Systemic Overexpression of Angiopoietin-2 Promotes Tumor Microvessel Regression and Inhibits Angiogenesis and Tumor Growth

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Abstract

Angiopoietin-2 (Ang-2) is a conditional antagonist and agonist for the endothelium-specific Tie-2 receptor. Although endogenous Ang-2 cooperates with vascular endothelial growth factor (VEGF) to protect tumor endothelial cells, the effect on tumor vasculature of high levels of exogenous Ang-2 with different levels of VEGF has not been studied in detail. Here, we report that systemic overexpression of Ang-2 leads to unexpected massive tumor vessel regression within 24 h, even without concomitant inhibition of VEGF. By impairing pericyte coverage of the tumor vasculature, Ang-2 destabilizes the tumor vascular bed while improving perfusion in surviving tumor vessels. Ang-2 overexpression transiently exacerbates tumor hypoxia without affecting ATP levels. Although sustained systemic Ang-2 overexpression does not affect tumor hypoxia and proliferation, it significantly inhibits tumor angiogenesis, promotes tumor apoptosis, and suppresses tumor growth. The similar antitumoral, antiangiogenic efficacy of systemic overexpression of Ang-2, soluble VEGF receptor-1, and the combination of both suggests that concomitant VEGF inhibition is not required for Ang-2–induced tumor vessel regression and growth delay. This study shows the important roles of Ang-2–induced pericyte dropout during tumor vessel regression. It also reveals that elevated Ang-2 levels have profound pleiotropic effects on tumor vessel structure, perfusion, oxygenation, and apoptosis. [Cancer Res 2007;67(8):3835–44]

Introduction

Because continued tumor growth highly depends on angiogenesis (1), a small degree of vascular destruction may translate into large-scale tumor kill by shutting off crucial blood supplies and depriving the tumor of oxygen and nutrients (2). Recent successes of the landmark phase III antiangiogenic clinical trial of bevacizumab aroused strong interests in exploring novel therapeutic molecules and strategies targeting tumor vasculature (3). Most antiangiogenic therapies have focused on inhibition of individual proangiogenic factors, such as vascular endothelial growth factor (VEGF). Although disrupting a single proangiogenic signal has met with some successes, an increasing body of evidence shows that this strategy is challenged by the existence of multiple redundant proangiogenic factors in tumors that may salvage tumor endothelial cells from death. One strategy to overcome this therapeutic obstacle is to inhibit as many proangiogenic factors as possible. Alternatively, the other strategy is to overexpress antiangiogenic factors to destroy tumor vasculature. Theoretically, if the rate of tumor vessel destruction exceeds the rate of angiogenesis, the overall tumor vasculature would regress and the devascularization may inhibit tumor growth.

The angiopoietin/Tie-2 family plays an important role in regulating vessel stability (4). Of the four identified angiopoietins (Ang-1 to Ang-4), Ang-1 and Ang-2 are the most studied (5). Ang-1, the primary physiologic ligand for the Tie-2 receptor, promotes angiogenesis, recruits pericytes, and stabilizes vessels (6). Ang-2 shows context-dependent proangiogenic and antiangiogenic activities. Ang-2 was first identified as a natural antagonist for Tie-2 that disrupts in vivo angiogenesis (7). Studies of Ang-2 knockout animals showed that Ang-2 is not required for embryonic vascular development but is needed for postnatal angiogenic remodeling (8). Ang-2 is only up-regulated at sites of active vascular remodeling, which involves vessel destabilization and regression (7). Transgenic overexpression of Ang-2 impairs vasculogenesis and angiogenesis, which is similar to the phenotypes of animals deficient in proangiogenic factors or their receptors, such as VEGF, VEGF receptor (VEGFR), Ang-1, and Tie-2 (8–11). Therefore, it is interesting to investigate whether high systemic Ang-2 levels may impair tumor vasculature and angiogenesis. The rationale of systemic Ang-2 overexpression is different from previous paracrine models using transduced Ang-2–secreting tumor, in which endothelial cells adapted to local high levels of Ang-2 from the beginning of tumor establishment. These Ang-2–transduced tumors showed different patterns of angiogenesis and growth. Ang-2–transduced HT-29 colon adenocarcinoma xenografts showed enhanced angiogenesis and proliferation (12). However, Ang-2 overexpression did not stimulate the growth of mouse BNL hepatocellular carcinoma unless VEGF was simultaneously up-regulated (13). In contrast, Ang-2–overexpressing Lewis lung carcinoma and TA3 mammary carcinoma showed disrupted angiogenesis, enhanced apoptosis, and suppressed growth and metastasis (14). Although these discrepancies might be due to the different interactions between Ang-2 and other growth factors, it is generally believed that Ang-2 destabilizes tumor vasculature (15). Several recent studies emphasize the complementary and

