Angiopoietin-2 TIEs Up Macrophages in Tumor Angiogenesis

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Abstract

Angiopoietin-2 (ANG2), a ligand of the TIE2 receptor, modulates endothelial cell biology and destabilizes blood vessels to facilitate angiogenesis. Recent reports have shown that ANG2 inhibition, for example, by monoclonal antibodies, peptibodies, or CovX-Bodies, may achieve substantial antiangiogenic and antitumor responses in a variety of mouse tumor models, including spontaneous MMTV-PyMT mammary and RIP1-Tag2 pancreatic islet adenocarcinomas. There is also evidence that targeting the ANG2/TIE2 signaling pathway may inhibit the functions of TIE2-expressing macrophages (TEM), a tumor-associated macrophage subset endowed with proangiogenic activity in mouse tumor models. The clinical opportunities afforded by simultaneously targeting the effects of ANG2 on tumor angiogenesis and the proangiogenic activity of TEMs are discussed. Clin Cancer Res; 17(16); 5226–32. ©2011 AACR.

Background

The ANG-TIE system

Angiopoietins (ANG) are cytokines that play an important role in regulating both the quiescent and the angiogenic microvasculature. The ANG-TIE system consists of 2 cell-surface tyrosine kinase receptors (TIE1 and TIE2) and 3 ligands (ANG1, ANG2, and ANG3/ANG4). TIE2 is primarily expressed on endothelial cells (EC) and binds all the known ANGs, whereas TIE1 is an orphan receptor that can regulate TIE2 activity via heterodimerization with it (1).

ANG1 and ANG2 bind TIE2 with comparable affinity but elicit markedly different responses from ECs. ANG1 is primarily expressed by perivascular cells, such as pericytes and smooth muscle cells, and maintains EC survival and quiescence in mature blood vessels. Conversely, ANG2 is secreted by activated ECs in tissues undergoing vascular remodeling and promotes angiogenesis by antagonizing ANG1-mediated EC quiescence (1). In quiescent, nontransformed tissues, ANG2 levels are low and ANG1 binding to TIE2 signaling is favored (2).

Tie2-deficient embryos develop a primary capillary plexus, indicating that TIE2 is not required for EC proliferation and initial vascular network formation. However, the primary capillary plexus remains poorly organized and fails to form extensive branches, remodel, and mature, leading to retarded embryonic growth and lethality between E9.5 and E12.5 (3). The vascular defects in Tie2-deficient embryos consist primarily of impaired association of pericytes and smooth muscle cells with the newly formed vascular sprouts (4). Ang1-deficient embryos die in utero with a similarly defective vascular phenotype (5). On the other hand, ANG2 destabilizes the vasculature to promote angiogenesis in concert with other proangiogenic factors (2, 6, 7). Together, these data suggest that the ANG-TIE system functions mostly as a gatekeeper of the quiescent EC phenotype, with constitutive ANG1 signaling promoting EC survival/quiescence and vascular maturation, and acute ANG2 antagonism promoting EC sensitization to proangiogenic stimuli and consequent angiogenesis (1).

The molecular and cellular responses regulated by autocrine or paracrine ANG signaling in the ECs of the vascular and lymphatic system were reviewed recently (1, 8). However, it is still unclear how ANG1 and ANG2 signal different responses in ECs. ANG1 and ANG2 bind TIE2 with similar affinities and apparently to the same site (9, 10). Both ANGs are known to bind TIE2 as multimers. However, potential differences in the multimerization states of ANG1 and ANG2 are unlikely to account for their different effects on target cells, as both ANGs form similar high-order oligomers in solution (10). It is thus likely that surface coreceptors, such as integrins, exist that could transduce or modulate ANG1- and ANG2-specific signals in ECs (11, 12) and, possibly, other cell types. Nevertheless, the concept that ANG2 may function as an antagonist for ANG1 is supported by the observation that unlike ANG1, ANG2 stimulates only weakly the tyrosine kinase activity of Tie2 in cultured ECs. Furthermore, the phenotypes of Ang1-deficient and Ang2-overexpressing mice are similar (5, 6). However, it is now clear that ANG2 can function as a partial TIE2 agonist under certain experimental conditions, for...
example, at high concentrations and/or in the absence of ANG1 (1). Contrary to quiescent organs, tumors often express high levels of ANG2, suggesting that ANG2-TIE2 signaling may prevail over ANG1-TIE2 signaling in the highly angiogenic tumor-associated vasculature (2). In the absence of ANG1, or upon acute release of ANG2 from activated ECs (13), pericytes disengage from ECs and the blood vessels become exposed to proliferation signals mediated by vascular endothelial growth factor (VEGF) and other proangiogenic factors (2, 7). Thus, the net outcome of ANG2-induced EC activation depends on the local cytokine milieu. In the presence of VEGF, ANG2 enables EC migration, proliferation, and the sprouting of new blood vessels (angiogenesis). When VEGF is absent, ANG2 leads to EC death and vessel regression (7).

