Angiopoietin-2: Development of Inhibitors for Cancer Therapy

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Angiopoietin-2 (Ang2) is a member of the Ang family, which plays an important role in angiogenesis during the development and growth of human cancers. Ang2's role in angiogenesis generally is considered as an antagonist for Ang1, inhibiting Ang1-promoted Tie2 signaling, which is critical for blood vessel maturation and stabilization. Ang2 modulates angiogenesis in a cooperative manner with another important angiogenic factor, vascular endothelial growth factor A. Genetic studies have revealed that Ang2 also is critical in lymphangiogenesis during development. However, new evidence suggests more complicated roles for Ang2 in angiogenesis in physiologic processes and invasive phenotypes of cancer cells during progression of human cancers. This article discusses recent studies of Ang2 in angiogenesis and the implication of Ang2 as a therapeutic target as well as a potential inhibitor for antiangiogenesis treatment for cancer patients.

Introduction

It has been established that growth and progression of human tumors depend on pathologic growth of new blood vessels (neoangiogenesis). *Angiogenesis* generally refers to the growth of vessel sprouts from capillary blood vessels, including endothelial cell (EC) migration toward stimuli in the microenvironment, EC proliferation, tube formation, and intussusception, followed by recruitment of peri-ECs and synthesis of extracellular matrix (ECM) [1]. The process of tumor angiogenesis is orchestrated by an array of angiogenic stimulators and inhibitors. Within the tumor microenvironment, tumor cells and tumor-associated stromal cells, such as macrophages and other immune cells, produce angiogenic factors such as vascular endothelial growth factor (VEGF) and endogenous inhibitors such as thrombospondin-1 [1]. Angiopoietins (Ang), a family of angiogenic factors, have distinct functions in tumor angiogenesis and behavior. One Ang family member in particular, Ang2, has been the focus of studies not only because of its unique roles in modulating angiogenesis, but also because of some of its unconventional functions; the close link of Ang2 expression to tumor progression, growth, and invasion/metastasis; and its great potential as a therapeutic target, a prognostic marker, and an inhibitor of human cancers. This article reviews recent literature describing this special growth factor.

Ang2's Roles in Angiogenesis

Ang2 in physiologic angiogenesis and lymphangiogenesis
The Ang family has four members—Ang1, Ang2, Ang3, and Ang4—and two related receptors, Tie2 and Tie1 [2•]. In vivo, Ang1 and Ang2 have complementary functions in maintaining vascular function and integrity. Ang1 maintains the integrity of vasculature by recruiting peri-ECs into blood vessels [1]. Transgenic mice deficient in Ang1 had disrupted interactions between ECs and peri-ECs that severely impaired vascular function, leading to embryonic death of the mice [3,4]. Transgenic mice overexpressing VEGF-A in the skin induced hyperpermeable vessels. Moreover, vessels in Ang1-overexpressing mice were not leaky and were resistant to leaks caused by inflammatory agents [5]. Coexpression of Ang1 and VEGF-A had an additive effect on angiogenesis and resulted in leakage-resistant vessels, typical of Ang1-expressing vessels [6].

Initially, Ang2 was identified as an antagonist for Ang1 by inhibiting Ang1 activation of Tie2. Transgenic mice overexpressing Ang2 had phenotypes similar to those of Ang1-null mice, displaying disrupted interactions between ECs and peri-ECs that severely disrupted blood vessel formation in mouse embryos. In adult mice and humans, Ang2 is expressed only at the sites of vascular remodeling [7]. Ang2's major role in angiogenesis appears to be in cooperation with VEGF-A function. In the presence of VEGF-A, Ang2 promotes vascular sprouting and destabilizes blood vessels by disrupting interactions between ECs and peri-ECs, thus enhancing VEGF-A stimulation. In the

absence of VEGF-A, however, Ang2 acts as a suppressor that accelerates vessel regression [8]. This hypothesis was validated by a study using a model of a transient ocular microvessel network that surrounds the anterior part of the lens in the developing eye. In the presence of exogenous VEGF-A, Ang2 promoted a rapid increase in capillary diameter, remodeling of the basal lamina, and proliferation and migration of ECs and stimulated the sprouting of new blood vessels. When VEGF was inhibited in the system by soluble VEGF receptor-1, Ang2 induced EC apoptosis, resulting in vessel regression [9].

