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The International Journal of Biochemistry & Cell Biology 36 (2004) 1070–1078
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Medicine in focus

Thrombospondin modules and angiogenesis

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Received 10 December 2003; received in revised form 25 January 2004; accepted 26 January 2004

Abstract

Angiogenesis is a complex, multifactorial process that involves signals from endothelial cells and from the stoma. Extracellular matrix proteins participate in the modulation of growth factor response, contribute to the architecture of the vasculature and provide signals for the stabilization of mature capillary beds. The identification of the relevant extracellular matrix molecules and the characterization of their effects has been a central focus of research in vascular biology. Thrombospondin-1 is an extracellular glycoprotein first to be recognized as an inhibitor of angiogenesis more than a decade ago. Since then, much has been learned about its ability to regulate vascular growth in several angiogenesis models, functional domains have been identified, and mechanisms of action determined. This review summarizes current understanding on the effects of thrombospondin-1 and -2 during the process of angiogenesis. We will also extend our comments to ADAMTS1, a member of a relatively novel group of matrix metalloproteinases with thrombospondin repeats and shown to affect endothelial cell function and angiogenesis.

Keywords: ADAMTS; Blood vessels; Capillaries; Endothelial cells; Matrix metalloproteinases

1. Introduction

The term angiogenesis refers to the expansion of the vasculature from existing capillary beds by progressive invasion and sprouting of endothelial cells. This process contrasts that of vasculogenesis that relies on precursor cell differentiation (Carmeliet, 2000; Risau, 1997). During sprouting, the endothelial cell must leave the vessel wall and invade surrounding tissue. Many studies have shown that this process must coordinate not only cytoskeletal changes for motility, but also secretion of proteolytic enzymes to promote matrix degradation and invasion (Carmeliet, 2000; Folkman & D'Amore, 1996; Risau, 1997; Varner & Cheresh, 1996). During this initial phase, specific integrins play a critical role for the progress of angiogenesis. Binding to the extracellular matrix allows anchorage during migration and, in addition, supports survival to the invasive endothelial cells (Varner & Cheresh, 1996). Once lumen formation has occurred, a provisional basement membrane is secreted by the endothelial cells resulting in a primitive functional blood vessel. This basement membrane engages a more comprehensive set of integrins that provides differentiation signals, inhibits proliferation and facilitates cell–cell adhesion (Form, Pratt, & Madri, 1986). It is accepted that the integrity of this basement membrane is essential to the stability of

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1357-2725/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2004.01.025

capillaries (Folkman & D'Amore, 1996; Form et al., 1986). Many of the proteins that comprise the basement membrane, such as type IV collagen, tenascin, laminin and thrombospondin-1 (TSP1) modulate endothelial cell function affecting attachment, proliferation and survival (Kalluri, 2003). Interestingly, their effects might vary depending on whether they are presented in solution or solid-state and whether they are intact or cleaved (Kalluri, 2003). Nevertheless, once these molecules are organized within the context of a basement membrane, they offer stable morphogenic signals to the differentiated endothelium (Folkman & D'Amore, 1996).

As vascular maturation proceeds, vessels undergo remodeling into a more permanent vasculature. The remodeling phase includes selective vascular fusion, hierarchic growth (thick to thin vessels) and coordinated vessel regression (Dor et al., 2003).

As part of the normal vascular developmental program, the newly formed microvasculature is adjusted by supply and demand during the remodeling process. The final size and density of the microvasculature is not genetically programmed, but fine-tuned according to the physiological needs of specific organs (Dor et al., 2003). In fact, the vasculature retains the ability to expand when hypoxia or other signs of vascular insufficiency are sensed. For example, an increase in tissue mass in the adult (i.e. new muscle or fat) stimulates the new formation of microvessels to supply the growing tissue. The oxygen-sensing mechanisms triggered by tissue hypoxia stimulate VEGF-A mRNA stability, transcription and translation making this process highly responsive and efficient (Forsythe et al., 1996; Levy, Levy, Wegner, & Goldberg, 1995; Shweiki, Itin, Sofeer, & Keshet, 1992; Stein et al., 1998).