The role of ANG2 in tumor angiogenesis and its significance as a therapeutic target remain controversial and poorly defined. In agreement with its purported proangiogenic role, the pharmacologic blockade of ANG2 using ANG2-specific inhibitors delayed the growth of s.c. tumor models to various extents in different studies, often by decreasing intratumoral vascular density (14–18). Our recent studies further indicate that ANG2 neutralization by an ANG2-specific, monoclonal antibody (15) has profound antiangiogenic and antitumor effects in both spontaneous and metastatic tumor models, irrespective of whether the treatment is administered to early- or late-stage tumors (19). However, tumors implanted s.c. in C57/Bl6/Angpt2-deficient mice (20) showed no alteration in tumor growth during the initial growth phase, suggesting that ANG2 may be dispensable for angiogenesis and growth in established tumors (21). Although the reasons for these discrepancies are currently unclear, we cannot exclude the possibility that the genetic knockout of Angpt2 may elicit a compensatory adaptation in C57/Bl6 mice.

TIE2-expressing monocytes/macrophages

In addition to ECs, some monocytes/macrophages express TIE2, and thus are called TIE2-expressing monocytes/macrophages (TEM). TIE2 is weakly expressed by circulating resident monocytes but is upregulated upon their homing to tumors and differentiation into a subset of perivascular macrophages (22–27). Such perivascular TEMs have been identified in a variety of mouse tumor models, including intracranial gliomas (23), MMTV-PyMT spontaneous mammary carcinomas (19, 24), and RIP1-Tag2 pancreatic islet adenocarcinomas (19, 23). The association of TEMs with tumor blood vessels suggests that these cells may cross-talk with ECs to provide paracrine support to the angiogenic vasculature. This has been inferred primarily from TEM-specific depletion studies showing that the absence of TEMs from tumors markedly impairs angiogenesis and delays tumor growth (22, 23). Furthermore, TEMs isolated from mouse mammary tumors display a distinctive gene expression signature that is consistent with enhanced proangiogenic activity and lower proinflammatory activity compared with TIE2-negative tumor macrophages (25). In a variety of mouse tumor models, TEMs express higher levels of mannose receptor C type 1 (MRC1), hemoglobin/haptoglobin scavenger receptor CD163, and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1) than do TIE2-negative, classic tumor macrophages (19, 25).

In the following sections, we outline the role of ANG2 in regulating the phenotype and functions of TEMs. We then discuss the therapeutic implications of targeting ANG2 in tumors to inhibit both the tumor-associated vasculature and prevent the insidious proangiogenic and protumoral functions of TEMs.

ANG2 regulates leukocyte extravasation in inflamed tissues

Studies have shown that leukocyte adhesion and extravasation are impaired in C57/Bl6/Angpt2-deficient mice challenged with experimental peritonitis (28). In vitro experiments further showed that ANG2 promotes leukocyte adhesion to ECs by enhancing the expression of the adhesion molecules intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) on ECs in the presence of the proinflammatory cytokine TNFα. Conversely, ANG1 limits the transmigration of circulating leukocytes by inhibiting NF-kB activation in ECs (29). Although these studies did not address tumor angiogenesis, the data indicate that ANGs modulate leukocyte extravasation via their effects on ECs, and further suggest that they may regulate monocyte infiltration in tumors.

ANG2 activates TIE2 in TEMs and enhances their proangiogenic activity

ANG2 induces TIE2 phosphorylation in cultured human monocytes and increases their expression of the proangiogenic genes thymidine phosphorylase and cathepsin-B (26). Furthermore, a medium conditioned by ANG2-stimulated, TIE2+ monocytes induced significantly more EC sprouts and tubules in EC-spheroids and human umbilical vein endothelial cell monolayers, respectively, compared with medium from unstimulated cells. In addition, the use of a thymidine phosphorylase inhibitor showed that this enzyme mediated, in part, the proangiogenic activity of ANG2-stimulated, TIE2+ monocytes (26). Such studies indicate that ANG2 can function as a TIE2 agonist on human TIE2+ monocytes and enhances their proangiogenic activity in vitro. Furthermore, they suggest that cell-to-cell contacts are not essential for TEMs to exert their proangiogenic activity on ECs, at least not in in vitro assays.

