Structural Basis for the Functions of Endogenous Angiogenesis Inhibitors

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Tipping the angiogenic balance between pro- and antiangiogenic stimuli to favor vasculature induction and enhanced angiogenesis is a key event in the growth and progression of tumors. Recently, we demonstrated that the genetic loss of normal physiological levels of individual endogenous inhibitors of angiogenesis leads to a change in the balance between proangiogenic stimulators and their inhibitors, thus favoring enhanced angiogenesis and increased tumor growth. Therefore, these endogenous angiogenesis inhibitors provide a physiological threshold against the induction of angiogenesis. The antiangiogenic activities of endostatin, tumstatin, and thrombospondin-1 are evaluated and correlated with their three-dimensional structure and active sites, deriving a structural basis for their activities. Collectively, structural analysis of all three inhibitors demonstrates that the active antiangiogenic sites on these molecules are exposed on the surface and available to bind their putative integrin receptors on proliferating endothelial cells.

The development of new blood vessels (neovascu-
lature) from preexisting blood vessels is generally referred to as angiogenesis (Folkman 1972). In adult mammals, the vasculature remains quiescent, except in tissues involved in wound repair and the female reproductive system, which undergo transient neovascularization to grow new capillaries (Folkman and Shing 1992). Quiescence depends on the delicate local balance between endogenous angiogenic stimulators and inhibitors. Tipping the balance between pro- and antiangiogenic stimuli to sufficiently “turn on” the angiogenic phenotype requires up-regulation of proangiogenic factors and simultaneous down-regulation of angiogenic inhibitors (Fig. 1) (Folkman 1995; Hanahan and Folkman 1996; Colorado et al. 2000; Bergers and Benjamin 2003). Induction of the “proangiogenic switch” enables endothelial cells to de-

grade the local basement membrane, change morphology, proliferate, invade the surrounding stromal tissue, form microtubes, sprout new capillaries, and reconstitute new basement membrane (Dvorak et al. 1995; Folkman 1995; Hanahan and Folkman 1996; Yancopoulos et al. 2000)—all essential processes in the growth of new blood vessels.

Uncontrolled neovascularization is associated with a number of pathological disorders, including diabetic retinopathy, psoriasis, rheumatoid arthritis, as well as cancer growth and metastasis. The growth of solid tumors, beyond a few cubic millimeters in size, and tumor metastasis are dependent on angiogenesis (Folkman 1995; Hanahan and Folkman 1996). The angiogenic switch involving conversion of quiescent endothelial cells to an active proangiogenic phenotype is a key early event in tumor progression. Therefore, antiangiogenic

Figure 1. Schematic illustration of the proangiogenic switch favoring tumor vasculature growth and progression. The onset of the angiogenic switch is regulated by a shift in the balance of negative (red) and positive (blue) regulators of angiogenesis. The positive regulators are most commonly growth factors, which stimulate endothelial cell migration and proliferation. Negative regulators of angiogenesis include components or protein fragments of the extracellular matrix and vascular basement membrane, which inhibit endothelial cell migration and protein synthesis, and induce apoptosis.

targeting of tumor vasculature could limit tumor expansion and be an efficient therapy for cancer progression (Folkman 1971). Clues for potential antiangiogenic targeting of tumor vasculature may arguably lie in a better understanding of the structure and function of physiologically active, endogenous antiangiogenic molecules.

Endogenous inhibitors of angiogenesis include various antiangiogenic peptides, hormone metabolites, and apoptosis stimulators (Fig. 1) (for review, see Folkman 1995; Cao 2001; Nyberg et al. 2005). Currently, nearly 30 different protein and small-molecule inhibitors are known to exist in the body that function as inhibitors of angiogenesis (Nyberg et al. 2005). Like angiogenic stimulators, endogenous inhibitors influence one or more processes during angiogenesis. They are known to antagonize the angiogenic activity induced by growth factors or to inhibit the proteolytic activity of angiogenic proteinases, endothelial cell proliferation, migration, or microtube formation. In addition, whereas some angiogenic inhibitors affect a variety of cell types, others specifically inhibit the growing population of endothelial cells in new blood vessels. An essential source of endogenous inhibitors of angiogenesis is the vascular basement membrane surrounding endothelial cells (VBM) (Fig. 1). VBM are specialized extracellular matrix organized as thin, ultrastructure layers to provide a supporting scaffold for epithelial and endothelial cells (Paulsson 1992). It is well demonstrated that VBM not only provide mechanical support, but also influence cellular behavior such as differentiation, proliferation, and migration of various cells, including endothelial cells during the sprouting of new capillaries. They play an important role in regulating angiogenesis (Tsilibary et al. 1990; Paulsson 1992; Madri 1997; Darland and D'Amore 1999; Colorado et al. 2000). A series of potent endogenous antiangiogenic factors have been described, of which many are fragments of naturally occurring extracellular matrix (ECM) and basement membrane (BM) proteins (for review, see Cao 2001; Folkman 2004; Nyberg et al. 2005). Thus, molecules that are needed to assemble BM to maintain the integrity of blood vessels, under different circumstances, can have antiangiogenic activities.