During pathological angiogenesis, the coordinated effort between growth factors, extracellular matrix proteins, proteases, and cell-adhesion molecules lacks organization and it is rather chaotic (Hanahan & Weinberg, 2000; Jain, 2003; McDonald & Choyke, 2003). Gradients of matrix molecules and growth factors are missing and the coordination between adhesion and proliferative signals (i.e. cell density) is lost. Consequently, although the basic process of angiogenesis is retained (that is, sprouting, lumen formation and stabilization) lack of coordinated regulation results in a tortuous, poorly organized and dysfunctional vascular supply. Yet, this vasculature is essential for tissue viability, as interfering with endothelial growth or survival signals results in tumor size reduction (Hanahan & Weinberg, 2000; Jain, 2003).

Because adhesion molecules, either membrane-bound or secreted, are so intrinsic to many of the events in angiogenesis, much effort has been placed in dissecting their specific effects on endothelial cells. A group of molecules that has received considerable attention has been TSP1 and 2. This review focuses on these and other endothelial modulatory proteins that contain the thrombospondin module (also known as TSR).

2. Thrombospondin-1 and -2 and their contribution to the formation of vascular beds

The thrombospondin family comprises a group of five members characterized by a specific modular organization (Adams, 2001; Lawler, 2000). Among these five members, TSP1 and TSP2 are the most similar in structure and amino-acid identity. Their structure includes a globular amino-terminal motif, followed by a pro-collagen homology region, three so-called "thombospondin" repeats (also known as TSR or properdin-like domains), three EGF or type 2 repeats, five calcium-binding or type 3 repeats and a globular carboxy-terminal end. Although highly similar in primary sequence, TSP1 and 2 differ significantly in expression patterns both during development (Iruela-Arispe, Liska, Sage, & Bornstein, 1993) and in the adult (Adams, 2001; Bornstein, 1992; Iruela-Arispe, Porter, Bornstein, & Sage, 1996; Kyriakides, Zhu, Yang, & Bornstein, 1998). Thus, in general terms, TSP1 has been shown to be more epithelial/endothelial, in contrast to the "mesenchymal" nature of TSP2. The regulation of both promoters is also notably different. TSP1 rapidly induced by injury and inflammatory mediators, in contrast, expression of TSP2 does not seem to be affected by the same cohort of inflammatory cytokines (Bornstein, 1992; Bornstein, Armstrong, Hankenson, & Kyriakides, 2000; Chen, Herndon, & Lawler, 2000). From all five family members, only TSP1 and 2 have been shown to influence endothelial cell behavior and angiogenesis (Armstrong & Bornstein, 2003).

The effects of TSP1 have been well documented in several in vivo and in vitro models (Armstrong &

Bornstein, 2003; Dawson et al., 1997; Iruela-Arispe, Lombardo, Krutzsch, Lawler, & Roberts, 1999; Taraboletti et al., 1997; Tolsma et al., 1993). In vivo, TSP1 suppresses FGF-2-mediated angiogenesis in the cornea pocket assay and inhibits growth of blood vessels in the CAM assay (Armstrong & Bornstein, 2003; Iruela-Arispe et al., 1999). Experiments in vitro performed by many laboratories have shown that the protein modulates adhesion, suppresses migration, inhibits proliferation, and signals apoptosis of endothelial cells via activation of CD36 (reviewed in Armstrong & Bornstein, 2003; Volpert, 2000). In support of this wealth of data, the effects of genetic manipulations that result in TSP1 overexpression using tissue-specific promoters strongly support the participation of this protein in the regulation of vascular growth and vessel diameter. In particular, two transgenic studies using the K14 promoter to drive TSP1 in the skin (Streit et al., 2000) and, the MMTV-promoter, to target TSP1 expression to the mammary epithelium (Rodriguez-Manzaneque et al., 2001), support a role for this protein in the regulation of vascular morphogenesis in whole animal settings.