Ang2's role in developmental angiogenesis was further investigated by genetic knockout studies. Unexpectedly, mice lacking Ang2 exhibited major lymphatic vessel defects with no effect on vascular development. At birth, the vasculature in newborn Ang2-null pups was indistinguishable from that in wild-type pups. However, postnatal Ang2$^{-/-}$ mice had dramatic defects in subsequent vascular remodeling. In the eye, the hyaloid vasculature normally had largely regressed in wild-type and heterozygous mice by postnatal day 10. In contrast, the hyaloid vasculature was maintained aberrantly in Ang2-deficient mice at this age. This and other postnatal defects in the vasculature of Ang2-null mice revealed that Ang2 is absolutely required for the coupled vascular regression and sprouting events that comprise normal angiogenic remodeling of the ocular vasculature. Genetic rescue with Ang1 corrected the lymphatic but not the postnatal angiogenesis defects, suggesting that Ang2 acts as a Tie2 agonist in the former setting but as an antagonist in the latter [10]. Two new studies provide further details about these phenomena. In Ang2-deficient mice, lymphatic vessels fail to mature and do not exhibit a collecting vessel phenotype. Dermal lymphatic vessels in Ang2-null pups prematurely recruit smooth muscle cells and do not undergo proper postnatal remodeling. However, genetic rescue with Ang1 in Ang2-null mice restores a normal hierarchic lymphatic structure, suggesting that Tie2 activation is required for normal lymphatic development [11]. In wild-type mice, Ang2 exerts a dual role in scheduled hyaloid vascular EC death (vessel regression) in the eye. Ang1 stimulation of Tie2 activates Akt signaling and suppresses death of hyaloid vascular ECs. In contrast, Ang2 induces hyaloid vascular EC death by suppressing Akt in ECs and upregulates macrophage Wnt7b, a Wnt receptor ligand that associates with hyaloid vessels. Wnt7b stimulates cell cycle entry of vascular ECs and couples cell death. Because this second pathway is macrophage derived and Wnt7b dependent, genetically engineered Ang1 could not rescue the postnatal angiogenesis defects in Ang2-deficient mice [12]. These observations also explain sustained hyaloid vasculature in the eyes of Ang2-deficient mice [10].

In vitro, Ang2 was shown to be an antagonist and agonist for Tie2-mediated signaling, enhancing EC migration, survival, and tubelike structure formation [2,13]. Moreover,

stimulated release of endogenous Ang2 or overexpression of Ang2 in ECs destabilizes quiescent ECs through an internal autocrine loop, leading to EC detachment and a vessel regression phenotype in vitro and ex vivo. Such a perturbation could be rescued by introducing exogenous Ang1 and VEGF-A, but not a soluble Tie2 [14]. Ang2 also cooperates with Ang1 in modulating endothelial development from circulating cord blood CD34$^+$ progenitors. Endogenous Ang1 regulates initial EC commitment of the CD34$^+$ progenitor cells, whereas Ang2 contributes to expansion of the endothelial progeny [15]. Additionally, combining Ang2 and Ang1 directly activates ECs and neutrophils to promote a proinflammatory response through Tie2- and β$_2$ integrin–mediated signaling [16].

Ang2 in tumor angiogenesis

Ang2's role in tumor angiogenesis has been controversial [2•]. Upregulation of Ang2 correlates with the malignancy of various types of human cancers. Ang2 overexpression in various types of human cancer cell lines augmented tumor angiogenesis and growth in mice. In contrast, other studies reported that specific induction of Ang2 in gliomas, mammary carcinomas, and lung carcinomas inhibited tumor growth and metastasis [2•,17]. Systemic Ang2 overexpression led to unexpected massive tumor vessel regression within 24 hours, even without concomitant VEGF inhibition. By impairing pericyte coverage of the tumor vasculature, Ang2 induced massive tumor vascular regression while improving perfusion in surviving tumor vessels and transiently exacerbating tumor hypoxia, without affecting adenosine triphosphate levels. This significantly inhibited tumor angiogenesis, promoted tumor apoptosis, and suppressed tumor growth [18•]. Ang2 was shown to prevent ECs from forming a tubelike structure in vitro and to impair angiogenesis in a chorioallantoic membrane chicken model system. In a glioma xenograft model, sustained Ang2 expression disrupted blood vessel formation and inhibited glioma growth in mouse brain [19].

