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Tumorigenesis and Neoplastic Progression

Contrasting Actions of Selective Inhibitors of Angiopoietin-1 and Angiopoietin-2 on the Normalization of Tumor Blood Vessels

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Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) have complex actions in angiogenesis and vascular remodeling due to their effects on Tie2 receptor signaling. Ang2 blocks Ang1-mediated activation of Tie2 in endothelial cells under certain conditions but is a Tie2 receptor agonist in others. We examined the effects of selective inhibitors of Ang1 (mL4-3) or Ang2 (L1-7[N]), alone or in combination, on the vasculature of human Colo205 tumors in mice. The Ang2 inhibitor decreased the overall abundance of tumor blood vessels by reducing tumor growth and keeping vascular density constant. After inhibition of Ang2, tumor vessels had many features of normal blood vessels (normalization), as evidenced by junctional accumulation of vascular endothelial-cadherin, junctional adhesion molecule-A, and platelet/endothelial cell adhesion molecule-1 in endothelial cells, increased pericyte coverage, reduced endothelial sprouting, and remodeling into smaller, more uniform vessels. The Ang1 inhibitor by itself had little noticeable effect on the tumor vasculature. However, when administered with the Ang2 inhibitor, the Ang1 inhibitor prevented tumor vessel normalization, but not the reduction in tumor vascularity produced by the Ang2 inhibitor. These findings are consistent with a model whereby inhibition of Ang2 leads to normalization of tumor blood vessels by permitting the unopposed action of Ang1, but decreases tumor vascularity primarily by blocking Ang2 actions. ***(Am J Pathol 2009, 175:2159–2170; DOI: 10.2353/ajpath.2009.090391)***

Solid tumors require angiogenesis—the formation of new blood vessels from existing vessels—for survival, growth, and metastasis.[1] Tumor vessels are structurally and functionally abnormal.[1,2] They exist in a constantly dynamic state of sprout formation, proliferation, remodeling, or regression. Structurally, tumor vessels tend to be leaky and tortuous, lacking the hierarchical arrangement of arterioles, capillaries, and venules.[2] Pericytes that attach to and help stabilize normal vessels are loosely associated with the endothelium of tumor vessels.[1,2] These vascular abnormalities result in impaired and heterogeneous blood flow. In tumors, angiogenesis inhibitors not only cause vessel regression or retardation of vessel growth, but they can also induce vascular normalization.[1–3]

The complicated regulation of angiogenesis and vascular maturation involves multiple signaling cascades driven by endothelial-cell specific growth factors and their receptors. One of these, vascular endothelial growth factor (VEGF) has been extensively studied,[4] but angiopoietins and other growth factors are also involved.[5,6] The angiopoietin ligands (Ang1 and Ang2) and their receptor (Tie2) have essential roles in vascular development.[7,8] Ang1 is produced by vascular mural cells, pericytes, and certain other cells, whereas Ang2 and Tie2 are expressed primarily by endothelial cells.

Angiogenesis and vascular remodeling involve a complex coordination of Ang1 and Ang2 signaling through Tie2.[5] The traditional view of Ang1 and Ang2 signaling is

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that the growth factors have opposing effects on Tie2 receptor activation: Ang1 binds to Tie2 to promote vascular maturation and integrity, whereas Ang2 acts as a naturally occurring antagonist of Ang1.[7–11] Although a number of studies indicate an antagonistic role of Ang2, recent studies have shown that Ang2 can have an agonistic role depending on the experimental environment.[12–15] If expressed at high concentrations or for long durations in cultured endothelial cells, Ang2—like Ang1—can induce Tie2 receptor phosphorylation.[13,16] Ang2 can also promote chemotaxis, tube formation, migration, and sprouting of endothelial cells in the absence of Ang1,[14] which support the view that Ang2 actions are context-dependent.