The generation of TSP1 null mice provided evidence that TSP1 is required for the regulation of epithelial growth in the lung (Lawler et al., 1998). In the absence of the protein, animals develop multifocal pneumonia and are more prone to inflammatory events. In addition, the animals display hyperplasia of pancreatic islands and show delayed wound healing (Lawler et al., 1998). This homeostatic function for TSP1 in epithelia was previously unknown. The combined conclusion from many transgenic studies is that TSP1 acts as a pleiotropic growth regulator with effects that impact morphogenesis and homeostasis of both vessels and epithelial cells.

Mechanistic insights for the effects of systemic lack of TSP1 appeared linked, at least in part, to its role as an activator of TGF-beta (Murphy-Ullrich & Poczatek, 2000). Certain pathologies displayed by the TSP1 null animal were overcome by treatment with TSP1 peptides that mediate activation of TGF-beta (Crawford et al., 1998). These results underscore the biological relevance of TSP1 in the activation of TGF-beta in vivo, but also bring to light other functions of this molecule that are independent of TGF-beta activation. Particularly interesting is the observation that TSP1 null animals show a reduced litter number and increased blood vessel profiles in the retina (Lawler et al., 1998; Wang, Wu, Sorenson, Lawler, & Sheibani, 2003). These defects are possibly related to the role of TSP1 in the modulation of angiogenesis that are independent of TGF-β. The effects might parallel to the mechanism of action of TSP2 which do not include activation of TGF-β.

Lessons from the TSP1 knock-out mouse have provided biological support for an important facet in understanding TSP function, which is its interaction with a multitude of matrix proteins. In fact, by nature of its interaction with proteoglycans, extracellular matrix molecules and growth factors, TSP1 directs the assembly of multiprotein complexes that modulate cellular phenotype (Adams, 2001). TSP1 also directly affects the activity of plasmin (Anonick, Yoo, Webb, & Gonias, 1993; Hogg, Stenflo, & Mosher, 1992), and can, therefore, indirectly work through modulation of this enzyme. Understanding the biological significance of these interactions and multitude of cell signaling pathways has become a challenge for investigators in this field.

Clues obtained from the overexpression of TSP1 have directed our efforts to further explore the role of TSP1 as a modulator of enzymatic activity; a previously reported function of this protein. Regulation of enzymatic activity by TSP1 is a well documented effect attributed to this protein. TSP1 has been shown to be slow (rate constant approximately $6.3 \times 10^3\,M^{-1}\,s^{-1}$), tight-binding (kDa 10^{-9} M) inhibitor or plasmin as determined by loss of amidolytic activity, loss of ability to degrade fibrinogen, and decrease lysis zones in fibrin plate assays (Anonick et al., 1993; Hogg et al., 1992; Mosher, Misenheimer, Stenflo, & Hogg, 1992; Silverstein, Nachman, Pannell, Gurewich, & Harpel, 1990). Stoichiometric titrations indicate that approximately 1 mol of plasmin interacts with 1 mol of TSP1. Plasmin in a complex with streptokinase or bound to epsilon-aminocaproic acid is protected from inhibition by TSP1, thereby implicating the lysine-binding kringe domains of plasmin in the inhibition process (DePoli, Bacon-Baguley, Kendra-Franczak, Cederholm, & Walz, 1989). TSP1 also inhibits urokinase plasminogen activator, but more slowly than plasmin, stimulates the amidolytic activity of tPA and has no effect on the amidolytic activity of alpha-thrombin or factor Xa (Silverstein et al., 1990). In addition, TSP1 has been shown to

be a competitive inhibitor of neutrophil elastase. In competitive binding assays, neutroplil elastase bound to TSP1 with a dissociation constant of 17 nM. Although TSP1 is cleaved by neutrophil elastase, the site(s) of the limited cleavage are independent of the competitive inhibition of elastase activity by TSP1. TSP1 can protect fibronectin from cleavage by neutrophil elastase and thus influence endothelial cell behavior indirectly via this function (Hogg, Owensby, Mosher, Misenheimer, & Chesterman, 1993; Hogg, Jimenez, & Chesterman, 1994).