ANG2 overexpression in tumors enhances TEM infiltration

Lewis lung carcinomas grown in transgenic mice that overexpress ANG2 specifically in ECs contain more TEMs than tumors grown in wild-type mice (26). This is consistent with cell migration/invasion assays that showed that human TIE2+ monocytes, but not their TIE2-negative
counterparts, responded to ANG2 stimulation in vitro (30, 31), and this effect was abrogated by an anti-TIE2 blocking antibody (31). However, the enhanced TEM infiltration in ANG2-overexpressing tumors could be due to either direct chemoattraction of TEMs or indirect effects via changes in the tumor-associated vasculature induced by ANG2. Indeed, ANG2 overexpression slightly increased microvesSEL density, decreased pericyte coverage of the blood vessels, and enhanced hypoxia in the tumors (26). These changes may have augmented monocyte extravasation, attraction, and/or retention in the tumor microenvironment in a TIE2-independent manner, as implied by previous studies (28). Of note, the frequency of all CD45⁺ leukocytes was increased in the ANG2-overexpressing tumors (26).

**ANG2 blockade inhibits tumor angiogenesis and growth while impeding TEMs’ upregulation of Tie2**

As mentioned above, there is increasing evidence that ANG2-specific inhibition produces remarkable antiangiogenic and antitumor effects in mouse tumor models (14–19). Two recent studies further showed that ANG2-specific blockade in tumors reduces Tie2 expression by tumor-infiltrating macrophages/myeloid cells (18, 19). Huang and colleagues (18) developed and characterized a new class of biotherapeutics referred to as CovX- Bodies, which are created by chemical fusion of a peptide and a carrier antibody scaffold. An ANG2-specific CovX-Body was then tested in tumor xenograft studies. The authors found that ANG2 blockade reduced ANG2 protein levels in tumors by approximately 50%, and that this was accompanied by a reduction in tumor microvesSEL density and significant inhibition of tumor growth. Of interest, the ANG2-specific CovX-Body reduced tumor infiltration by Tie2-expressing myeloid cells (18). Based on these findings, the authors speculated that ANG2 blockade may induce antiangiogenic effects in tumors that are achieved, at least in part, via the inhibition of proangiogenic TEM recruitment.

We recently showed that ANG2 neutralization by a fully humanized, ANG2-specific monoclonal antibody (15) regresses the tumor vasculature and inhibits the progression of MMTV-PyMT mammary carcinomas and RIP1-Tag2 islet insulinsomas (19). In the mammary tumors, vascular regression was accompanied by enhanced intratumor hypoxia and, unexpectedly, increased recruitment of MRC1⁺ TEMs. The latter effect may be mediated by the hypoxic upregulation of stromal cell–derived factor-1 (SDF1, or CXCL12; ref. 19), which is a potent TEM chemoattractant in various tumor models (32–34). We further showed that, following ANG2 depletion, the tumor-infiltrating MRC1⁺ TEMs expressed significantly lower Tie2 mRNA levels than their counterpart in untreated mammary tumors (19). This could explain why Huang and colleagues (18) detected significantly fewer Tie2⁺ myeloid cells (i.e., TEMs) in CovX-Body–treated tumors.

Because in our study the MRC1⁺ TEMs recruited to ANG2-depleted mammary tumors expressed much lower Tie2 levels than those of untreated tumors (19), it is possible that EC-derived ANG2 stimulates Tie2 expression on perivascular TEMs at the transcriptional level. Although it cannot be excluded that Tie2 upregulation in TEMs is mediated indirectly by ANG2, ANG2 blockade specifically modulated the Tie2 mRNA among several genes analyzed in TEMs, suggesting that this response is intimately linked to Tie2 signaling (19). Of note, several growth factors, including ANGs, can regulate the expression of their receptor tyrosine kinases at the transcriptional level via autoregulatory feedback loops (35).