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coordinated roles of Ang-2 and VEGF during angiogenesis (16). Lobov et al. (17) showed that VEGF converts Ang-2 from an antiangiogenic to a proangiogenic stimulus. On one hand, in the presence of VEGF, Ang-2 promotes endothelial proliferation and migration, which facilitates the remodeling of neovascularization. Because tumor hypoxia up-regulates both VEGF and Ang-2 (18) and because VEGF itself also stimulates Ang-2 expression (19), concomitant up-regulation of Ang-2 and VEGF has been found in many tumors (19, 20). On the other hand, in the absence of significant levels of VEGF, Ang-2 leads to endothelial apoptosis and vessel regression (10, 17). Recent clinical studies show that high Ang-2 levels with low VEGF levels correlate with improved long-term prognosis in non–small cell lung cancer (21) and acute myelogenous leukemia (22).

Structural and functional abnormalities are hallmarks of tumor vessels (4). The instability and vulnerability of tumor neovascularization may provide a targeting advantage for Ang-2 treatment because new vessels are most sensitive to elevated Ang-2 levels (10). Therefore, we hypothesized that systemic Ang-2 overexpression may inhibit tumor growth by selectively destroying tumor vasculature. To determine whether concomitant anti-VEGF therapy is required for Ang-2–induced tumor vessel regression, we systemically overexpressed truncated soluble mouse VEGFR-1 to neutralize VEGF. We characterized the time course of morphologic, cellular, and functional changes in the tumor vasculature of HCT116 human colon adenocarcinoma xenografts in response to systemic overexpressions of Ang-2, soluble VEGFR-1 (sVEGFR-1), and a combination of both. We also investigated the relationship between vascular changes, tumor vessel perfusion, hypoxia, and bioenergetics. We found that single-dose and multiple-dose Ang-2 treatments have different effects on tumor hypoxia. Finally, we studied the pleiotropic effects of sustained systemic Ang-2 overexpression on tumor angiogenesis, apoptosis, proliferation, and growth.

Materials and Methods

Adenoviral vectors. Adenoviral vectors were first-generation E1-deleted replication-defective vectors made by AdEasy system. Gene expression of the protein/peptide of interest was driven by human cytomegalovirus immediate/early promoter. Empty adenoviral vector was used as control (AdControl). AdAng-2 encodes mouse Ang-2. AdsVEGFR-1 encodes a soluble mouse VEGFR-1 (the first three IgG loops of the extracellular domain of mouse VEGFR-1) with 6× His tags.

Cell lines. HEK293 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotic and antymotic solution. HCT116 cells were cultured in McCoy5A medium with 10% FBS and 1% antibiotics/serum (FBS) and 1% antibiotic and antymotic solution. HCT116 cells were immediately excised and snap frozen. In the tumorgrowth delay study, adenoviruses were injected every other day until day 6. We used 5 × 108 IFU of AdControl to equalize the total viral particle number (1 × 109 IFU/animal). Total tumor vascular length and red fluorescent tumor area were measured with Scion Image. Total tumor vascular length density (total tumor vascular length / tumor area) was normalized to its value at day 0 in each animal.

In vivo tumor studies. HCT116 cells (1 × 106) were s.c. injected into the mouse right flank. When the tumor volume reached 40 to 60 mm3 (length × width / height / 2), animals were randomized into four groups. Forty-eight hours after a single-dose adenoaviral administration, animals were injected with Hoechst 33342 dye (20 mg/kg i.v.) 2 min before sacrifice. The tumors were immediately excised and snap frozen. In the tumor growth delay study, adenoviruses were injected every other day until day 16. Tumor volumes were measured every 2 to 3 days. Pimonidazole was injected as described (23). Tumor tissues were formalin fixed and embedded in paraffin.

Ex vivo ATP bioluminescence. ATP bioluminescence was carried out as described (24).