Normalization of tumor vascular morphology and function has been demonstrated with numerous angiogenesis inhibitors.[1,17,18] Ang1 and Ang2 regulate vascular maturation and integrity during development; however, their effects on normalization of tumor vessels are not known. Tumors grown in mice lacking Ang2 have a more mature vascular phenotype, but it is not known whether Ang1 plays a role.[19] The effects of individual angiopoietins on the tumor vasculature have not been studied extensively in loss-of-function experiments, due largely to the limited availability of selective angiopoietin inhibitors. Some clues to the effects of Ang1 and Ang2 on tumor vessels have been garnered through overexpression of the ligands in tumor cell xenografts.[20–26] These studies, however, have yielded conflicting data,[20–26] the ligands were administered at nonphysiological levels, and the results were restricted to prevention studies. Studies blocking the Tie2 receptor have shown reduced tumor angiogenesis,[27–30] but the specific roles of each ligand cannot be differentiated. Pharmacological angiopoietin inhibitors using antisense, aptamer, and peptide-Fc fusion protein (peptibody) technologies are currently being developed, but published studies have been restricted to inhibition of Ang1 or Ang2 alone.[31–33] Studies using aptamers or peptibodies that potently neutralize Ang2 activity showed that Ang2 antagonism resulted in inhibition of angiogenesis and tumor growth.[31,32] Inhibition of Ang1 in a cell line stably transfected with antisense RNA resulted in reduced tumor growth and angiogenesis.[33]

To gain a better understanding of the effects of Ang1 and Ang2 on blood vessels in tumors, we used selective inhibitors (peptibodies) of Ang1 and Ang2, alone or in combination, in Colo205 tumors. These studies focused on Colo205 tumors, as this model is sensitive to angiopoietin inhibitors.[31] We found that inhibition of Ang1 alone had little effect on the tumor vasculature, whereas inhibition of Ang2 resulted in fewer tumor vessels and normalization of the surviving tumor vessels. When the Ang2 inhibitor was administered with the Ang1 inhibitor, tumor vessel normalization did not occur, but the Ang2 inhibitor-mediated reduction in vascularity was unaffected. These findings suggest that inhibition of Ang2 leads to unopposed Ang1 activity and results in normalization of tumor vessels. In contrast, the Ang2 inhibitor-mediated reduction in tumor vascularity was Ang1-independent.

Materials and Methods

Animals and Treatment

The Colo205 colorectal tumor model was used as previously described.[31] Colo205 tumors were chosen because they express both Ang1 and Ang2 and are sensitive to Ang2 inhibitors.[31] Human Colo205 tumors grown in nude mice express human Ang1 (95 copies mRNA), mouse Ang1 (2683 copies mRNA), human Ang2 (11,758 copies mRNA), and mouse Ang2 (28,721 copies mRNA) (all copy numbers per 100 ng total mRNA measured by TaqMan real-time PCR using species-specific probe sets and recombinant mRNA standard curves; D. Yu, A. Coxon, and J. Oliner, unpublished data). CD1 nude mice (Charles River, Wilmington, MA) were injected with 0.2 ml of tumor cell suspension in RPMI medium plus Cultrex (R&D Systems, Minneapolis, MN) (3:1) containing 2×10^6 Colo205 tumor cells (ATCC, Manassas, VA). Colo205 tumors were allowed to grow for 2 to 3 weeks before treatment. Mice were treated with an Ang1-specific peptibody (mL4-3) having an IC_{50} value of 33 pM against murine Ang1 (T. Lee, A. Coxon, and J. Oliner, unpublished data) or an Ang2-specific peptibody (L1-7[N]) displaying an IC_{50} of 71 pM against murine Ang2.[31] Mice bearing Colo205 tumors were injected subcutaneously daily for 7 or 26 days with normal human IgG1 Fc (hFc, control, 550 μg), mL4-3 (500 μg), L1-7(N) (50 μg), or these doses of both inhibitors. The control hFc protein was added to the treatment groups to match the total amount of protein delivered in the combination group (550 μg). All experimental procedures were approved and conducted in accordance with institutional guidelines established by the Institutional Animal Care and Use Committees of the University of California, San Francisco and Amgen, Inc.