**ANG2 blockade in tumors, or Tie2 knockdown in TEMs, impedes TEM association with tumor blood vessels**

In both MMTV-PyMT and RIP1-Tag2 tumors, ANG2 blockade impeded the association of MRC1⁺ TEMs with blood vessels (19). We also observed a lack of TEM-EC association in MMTV-PyMT tumors after a conditional knockdown of Tie2 in hematopoietic-lineage cells (19). Together, these findings suggest that ANG2-Tie2 signaling, or the upregulation of Tie2 receptors on the surface of TEMs per se, is important for enabling TEM interactions with angiogenic blood vessels. This supports the tentative model that activated ECs in tumors release ANG2, which in turn induces TEMs to associate with sprouting blood vessels via (1) TEM upregulation of Tie2 and (2) the formation of EC-TEM cell-to-cell contacts mediated by Tie2 and ANG2 (Fig. 1). It should be noted that recent studies demonstrated that ANGs mediate the formation of homotypic Tie2-Tie2 complexes at endothelial cell-to-cell contacts (36). It is tentatively speculated that ANG2 similarly mediates homotypic Tie2-Tie2 complexes between ECs and TEMs in angiogenic tissues. In this regard, other studies have suggested that, during embryonic development, Tie2⁺ hematopoietic cells (e.g., macrophages) physically interact with Tie2⁺ ECs to promote blood vessel formation (37, 38). The ability of Tie2 to interact with distinct coreceptors, including Tie1 and several integrins (11, 12), may account for cell-type–specific effects of ANGs on ECs and macrophages.

**ANG2 enhances the immunosuppressive activity of TEMs**

In addition to promoting angiogenesis, TEMs may contribute to generate an immunosuppressive environment in experimental tumors via their responses to ANG2 (26, 39). ANG2 was found to augment the expression of the immunosuppressive cytokine interleukin-10 (IL-10), and a chemokine for regulatory T cells (Treg), CCL17, by TEMs in vitro (26). Further studies showed that TEMs express higher levels of IL-10 than do Tie2-negative macrophages in mouse tumors, and that ANG2-stimulated release of IL-10 by human Tie2⁺ monocytes suppresses T-cell proliferation and promotes the expansion of CD4⁺CD25⁺FOXP3⁺ Tregs in vitro (39). These interesting observations were verified in mouse tumors overexpressing ANG2 (39), suggesting that ANG2 may stimulate TEM to acquire an immunosuppressive function in tumors.
ANG2 blockade: an antiangiogenic strategy that limits tumor metastasis

There is growing experimental evidence that ANG2 may represent a promising target for effective antiangiogenic and antitumor therapy (40). Our recent studies showed that specific ANG2 neutralization regresses the tumor-associated vasculature, augments pericyte coverage of the remaining blood vessels, and induces durable antitumor responses in spontaneous tumor models. Furthermore, ANG2 blockade suppressed the metastatic spread of spontaneous MMTV-PyMT mammary tumors, as well as the growth of preestablished pulmonary metastases (19). These findings suggest that ANG2 blockade, at variance with certain forms of VEGF inhibition (41–43), does not enhance tumor malignancy and instead suppresses metastasis in the mouse tumor models tested.

The mechanistic basis for the reduced rate of metastasis in ANG2-depleted mammary tumors may be 3-fold. First, ANG2 blockade may limit tumor cell intravasation at the primary tumor site by decreasing the numbers of tumor blood vessels and augmenting their coverage by pericytes, which can limit tumor cell dissemination (44). The enhanced pericyte coverage of blood vessels in ANG2-depleted tumors (19) may result from the increased ANG1/ANG2 ratio in the tumor microenvironment. Second, ANG2 blockade displaced MRC1+ macrophages from the blood vessels (19). Based on previous studies showing that perivascular macrophages assist tumor cells during their intravasation in mammary tumors (45, 46), it is possible that ANG2 blockade may have decreased metastasis by impeding macrophage-assisted tumor cell intravasation in the primary tumors. Third, ANG2 blockade may directly inhibit metastasis outgrowth by impairing angiogenesis.