On the opposing side of the angiogenic switch (Fig. 1), stimulators of angiogenesis include growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hypoxic conditions that activate hypoxia-inducible factor-1, as well as angiogenic oncogenes, such as Ras and Myc, and tumor suppressors, such as p53 and PTEN that are secreted or produced by a variety of cell types and aid in the development of the tumor vasculature. In tumors, the angiogenic phenotype is characterized by expression of proangiogenic proteins such as VEGF, basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), placenta-like growth factor (PLGF), transforming growth factor- β (TGF- β), PDGF, and others (Relf et al. 1997; Carmeliet et al. 1998; Fukumura et al. 1998; Rak et al. 2000; Yu et al. 2002; Pore et al. 2003; Watnick et al. 2003).

To initiate angiogenesis, endothelial cells have to break down the surrounding BM via secretion of proteolytic en-

zymes. Although metalloproteinases play critical proangiogenic roles in this BM degradation, they also generate specific proteolytic fragments of BM proteins that have antiangiogenic activities and, thus, contribute to the down-regulation of angiogenesis. One such endogenous inhibitor of angiogenesis is endostatin, a 20-kD proteolyzed fragment of the heparan sulfate proteoglycan collagen XVIII, which was identified as an endogenous antiangiogenic inhibitor and is currently in clinical trials (Schellens and Ratain 2002; Thomas et al. 2003). Another important matrix-derived inhibitor of angiogenesis, tumstatin, is a 28-kD proteolyzed fragment of the NC1 domain of the α 3 chain of type IV collagen and has demonstrated antiangiogenic and antitumor activities (Maeshima et al. 2000a,b, 2001a,b; Hamano et al. 2003; Pasco et al. 2004; Sund et al. 2005). In addition to matrix-derived angiogenesis inhibitors, several naturally occurring angiogenic antagonists have been identified (for review, see Carpizo and Iruela-Arispe 2000; Cao 2001; Bergers and Benjamin 2003; Nyberg et al. 2005). One such factor, thrombospondin-1 (TSP-1), was identified as one of the first inhibitors to be produced by normal fibroblast cells (Good et al. 1990).

The in vitro and in vivo antiangiogenic activities of endostatin, tumstatin, and TSP-1 have been the subject of intense study. The availability of putative three-dimensional structures of these endogenous inhibitors of angiogenesis provides molecular insight into their important mechanisms of action. Here we describe the antiangiogenic activities of full-length endostatin, tumstatin, and TSP-1, and regions within these factors that exhibit antiangiogenic activity as peptides isolated from their folded-parent proteins, and examine the molecular structure and surface expression of these naturally occurring antagonist subdomains.

ENDOSTATIN

Endostatin is a 20-kD fragment from the carboxy-terminal non-collagenous domain 1 (NC1) of collagen XVIII (O'Reilly et al. 1997; Sasaki et al. 1998). Collagen XVIII belongs to a subfamily of collagens, the multiplexin family, which have a domain organization that differs from other known collagen types in that non-triple-helical regions interrupt the triple-helical domains (Fig. 2). Collagen XVIII is ubiquitous and highly conserved among vertebrates, *Caenorhabditis elegans*, and *Drosophila*. It is a triple-helical hybrid molecule bearing several heparan sulfate side chains, and a major proteoglycan of endothelial and epithelial BM (Halfter et al. 1998; Musso et al. 2001). The carboxy-terminal NC1 of collagen XVIII includes a trimerization region and a hinge region that provides protease-sensitive sites and antiangiogenic fragments, such as endostatin. Physiological proteolytic cleavage of endostatin from the NC1 of collagen XVIII occurs through the activities of several enzymes, including pancreatic elastase-like enzyme (Wen et al. 1999), cathepsin (Felbor et al. 2000), and matrix metalloproteinases (MMP), for example, MMP-7 (Coussens et al. 2002; Egeblad and Werb 2002).

Figure 2. Schematic representation of the domain structure of endogenous angiogenesis inhibitors. The structural domains of endogenous inhibitors of angiogenesis at the focus of this review, namely the non-collagenous (NC1) domains from the $\alpha 1$ chain of type XVIII collagen and the $\alpha 3$ chain of collagen IV, and the type 1 repeats (TSRs) from thrombospondin-1 (TSP-1), are highlighted in black; all other protein domains are shown in gray. The collagens and TSP-1 are represented as monomers for simplicity, yet are known to form trimers. The collagen α chain NC1 domains provide antiangiogenic fragments endostatin and tumstatin, respectively.

