Tumor Angiogenesis

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In order to grow beyond minimal size and to metastasize, tumors need to induce the growth of new blood vessels (angiogenesis). Whereas in normal tissues, vascular quiescence is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli, tumor angiogenesis is induced by increased secretion of angiogenic factors and/or by downregulation of angiogenesis inhibitors. Recent evidence suggests vascular endothelial growth factor (VEGF) as the major tumor angiogenesis factor, promoting tumor growth, invasion, and metastasis. Conversely, blocking of VEGF function inhibits angiogenesis and suppresses tumor growth *in vivo*. Newly identified members of the VEGF family of angiogenesis factors include placental growth factor, VEGF-B, VEGF-C, and VEGF-D, and show overlapping binding patterns to specific endothelial cell receptors. VEGF-C appears to play a major role as a lymphangiogenesis factor and as a growth factor for Kaposi's sarcoma. In contrast, endogenous inhibitors prevent blood vessel growth in normal tissues. In particular, thrombospondin-1 (TSP-1) and TSP-2 are expressed in normal skin and, when introduced into squamous cell carcinomas, potently inhibit malignant tumor growth via inhibition of tumor angiogenesis. *Key words: skin/TSP-1/TSP-2/VEGF. Journal of Investigative Dermatology Symposium Proceedings 5:20–23, 2000*

Angiogenesis, the growth of new capillaries from pre-existing blood vessels, is essential for cancers to grow beyond minimal size (Folkman, 1992), providing a lifeline for tumor sustenance and waste disposal. It has been suggested that expression of an angiogenic phenotype may even precede the development of other traits that contribute to the malignant phenotype ("angiogenic switch") (Hanahan and Folkman, 1996), and there is convincing evidence that therapeutic inhibition of angiogenesis leads to inhibition of tumor growth and metastatic spread in several mouse tumor models (O'Reilly *et al*, 1994, 1997; Folkman, 1997). Angiogenesis is a complex multistep process involving extravasation of plasma proteins, degradation of extracellular matrix, endothelial cell migration and proliferation, and capillary tube formation. Recent advances in vascular biology have identified some of the key factors that control vascular growth, and have led to the concept that in normal tissues, vascular quiescence is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli, whereas tumor angiogenesis is induced by increased secretion of angiogenic factors and/or by downregulation of angiogenesis inhibitors. The following review identifies some of the major mediators involved in the control of tumor angiogenesis and presents evidence for an important role of the non–neoplastic tumor stroma in the control of tumor growth and invasion.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

Vascular endothelial growth factor (VEGF) is regarded as the major tumor angiogenesis factor during epithelial carcinogenesis, in a large number of malignant human cancers, and in tumor metastases (Brown *et al*, 1997; Dvorak *et al*, 1995). Originally identified as a tumor cell-derived factor that induced vascular hyperpermeability to plasma proteins (Senger *et al*, 1983) and therefore named vascular permeability factor, subsequent studies characterized VEGF as an endothelial cell-specific mitogen. VEGF is a homodimeric, heparin-binding glycoprotein occurring in at least four isoforms of 121, 165, 189, and 201 amino acids, due to alternative splicing (Houck *et al*, 1991; Tischer *et al*, 1991). VEGF binds to two type III tyrosine kinase receptors on vascular endothelial cells, Flt-1/VEGF receptor-1 (VEGFR-1) and KDR/Flk-1/VEGFR-2 (Terman *et al*, 1991; deVries *et al*, 1992; Quinn *et al*, 1993). Moreover, VEGF165 additionally binds to the neuropilin receptor on endothelial and other cells (Soker *et al*, 1996). *In vivo*, VEGF enhances microvascular permeability (Senger *et al*, 1983) and angiogenesis (Connolly *et al*, 1989; Leung *et al*, 1989; Claffey *et al*, 1996; Detmar *et al*, 1998). The importance of VEGF in promoting angiogenesis and vascular permeability *in vivo* was recently confirmed in a transgenic mouse model, using the human keratin 14 promoter to target selective overexpression of the murine VEGF gene to basal epidermal keratinocytes and follicular outer root sheath keratinocytes (Detmar *et al*, 1998). VEGF transgenic mice display markedly enhanced skin vascularization with increased numbers of tortuous and leaky blood vessels.