Immunohistochemistry and analysis. Tumor vasculature in 10-µm cryosections was detected by fluorescein-labeled Griffonia (Bandeiraea) simplicifolia lectin I (isolectin B4) staining (1:200 dilution; Vector Laboratories, Burlingame, CA). Five to eight random fields in each cryosection (three sections per tumor, five tumors per group) were analyzed. Pericites were detected with a rabbit anti-Ng2 polyclonal antibody (Chemicon International, Temecula, CA) and a goat anti-rabbit IgG conjugated with Alexa 594 (Invitrogen, Carlsbad, CA). Confocal microscopic images of three to five random fields were taken in each section (three sections per tumor, six tumors per group). Colocalized and noncolocalized areas of stained pericytes and endothelial cells were analyzed using Photoshop. Carboxy anhydrase IX (CA IX) was detected with a rabbit polyclonal antibody against human CA IX (five tumors per group; 1:200 dilution; AbCam, Cambridge, MA). Tumor vessels in 10-µm paraffin sections were detected by a rat monoclonal antibody against mouse CD34 (clone MECA-44; AbCam). Vessels at the hotspots in five fields per tumor were counted. Cell apoptosis was detected by DeadEnd colorimetric terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) system (Promega, Madison, WI). Apoptotic index was quantified as the percentage of TUNEL-positive area in each field. Cell proliferation was detected with a monoclonal mouse anti-human Ki-67 antibody (clone Ki-67; DAKO, Carpinteria, CA) with DAKO animal research kit to exclude nonspecific staining. Ki-67 labeling index was quantified as the percentage of Ki-67–positive nuclei in each field. Tumor hypoxia was detected by Hypoxprobe-1 kit (23). The hypoxic fraction of necrotic tumor tissue was calculated as the percentage of pimonidazole–positive area per field. All slides were counterstained with hematoxylin. Omission of the primary antibody served as a staining control. Five tumors in each group were analyzed.

Statistics. Groups were first tested for normality and variance homogeneity. Student’s t test was applied for comparison of two means and one-way ANOVA test for pairwise multiple comparisons. When variance was not homogeneous, the nonparametric Kruskal-Wallis test was applied. Tukey test was applied for microscopic results and Student-Newman-Keuls (SNK) test for nonmicroscopic results. Differences were considered significant when P < 0.05.

Results

Systemic Ang-2 overexpression by single-dose adenoviral treatment causes tumor vessel regression. To systemically overexpress Ang-2 and to neutralize VEGF, we synthesized adenoviral vectors encoding mouse Ang-2 (AdAng-2) and 6× His-tagged
soluble truncated mouse VEGFR-1 (AdsVEGFR-1). We used an empty adenoviral vector as the negative control (AdControl). Western blots showed strong expression of Ang-2 and sVEGFR-1 in vitro (Fig. 1A). Single-dose i.v. administration of AdAng-2 and AdsVEGFR-1 significantly increased the plasma levels of Ang-2 and sVEGFR-1 (Fig. 1B and C). Because the levels of sVEGFR-1 in circulation were sufficient to block VEGFR activity as described before (25, 26), the AdsVEGFR-1 treatment also served as a positive control for antiangiogenic therapy. We selected HCT116 human colon carcinoma as the tumor model because HCT116 cells do not endogenously express Ang-2 under normoxic conditions (18). To distinguish the tumor vasculature from the vessels in surrounding normal tissues, we established an HCT116 cell line that constitutively expressed RFP. Mouse dorsal skin fold window chambers permitted continuous noninvasive observation of tumor vascular responses. Using intravital microscopy, we found intensive angiogenesis with continued growth of HCT116RFP tumors 10 days after tumor inoculation (day 0). At that time, we i.v. injected a single-dose of AdControl, AdAng-2, AdsVEGFR-1, or the combined AdAng-2+AdsVEGFR-1. AdControl treatment did not interfere with tumor angiogenesis as shown by the continued formation of new vessels from the preexisting vasculature. In contrast, the AdAng-2