In addition to Ang2's role in tumor angiogenesis, its expression by tumor cells has been linked to invasive and metastatic phenotypes of gliomas and gastric, colon, prostate, and breast cancers [20,21•]. Recently, we reported that Ang2 is co-upregulated with matrix metalloprotease (MMP)-2, membrane type-1 MMP, and laminin-γ-2 in invasive areas but not in the central regions of clinical primary glioma specimens. Statistical analysis revealed a significant link between the preferential expression of these molecules and invasiveness [22]. Consonant with these features, intracranial xenografts of U87MG glioma cells engineered to express Ang2 were highly invasive into adjacent brain parenchyma compared with isogenic control tumors. In regions of Ang2-expressing tumors actively invading the brain, high levels of MMP-2 expression and increased angiogenesis also were evident. A link

between these two features became apparent when stable Ang2 expression by U87MG cells or treatment of several glioma cell lines with recombinant Ang2 in vitro caused MMP-2 activation and increased invasiveness. Conversely, MMP inhibitors suppressed Ang2-stimulated activation of MMP-2 and Ang2-induced cell invasion [23]. Our observations of Ang2 promotion of glioma cell invasion were supported by other studies in gliomas and gastric cancers and by our own results using various breast cancer xenograft models in animals [20,21•].

Ang2 Signaling and Modulation
Tie2-mediated signaling
Ang1 and Ang2 interact with their cognate receptor, Tie2, which is most frequently expressed in ECs. Upon binding Angs, the Tie2 receptor dimerizes and becomes phosphorylated in tyrosine residues at its C-terminal [24]. Activated Tie2 stimulates phosphatidylinositol 3-kinase (PI3K)-dependent activation of Akt, leading to increases in cell survival, suppression of caspase 3, 7, and 9 activity, and attenuation of mitochondrial-derived activator of caspase (Smac) release. Ang1 stimulates Tie2, activating the DokR-Pak, Nck, and Grb14 pathways; inhibiting EC proliferation; and displaying an antiangiogenic property [13]. Ang1 also upregulates cyclin D1 expression and activates mitogen-activated protein kinase (MAPK) and PI3K in murine IBE and human umbilical vein endothelial cells [25]. In addition, COMP-Ang1–induced vascular enlargement and blood flow are mediated mainly through Tie2 activation, and sustained Tie2 overexpression may participate in the maintenance of vascular changes [26]. The protective role of ECs from apoptosis by Ang1 or in synergy with VEGF-A has been widely observed in vitro, in vivo, and under various stress conditions [2•,17]. Ang2 has been found to be an antagonist as well as an agonist for Tie2 receptor signaling in modulating EC migration and survival [13]. However, unexpected data of Ang2 function in ECs have been documented. For example, Harfouche and Hussain [27] demonstrated that treatment of ECs with Ang2 (150–300 ng/mL) increases the phosphorylation of Tie2, Akt, extracellular signal–regulated kinase 1/2 (ERK1/2), and p38 members of MAPKs but inhibits JNK phosphorylation. When ECs were stimulated with a combination of Ang2 and VEGF, ERK1/2 activation also was inhibited. In contrast, augmentation of Ang1-induced ERK1/2 phosphorylation was triggered by VEGF treatment. Moreover, Ang2 treatment did not stimulate cell migration in vitro but significantly attenuated serum deprivation–induced EC apoptosis and promoted cell survival. In non-ECs that were found to express Tie2, such as human glioma cell lines, Ang2 inhibited VEGF-A expression at the mRNA and protein levels in Tie2-expressing glioma cells but not in Tie2-negative tumor cells. Ang2 regulated VEGF expression at the transcriptional level in relation to a decrease in hypoxia-inducible factor (HIF)-1α expression and HIF-DNA–binding activity. Tie2 silencing by small interfering RNA (siRNA) rescued the Ang2-mediated down-modulation of VEGF, suggesting an essential role for Tie2 in this regulatory loop [28].

Interactions with integrins
Although Ang2 modulates angiogenesis through interaction with the Tie2 receptor [29], it contains a highly conserved N-terminal fibrinogen-like receptor–binding domain that may form a functional association with certain integrin receptors [2•]. Ang1 and Ang2 were shown to stimulate Tie2-independent cell adhesion of ECs and fibroblasts to Ang1- or Ang2-coated surfaces through $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrin–mediated activation of ERK and focal adhesion kinase (FAK) signaling [30]. Skeletal myocytes lacking Tie2 adhere to Ang1- and Ang2-coated surfaces in a similar manner as to laminin, fibronectin, and vitronectin. The Ang-stimulated skeletal myocyte adhesion is mediated by integrin receptors such as $\alpha_5\beta_1$, activating ERK and FAK signaling and promoting cell survival [31]. It has been postulated that Ang's interaction with integrins likely occurs through the fibrinogen-like receptor–binding domain present in the Ang protein structure [32]. This theory was examined recently, showing that a monomeric Ang1 variant (ΔAng), composed only of the fibrinogen-like receptor–binding domain that also exists in Ang2, ligates Tie2 without activating the receptor. Moreover, ΔAng binds to $\alpha_5\beta_1$ integrin with an affinity similar to that of Tie2. When ECs were plated on ΔAng-coated surfaces, ΔAng displayed biologic effects similar to those of the full-length Ang1, stimulating cell adhesion, ERK signaling, and vascular maturation [33].