ANG2 blockade: clinical development

Distinct ANG2 inhibitors have been tested in preclinical mouse tumor models and are currently under clinical development or testing (see ref. 40 for a timely review). AMG-386, a peptibody that targets both ANG1 and ANG2 (47), reduced tumor blood flow (measured by dynamic contrast-enhanced magnetic resonance imaging) while inducing some antitumour responses in a dose-escalation study of patients with advanced solid tumors (48). In a phase I combination trial with 3 distinct chemotherapeutic treatments, AMG-386 plus chemotherapy achieved complete and partial responses in some patients with advanced solid tumors (49). Nevertheless, clinical data are not yet

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Figure 1. Modeling the effects of ANG2 inhibition on angiogenesis and TEMs in mouse mammary tumors. In mature blood vessels, ANG1 is constitutively expressed by perivascular cells, such as pericytes and smooth muscle cells, and promotes EC quiescence by interacting with TIE2 on ECs. In these conditions, ECs release little or no ANG2 (not shown). A, in growing tumors, ECs proliferate and form new blood vessels. This process is accompanied by the dissociation of pericytes from, and association of TEMs with, the sprouting ECs. B, at sites of sprouting angiogenesis (inset in A and B), activated ECs upregulate and release ANG2 (which competes with ANG1 for binding to TIE2) in the peri-EC spaces, making it available for binding to TIE2 on ECs and perivascular TEMs. The net outcome of the process, which occurs in the presence of VEGF and other proangiogenic factors, is stimulation of angiogenesis. C, ANG2-specific inhibition leads to regression of tumor blood vessels. This is accompanied by enhanced pericyte coverage of, and displacement of TEMs from, the remaining blood vessels. The former effect may be mediated, at least in part, by ANG1, which outcompetes ANG2 for binding to TIE2. Note that TEMs express much lower Tie2 levels in ANG2-depleted tumors than in untreated tumors, suggesting that ANG2 directly stimulates Tie2 expression by TEMs.
available for ANG2-specific inhibitors that, given the opposing and context-dependent functions of the 2 ANGs in preclinical models, may produce substantially different antitumor responses in cancer patients compared with those seen with ANG1/ANG2 bispecific inhibitors (14, 16, 17). However, only clinical trials will establish whether any of the ANG2-specific inhibitors currently under preclinical or clinical development (40) have the marked antitumor activity predicted by studies in mouse tumor models (15, 18, 19).

**Blocking TEMs to enhance tumor responses to conventional anticancer therapies**

Several recent reports have provided compelling evidence that damaging the tumor-associated vasculature (e.g., by using distinct angiogenesis inhibitors, vascular-disrupting agents, or radiation therapy) may enhance tumor hypoxia and upregulate the expression of several myeloid cell chemokine receptors, which attract proangiogenic myeloid cells into the treated tumors (27, 32–34, 50–52). Because myeloid cells are a major source of various proangiogenic factors in tumors, anticancer therapies that also enhance myeloid cell recruitment to the treated tumors should be combined with drugs that concomitantly target the tumor-infiltrating myeloid cells.

In this regard, we recently showed that blood vessel collapse, increased hypoxia, and hemorrhagic necrosis in murine mammary tumors treated with the vascular disrupting agent combretastatin A4 phosphate (CA4P) were accompanied by elevated tumor levels of SDF1 and enhanced tumor infiltration by CXCR4+ TEMs (34). Of interest, inhibiting TEM recruitment to CA4P-treated tumors by either interfering pharmacologically with the SDF1/CXCR4 axis (using AMD3100) or genetically depleting TEMs in tumor-bearing mice (22) markedly increased the efficacy of CA4P treatment. These data suggest that TEMs prevent the full antitumor activity predicted by studies in mouse tumor models (15, 18, 19).

**References**


The pharmacologic inhibition of the SDF1/CXCR4 axis prevented their influx to irradiated tumors and inhibited postirradiation development of a functional tumor vasculature, resulting in abrogation of tumor relapse (33).

A recent report showed that vaccination with irradiated, autologous tumor cells engineered to secrete granulocyte-macrophage colony stimulating factor and antibody blockade of cytotoxic T lymphocyte–associated antigen-4 triggered a tumor vasculopathy in some long-term responding patients with solid cancer (53). These reactions were associated with important humoral reactions against multiple angiogenic cytokines, including ANG2. Of note, the sera of these patients inhibited ANG2-induced TEM migration in vitro, suggesting the intriguing scenario that the impeded recruitment of TEMs to the tumors of long-term responding patients (i.e., patients with durable antitumor responses and/or increased survival) may have contributed to the observed immunontherapeutic responses (53).

In conclusion, increasing data suggest that the ANG2-TIE2 axis regulates the protumoral functions of TEMs in a number of preclinical mouse tumor models. Because TEMs are known to convey protumoral and proangiogenic programs that can counteract the efficacy of both antivascular and radiation treatments, the combined targeting of angiogenic ECs and proangiogenic TEMs by selective ANG2/TIE2-pathway inhibitors may extend the reach of vascular-targeting therapies in cancer patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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