Figure 1. Effects of adenoviral treatments on tumor vasculature. A, Western blots show the expression of mouse Ang-2 and 6×His-tagged sVEGFR-1 in conditioned medium of infected HEK293 cells. B, mouse plasma Ang-2 was quantified by ELISA 48 h after a single-dose of AdControl, AdAng-2, AdsVEGFR-1, or the combined AdAng-2+AdsVEGFR-1 treatment. Columns, median of mouse plasma Ang-2 concentration; bars, SE. C, mouse plasma sVEGFR-1 was quantified by ELISA 48 h after a single-dose of AdControl, AdAng-2, AdsVEGFR-1, or the combined AdAng-2+AdsVEGFR-1 treatment. Columns, median of mouse plasma sVEGFR-1 concentration; bars, SE. * P < 0.05, all ELISA results were analyzed by Kruskal-Wallis one-way ANOVA on ranks and SNK test, n = 6. D, AdAng-2–induced vessel regression in HCT116RFP window chamber tumors. Red fluorescence shows the tumor region. Arrows, new capillaries formed from preexisting tumor vessels (angiogenesis); arrowheads, dilated tumor vessels after AdAng-2 treatment. Asterisk, tumor capillary plexus regressed after adenoviral treatments. Bar, 0.5 mm. E, time course study of the relative tumor vascular length density in HCT116RFP window chamber tumors treated with AdControl, AdAng-2, AdsVEGFR-1, or the combined AdAng-2+AdsVEGFR-1. Points, relative tumor vascular length density; bars, SE. *, P < 0.05, one-way ANOVA followed by Tukey test, n = 6.
treatment was sufficient to cause massive tumor vascular regression without concomitant VEGF neutralization (Fig. 1D). Vascular destruction occurred in the red fluorescent tumor regions within the first 24 h after AdAng-2 treatment (Fig. 1D, asterisk). There was no apparent vascular regression in adjacent normal tissues. Many surviving tumor vessels became dilated, whereas the vessels in adjacent normal tissues did not show any apparent change in diameter. These results show that tumor vasculature is more sensitive to systemic Ang-2 overexpression than vessels in normal tissues. Administration of AdsVEGFR-1 or the combined AdAng-2+AdsVEGFR-1 also inhibited tumor angiogenesis compared with AdControl treatment (Fig. 1D).

To determine the time course of tumor vasculature remodeling in response to the treatments, we quantified relative tumor vascular length density by normalizing tumor vascular length density at each time point to the measurement on day 0. The AdAng-2, AdsVEGFR-1, and the combined treatment significantly inhibited tumor angiogenesis compared with the AdControl treatment, as shown by continued decrease in relative tumor vascular length density (Fig. 1E). There was no significant difference in relative tumor vascular length density between AdAng-2, AdsVEGFR-1, and AdAng-2+AdsVEGFR-1 groups. These results suggest that systemic Ang-2 overexpression does not require concomitant anti-VEGF treatment to induce tumor vessel regression.

**Ang-2 treatment impairs the association between tumor pericytes and endothelial cells.** Our window chamber study showed that many tumor vessels that survived the Ang-2 treatment were dilated (Fig. 1D, arrowheads). Previous studies suggest that the loss of pericytes is one of the causative factors for capillary dilation (27) and that Ang-2 may mediate this effect (28). In seeking the mechanism underlying the rapid tumor vascular regression on Ang-2 treatment, we investigated whether there was pericyte loss and the coverage of tumor vessels by pericytes 48 h after a single-dose treatment of AdControl or AdAng-2. The colocalization of tumor pericytes and endothelial cells was evaluated by staining pericytes with the specific marker NG2 (29) and endothelial cells with fluorescein-conjugated endothelium-specific *Griffonia* isoelectric B4 (30). Confocal microscopic images showed that systemic Ang-2 overexpression led to a pronounced loss of tumor endothelial cells (Fig. 2A). This result is consistent with the tumor vessel regression in our window chamber experiments. The majority of pericytes in AdControl-treated tumors colocalized with endothelial cells (Fig. 2A). In contrast, the coverage of tumor microvessels by pericytes was severely reduced after AdAng-2 treatment. Quantitative image analysis showed that Ang-2 overexpression did not cause significant pericyte loss; however, it did result in significant loss from the total tumor endothelial cell population and the endothelial subpopulation covered by pericytes (Fig. 2B). Thus, systemic overexpression of Ang-2 disrupts the attachment between tumor endothelial cells and pericytes, which is associated with dramatic tumor vessel regression.

**Systemic Ang-2 overexpression improves perfusion in surviving tumor vessels.** Although antiangiogenic therapies are generally believed to damage tumor vasculature and deprive tumors of oxygen and nutrients (1), emerging evidence indicates that some antiangiogenic therapies transiently normalize the structure and function of tumor vasculature. Vascular normalization is theorized to correct chaotic tumor blood flow, improve perfusion, and enhance tumor accessibility to antitumor drugs and oxygen (31). To investigate the effects of systemic Ang-2 overexpression on tumor vessel perfusion, we investigated the labeling of perfusion marker Hoechst 33342 in the tumors 48 h after a single-dose adenoviral treatment as mentioned above. There was scarce perivascular Hoechst 33342 labeling in the AdControl-treated tumors in spite of high vascularization (Fig. 3A). It indicated that a large portion of tumor vasculature was not efficiently perfused. In contrast, most blood vessels in the tumors treated with AdAng-2, AdsVEGFR-1, and the combination of both showed intensive perivascular Hoechst 33342 labeling (Fig. 3A).