Modulation of EC function and angiogenesis by Ang1 through direct interaction with integrins also was demonstrated in ECs expressing the Tie2 receptor. Immobilized Ang1 was shown to selectively mediate $\alpha_5\beta_1$ integrin outside-in signaling, leading to cross-talk between Tie2 and $\alpha_5\beta_1$ and promotion of angiogenesis [34]. In tumor cells lacking Tie2 expression, we showed that Ang2 induces glioma cell invasion by stimulating MMP-2 expression through the $\alpha_v\beta_1$ integrin and FAK pathways [35•]. We also reported that Ang2 associates with $\alpha_5\beta_1$ integrin in Tie2-deficient breast cancer cells. Ang2 activates an integrin-mediated signaling pathway, leading to breast cancer cell invasion and metastasis. Inhibition of β_1 or α_5, but not other integrins, attenuates Ang2 modulation of integrin-linked kinase (ILK), Akt, glucose synthase kinase-3β, Snail, E-cadherin, and vimentin, and Ang2-stimulated breast cancer cell motility [21•]. Furthermore, similar to the association of Ang2 with integrins in skeletal myocytes [31] and of PG-MV/vesican with integrin in glioma cells [36], the association of Ang2 with integrins was highly calcium and manganese dependent in our system. These studies established Ang2's critical role in promoting tumor

cell invasion and metastasis by stimulating cell motility and invasion mediated by the $\alpha_v\beta_1$ integrin/FAK pathway in glioma cells and the $\alpha_5\beta_1$ integrin/ILK pathway in breast cancer cells independent of Tie2.

Regulation of Ang2 expression
Ang2 expression is regulated by multiple stimuli produced by tumors and their microenvironment. Tumor-derived VEGF increases Ang2 expression in tumor ECs in vivo and in vitro [20]. Insulin-like growth factor-1 and platelet-derived growth factor (PDGF) induce Ang2 expression at transcriptional and posttranslational levels in vascular smooth muscle cells [37]. Hypoxia (low oxygen tensions), which occurs frequently in human cancers, increases Ang2 mRNA expression and protein synthesis involving MAPK-, protein kinase C–, and cyclooxygenase-2–mediated pathways [2•,17]. In human umbilical vein ECs, Ang2 expression also is induced by tumor necrosis factor-α and thrombin through distinct pathways [38,39]. Estrogen was shown to regulate Ang2 expression in nonreproductive tissues such as heart, kidney, and lung in vivo [40]. In adipose tissues, leptin treatment of ECs induces Ang2 expression without a concomitant increase in VEGF. The leptin upregulation of Ang2 coincided with the initiation of apoptosis in adipose ECs, suggesting a causal effect of Ang2 upregulation in adipose tissue regression [41]. Recently, Yao et al. [42] reported that high glucose regulated Ang2 transcription in mouse kidney microvascular ECs indirectly through methylglyoxal modification of a transcription cosuppressor, mSin3A, resulting in increased recruitment of O-GlcNac transferase, with consequent increased modification of Sp3. The modified Sp3 lost its capacity to bind to a glucose-responsive GC box in the Ang2 promoter, leading to increases in Ang2 expression. Additionally, the forkhead box transcription factors FOXO1 and FOXC2 specifically regulate Ang2 expression. FOXO1, one of the major FOXO factors in ECs, inhibits EC tube formation and migration. Suppression of FOXO1 by siRNA significantly downregulates expression of Ang2 as well as several other angiogenic factors in ECs, such as PDGF, leading to enhanced angiogenic activity of these cells [43]. In adipose tissues of Ang2 transgenic mice, FOXC2 overexpression transcriptionally upregulated expression of Ang2 and other angiogenic factors. Increased Ang2 switched on an angiogenic phenotype by altering vessel patterning, remodeling, and maturation of adipose vasculature. Such vascular phenotypes could be reversed when Ang2 activity was blocked by an Ang2 inhibitor in 3-week-old FOXC2 transgenic mice [44].

