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The enigmatic role of angiopoietin-1 in tumor angiogenesis

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ABSTRACT

A tumor vasculature is highly unstable and immature, characterized by a high proliferation rate of endothelial cells, hyper-permeability, and chaotic blood flow. The dysfunctional vasculature gives rise to continual plasma leakage and hypoxia in the tumor, resulting in constant on-sets of inflammation and angiogenesis. Tumors are thus likened to wounds that will not heal. The lack of functional mural cells, including pericytes and vascular smooth muscle cells, in tumor vascular structure contributes significantly to the abnormality of tumor vessels. Angiopoietin-1 (Ang1) is a physiological angiogenesis promoter during embryonic development. The function of Ang1 is essential to endothelial cell survival, vascular branching, and pericyte recruitment. However, an increasing amount of experimental data suggest that Ang1-stimulated association of mural cells with endothelial cells lead to stabilization of newly formed blood vessels. This in turn may limit the otherwise continuous angiogenesis in the tumor, and consequently give rise to inhibition of tumor growth. We discuss the enigmatic role of Ang1 in tumor angiogenesis in this review.

Key words: *angiopoietin, angiogenesis, cancer, endothelial cells, mural cells*.

INTRODUCTION

Normal blood vessels are composed of two distinct cell types: endothelial cells, the most well studied component of blood vessels with regard to cancer angiogenesis, and mural cells. The mature quiescent vasculature of most organs is characterized by extensive coverage by mural cells; in capillaries and smaller vessels, the mural cell component is comprised of pericytes, whereas in larger vessels this role is fulfilled by smooth muscle cells. This investiture of the endothelial tubule by mural cells is thought to play a major role in maintenance of the quiescent state. For new blood vessel formation to occur, the mural cell coating of the preexisting vessel must first be dissociated, followed by matrix degradation and extravascular fibrin deposition, and freeing of endothelial cells to respond to angiogenic signals with proliferation, migration and tubule formation (reviewed in[1]). Remodeling occurs to prune vessels to fit the needs of the tissue. This is then followed by a maturation phase characterized by investiture of the endothelial tubule with mural cells leading to quiescence of both cell types, with subsequent basement membrane reconstitution, establishment of cell-cell junctional complexes, and stabilization of the vessel. Coordinated regulation of pro- and anti-angiogenic factors is necessary for each stage to ensure the development of a normal, functional vessel.

It is well established that tumors must acquire the ability to stimulate capillary formation to progress from a small localized growth with a limited oxygen and nutrient supply to a well-vascularized enlarged tumor[2]. The primary driving force for tumor angiogenesis is the combination of the demand for oxygen and nutrients by the growing cancer cells, and the physical limit of the distances for small molecules to diffuse across the stroma between a nearby capillary and the cell making the demand, a physical limit of about 100 mm[3]. Folkman and colleagues suggested that once the radius of the tumor reaches this limit, hypoxic conditions occur in the center of the cell mass. An `angiogenic switch' then takes place in favor of new blood vessel growth[4].

Several different mechanisms have been proposed to

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lead to vascularization of tumors. Folkman and colleagues suggested that the tumor induces capillary sprouts from surrounding vasculature by altering the local balance of angiogenic promoters and inhibitors[2], in a process known as angiogenesis. Alternatively, Yancopolous and colleagues proposed that cancer cells initially encroach upon existing microvessels (co-opting), this being followed by destabilization and regression of the vessels in the center of the tumor mass, and initiation of new capillary growth at the periphery of the tumor[5]. Moreover, Dvorak and colleagues suggested that a tumor prior to its own expansion would prepare a microvascular network by stimulating angiogenesis in its immediate surrounds, then utilize these vessels in the expansion phase[6]. A considerable collection of growth factors and cytokines are shown to take part in the modulation of tumor angiogenesis. However, among the most important components of the potential to initiate angiogenesis in a tumor are plasma leakage and hypoxia.