**Figure 2.** Changes in tumor pericytes and endothelial cells 48 h after AdControl and AdAng-2 treatments. A, representative confocal microscopy images showing that AdAng-2 treatment led to severe loss of tumor endothelial cells (green) with many isolated pericytes (red). Bar, 150 μm. B, systemic Ang-2 overexpression did not cause significant decrease on the areas of total pericytes but significantly decreased the areas of both total endothelial cells (EC) and endothelial cells covered by pericytes. Columns, mean of area; bars, SE. *, P < 0.05. Student t test, n = 5.
Quantification of isolectin-Hoeschst 33342 double labeling revealed that systemic overexpression of Ang-2 led to a significant decrease in tumor vessel area compared with other treatments (Fig. 3B). Similarly, the combined Ang-2+sVEGFR-1 treatment significantly decreased tumor vessel abundance in comparison with the AdControl and the AdsVEGFR-1 treatments (Fig. 3B). The AdAng-2 treatment led to more tumor vessel loss than the combined Ang-2+sVEGFR-1 treatment, which was consistent with the higher
plasma level of Ang-2 in the AdAng-2 group compared with the combined AdAng-2+AdsVEGFR-1 group (Fig. 1B). Besides, there was no difference in the plasma level of Ang-2 between the AdControl group and the AdsVEGFR-1 group, neither was there difference in tumor vessel area between the two groups (Fig. 3B). Therefore, it suggests that the plasma level of Ang-2 may be a determining factor on tumor vascular regulation. In addition, there were significant increases in isolectin-Hoechst 33342 double-positive area (Fig. 3C) and in the percentage of perfused tumor vessels 48 h after AdAng-2, AdsVEGFR-1, or AdAng-2+AdsVEGFR-1 treatment (Fig. 3D). Taken together, these results revealed that all three therapeutic regimens destroy a fraction of the tumor vasculature while improving the perfusion of the surviving vessels.

**Systemic Ang-2 overexpression exacerbates tumor hypoxia shortly after the treatment.** The above results reveal that systemic Ang-2 overexpression causes tumor vascular regression while improving perfusion in surviving tumor vessels. The degree of tumor hypoxia depends on the net balance between relative changes in vascular density and in the fraction of perfused vessels. To examine this point, we evaluated the extent to which the above treatments influenced tumor hypoxia and energy status after 48 h. We examined the expression of CA IX, an endogenous hypoxia marker (32). Compared with the other three treatments, the AdAng-2 treatment led to significantly higher CA IX expression, particularly in the central regions (Fig. 4A). There was no difference in tumor CA IX expression between the AdControl, AdsVEGFR-1, and AdAng-2+AdsVEGFR-1 treatments (Fig. 4B). Thus, despite the improved perfusion in surviving tumor vessels, systemic Ang-2 overexpression causes tumor vessel regression and exacerbates tumor hypoxia. However, all the single-dose treatments did not lead to significant changes in overall tumor ATP levels 48 h after adenoviral administration (Supplementary Fig. S1).

**Sustained systemic Ang-2 overexpression suppresses tumor growth.** Because the single-dose AdAng-2 therapy markedly impaired tumor vasculature and exacerbated tumor hypoxia within 48 h after treatment, we speculated that sustained systemic Ang-2 overexpression might be able to inhibit tumor growth. To test this hypothesis, we i.v. injected AdControl, AdAng-2, AdsVEGFR-1, or AdAng-2+AdsVEGFR-1 adenoviruses every other day into animals with HCT116 flank tumors. Treatment regimens started on day 0 when tumor volumes had reached $51 \pm 12$ mm$^3$ and ended on day 14. AdAng-2, AdsVEGFR-1, and the combined AdAng-2+AdsVEGFR-1 treatments significantly inhibited tumor growth compared with the AdControl treatment. Ang-2 and the combined Ang-2+sVEGFR-1 treatments showed significant inhibitory effects on tumor growth on day 4 (Fig. 5A). The AdsVEGFR-1 treatment did not inhibit tumor growth before day 7. All three therapeutic regimens continued to suppress tumor growth with the same efficacy until day 16 (Fig. 5A). On day 16, the average tumor weight of the control group was significantly higher than that of the other three groups (Fig. 5B). There was no difference in the average tumor weight between the three therapeutic groups. The tumor weight results were consistent with tumor volumes. In addition, most tumors in the three treated groups developed crater-shaped cavitations and gross central necrosis in spite of the smaller size (Fig. 5C). The three treated groups showed a higher percentage of gross necrosis than the control group, in which no gross necrosis was found (Supplementary Fig. S2). The higher frequency of gross central necrosis in small tumors suggested that this necrosis was caused by the adenoviral therapies rather than fast tumor growth.