The majority of human cancers studied are characterized by overexpression of VEGF by tumor cells and by overexpression of VEGF receptors on tumor-associated blood vessels (Dvorak *et al*, 1995). Blocking of VEGF function inhibits angiogenesis and suppresses tumor growth *in vivo* (Kim *et al*, 1993; Millauer *et al*, 1994, 1996; Warren *et al*, 1995; Cheng *et al*, 1997), and antibody inhibition of the VEGF receptor Flk-1 prevented tumor cell

invasion *in vivo* (Skobe *et al*, 1997). Tumor cell VEGF expression is induced by at least three distinct molecular pathways: (1) Tumor-secreted growth factors such as transforming growth factor-α directly stimulate tumor growth in an autocrine loop and, simultaneously, induce secretion of tumor-derived VEGF, acting – in a paracrine way – as a potent pro-angiogenic factor to recruit stromal blood vessels into the tumor for enhanced nutritional support (Detmar *et al*, 1994). (2) Hypoxia directly induces tumor cell expression of VEGF, increasing both VEGF gene transcription and mRNA stability (Detmar *et al*, 1997; Claffey *et al*, 1998). The importance of this mechanism is demonstrated by the high VEGF expression in malignant tissue in the immediate vicinity of tumor necroses. (3) Oncogenes are directly involved in the control of VEGF expression, with a prominent inductive role of the ras oncogene and a suppressive function of the p53 tumor suppressor gene (Grugel *et al*, 1995; Kieser *et al*, 1994).

OTHER VEGF FAMILY MEMBERS

Within the last few years, several additional members of the VEGF family of angiogenesis factors have been identified. Placental growth factor (PlGF) and VEGF-B bind to the VEGF receptors Flt1 and neuropilin (**Fig 1**), whereas VEGF-C and VEGF-D bind to KDR and, in addition, to the recently identified Flt4 receptor that is predominantly expressed on lymphatic endothelium, suggesting an important role in lymphangiogenesis. Indeed, overexpression of VEGF-C in the skin of transgenic mice resulted in an increased number of enlarged cutaneous lymphatic vessels, but not blood vessels (Jeltsch *et al*, 1997). Whereas little is known
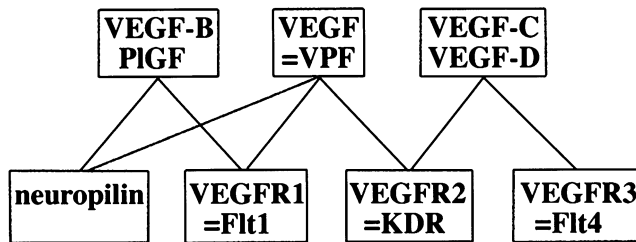
Figure 1. Overlapping binding patterns of members of the VEGF family of angiogenesis factors to endothelial cell VEGF receptors (VEGFR).
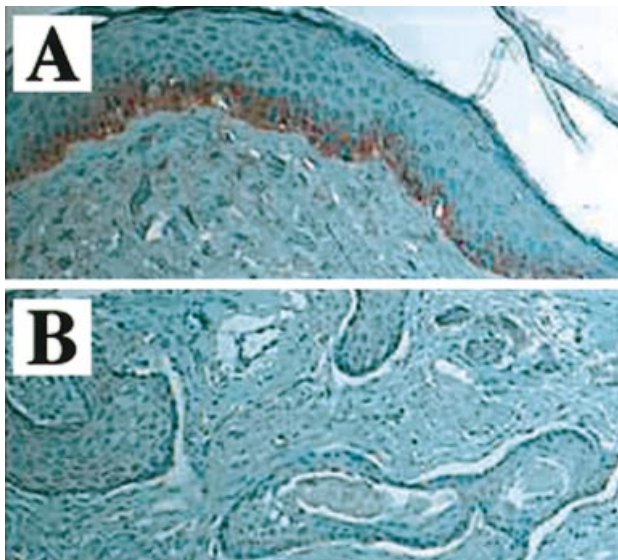
Figure 2. TSP-2 is expressed in normal human skin. Immunohisto-chemical detection of TSP-2 in the basal layer of normal epidermis (*A*) and absence of TSP-2 immunoreactivity in invasive cutaneous squamous cell carcinoma (*B*). Immunohistochemistry was performed as described in Streit *et al* (1999).