Abnormality of tumor blood vessels

Due to the aberrant expression of angiogenic factors, tumor vessels develop very abnormally, giving rise to a highly dysfunctional vasculature[7]. Tumor blood vessels are dilated, with uneven diameters and excessive branching and shunts. The tortuous blood flow is inadequate and leads to hypoxia and acidic regions. The vessel walls are also abnormal, characterized by the presence of a large number of endothelial fenestrae and trans-cellular openings, widened intercellular junctions, and a discontinuous basement membrane. Finally, tumor blood vessels are characterized by decreased mural cell investiture[8-10]; ultrastructural studies demonstrate that even when mural cells are present, they exhibit abnormal association with the underlying endothelial tubule[11, 12]. Consequent to this lack of maturation, tumor vessels are highly permeable with significant plasma extravasation [13, 14].

As a result of this permeability, Dvorak likened tumors to wounds that do not heal [15]. In a wound, plasma leakage is the result of tissue injury, whereas tumor cells secrete vascular permeability factor (VPF), perhaps better now known as Vascular Endothelial Growth Factor (VEGF), which renders vessels hyperpermeable. During wound healing, platelets facilitate generation of the provisional matrix and initiate the wound healing process; platelets are also important in the later stage of wound healing because they produce platelet-derived growth factor (PDGF), a potent mitogen and chemoattractant for precursors of smooth muscle cells and pericytes [16]. In contrast, platelets have not been found outside of blood vessels of solid tumors. Moreover, fibrin and fibronectin appear only transiently in wounds that heal normally, being replaced by type I and III collagen in which the density of blood vessels diminishes[17]. Fibrin and fibronectin persist in tumor stroma, however, probably due to constitutive tumor production of VPF/VEGF, which results in protracted vessel leakage and continuing clotting of extravasated fibrinogen and fibronectin; in wounds, by contrast, vascular permeability is repaired within a few days after injury. Tumors are thus likened to an unending series of wounds that continually initiate healing and angiogenesis but are unable to heal completely.

Angiopoietins and Tie2

Many growth factors are proposed to play a role in both physiological and pathological angiogenesis. One family of vascular regulatory molecules which has been the subject of intense investigation in both physiological and pathological blood vessel generation are the angiopoietins. The Angiopoietin family of growth factors is comprised of four family members that bind to the Tie2 tyrosine kinase receptor with different outcomes. Angiopoietin-1 (Ang1), the main ligand for Tie2[18,19], and -4[20] are agonistic ligands, whereas Angiopoietin-2 (Ang2) and -3 can serve as antagonistic ligands[20, 21]. Although Ang1 does not stimulate proliferation of endothelial cells [18], in vitro Ang1 can induce endothelial migration[22], tubule formation[23] and sprouting[24,25], and survival from a variety of apoptotic insults[26-29], suggesting that Ang1 can be a potent pro-angiogenic factor. Transgenic null mutation of the Ang1 gene confirms an angiogenic role for Ang1, as Ang1 null embryos are unable to form a complex vascular network and exhibit decreased vessel support by mural cells[19]. These results gave the first indication that Ang1 may play a role in recruitment of mural cells to support the primitive endothelial tubule and enhance vessel maturation. Transgenic Ang1 overexpression or systemic adenoviral delivery resulted in increased vascular branching[30-32]. Since Ang1 is not a mitogen, the increased vascular branching may arise from reinforcement of VEGF-induced angiogenesis. Indeed, Ang1 has been shown to synergise with VEGF to enhance angiogenesis in the rat

aorta model[33] and increase vessel density in the corneal implant assay[34] and several other in vivo assays [35-38]. While overexpression of VEGF alone gives rise to increased vascular branching, the vessels induced by VEGF are leaky[32]. By contrast, the vessels induced in the presence of Ang1 are not leaky and resist leakage induced by inflammatory agents[32,39,40]. Part of the means by which this resistance to leakiness occurs may be attributed to markedly enhanced pericyte coverage of the nascent vessels.

The role of Ang2 in blood vessel regulation is quite complex. Transgenic overexpression of Ang2 leads to a phenotype essentially the same as that seen in the Ang1 knockout, suggesting that Ang2 serves as an antagonist for Ang1[21]. This prediction has held true in vitro, as Ang2 can prevent Ang1-stimulated effects on endothelial cells including phosphorylation of Tie2[21] and migration[22]. Interestingly, however, it has been shown that Ang2 can activate ectopically-expressed Tie2 on fibroblasts[22] and can activate endothelial Tie2 at high concentrations[41] or when cells are plated on fibrin[42] or collagen matrix[43]. Indeed, the Ang2 knockout demonstrates that while Ang2 is dispensable for embryonic vascular development, Ang2 is required for both the vascular regression and sprouting events involved in postnatal ocular angiogenesis[44].