**Sustained systemic overexpression of Ang-2, sVEGFR-1, or both inhibits tumor angiogenesis and promotes apoptosis but does not affect tumor proliferation and hypoxia.** To identify the mechanisms underlying tumor growth suppression, we investigated...
Systemic Ang-2 Overexpression and Tumor Vasculature

Figure 5. Effects of sustained systemic overexpression of Ang-2, sVEGFR-1, or Ang-2+sVEGFR-1 on tumor growth. A, changes of the relative tumor volume during treatment. The tumor volume at each time point was normalized to the corresponding volume on day 0. Points, relative tumor volume; bars, SE. *, P < 0.05, one-way ANOVA analysis followed by SNK test, n = 5. B, tumor weights at the end point of the tumor growth delay study (day 16). Columns, mean tumor weight of each group; bars, SE. *, P < 0.05, one-way ANOVA analysis followed by SNK test, n = 5. C, representative tumors in each group on day 16. Dashed lines, flank tumors; arrows, crater-shaped cavitations and central necrosis. Notice the difference in tumor size between the control tumor and the treated tumors. Bar, 0.5 cm.

Discussion

Research interest in antiangiogenic therapies has been reignited since recent clinical trials showed that anti-VEGF therapy prolonged survival of cancer patients (2, 3). Most efforts are currently focusing on VEGF, the primary driver of angiogenesis, with two strategies: one strategy targets VEGF with VEGF antibodies, high-affinity sVEGFR fusion proteins (36), and aptamers (37); the other targets VEGF signaling cascade with VEGFR antibodies that disrupt ligand binding or small-molecule tyrosine kinase inhibitors that inhibit phosphorylation and activation of VEGFRs (38). To investigate whether a concomitant inhibition of VEGF is required for Ang-2 to induce tumor vascular regression, we neutralized VEGF by adenovirus-mediated systemic overexpression of sVEGFR-1. Ang-2 renders new vessels unstable, fragile, and predisposed to destruction. Vascular destabilization is important not only for vessel adaptation and remodeling, but also for angiogenesis because endothelial cells need to be freed from pericyte coverage to proliferate and migrate. After vessel destabilization, Ang-2 exquisitely cooperates with other proangiogenic factors such as VEGF and Ang-1 to regulate dynamic angiogenesis (15, 17). Therefore, inhibition of Ang-2 (39, 40) or VEGF (13), respectively, shows antiangiogenic effects by suppressing either the destabilization of preexisting vessels or the reconstruction of newly formed endothelial tubes. Because high levels of Ang-2 cause neovascularization but not mature vessels to regress (10), we hypothesized that sustained high levels of Ang-2 may exploit the fragility of tumor neovasculature to destabilize and destroy tumor microvessels. Although hypoxia promotes local Ang-2 expression in tumors (18), it does not elevate Ang-2 level in circulation (41). The AdAng-2 treatment substantially increased the Ang-2 level in circulation compared with the AdControl or AdsVEGFR-1 treatment (Fig. 1B). It was found that Ang-2 promoted endothelial cell death and vascular regression in pupillary membranes when VEGF was inhibited (17). Our results reveal that AdAng-2–induced massive tumor vascular regression and dilatation were similar to the vascular responses in Ang-2–treated pupillary membranes. However, it does not depend on VEGF inhibition. The discrepancy in the requirement of concomitant VEGF inhibition may be due to different structural stability, maturity, or sensitivity to Ang-2 between normal vessels and abnormal tumor vasculature. More importantly, this study provides new evidence on the mechanism of Ang-2–induced tumor vessel regression—a disrupted attachment between tumor pericytes and endothelial cells. Pericytes are essential in stabilizing vascular tumor angiogenesis, apoptosis, proliferation, and hypoxia after multiple-dose treatments. CD34 staining showed significant decrease in tumor microvessel density, which suggested that sustained systemic overexpression of Ang-2, sVEGFR-1, or the combination efficiently inhibited tumor angiogenesis (Fig. 6A and B). TUNEL staining further showed that all three therapeutic regimens promoted tumor cell apoptosis (Fig. 6A and C). However, Ki-67 staining did not reveal significant differences in cell proliferation in nonnecrotic tumor areas (Fig. 6A and D). Thus, the above tumor growth suppression was caused, at least partially, by enhanced tumor cell apoptosis rather than inhibited cell proliferation.