about the expression and biologic activity of PlGF and VEGF-B in malignant tumors, we have recently identified VEGF-C as a novel growth factor for AIDS-associated Kaposi's sarcoma (Skobe *et al*, 1999). Moreover, we found strong expression of both VEGF-C receptors, KDR and Flt4, on spindle-shaped Kaposi's sarcoma cells *in situ*, suggesting that the tumor cells of Kaposi's sarcomas are of lymphatic rather than blood vessel origin and/or differentiation (Jussila *et al*, 1998; Skobe *et al*, 1999). These findings identify Flt4 as a novel molecular marker for Kaposi's sarcomas and may yield new insights into the histogenetic differentiation of this tumor. There is increasing evidence that tumor cell expression of VEGF-C (and, possibly, VEGF-D) might be related to the metastatic risk of several human cancers; however, the direct biologic roles of VEGF-C and VEGF-D for tumor growth and tumor (lymph)angiogenesis have remained largely elusive.

THROMBOSPONDIN-1 (TSP-1)

In contrast to the large number of reports on tumor angiogenesis factors, much less is known about the expression and the biologic role of endogenous inhibitors of angiogenesis during carcinogenesis, tumor growth, and tumor metastasis. Several naturally occuring inhibitors of tumor angiogenesis have been identified (**Table I**), including thrombospondin-1 (TSP-1) (Weinstat-Saslow *et al*, 1994), angiostatin (O'Reilly *et al*, 1994), endostatin (O'Reilly *et al*, 1997), vasostatin (Pike *et al*, 1998), and thrombospondin-2 (Streit *et al*, 1999), and they have been shown to inhibit tumor growth and tumor angiogenesis in several experimental models of human tumor xenotransplants in immunodeficient mice. Despite this proven antitumoral activity in mouse models, the mechanisms of action of these endogenous inhibitors have remained largely unknown.

TSP-1 is a member of a family of matricellular proteins (TSP-1 to TSP-5) that are encoded by separate genes (Bornstein, 1992). TSPs play an important role in a variety of biologic processes, including cell–cell and cell–matrix interactions. TSP-1 is a 450 kDa homotrimeric glycoprotein that regulates attachment, proliferation, migration, and differentiation of various cell types (for review, see Bornstein, 1995). TSP-1 inhibits proliferation and migration of vascular endothelial cells *in vitro* and inhibits neovascularization *in vivo*, contributing to the normal quiescence of the vasculature (Tolsma *et al*, 1993). TSP-1 expression was inversely correlated with malignant progression in human lung, breast, and bladder carcinoma cell lines (Zabrenetzky *et al*, 1994; Campbell *et al*, 1998). In human skin, TSP-1 is deposited in the dermo–epidermal basement membrane (Wight *et al*, 1985), contributing to the antiangiogenic barrier that separates the avascular epidermis from the vascularized dermis. Recently, we found that TSP-1 expression was downregulated in squamous cell carcinomas (SCC) of the skin, as compared with normal skin. Based on these findings, we studied the biologic role of TSP-1 for cutaneous carcinoma growth, using stably transfected A431 or SCC-13 squamous cell carcinoma cells in an intradermal xenograft model (Streit *et al*, 1999). These investigations demonstrated that TSP-1 overexpression significantly reduced intradermal tumor growth of A431 squamous cell carcinoma cells and completely inhibited tumor formation of the slowly growing squamous cell carcinoma cell line SCC-13. TSP-1