Antiangiogenic Activity of Endostatin

Endostatin was first isolated from the conditioned media of nonmetastatic mouse hemangioendothelioma cells as an inhibitor of endothelial proliferation, angiogenesis, and tumor growth in mice (O'Reilly et al. 1997) and later characterized in mice (Standker et al. 1997). The current hypothesis surrounding endogenous endostatin activity is that during endothelial activation, the production of proteolytic enzymes from the BM leads to a release of antiangiogenic fragments to serve as local inhibitors of angiogenesis (Fig. 1). Recombinant endostatin potently blocks angiogenesis and suppresses primary tumor growth in experimental animal models (Table 1). Endostatin interferes with FGF-2-induced signal transduction, inhibiting cell migration (Dixelius et al. 2002), and induces apoptosis (Dhanabal et al. 1999) and cell-cycle arrest (Hanai et al. 2002a), leading to reduced vascularization of tumors (O'Reilly et al. 1997). Endostatin also suppresses growth factor expression, predominantly VEGF (Hajitou et al. 2002), and blocks VEGF receptor-2 (VEGF-R2), via direct interactions (Kim et al. 2002). Endostatin rapidly down-regulates many genes in growing endothelial cells and several signaling pathways in human microvascular endothelium associated with proangiogenic processes (Fig. 3) (Abdollahi et al. 2004). In addition, endostatin can induce Src-dependent disassembly

of the actin cytoskeleton (Wickstrom et al. 2002, 2004) and inhibit Wnt signaling (Hanai et al. 2002b). Genetic loss of normal physiological levels of endostatin enhances angiogenesis and increases tumor growth 2- to 3-fold in a mechanism dependent on $\alpha 5 \beta 1$ integrin (Sund et al. 2005). Additionally, tumor growth in transgenic mice overproducing endostatin in endothelial cells, a 1.6-fold increase in circulating levels, is 3-fold slower than growth in wild-type mice. Together, these findings suggest that physiological levels of endostatin serve as a source of endothelial-specific tumor suppressor.

Endostatin's Mechanism(s) of Action

Endostatin binds to many endothelial cell-surface proteins, including heparan sulfate proteoglycans, glypicans, VEGF-R2, and $\alpha 5$ and αV containing integrins (Table 1). Endostatin lacks RGD sequences known to bind integrins, and specific integrin-binding sites have not been identified yet. Human endostatin binding to $\alpha 5 \beta 1$ integrin leads to the inhibition of focal adhesion kinase/c-Raf/MEK1/2/p38/ERK1 mitogen-activated protein kinase pathway (Fig. 3) (Sudhakar et al. 2003). Endostatin also induces clustering of $\alpha 5 \beta 1$ integrin associated with actin stress fibers and colocalization of caveolin-1 (Cav1) to activate phosphatase-dependent Src family kinases (Wickstrom et al. 2002). Recently, endostatin activity was linked

Table 1. Endogenous Angiogenesis Inhibitors

Endogenous inhibitor (MW)	Parent protein	Domain	Receptors	Antiangiogenic effects
Endostatin (20 kD)	$\alpha 1$ chain of type XVIII collagen	NC1 domain	$\alpha 5 \beta 1$ integrin, proteoglycans, VEGFR2, tropomyosin	inhibits EC proliferation, migration, induces apoptosis, suppresses tumor growth
Tumstatin (28 kD)	$\alpha 3$ chain of type IV collagen	NC1 domain	$\alpha V \beta 3$ integrin $\alpha 6 \beta 1$ integrin	inhibits EC proliferation, induces apoptosis, suppresses tumor growth
Thrombospondin-1 (40 kD)	thrombospondin-1 (TSR)	type 1 repeats	CD36, CD47, $\alpha \beta 1$ integrin $\alpha V \beta 3$ integrin $\alpha V \beta 5$ integrin	inhibits EC proliferation, induces apoptosis, induces cell adhesion, suppresses tumor growth, activates TGF- β

(NC1) Non-collagenous domain; (VEGFR2) vascular endothelial growth factor receptor-2; (EC) endothelial cell; (TGF- β) transforming growth factor β .

Figure 3. Schematic of the downstream signaling effects of interactions between human endogenous angiogenic inhibitors and their associated cell surface receptors. The effects of endostatin through $\alpha_5\beta_1$ integrin include inhibition of focal adhesion kinase and Src-family kinase phosphorylation to down-regulate key processes in endothelial cell migration and adhesion. The effects of tumstatin are mediated by $\alpha_v\beta_3$ integrin and include inhibition of focal adhesion kinase phosphorylation that affects signaling through phosphatidylinositol 3-kinase (PI3K), the serine/threonine kinase Akt, mammalian target of rapamycin (mTOR), and eukaryotic translation initiation factor (eIF4E) complexes leading to decreased protein synthesis and endothelial cell proliferation. The effects of thrombospondin-1 (TSP-1) via interaction between the type 1 repeat (TSR) sequence motifs and CD36 induce apoptosis through regulation of the signaling pathway from Src kinase pp59^{lyn} to mitogen-activated protein kinase (p38MAPK).

to E-selectin expression on endothelial cells (Yu et al. 2004). Clearly, more studies are needed to elucidate the exact antiangiogenic mechanisms of endostatin.