Tumor hypoxia not only up-regulates both VEGF and Ang-2 to promote angiogenesis in a concerted way, but also results in resistance to chemotherapy and radiotherapy by activating multiple survival signaling pathways (33, 34). Tumor hypoxia is determined by both oxygen supply and consumption rates. Vascular regression and/or lower vascular perfusion decrease oxygen supply. Oxygen consumption depends on the number of living cells, cell proliferation, and metabolic status (35). Because sustained systemic Ang-2 overexpression caused tumor vascular regression, inhibited angiogenesis, and promoted tumor cell apoptosis without changing cell proliferation, we finally sought to determine whether the multiple-dose treatments affected tumor hypoxia in a way similar to single-dose treatments. Staining of the hypoxia marker drug pimonidazole revealed no significant changes in tumor hypoxia in all groups (Fig. 6A and E). Thus, in contrast to single-dosed treatments, sustained systemic overexpression of Ang-2, sVEGFR-1, or the combination of both did not exacerbate tumor hypoxia.
structure by secreting endothelial survival factors and sensing hemodynamic and physiologic changes (42). Local high Ang-2 levels cause pericyte dropout (28) and endothelial apoptosis in retinal and brain vascular beds (11, 43). Loss or disruption of pericytes causally precedes blood vessel loss (44). Our data reveal that systemic Ang-2 overexpression severely impairs pericyte coverage on tumor vessels even without significant pericyte loss. Thus, the efficacy of the pericyte-endothelial interaction could be more crucial than the number of pericytes to stabilize tumor vessels.

**Figure 6.** Effects of sustained systemic overexpression of Ang-2, sVEGFR-1, or Ang-2+sVEGFR-1 on tumor angiogenesis, apoptosis, proliferation, and hypoxia. A, immunohistochemical stainings of angiogenesis (CD34), apoptosis (TUNEL), proliferation (Ki-67), and hypoxia (pimonidazole) in tumors 16 d after AdControl, AdAng2, AdsVEGFR-1, or AdAng2+AdsVEGFR-1 treatment. Bar, 100 \( \mu \)m. B, lower tumor microvessel density after the AdAng2, AdsVEGFR-1, or AdAng2+AdsVEGFR-1 treatment compared with the AdControl treatment. Columns, mean of microvessel density; bars, SE. *, \( P < 0.05 \), one-way ANOVA analysis followed by Tukey test, \( n = 5 \). C, TUNEL staining revealing that AdAng2, AdsVEGFR-1, or AdAng2+AdsVEGFR-1 treatment significantly increased tumor apoptosis index compared to the AdControl treatment. Columns, mean of apoptotic index; bars, SE. *, \( P < 0.05 \), one-way ANOVA analysis followed by Tukey test, \( n = 5 \). D, quantitative analysis showing no significant difference in tumor cell proliferation after the four treatments. Columns, mean of Ki-67 labeling index; bars, SE. \( P = 0.486 \), one-way ANOVA analysis, \( n = 5 \). E, quantitative analysis showing no significant difference in tumor hypoxic fraction after the four treatments. Columns, mean of tumor hypoxic fraction; bars, SE. \( P = 0.761 \), one-way ANOVA analysis, \( n = 5 \).
This study shows that systemic Ang-2 overexpression impairs tumor vasculature, inhibits angiogenesis, and decreases tumor oxygenation shortly after initial treatment, although it improves perfusion in the surviving tumor vessels. Jain et al. (31) advocated the concept that certain antiangiogenic therapies can trim and normalize malformed tumor vessels, leaving a more orderly and better perfused vascular bed with more efficient transport properties. If this occurs, tumor oxygenation should improve. Within 48 h after a single-dose Ang-2 adenoviral treatment, a substantial proportion of tumor vessels regressed and the remaining microvessels appeared more dilated and better perfused. However, the temporarily exacerbated hypoxia suggests that the extent of improved vessel perfusion is not sufficient to compensate for the decrease in blood transport capacity caused by massive vascular regression. It is important to note that changes in vascular density and microvessel perfusion cannot compensate for disorderly distribution of microvessels with respect to the efficiency of oxygen transport (45). In contrast, the AdsVEGFR-1 and the combined AdAng-2+AdsVEGFR-1 therapies caused a trend toward alleviated tumor hypoxia ~48 h after treatment. The timing is consistent with a previously reported tumor vessel normalization window after VEGFR-2 blockade with DC101 antibody (46). Inhibition of VEGFR-2 creates a transient drop of tumor hypoxia that occurs within 2 to 5 days. Taken together, the trend to improved tumor oxygenation by blocking VEGFR-1 or VEGFR-2 suggests that VEGFR inhibition plays an important role in tumor vascular normalization.