Table I. Endogenous inhibitors of tumor angiogenesis

Year	Inhibitor	Fragment of	MW	Authors
1994	thrombospondin-1	–	450 kDa	Weinstat-Saslow *et al*
1994	angiostatin	plasminogen	38 kDa	O'Reilly *et al*
1997	endostatin	collagen XVIII	20 kDa	O'Reilly *et al*
1998	vasostatin	calreticulin	≃30 kDa	Pike *et al*
1999	thrombospondin-2	–	450 kD	Streit *et al*

overexpressing A431 tumors were characterized by extensive areas of necrosis and by decreased tumor vessel numbers and sizes, clearly demonstrating its antiangiogenic activity. Several lines of evidence suggest that the antitumoral effects of TSP-1 are predominantly mediated through interaction with vascular endothelium, with little or no direct activity on tumor cell growth. In vitro, TSP-1 did not affect anchorage-dependent tumor cell growth in monolayer culture or anchorage-independent cell growth in soft agar colony forming assays, and TSP-1 did not increase tumor cell susceptibility to induction of apoptosis (Streit et al, 1999). Moreover, we did not detect any changes in the rate of cell proliferation in TSP-1 overexpressing A431 tumors in vivo. The mechanisms of TSP-1's antiangiogenic activity are the subject of ongoing research. Interaction of the CSVTCG sequence contained within TSP-1's type I repeats with the CD36 receptor on endothelial cells has been reported to induce endothelial cell apoptosis (Tolsma et al, 1993; Volpert et al, 1995; Dawson et al, 1997). Moreover, distinct heparin-binding sequences within the TSP-1 type I repeats have been identified that inhibit endothelial cell migration in vitro (Iruela-Arispe et al, 1999). Recent evidence also suggests an important role of the RFK sequence, contained within the type I repeats, as a physiologic mediator of TGF-β activation. Whereas several of the abnormalities found in TSP-1 deficient mice could be normalized by administration of a TGF-β activating synthetic peptide (Crawford et al, 1998), it remains to be established whether and to what extent activation of TGF-β is also involved in the antitumoral activity of TSP-1.

THROMBOSPONDIN-2 (TSP-2)

TSP-2 is a 450 kDa trimeric, modular glycoprotein that has a considerable structural similarity with TSP-1 (Bornstein et al, 1991). Similar to TSP-1, TSP-2 is secreted as a disulfide-bonded homotrimer (Bornstein, 1992) and interacts with several cell surface receptors, including the integrin $\alpha_v\beta_3$, low-density lipoprotein-related receptor protein, and heparan sulfate proteoglycans (Chen et al, 1994, 1996). The expression of TSP-2 is spatially and temporally different from TSP-1 during embryonic development and in adult tissues, with predominant TSP-2 expression in areas of chondrogenesis and in early connective tissues (Iruela-Arispe et al, 1993; Kyriakides et al, 1998). Moreover, the regulation of TSP-2 gene expression by growth factors and hormones is distinct from TSP-1 (Bornstein et al, 1991; Lafeuillade et al, 1996). Previously, TSP-2 was reported to diminish the angiogenic activity of basic fibroblast growth factor (Volpert et al, 1995) and the formation of focal adhesions in aortic endothelial cells (Murphy-Ullrich et al, 1993). Mice deficient in TSP-2 were characterized, among other abnormalities, by increased numbers of blood vessels in several tissues including the skin (Kyriakides et al, 1998), suggesting a role of TSP-2 in the control of blood vessel growth.

Recently, we found that TSP-2 mRNA is expressed in basal epidermal keratinocytes and that TSP-2 protein is found in the basal epidermal layer as well as in the dermal–epidermal basement membrane area (**Fig 2**). TSP-2 probably contributes to the anti-angiogenic barrier function that prevents vessel ingrowth into the epidermis. It is of interest that we found both TSP-2 mRNA and protein expression to be downregulated in invasive human squamous cell carcinomas (SCC) of the skin, lesions that are characterized by richly angiogenic stroma (**Fig 2**). These findings suggested a protective role of TSP-2 that is lost during carcinogenesis. To test this hypothesis, we stably overexpressed TSP-2 in human A431 squamous cell carcinoma cells, either alone or in combination with the related inhibitor TSP-1. Indeed, we found a potent, more than 90% inhibition of in vivo tumor growth by TSP-2 (Streit et al, 1999), most likely caused by the potent inhibition of tumor vascularization that was observed in TSP-2 expressing tumors. The TSP-2 mediated antitumoral effect was significantly more potent than the effect of TSP-1 that resulted in tumor growth inhibition of approximately 50%. Importantly, when TSP-2 was coexpressed with TSP-1, tumor development was completely inhibited over an observation period of