Three-Dimensional Structure of Endostatin

The crystal structure of recombinant mouse endostatin has been determined at 1.5 Å resolution and reveals a compact fold, similar to the C-type lectin carbohydrate-recognition domain and the hyaluronan-binding link module (Hohenester et al. 1998, 2000). The subsequent crystal structure of human endostatin revealed a zinc-binding site in the amino terminus of endostatin at pH 8.5 (Ding et al. 1998) that is coordinated through H132, H134, H142, and D207, and thus, is likely to serve a role in endostatin structure and not catalytic function (Fig. 4). Interestingly, the antiangiogenic activity of recombinant endostatin was reported to require zinc binding (Boehm et al. 1998); however, subsequent studies failed to show an effect of zinc binding on the inhibition of endothelial cell migration by endostatin (Yamaguchi et al. 1999). Surface arginine residues in endostatin have been suggested to act as binding sites for heparin and heparan sulfate (Hohenester et al. 1998; Sasaki et al. 1999).

A very recent report shows that the entire antitumor and antimigration activities of endostatin are mimicked by a 27-amino-acid peptide (hP1) corresponding to the amino-terminal domain of human endostatin (Fig. 4), and are dependent on the three zinc-binding histidines in the amino-terminal domain (Tjin Tham Sjin et al. 2005). Also recently, an arginine-rich sequence motif (ES-2, IVRRADRAAVP) of human endostatin (Fig. 4) was shown to bind endothelial cell surface β_1 integrin and to

heparin, and inhibit endothelial cell migration and tube formation (Wickstrom et al. 2004). It is likely, then, that endostatin has several biological functions mediated by different regions of the protein. Interestingly, examination of the known human endostatin structure reveals that the integrin/heparin-binding sequence (ES-2) and the amino-terminal antiangiogenic sequence (hP1) of endostatin are adjacent to one another in the tertiary structure (Fig. 4). Thus, although these two sites of endostatin activity are discontinuous in primary sequence, together they form a continuous “antiangiogenic active face” on the globular structure of endostatin, where the histidine-dependent zinc-binding site within the most amino-terminal strand neighbors the arginine-rich, surface-exposed, receptor-binding motif to potentially coordinate heparin-dependent and heparin-independent mechanisms of the antiangiogenic action associated with endostatin.

TUMSTATIN

The entire 28-kD fragment of the carboxy-terminal globular non-collagenous (NC1) domain of the α_3 chain of type IV collagen was named tumstatin (Fig. 2) (Maeshima et al. 2000a,b). This proteolyzed collagen fragment is likely liberated from the basement membranes of the kidney, lung, and testis, which contain abundant amounts of the α_3 chain containing type IV collagen. VBM organization is dependent on the assembly of a type IV collagen network, which is believed to occur through the carboxy-terminal NC1 domain (Madri and Pratt 1986; Tsilibary et al. 1990; Zhang et al. 1994; Timpl 1996; Madri 1997; Zeisberg et al. 2001). Type IV collagen is one of the major macromolecular constituents of all mam-

Figure 4. The structure of human endostatin. (A) Primary sequence of the proteolytic fragment endostatin from the non-collagenous domain (NC1) of the $\alpha 1$ chain of human collagen XVIII. (B) Three-dimensional structure, front and side view, of human endostatin from X-ray crystallographic data (Protein Data Bank ID 1bnl). The regions of endostatin corresponding to antiangiogenic active fragments hP1 and ES2 are rendered in magenta and yellow, respectively, and the remainder of the endostatin backbone is colored green. (C) The antiangiogenic active fragments of endostatin, hP1 and ES2, are shown as they exist folded in the intact endostatin structure, with side chains labeled by single-letter amino acid code.

malian basement membranes, including VBM, and is expressed as six distinct α chains, $\alpha 1$ – $\alpha 6$ (Hudson et al. 1993; Prockop and Kivirikko 1995). These α chains are assembled into triple helices that further form a network to provide a scaffold for other BM macromolecules. The $\alpha 1$ and $\alpha 2$ chain isoforms are ubiquitously present in human VBM, whereas the other four isoforms exhibit restricted distributions (Hudson et al. 1993; Kalluri et al. 1997). Type IV collagen promotes cell adhesion, migration, differentiation, and growth, while playing a crucial role in angiogenesis (Madri and Pratt 1986; Ingber and Folkman 1988; Madri 1997; Haas and Madri 1999). The non-collagenous NC1 domains of type IV collagen have been implicated as important for the assembly of type IV collagen and also other functions that regulate cell behavior directly (Furcht 1984, 1986; Herbst et al. 1988; Tsilibary et al. 1988, 1990; Chelberg et al. 1990; Cameron et al. 1991; Miles et al. 1995; Zeisberg et al. 2001).