An intimate relationship between angiopoietins and VEGF in angiogenesis was predicted by analysis of Ang1, Ang2, and VEGF expression in cyclical rat ovary angiogenesis. In this study, Ang2 and VEGF mRNA were co-expressed at the front of invading sprouts during active angiogenesis, whereas Ang2 is upregulated and VEGF is downregulated during vessel regression[21]. While Ang1 mRNA expression is relatively stable throughout the process, the Ang2 to Ang1 ratio is drastically elevated during corpus luteum vessel regression compared to angiogenesis during corpus luteum formation[45]. These studies lead to the current dogma that Ang1 and VEGF promote angiogenesis and vessel maturation, whereas Ang2 serves to antagonize the mural cell contact induced by Ang1; in the presence of VEGF, angiogenesis ensues while in the absence, vessels regress[21]. This notion is further supported by analysis of angiogenesis in the pupillary membrane. Ang2 induces proliferation and migration of endothelial cells and stimulates sprouting of new blood vessels when VEGF is present, whereas it promotes endothelial cell death and vessel regression when the activity of endogenous VEGF is inhibited[46].

Null mutation of the gene for the Tie2 angiopoietin rereceptor gave rise to a phenotype similar to both Ang1 null and Ang2 transgenic mice[47,48]. Since Tie2 is thought to be largely specific to endothelial cells, it has been suggested that Ang1 activates the Tie2 receptor on endothelial cells, resulting in a yet uncharacterized paracrine loop between EC and SMC. As transgenic knockout approaches targeting PDGF-B/bR give rise to vessels that similarly lack sufficient mural cell investiture[16,49], PDGF has been suggested as a candidate for such a paracrine loop but this has yet to be documented experimentally. We and others have recently reported that mesenchymal mural cell precursor cells, smooth muscle cells, and pericytes express Tie2[50-53], and that Tie2 levels can be further upregulated on smooth muscle cells[53] and pericytes[52] by VEGF. Further, Ang1 can induce migration of mural cell precursors[51] and VEGF-preconditioned smooth muscle cells[53]. These data suggest that part of the mechanism of Ang1-induced vessel maturation may be direct stimulatory action on mural cells.

Angiopoietin expression in tumors

Given the importance of angiopoietins in vascular development, it was of interest to determine what role these factors may play in tumor angiogenesis. In general, high levels of Ang2 by tumor or vascular tissues have been documented in a wide variety of highly vascularized tumors such as malignant glioblastoma[54-56], non-small cell lung cancer[57, 58], hepatocellular carcinoma [59-62], gastric carcinoma[63], Kaposi's sarcoma and angiosarcoma[64], neuroblastoma[65] and thyroid tumor [66] (see Tab 1). Further, Ang2 expression has been correlated with poor prognosis in NSCLC [67], HCC[62], gastric[63] and breast[68] cancers. In addition, HT29 colon cancer, hepatocellular carcinoma, and MKN-7 gastric cancer cells engineered to overexpress Ang2 demonstrated augmented tumor growth and vessel count compared to vector controls[59,63,69]. In many cases, VEGF overexpression is observed as well, suggesting that destabilization by Ang2 permits VEGF-induced angiogenesis to proceed (see Tab 1). Indeed, VEGF has been reported to upregulate endothelial Ang2 in vitro[70] and in vivo[71], although in a C6 brain tumor model, Ang2 expression precedes the appearance of VEGF during the initiation of tumor angiogenesis[72, 73]. Increased ex-

Tab 1. Expression of angiopoietin-1 and angiopoietin-2 in human cancers in clinical settings

