chiarugi, A., and Gallo, O. (1999). Hypoxia induces pivotal tumor angiogenesis control factors including p53, vascular endothelial growth factor and the NF κ B-dependent inducible nitric oxide synthase and cyclooxygenase-2. *J. Cancer Res. Clin. Oncol.* **125**, 525–528.
- Dvorak, H. F., Brown, L. F., Detmar, M., and Dvorak, A. M. (1995). Vascular permeability factor: Vascular endothelial growth factor microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.* **146**, 1029–1039.
- Fibbi, G., Caldini, R., Chevanne, M., Pucci, M., Schiavone, N., Morbidelli, L., Parenti, A., Granger, H. J., Del Rosso, M., and Ziche, M. (1998). Urokinase-dependent angiogenesis *in vitro* and diacylglycerol production are blocked by antisense oligonucleotides against the urokinase receptor. *Lab. Invest.* **78**, 1109–1119.
- Furie, B., and Furie, B. C. (1995). Molecular basis of blood coagulation. In "Hematology: Basic Principles and Practice" (E. J. Hoffman, R. S. Benz, Jr., B. Shattil, B. Furie, L. Cholmen, and H. J. Choen, Eds.), 2nd ed., pp. 1566–1587. Churchill-Livingstone. New York.
- Guillemin, K., and Krasnow, M. A. (1977). The hypoxic response: Huffing and HIFing. *Cell* **89**, 9–12.
- Haralabopoulos, G. C., Grant, D. S., Kleinman, H. K., and Maragoudakis, M. E. (1997). Thrombin promotes endothelial cell alignment in Matrigel *in vitro* and angiogenesis *in vivo*. *Am. J. Physiol.* **42**, C239–245.
- Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J. (1998). Matrix metalloproteinase regulate neovascularization by acting as pericellular fibrinolysis. *Cell* **95**, 365–377.
- Korpelainen, E. I., and Alitalo, K. (1998). Signalling angiogenesis and lymphangiogenesis. *Curr. Opin. Cell. Biol.* **10**, 159–164.
- Kudelka, A. P., Verschraegen, C. F., and Loyer, E. (1998). Complete remission of metastatic cervical cancer with the angiogenesis inhibitor TNP-470. *N. Engl. J. Med.* **338**, 991–992.
- Pepper, M. S., Montesano, R., Mandriota, S. J., Orci, L., and Vassalli, J. D. (1996). Angiogenesis: A paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. *Enzyme Protein* **49**, 138–162.
- Sato, H., Kinoshita, T., Takino, T., Nakayama, K., and Seiki, M. (1996). Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinase (TIMP)-2. *FEBS Lett.* **393**, 101–104.
- Sato, T., Iwai, M., Sakai, T., Sato, H., Seiki, M., Mori, Y., and Ito, A. (1999). Enhancement of membrane-type 1-matrix metalloproteinase (MT1-MMP) production and of sequential activation of progelatinase-A on human squamous carcinoma cells co-cultured with human dermal fibroblasts. *Br. J. Cancer* **80**, 1137–1143.
- Srinivas, V., Zhu, X., Salceda, S., Nakamura, R., and Caro, J. (1998). Hypoxia inducible factor 1 α (HIF-1 α) is a non-heme iron protein. Implications for oxygen sensing. *J. Biol. Chem.* **273**, 18019–18022.
- Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995). Mechanism of cell surface activation of 72-kDa type IV collagenase. *J. Biol. Chem.* **270**, 5331–5338.
- Tsujii, M., Kawano, S., Tsuji, S., Sawaoka H., Hori, M., and DuBois R. M. (1998). Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **93**, 705–716.
- Werb, Z. (1997). ECM and cell surface proteolysis: Regulating cellular ecology. *Cell* **91**, 439–442.
- Westermarck, J., and Kahari, V. M. (1999). Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J.* **13**, 781–792.
- Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994). Role of transcription factor NF- κ B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**, 4705–4708.
- Zucker, S., Conner C., DiMassmo, B. J., Ende, H., Drews, M., Seiki, M., and Bahou, W. F. (1995). Thrombin induces the activation of progelatinase-A in vascular endothelial cells: Physiologic regulation of angiogenesis. *J. Biol. Chem.* **270**, 23730–23738.
- Zucker, S., Mirza, H., Conner, C. E., Lorenz, A. F., Drews, M. H., Bahou, W. F., and Jesty, J. (1988). Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: Conversion of prothrombin to thrombin results in progelatinase-A activation and cell proliferation. *Int. J. Cancer* **75**, 780–786.