Antiangiogenic and Antitumor Activity of Tumstatin

The full-length endogenous $\alpha 3$ chain NC1 domain fragment, tumstatin, and tumstatin fragments containing residues 54–132 and 74–98, inhibit the formation of new blood vessels in Matrigel plug assays, suppress tumor growth of renal cell and prostate carcinomas in xenograft mouse models, and induce apoptosis of endothelial cells

(Table 1) (Maeshima et al. 2000a,b, 2001a,b), specifically by inhibiting protein synthesis in vascular endothelial cells in an integrin-dependent manner leading to endothelial cell-specific apoptosis (Fig. 3) (Maeshima et al. 2002). Overexpression of tumstatin by tumor cells inhibits their invasive properties in a mouse melanoma model (Pasco et al. 2004). Absence of normal physiological levels of tumstatin circulating in the blood facilitates pathological angiogenesis and increased tumor growth (Hamano et al. 2003; Sund et al. 2005). Mice genetically deficient in collagen IV $\alpha 3$ chain show accelerated tumor growth and vascularization, and supplementing these mice with recombinant tumstatin at normal physiological levels abolishes the increased rate of tumor growth. These studies provide compelling evidence for the role of tumstatin as an endogenous inhibitor of angiogenesis and tumor suppressor functioning at physiological levels.

The *in vitro* activity of tumstatin was evaluated using recombinant human protein produced in bacteria and tumstatin-derived synthetic peptides. Recombinant tumstatin inhibits proliferation of endothelial cells, causes G₁ arrest of growth-factor-stimulated endothelial cells, and induces apoptosis of proliferating endothelial cells via regulation of caspase 3 (Maeshima et al. 2000a,b, 2001a,b). Synthetic peptide derived from the $\alpha 3$ chain of the NC1 domain of type IV collagen, residues 183–205, has been shown to inhibit the proliferation of melanoma and other epithelial tumor cell lines *in vitro* (Monboisse

et al. 1994; Han et al. 1997) and bind to the $\alpha V\beta 3$ integrin complex (Fig. 3) (Shahan et al. 1999a,b, 2000). This interaction appears to stimulate focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K) phosphorylation (Pasco et al. 2000). The 183–205 peptide does not affect endothelial cells, but rather demonstrates an anti-melanoma cell activity (Maeshima et al. 2000b). Specifically, the 183–205 fragment of tumstatin binds both endothelial and melanoma cells but only inhibits the proliferation of melanoma cells (Shahan et al. 1999b; Maeshima et al. 2000b; Floquet et al. 2004).

In contrast to this anti-melanoma activity, the antiangiogenic activity of tumstatin was localized to residues 54–132 (tum-5) using deletion mutagenesis (Maeshima et al. 2000b, 2001a; Petitsclerc et al. 2000). Tumstatin fragment 54–132 binds both endothelial cells and melanoma cells, but only inhibits the proliferation of endothelial cells, with no effect on tumor cell proliferation. The antiangiogenic site was further defined using overlapping synthetic peptides to a 25-amino-acid region comprising residues 74–98, namely the T7 tumstatin peptide (Maeshima et al. 2001b). The 54–132 tumstatin fragment inhibits the activation of FAK/PI3K/protein kinase B (PKB)/mammalian target of rapamycin (mTOR) signaling and inactivates eukaryotic initiation factor 4E protein (eIF4E), leading to inhibition of cap-dependent protein synthesis (Fig. 3) (Maeshima et al. 2002). Thus, two separate tumstatin activities, one antiangiogenic and the other antitumor cell, have been localized to distinct regions of the tumstatin molecule.

Tumstatin's Mechanism(s) of Activity

Tumstatin has two binding sites for $\alpha V\beta 3$ integrin, one in the amino-terminal end of the molecule (residues 54–132) that is associated with antiangiogenic activity, and the other in the carboxy-terminal end (residues 185–203) that is associated with antitumor cell activity (Shahan et al. 1999b; Maeshima et al. 2000b; Floquet et al. 2004). The presence of cyclic RGD peptides does not compete for the $\alpha V\beta 3$ integrin-dependent activity of tumstatin, suggesting unique $\alpha V\beta 3$ integrin-mediated mechanisms governing these two distinct antiangiogenic and antitumor activities (Maeshima et al. 2000a). $\alpha V\beta 3$ integrin was been identified as a receptor for tumstatin (Maeshima et al. 2000a, 2001b), and tumstatin fails to suppress neovascularization of Matrigel plugs in $\beta 3$ integrin-deficient mice (Hynes 2002; Reynolds et al. 2002). Tumstatin has also been shown to interact with $\alpha 6\beta 1$ integrin; however, the downstream consequences of such interaction have yet to be determined.