We found that TSP1 can inhibit activation of pro-MMP-9 by MMP-3 and therefore restrict the availability of active MMP-9 (Rodriguez-Manzaneque et al., 2001). These effects impact directly the process of angiogenesis. MMP-9 has been shown to regulate release of circulating endothelial cell progenitors via proteolytic cleavage of c-kit (Heissig et al., 2002). Based on these data, it is likely that TSP1 also affects endothelial cell migration and vascular morphogenesis, at least in part, by its ability to suppress MMP-9 processing. This blockade likely limits the availability of active protease with consequences to extracellular matrix remodeling and endothelial cell migration.

A role for TSPs in the regulation of MMP function has received support from the work of several groups that independently demonstrated different facets of functional interplay between MMPs and TSPs. Using the properdin/type 1 repeats of TSP1 in a yeast-two-hybrid screen, both MMP-2 and MMP-9 were found to bind to the baits, suggesting that, in fact, these proteins might interact (Bein & Simons, 2000). Fibroblasts from TSP2 knock-out animals displayed adhesion/attachment defects, as these cells do not attach well and are removed from culture dishes very easily (Yang, Kyriakides, & Bornstein, 2000). In the case of TSP-2 null fibroblasts, this phenomenon has been attributed to an excess of MMP-2, a finding confirmed in the dermis of the TSP2 null mouse (Kyriakides, Zhu, Smith, & Bornstein, 1998; Yang, Strickland, & Bornstein, 2001). Finally, increase of pro-MMP-9 upon addition of TSP1 was also reported in cultures of cytotrophoblasts. The authors also noted that the presence of TSP1 modulated responses in invasion and motility of these cells (Zhou, Frazier, Lawler, Damsky, & Fisher, 2000). Interestingly high levels of TSP1 have also been correlated with high levels of MMP-9, however the ratio of pro versus active MMP-9 were not evaluated in that study (Qian, Wang, Rothman, Nicosia, & Tuszynski, 1997). Consequently it remains to be determined whether soluble TSP1 can also suppress MMP-9 activation in this system.

Modulation of MMP activity by TSPs can likely explain the effects of this protein on the inhibition of endothelial cell migration and angiogenesis. Like many matrix proteins, TSP1 and 2 appear to function "contextually" as adapters and modulators of cell-matrix interactions, a concept that contributed to its "matricellular" classification (Bornstein, 2001).

3. Thrombospondin in anti-angiogenic therapy

Overexpression of TSP1 and TSP2 in several tumor cell lines has been associated with reduced tumor burden of xenografts due to a decrease in vascular density in comparison to control tumors (reviewed in Armstrong & Bornstein, 2003). Furthermore, transgenic overexpression studies in which the effect of absence or excess of TSP1 was evaluated in tumors that arise "spontaneously" in the mammary gland support the earlier xenograft results. Tumor-prone MMTV-cneu transgenic animals crossed with TSP1 nulls and with MMTV-TSP1 overexpressing mice were used to ascertain the contribution of endogenous levels of TSP1 in tumor growth (Rodriguez-Manzaneque et al., 2001). The c-neu transgenic animal has been extensively characterized as an endogenous tumor model in which stochastic appearance of mammary tumors which occurs due to expression of constitutively active erb2 (c-neu) is driven by the MMTV promoter. Consistently with previous studies, excess of TSP1 was associated with delayed incidence and reduction in tumor burden in comparison to animals with no TSP1 protein. Interestingly, tumors of animals with no TSP1 had large dilated vessels, in marked contrast with tumors from overexpressing animals that showed a small lumen (Rodriguez-Manzaneque et al., 2001).

Pertinent to a therapeutic approach and consistent with the findings above, systemic treatment of tumor-bearing mice with TSP1 or peptides derived from the TSP1 TSR repeats are capable to inhibit tumor growth (Guo, Krutzsch, Inman, & Roberts, 1997).