Therapeutic Implications for Ang2
A close link between increased Ang2 expression and tumor progression, invasion, and metastasis in various types of human cancers has established Ang2's prognostic value in predicting cancer progression and clinical outcome [17,20,21•]. Indeed, in 69 patients with acute myeloid leukemia (AML), levels of circulating Ang2 were increased by an average of 3.57-fold compared with normal controls. In a univariate Cox proportional hazards model, higher Ang2 levels predicted poor survival in AML patients [45]. In a retrospective study of 131 patients with hepatocellular carcinoma (HCC), mean Ang2 serum levels were approximately 2.3-fold higher compared with the levels measured in 40 normal controls. The combination of Ang2 serum levels and α-fetoprotein, which is commonly used in screening for HCC, led to improved discrimination between HCC and cirrhosis, a liver disease often associated with HCC development [46]. In 136 patients with lung cancer, Ang2 serum levels were an average of approximately 1.61-fold higher than those in 40 controls. Levels of serum Ang2 correlate with those of VEGF in patients with lung cancer, especially non–small cell lung cancer (NSCLC), but not small cell lung cancer. Significantly, NSCLC patients with distant metastases had serum Ang2 levels higher than those in patients without metastases. The low-Ang2 group had a better overall survival rate than the high-Ang2 group [47]. Taken together, serum Ang2 levels may be a useful clinical marker for progression of human cancers and a predictor of survival in cancer patients.

Ang2's unique roles in angiogenesis in development and progression of various types of human cancers also make Ang2 an attractive therapeutic target. Two recent studies explored such options. In a rat corneal angiogenesis model, White et al. [48] developed an iterative in vitro selection strategy called *systematic evolution of ligand by exponential enrichment* (SELEX) to generate nuclease-resistant RNA ligands (aptamers) that bind Ang2 with high affinity, but not other related Tie2 agonists, such as Ang1 and basic fibroblast growth factor (bFGF). This aptamer specifically inhibits the binding activity of Ang2 to Tie2 and Ang2 stimulation of tyrosine phosphorylation of Tie2. When Ang2-specific aptamer was delivered together with bFGF as bilateral corneal micro-pockets (pellets) into rat corneas, bFGF-stimulated angiogenesis in the corneas was significantly inhibited. Using various models, including in vitro ECs, tumor xenografts, and rat cornea angiogenesis, Oliner et al. [49] developed a neutralizing anti-Ang2 antibody that specifically disrupts the interaction of Ang2 and Tie2 receptor. Treatment of mice bearing xenografts of human A431 epidermoid tumor and Colo205 colon cancer using the neutralizing anti-Ang2 antibody caused static growth and eventually regression of large established tumors by suppressing cell proliferation and inducing apoptosis of tumor-derived ECs. In rats, injection of this anti-Ang2 antibody inhibited VEGF-induced corneal angiogenesis in the eye. Lastly, Cao et al. [18•] and Lee et al. [28]

demonstrated that Ang2 also may be considered an inhibitor for suppression of tumor angiogenesis because systemic expression or overexpression of Ang2 attenuated VEGF expression in glioma cells expressing Ang2, impaired pericyte coverage of the tumor vasculature, inhibited EC proliferation, and induced EC apoptosis, resulting in a massive regression of tumor vasculature and tumor growth.

Conclusions

Accumulated studies on Ang2's role and therapeutic implications in angiogenesis using models of animal development, cell culture, and tumor xenografts, as well as clinical investigations, have depicted complicated functions of Ang2 in tumor angiogenesis and progression. It is well recognized that Ang2 regulates tumor angiogenesis in cooperation with VEGF as well as Ang1 through the Tie2-dependent pathways. In addition, Ang2 stimulates tumor angiogenesis, invasion, and metastasis through Tie2-independent pathways involving integrin-mediated signaling. Prognostic values of Ang2 expression in various types of human cancers and elevated Ang2 levels in the blood of cancer patients have established Ang2 as an attractive therapeutic target, and this was corroborated by recent studies using a neutralizing anti-Ang2 antibody. Ang2 also may be used to inhibit tumor angiogenesis and growth directly. However, the challenge of studying tumor angiogenesis is to understand the underlying mechanisms of the angiogenic process within the proper biologic context. As described in this article, the complexity of Ang2 functions and Ang2's regulation are highly cell context dependent. A better understanding of Ang2 functions in tumor angiogenesis and progression will lead to future advances in effective antiangiogenic and anticancer therapies.

Acknowledgments

We apologize that because of space limitations, we could not cite as many original research articles as we would have liked. However, all the information in this report is included in several recent reviews listed in "References and Recommended Reading."

Disclosures

No potential conflicts of interest relevant to this article were reported.

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