Three-Dimensional Homology Modeled Structure of Tumstatin

From crystallographic data it has been shown that the NC1 domains of type IV collagen $\alpha 1$ and $\alpha 2$ chains ($\alpha 1[IV]NC1$ and $\alpha 2[IV]NC1$) are folded virtually identically, and their topologies very closely resemble one another (Than et al. 2002). Based on the high sequence identity between all type IV collagen NC1 domains, the NC1

domain of type IV collagen $\alpha 3$ chain ($\alpha 3[IV]NC1$) is expected to fold with the same topology as the $\alpha 1(IV)NC1$. Therefore, by aligning the $\alpha 3(IV)NC1$ and $\alpha 1(IV)NC1$ sequences and using the known $[(\alpha 1)_2\alpha 2]_2$ NC1 hexamer structure (Protein Data Base ID 1LI1), a backbone homology $\alpha 3(IV)NC1$ structure has been made (Fig. 5).

In analyzing the α -chain (IV)NC1 alignments and the backbone $\alpha 3(IV)NC1$ homology structure, we have determined that the two distinct regions of tumstatin that exhibit antiangiogenic (T7) and antitumor cell (185–203) activities share partial sequence and structural homology, unidentified as of yet. Seven residues in the amino terminus of the 185–203 sequence align with carboxy-terminal residues in the T7 peptide (Fig. 5). The NC1 domain is composed of two homologous subdomains, and the T7 peptide and the 185–203 sequences are partially homologous regions between the NC1 subdomains (Than et al. 2002). In a recent report, a shorter peptide corresponding to residues 185–191 (CNYYSNS) shared the same inhibitory properties in a mouse melanoma model as the 185–203 sequence (Floquet et al. 2004). The inhibitory effects were conformation-dependent, and residues YYSNS, which form a β turn, were crucial for activity. Interestingly, the analogous residues in T7, the sequence SRND, appear to form a β -turn structure in the homology model of $\alpha 3(IV)NC1$ (Fig. 5). Monoclonal antibodies against the αV and $\beta 3$ integrin subunits were used to determine that the 185–203 peptide binds directly to the $\beta 3$ subunit (Pasco et al. 2000). Therefore, the β turns located in these regions likely expose tumstatin residues within the turn for interaction and/or provide a recognizable structural ligand for interaction with the $\beta 3$ integrin subunit. One might also conclude that the distinct activities identified for the T7 and 185–203 peptides are determined by residues, unique to each peptide, that flank the common β -turn structures.

THROMBOSPONDIN

Thrombospondin-1 (TSP-1) was identified as one of the first endogenous angiogenesis inhibitors discovered in normal fibroblast cell cultures (Good et al. 1990). Thrombospondins (TSPs) are multimeric, calcium-binding, modular glycoproteins that modulate ECM structure and cell behavior (for review, see Adams 1997, 2001; Tucker 2004). The best-characterized TSP of the 5 known proteins is TSP-1, which functions in platelet aggregation, inflammation, and regulation of angiogenesis by inducing cell attachment, cell motility, cell proliferation, apoptosis, extracellular protease activation, and growth factor inhibition (Table 1) (for review, see Adams 2001). TSP-1 forms homotrimers, and each TSP subunit contains multiple domains and a coiled-coil oligomerization region. The common feature of all TSPs is a cassette of domains around 650 amino acids in length containing a variable number of type 2 calcium-binding EGF-like domains that are contiguous with seven TSP type 3 repeat regions and a globular carboxyl terminus (Fig. 2). Unique to TSP-1 and TSP-2 are the procollagen domain and distinctive type 1 repeat domains (TSRs), which have specific functions in signaling inhibition of angiogenesis by

Figure 5. Homology-based model of tumstatin. (A) Primary sequence of the proteolytic fragment tumstatin, the entire non-collagenous domain (NC1) of the $\alpha 3$ chain of human collagen IV. (B) Homology-based three-dimensional model of tumstatin, shown in front and side views, based on the known trimer structure ($[\alpha 1]_2\alpha 2$) of the homologous $\alpha 1$ and $\alpha 2$ chain NC1 domains of human type IV collagen from X-ray crystallographic data and homology modeling. The structures of the ($[\alpha 1]_2\alpha 2$)₂ NC1 hexamers from human placenta basement membranes (Protein Data Bank ID 1L11) were rendered using the molecular graphics visualization program YASARA (YASARA Biosciences) and analyzed to generate modeled structures using the molecular graphics software InsightII (Accelrys). The regions of tumstatin corresponding to antiangiogenic active fragments T7 and the 183-205 sequence are rendered yellow and magenta, respectively, and the remainder of the tumstatin backbone is colored blue. (C) The antiangiogenic active fragments of tumstatin, shown as they exist folded in the intact homology tumstatin model, with side chains labeled by single-letter amino acid code.