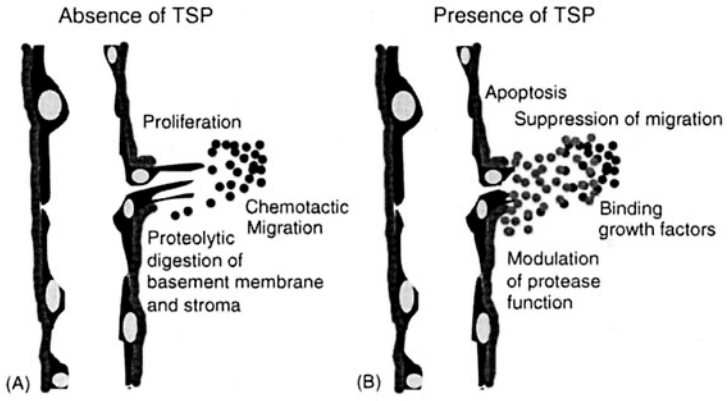

Fig. 1. Schematic representation of the multiple effects of TSPs on endothelial cells. (A) Breakdown of vascular basement membranes (in red lining the blood vessels) in response to a gradient of growth factors (blue dots) promotes directional growth of sprouting vessels. (B) Higher levels of TSP1 and/or TSP2 (green dots) within the stroma may attenuate the sprouting response by several mechanisms, including activation of TGF-beta, initiation of apoptosis, binding and sequestration of growth factors and suppression of extracellular proteases.

In conclusion, TSP1 and TSP2 provide multiple signals to the endothelium (Fig. 1). In mature/quiescent vascular beds, presence of TSP1 in the basement membrane is likely involved in the provision of stable signals. Experimental support for a role of TSP in the stability of vessels was initially indicated by studies performed in transformed bEnd cells. Introduction of TSP1 in these cells reverted the transformed phenotype to a more "normal" endothelial phenotype (Sheibani & Frazier, 1995). During angiogenesis, however, excess of basement membrane-free TSP1 suppresses migration, mediates apoptosis via activation of CD36, regulates activation of TGF-beta and modulates activity of plasmin and MMPs. Combined these effects result in the suppression of capillary sprouting and negative modulation of the angiogenic response.

The identification of TSR modules in other proteins has prompted the obvious question as to their potential ability to regulate angiogenesis. ADAMTS1 is a protease with three TSP repeats. Initially cloned from a cachexic colon adenocarcinoma (Kuno, Kanada, Nakashima, Ichimura, & Matsushima, 1997), it was later shown to display angioinhibitory activities (Vazquez et al., 1999).

4. ADAMTS family of proteases

The ADAMTS family comprises a group of zinc-dependent metalloproteinases that play important roles in a variety of normal and pathological conditions. Currently there are nineteen members that display an identical domain organization and includes, a signal sequence, propeptide, metalloproteinase domain, disintegrin-like domain, one thrombospondin (TSP) repeat, a cysteine-rich region followed by a second group of TSP motifs. The presence of disintegrin and metalloproteinase domain is the signature of the ADAM family of proteases (A disintegrin and metalloproteinase domain). However, unlike most ADAMs, all 19 members of the ADAMTS family are secreted and contain from 1 to 14 TSP repeats in the carboxy-terminus. Substrates for some of these proteases has been identified, but most aspects of the biology are, not surprisingly, lagging behind considering that most of these proteases were cloned within the last 4 years.

Functional analysis of ADAMTS1 (also known as METH1) and ADAMTS8 (also known as METH2) demonstrated their ability to inhibit angiogenesis when used in the cornea pocket and CAM assays. In addition, ADAMTS1 was able to suppress endothelial cell proliferation in vitro (Vazquez et al., 1999). The results were interesting particularly in light of the fact that ADAMTS proteins have TSP motifs, previously validated anti-angiogenic modules. Whether the physiological function of ADAMTS1 is to regulate angiogenesis in vivo is yet to be determined, however, it has been demonstrated in a number of in vivo and in vitro assays that exogenous addition of ADAMTS1