Our results highlight several important issues about antiangiogenic and antivascular therapies. First, it is believed that antiangiogenic therapies must be combined with other cytotoxic therapies, such as radiation therapy or chemotherapy, to achieve efficient tumor killing. Oxygen is indispensable for efficient radiotherapy and some types of chemotherapy. Previous studies showed that VEGFR-2 targeting therapy provides a therapeutic window during which oxygen and drug transport could be enhanced (46). Here we show that the overexpression of Ang-2, but not sVEGFR-1, quickly exacerbates tumor hypoxia, although both therapies impair tumor vasculature and inhibit angiogenesis. This difference clearly shows that not all tumor vessel-targeting therapies create a vascular normalization window. Alternatively, at least, the time course of the vascular normalization window may vary between different antiangiogenic therapies. Therefore, it is important to characterize the vascular normalization window for each vessel-targeting therapy and to optimize the scheduling of a combined radiotherapy or chemotherapy. Second, the vascular normalization in anti-VEGFR-2 therapy was attributed to temporarily improved pericyte coverage of tumor vessels (46). AdAng-2 treatment results in severe loss of pericyte coverage of tumor vessels and transiently exacerbates hypoxia, which clearly shows that pericyte-targeting therapies do not normalize tumor vessels. Thus, the existence of a tumor vascular normalization window depends on the targeting molecule, cell, and mechanism of each antiangiogenic therapy. Third, this study shows that single-dose Ang-2, sVEGFR-1, and the combined adenoviral therapies do not cause significant changes in tumor ATP content in spite of tumor vascular regression. Although cell respiration is more efficient than anaerobic glycolysis to produce ATP, the unchanged ATP levels 48 h after adenoviral treatments indicate strong and quick compensations in tumor bioenergetics. Although single-dose Ang-2 adenoviral therapy exacerbates tumor hypoxia after 48 h, sustained multiple-dose Ang-2 therapy, anti-VEGF therapy, and the combined therapy do not affect the steady state of tumor hypoxia compared with the control treatment. This may be because hypoxic cells were pushed into apoptosis as shown by the increased apoptotic index in all three therapeutic groups (Fig. 6A and C). Therefore, most of the surviving cells should stay in the equilibrium between oxygen supply and consumption. HCT116 has fully functional p53, which promotes hypoxia-induced tumor apoptosis by transactivation of Fas/CD95 (47). It would be interesting to see whether Fas/CD95 signaling is activated during AdAng-2–induced apoptosis. Finally, the dispensable role of concomitant VEGF inhibition for tumor vascular regression by Ang-2 raises critical questions on how angiopoietins and VEGF orchestrate tumor angiogenesis and vascular regression. Previous studies suggest that combining antiangiogenic and antivascular therapies may prolong tumor control (48, 49). Therefore, we speculated that the combination of Ang-2 overexpression with VEGF neutralization would show additive or even synergistic inhibition of angiogenesis. However, the similar therapeutic effects between the single agents and the combined approach suggest the existence of a potential point of convergence between Ang-2 and VEGF signaling pathways. Shih et al. (50) reported that pericytes can protect endothelial cells by expressing transforming growth factor-β, which induces VEGFR-1 expression in endothelial cells. Because our results show that Ang-2 overexpression causes significant loss of pericyte coverage on tumor vessels, further studies to find the key molecular hubs between Ang-2 and VEGF signaling pathways would provide new therapeutic strategies to control pathologic angiogenesis.

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