Tumor	Reference	Ang1	Ang2	VEGF
Astrocytoma	76	Increased	Increased	Increased
	55	Increased	Absent	Increased
Glioblastoma	54	Increased	Increased	ND
	55	Increased	Increased	Increased
	56	Increased	Increased	ND
	74	ND	Increased	Increased
	101	Present	Present	Present
Breast	82	Absent	ND	ND
	83	Decreased	Decreased	Increased
	68	Present	Increased	ND
Ovarian	71	Present	Increased	Increased
	102	Decreased	Normal	ND
	103	Increased	Need paper	Increased
Prostate	104	Increased	Increased	ND
Colon	84	Decreased	Increased	ND
Colorectal	105	ND	Increased	ND
NSCLC	57	Increased	Increased	Increased
	58	Decreased	Increased	Increased
	112	Increased	ND	Increased
	67	Increased	Increased	Increased
HCC	60	ND	Increased	ND
	61	Normal	Increased	Increased
	62	Normal	Increased	Increased
	59	Normal	Increased	ND
Gastric	63	Normal	Increased	ND
RCC	106	Normal	Increased	ND
Esophageal	107	Present	ND	Present
Uveal melanoma	108	Absent	Present	Present
Thyroid	66	Variable	Increased	Increased
	109	Increased	ND	ND
Multiple Myeloma	110	Increased	Absent	ND
	111	Absent	Present	Present
Angiosarcoma	64	Normal	Increased	ND
Kaposi's Sarcoma	64	Normal	Increased	ND
Neuroblastoma	65	Increased	Increased	Increased

"Increased" or "Decreased" denotes expression levels or frequency were altered compared to normal controls;
"Present" indicates that the factor was detected but levels not compared to normal controls.
ND = Not Determined; NSCLC = Non-small cell lung cancer; HCC = hepatocellular carcinoma; RCC = renal cell carcinoma

pression of Ang2 is thought to play an integral role in both the proposed mechanism of vessel co-option[5,74] as well as sprouting angiogenesis[73]. Interestingly, Lewis Lung carcinoma or TA3 mammary carcinoma cells transfected with Ang2 result in decreased tumorigenesis, which the authors attributed to an imbalance with VEGF expression that allowed for vessel regression[75]. It should be noted that Ang2-overexpressing xenograft vessels lacked coverage by mural cells[54,63,71,75] and an inverse correlation between Ang2 upregulation and pericyte coverage has been observed in human gliomas[54, 74,76]. Finally, in a chemically induced skin carcinogenesis model, Ang2 is not expressed in normal skin, but is upregulated at an early stage during papillomagenesis[77]. Not surprisingly, Ang2 expression has been reported to be upregulated by hypoxia in microvascular endothelial [70,78] and glioma cells in vitro[74] and in capillary endothelium in vivo[78,79]. Taken together, these results suggest that Ang2 plays an important role in the initiation of tumor angiogenesis, presumably by its ability to antagonize Ang1-induced, mural cell-mediated vessel stabilization.

By contrast, the role of Ang1 in tumor angiogenesis is less clear. Overexpression of Ang1 has been documented in malignant glioblastoma[54,55], neuroblastoma [65], non-small cell lung cancer[57], and variably in other tumors as well (see Table I). In addition, in a Hela xenograft model, Ang1 antisense RNA lead to decreased tumor growth and angiogenesis[80] and overexpression of Ang1 promoted HeLa tumor angiogenesis[81], suggesting that Ang1 may stimulate angiogenesis in that model. Increasing evidence, however, is suggesting a lack of a role for Ang1 in several cancers in a clinical setting. Recently, we reported that breast cancer epithelial cells do not express Ang1[82]; others have confirmed decreased Ang1 expression in breast tumors compared to normal breast tissue[83]. Similarly, immunohistochemistry studies demonstrated that Ang1 is expressed in normal colonic epithelium, whereas colon tumors lack appreciable Ang1 staining[84]. Further, in a mouse skin carcinogenesis model, it was shown that Ang1 expression was completely abolished in papillomas compared to normal skin, and that Ang1 was downregulated in mutant ras-bearing keratinocyte cell lines[85]. In addition, hypoxia, a principal driver of tumor angiogenesis, has been shown to downregulate Ang1 production by glioblastoma cell lines[55] and fibroblasts[86]. These studies suggest a selective loss of Ang1 expression during the progression toward malignancy.