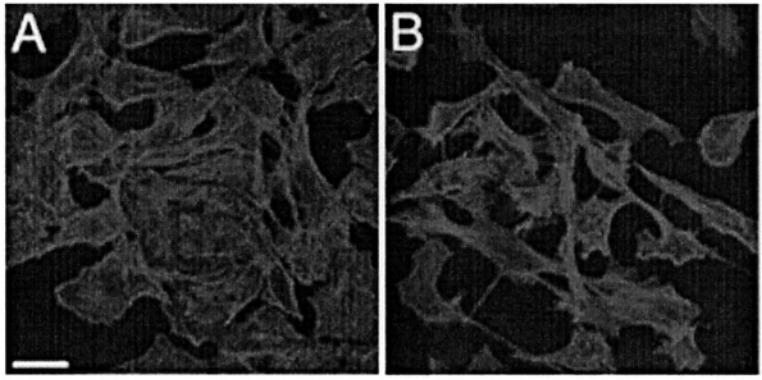

Fig. 2. Effects of ADAMTS1 in the actin cytoskeleton. Bovine aortic endothelial cells at 80% confluency were exposed to vehicle (A) or purified ADAMTS1 (200 ng/ml) for 4 h (B). Cultures were subsequently washed, fixed in 4% paraformadehyde, and the effects on actin cytoskeleton were evaluated by phalloidin staining: bar, 10 μm.

has a significant ability to block neovascularization (Vazquez et al., 1999). In parallel to these findings, inactivation of the gene by homologous recombination in mice resulted in decreased growth, renal anomalies, partial to complete obstruction of the ureteropelvic junction and alterations in adipose tissue and adrenal medullary architecture (Shindo et al., 2000). Both males and females died between 2 and 6 months probably due to renal abnormalities. In addition, female mice are infertile due to ovarian and uterine pathologies (Shindo et al., 2000). Similarly to TSPs, these data indicate that ADAMTS1 is likely to participate in an array of physiological and pathological conditions.

To gain further insights on the effects of ADAMTS1 in endothelial function, we have evaluated the effects of this protease on growth factor-mediated signaling events (Luque, Carpizo, & Iruela-Arispe, 2003). Interestingly, ADAMTS1 is capable of suppressing VEGFR2 phosphorylation via binding and sequestration of VEGF. This binding occurs via the carboxy-terminal TSP motifs (Luque et al., 2003). Interestingly, ADAMTS1 is processed in this region to release the last two TSP domains. The cleavage event can be blocked by MMP inhibitors, but not by other protease inhibitors. The physiological MMP or MMPs responsible for this processing are not known, however in vitro, several MMPs, including MMP-2; MMP-8; and MMP-15 can mediate this cleavage (Rodriguez-Manzaneque, Milchanowski, Dufour, Leduc, & Iruela-Arispe, 2000). Therefore, ADAMTS1 could potentially function as an anti-angiogenic molecule through the release of TSP repeats similarly to other proteins that harbor anti-angiogenic potential such as collagen XVIII and plasminogen (O'Reilly et al., 1994, 1997).

In addition to the effect of the TSP repeats, the catalytic activity of ADAMTS1 alters endothelial cell adhesion and migration. Fig. 2 illustrates the effect of ADAMTS1 in the actin cytoskeleton, a likely mediator of the effects in adhesion and migration. Exposure of either epithelial cells or endothelial cells to the protease results in stress fiber formation and changes in cell shape. This affects focal adhesion distribution and migration. The effects in cytoskeleton are not detected is a catalytically inactive form is used (data not shown).

Clearly, a detail profile of the substrates for this protease is needed to fully understand its effects on cellular processes. It has been demonstrated that ADAMTS1 is able to cleave the proteoglycans aggrecan and versican (Kuno et al., 2000; Sandy et al., 2001). Interestingly, cleavage of versican by ADAMTS1 appears essential to its effects on ovulation (Russell, Doyle, Ochsner, Sandy, & Richards, 2003). Cell surface biotinylation studies have shown that ADAMTS1 sheds proteins from the surface of endothelial cells (D. Carpizo and M.L. Iruela-Arispe, submitted). Future studies directed towards the identification of the spectrum of ADAMTS1 substrates and their function in the endothelium will expand our understanding of the

mechanism by which this protease suppresses vascular growth.

Acknowledgements

Experimental, unpublished aspects of this work were supported by a grant from the NIH (CA-65624). Alfonso Luque is a fellow of the Department of Defense (DAMD 17-02-1-0329). Nathan Lee holds a fellowship from the AHA (#0315010Y).

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