binding to CD36 and supporting attachment of varied cell types. TSPs appear to function at the cell surface to bring together membrane proteins and cytokines that regulate ECM structure and cellular phenotype. Known binding partners of TSPs include integrins, the integrin-associated protein CD47, CD36, proteoglycans, and the growth factors TGF- β and PDGF (Fig. 3).

TSPs are expressed in most adult tissues; however, TSP-1 and TSP-2 are highly expressed by stromal fibroblasts, tumor-associated endothelial cells, and tumor cells (Fig. 1) (Brown et al. 1999; Hawighorst et al. 2001). TSP-1 expression increases in response to growth factors PDGF, bFGF, and TGF- β , as well as heat shock and hypoxia. TSP-1 is secreted into the extra- and pericellular matrix by a multitude of different cell types including platelets; megakaryocytes; chondrocytes; osteocytes; epithelial, endothelial, and stromal cells (for review, see Carpizo and Iruela-Arispe 2000); as well as cancer cells, where loss of TSP-1 expression by tumor cells contributes to the angiogenic phenotype (Jimenez and Volpert 2001; Volpert et al. 2002; Watnick et al. 2003).

Antiangiogenic Activity of Thrombospondin-1

TSP-1 was the first protein to be identified as a naturally occurring inhibitor of angiogenesis (Good et al. 1990). TSP-1 has been shown to inhibit tumor growth and

metastasis, identifying it as a potent inhibitor of in vivo neovascularization and tumorigenesis. In addition, expression of TSP-1 has been inversely correlated with tumor progression in breast and lung carcinomas and melanomas (Zabrenetzky et al. 1994; Streit et al. 1999; Rodriguez-Manzanique et al. 2001). Collective in vivo studies evaluating the effects of TSP-1 on tumor growth have shown that TSP-1 acts to inhibit tumor growth via antiangiogenic mechanisms, and analysis of the tumors and tumor vasculature indicates that the observed anti-tumor effect of TSP-1 occurs through an inhibition of angiogenesis rather than a direct effect on tumor cells (Weinstat-Saslow et al. 1994; Zabrenetzky et al. 1994; Campbell et al. 1998; Bleuel et al. 1999; Streit et al. 1999). Genetic approaches to assess the functions of TSP-1 have been performed by targeted gene disruption. To evaluate the importance of TSP-1 for the progression of naturally arising tumors, TSP-1-deficient mice were crossed with p53-deficient mice, resulting in decreased survival. Furthermore, TSP-1-deficient mice showed faster tumor growth with increased vascular density (Lawler et al. 2001; Sund et al. 2005). Additionally, in tumstatin/TSP-1 double-knockout mice, tumors grow twofold faster compared with either tumstatin- or TSP-1-deficient mice. Both results strongly suggest that physiological levels of host-derived factors from the tumor microenvironment serve as endogenous inhibitors of angiogenesis and suppress tumor growth.

The Mechanisms of Thrombospondin-1 Antiangiogenic Activity

Since the initial observation that TSP-1 activates TGF- β , fusion proteins and peptide fragments of TSP-1 have been used to determine the molecular basis of this interaction. Fusion protein and synthetic peptides have shown that the WSHWSPW sequence in the second TSR binds to TGF- β , and the RFK sequence at the carboxyl terminus of the first TSR activates TGF- β (Schultz-Cherry et al. 1995). Nascent TGF- β is proteolytically processed into mature TGF- β and a latency-associated peptide (LAP). The RFK sequence of TSP-1 has been shown to interact to displace LAP and make TGF- β accessible for activity (Ribeiro et al. 1999).

Peptides from the procollagen domain and TSR domains have been shown to inhibit angiogenesis in the corneal pocket assay (Tolsma et al. 1993). Synthetic peptides of the second TSR exhibited antitumor effects that were mediated by an antiangiogenic mechanism (Guo et al. 1997). Using similar approaches, two subdomains within the TSR repeats were identified with antiangiogenic properties: (1) the tryptophan-rich sequence, described above in interactions with TGF- β , from TSR2 and 3 is an inhibitor of angiogenesis in the CAM assay, and (2) the CSVTCG sequence from TSR2 interacts with CD36, specifically, to inhibit angiogenesis (Iruela-Arispe et al. 1999). Interestingly, using larger peptides encompassing these sequences, the antiangiogenic activity of the Trp-rich sequence region was restricted to FGF-2-induced neovascularization, whereas the CD36-binding region's antiangiogenic activity was maintained in both FGF-2- and VEGF-induced neovascularization. It was previously shown that the Trp-rich region prevents FGF-2 binding to endothelial cells, and together these studies suggest that distinct TSP-1 antiangiogenic mechanisms may be growth-factor-dependent (Carpizo and Iruela-Arispe 2000).