Inhibition of tumor growth b Ang1 overexpression

In sharp contrast to the findings from the transgenic studies showing that Ang1 is a promoter of vasculogenesis and angiogenesis during embryonic development, studies using xenograft models demonstrate that ectopic expression of Ang1 in breast[82] and colon [69] cancer cells results in decreased tumor proliferation and angiogenesis. In addition, Ang1 inhibits colon cancer peritoneal[87] and hepatic metastases[88]. A similar tumor inhibitory role for Ang1 has been observed for squamous cell carcinoma (SCC), as stable overex-pression of Ang1 in A431 xenograft model showed inhibition of tumor growth[77], and the K14-HPV16/K14-Ang1 double transgenic developed fewer pre-malignant and tumorigenic lesions than K14-HPV16 parentals [89]. These data suggest that, despite its important stimulatory role in embryonic blood vessel formation, Ang1 may exert an inhibitory role for tumor angiogenesis.

What is the mechanism by which the angiogenic factor Ang1 can paradoxically inhibit tumor angiogenesis? Analysis of blood vessels from the Ang1-inhibited tumor models above suggest that the answer may lie in the ability of Ang1 to recruit mural cells to stabilize the blood vessel. Tumor vessels in the Ang1-transfected breast[50], colon[69], hepatic colon tumor[88], and squamous cell [77] xenografts mentioned above all demonstrate significantly increased association of pericytes with vessels, suggesting that enforced maturation of blood vessels may functionally inhibit tumor angiogenesis. First of all, the ability of Ang1 to inhibit vascular permeability is thought to be due partly to the enhancement of cell-cell junctions [40], as well stabilization of blood vessels by the promotion of mural cell recruitment[19,34,35,38]; it is interesting to note that the absence of pericytes leads to defects in endothelial junction formation[16]. In addition, peripheral blood vessels in the Ang1-transfected MCF7 tumors were not dilated, in contrast to those in the vector control counterparts[50]. This Ang1-mediated decline in vessel permeability may decrease the plasma extravasation that creates the permissive, or even stimulatory, environment for further angiogenesis.

Secondly, the presence of mural cells is postulated to be inhibitory for endothelial angiogenic responses. Indeed, several studies[90,91] have demonstrated that actively proliferating endothelium lacks coverage by mural cells,

and ultrastructural analysis of breast tumors showed that vessels in areas of low vascular density had greater pericyte coverage than areas of high vascular density[92]. Further, the loss of pericytes observed in PDGF-B/bR knockouts is concomitant with endothelial hyperplasia [16]. In addition, mural cell-endothelial cell interactions are reduced following stimulation of angiogenesis[93,94], and the arrival of pericytes coincides with the cessation of vessel growth during wound healing[95], suggesting that contact with pericytes leads to quiescence of endothelial cells. In vitro studies by culturing endothelial cells with smooth muscle cells or pericytes using a variety of models corroborate the decreased growth of endothelial cells under these conditions in a fashion that requires cell-cell contact[96,97]. Mural cells have been further implicated in the prevention endothelial cell migration[98] and sprouting[99], and activation of endothelial MT1-MMP[100].

Numerous studies have indicated the important role of VEGF in both physiological and pathological angiogenesis. The intimate interaction of VEGF with the angiopoietins leads to the ability to tightly control the angiogenic process. It is thought that the antagonism of Ang1-induced vascular stability by Ang2 leads to dissociation of the mural cell coating to initiate angiogenesis; the ratio of Ang1 to Ang2 is likely to be a critical determinant in this process. In the presence of VEGF, endothelial cells can proliferate, migrate, and form tubules, and in some cases this may act in synergy with Ang1 that is present[32,36,37]. It is tantalizing to suggest that the expression of Tie2 by mural cells and their precursors, and the ability of VEGF to upregulate Tie2 on these cells, renders them responsive to Ang1-mediated migration, leading to investiture of the endothelial tubule with mural cells. This stabilization makes endothelial cells unresponsive to further angiogenic cues and thereby terminates angiogenesis. Ang1-mediated stabilization of tumor blood vessels may therefore be desirable therapeutically to inhibit new vessel formation and thereby arrest tumor growth.

CONCLUSIONS

Researche on anti-angiogenesis as an anti-cancer approach has for some time focused on endothelial cells. It is not until recently that the role of mural cells has drawn more and more attention from cancer researchers, despite the fact that mural cells are an important component of the vascular wall, and their functions have been studied extensively in the cardiovascular field. It is plausible that the anti-angiogenesis effect of vascular stabilization in tumors may lead to new approaches to the development of cancer therapies.

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