up to 3 months, suggesting that combinations of angiogenesis inhibitors might have additive or synergistic antitumoral effects (Streit et al, 1999). Our results also suggest that the expression of TSP-2 in normal tissues exerts a protective effect against tumor development, and that re-introduction of TSP-2 into malignant tumor cells or treatment of tumors with exogenous TSP-2 or TSP-2 derived fragments might be a promising new approach for the treatment of human cancers and/or their metastases.

The molecular mechanisms of TSP-2 mediated inhibition of tumor angiogenesis and the molecular domain(s) responsible remain to be established. Similar to TSP-1, the TSP-2 protein also contains two CSVTCG sequences within the type I repeats that bind to the CD36 receptor on endothelial cells; however, in contrast to TSP-1, TSP-2 lacks the TGF-β-activating sequence RFK. Because the in vivo activity of TSP-2 appears to be more potent than that of TSP-1, as demonstrated by the more pronounced effects in tumor assays and in TSP-2 versus TSP-1 deficient mice, it is likely that additional sequences within the TSP-2 molecule are involved in the mediation of its angio-inhibitory activity. Using both recombinant fragments of human TSP-2 and synthetic peptides derived from the human TSP-2 sequence, we currently aim to identify additional bioactive domains within the human TSP-2 molecule.

CONTROL OF MALIGNANT TUMOR GROWTH AND INVASION BY TUMOR STROMA

Recent studies provide compelling evidence for a much more active role of the tumor stroma (nonmalignant mesenchymal cells in the vicinity of the tumor, including fibroblasts and endothelial cells) in the control of tumor progression and invasion than generally anticipated. Indeed, it appears that stromal cells are essential to provide the matrix needed for tumor invasion, and that inhibition of distinct stromal functions might prevent cancer progression. In 1997, Skobe et al demonstrated that inhibition of flk-1 (VEGFR-2), a VEGF receptor selectively expressed on endothelial cells, was sufficient to inhibit tumor invasion of malignant, transformed epidermal keratinocytes, despite the main-tenance of tumor cell proliferation (Skobe et al, 1997). More recently, we have shown that overexpression of VEGF in tumor cells, leading to selective induction of tumor angiogenesis and vascular hyperpermeability, was sufficient to induce invasiveness, including single cell invasion, of SCC-13 squamous cell carcinomas that are normally noninvasive after intradermal injection into nude mice (Detmar et al 2000).

It was previously shown that ras-transduced epidermal kerati-nocytes, isolated from the skin of newborn mice, had the capacity to form malignant, invasive squamous cell carcinomas after transplantation onto mouse skin, using a silicone transplantation chamber technique (Dotto et al, 1988). When normal dermal fibroblasts were admixed with these ras-transduced keratinocytes, squamous cell carcinoma development was inhibited. The exact molecular mechanisms responsible for this inhibitory effect have remained unknown; however, our recent studies show pronounced expression of TSP-1 and TSP-2 also by dermal fibroblasts in vitro and in vivo. Therefore, the inhibitory effect of normal fibroblasts on tumor growth might be due, at least in part, to the secretion of endogenous angiogenesis inhibitors by fibroblasts, resulting in inhibition of tumor growth and angiogenesis. Currently, we are investigating this hypothesis using genetic mouse models for transgenic overexpression or for deficiency of TSP-1 and TSP-2. In summary, increasing evidence suggests that the creation of an angiogenic stroma is required for tumor progression and invasion and that inhibition of angiogenesis, in addition to its effects on the growth and metastasis of established tumors, might also affect the early steps of tumor development.

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