CD36, a class B scavenger receptor, has been implicated as the cell surface receptor that mediates the action of TSP-1 on endothelial cells, initially through the CSVTCG motif from TSR2 (Dawson et al. 1997). Subsequently, a secondary binding motif within TSP-1, GVQXR, was shown to bind CD36 and to mediate the migratory inhibition action of TSP-1 (Dawson et al. 1997). Recently, the downstream intracellular interactions involving CD36 have been determined and reveal that TSP-1 induces apoptotic CD36-dependent mechanisms that activate Src-family tyrosine kinase pp59^{fyn}, group II caspases, and p38MAPK (Jimenez et al. 2000). A direct link between CD36 receptor and pp59^{fyn} activation has yet to be determined.

Three-Dimensional Structures of Thrombospondin-1 Repeat Domains

The three-dimensional structure of TSR domains 2 and 3 from human TSP-1 has been determined by X-ray crystallography (Fig. 6) (Tan et al. 2002). The structure (Fig. 6) shows that each TSR folds into a long, thin, stranded domain composed of three antiparallel strands, the first

without regular β -strand structure, and the second two with. Intervening loops between the strands are prominently solvent-exposed, and the structures are stabilized by cross-strand disulfide bridging between pairs of cysteines. Two of the active binding site sequences in TSR2, specifically WSHWSPWS and GVITRIR, which are both implicated in antiangiogenic effects that inhibit VEGF- or FGF-2-induced angiogenesis, can be mapped to exposed regions on the first and second strands of TSR2 (Fig. 6) and contribute side chains to a single positively charged groove on the same face of TSR2 (Tan et al. 2002; Lawler and Detmar 2004). The TSR represents a novel domain fold whereby interdigitating side-chain stacking of Cys, Trp, and Arg forms the core structure. In light of the complex core TSR structure, it is intriguing that isolated peptides from either of the interdigitating strands in the TSR2 domain have antiangiogenic inhibitory activities (Tolsma et al. 1993; Dawson et al. 1999; Iruela-Arispe et al. 1999; Jimenez et al. 2000). Recently, two designed, structurally modified fragments from the TSR2 domain of TSP-1 were shown to increase apoptosis in endothelial cells, block neovascularization in mouse Matrigel plug models, inhibit tumor growth in mouse lung carcinoma models, inhibit melanoma metastases, and block the growth of implanted human carcinoma in nude mice (Dawson et al. 1999; Reiher et al. 2002; Haviv et al. 2005). These molecules, which are now in phase II clinical trials, serve as important examples to demonstrate the potential that exists in small, peptide antiangiogenic therapy design that is based on the functional and structural data of endogenous proteins.

SUMMARY

An examination of the structural features and functional activities of the endogenous inhibitors of angiogenesis—endostatin, tumstatin, and TSP-1—amplifies a recurrent theme, that is, the exposed surfaces of naturally occurring endogenous antiangiogenic factors possess multiple, adjacent receptor- or protein-binding sites, often with distinct functional activities. Most of the motifs implicated in cell surface receptor binding by these endogenous factors are novel sequence- or conformation-dependent motifs for interaction. Interestingly, studies using peptide fragments of all three of these inhibitors demonstrate that these small sequence motifs maintain their potent functional activities apart from the structural constraints of their full-length parent molecules. It remains, then, of great interest to more fully elucidate the unique molecular structures inherent in these factors, their receptor-binding sites, and their mechanisms of action, in order to gain insight into their structure/function necessary to facilitate ongoing efforts to develop potent antiangiogenic cancer therapies targeting tumor vasculature.

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Figure 6. The structure of TSP-1 repeats 2,3 (TSR) of TSP-1. (A) Primary sequence of the second type 1 repeat (TSR2) domain of human TSP-1. Regions of TSR2 that have demonstrated antiangiogenic activity are underlined/bolded. (B) Three-dimensional structure of TSR2 and TSR3 from human TSP-1 from X-ray crystallographic data (Protein Data Bank ID 1LSL). Regions of the structure corresponding to the antiangiogenic active sequences are rendered using the same color as the underlining in A. The remainder of the TSR2 and TSR3 structure is colored cyan. (C) Enlarged image of the backbone secondary structure and side chain positions of adjacent antiangiogenic active sequences that comprise the strands and connective turn of a single positively charged groove on the surface of TSR2.

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