Vascular Perfusion and Tissue Preparation

At the end of the treatment period, mice were anesthetized with ketamine (100 mg/kg) and xylazine (12 mg/kg) i.p. and tissues were preserved by vascular perfusion of fixative (1% paraformaldehyde in PBS, pH 7.4) for 2 minutes at a pressure of 120 mmHg.[3] Tumors were removed, weighed, fixed in 1% paraformaldehyde for 1 hour at 4°C, immersed in 30% sucrose in PBS overnight, frozen in optimal cutting temperature compound on dry ice, and stored at −20°C.

Immunohistochemistry

Sections 60 to 80 μm in thickness were cut on a cryostat and dried on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA) for 5 hours or overnight. Sections were permeabilized with PBS containing 0.3% Triton X-100 (Lab Chem Inc., Pittsburg, PA) and blocked in 5% normal serum (Jackson ImmunoResearch, West Grove, PA) in PBS+ (PBS containing 0.3% Triton X-100, 0.2% bovine serum albumin [Sigma, St. Louis, MO, and 0.01% sodium azide [Sigma]) for 30 minutes to 1 hour. Sections were

incubated for 12 to 15 hours in primary antibodies diluted in 5% normal serum in PBS+. After rinsing with PBS containing 0.3% Triton X-100, sections were incubated at room temperature in fluorophore-conjugate secondary antibodies (fluorescein isothiocyanate, Cy3, or Cy5, Jackson ImmunoResearch) diluted in PBS plus 0.3% Triton X-100 for 3 to 5 hours. Sections were rinsed with PBS plus 0.3% Triton X-100, fixed in 4% paraformaldehyde for 5 to 10 minutes, rinsed in PBS, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Endothelial cells were stained with hamster anti-platelet/endothelial cell adhesion molecule (PECAM-1, CD31, Clone 2H8, 1:500, Thermo Scientific, Hudson, NH), rat anti-vascular endothelial (VE)-cadherin (1:500, BD Biosciences, Franklin Lakes, NJ), or rat anti-junctional adhesion molecule (JAM-A) (JAM-1, Clone BV12, 1:20, E. Dejana). Pericytes were stained with rat anti-platelet derived growth factor receptor-β (PDGFR-β, clone APB5, 1:2000, eBioscience, San Diego, CA) or Cy3-conjugated anti-α-smooth muscle actin (clone1A4, 1:1000, Sigma). Viable tumor cells were identified using the nuclear dye, YO-PRO-1 (1 μmol/L solution, Invitrogen/Molecular Probes, Carlsbad, CA).

Microscopy and Area Density Measurements

Stained sections were examined with a Zeiss Axiophot fluorescence microscope equipped with single, dual, and triple fluorescence filters and a low-light, externally cooled, three-chip charge-coupled device camera (480 $\times$ 640 pixel RGB-color images, CoolCam; SciMeasure Analytical Systems, Atlanta, GA) and with a Zeiss LSM 510 confocal microscope with argon, helium-neon, and UV lasers (512 $\times$ 512 or 1024 $\times$ 1024 pixel RGB-color images). Area density of PECAM and PDGFR-β immunoreactivities was measured with ImageJ software (*http://rsbweb.nih.gov/ij/*) on digital fluorescence microscopic images using empirically determined threshold values (30 to 40 for PECAM; 20 to 25 for PDGFR-β).[3] Area density was calculated as the proportion of pixels having a fluorescence intensity value equal to or greater than the corresponding threshold.

For CD31 area density measurement, nonviable or necrotic regions were excluded from the analysis by selecting the region of interest based on YO-PRO-1 staining. To measure the area density of PDGFR-β positive pericytes associated with tumor vessels, confocal images of tumor vessels and pericytes were taken with the $\times$40 objective and $\times$2 zoom and the region of interest was identified as the area 10 μm from the edge of the tumor vessels stained with PECAM.

PECAM area was used as a reflection of the total amount of PECAM found in the viable regions of the tumors. Images of the entire tumor stained with YO-PRO-1 were taken with the Zeiss Axiophot fluorescence microscope ($\times$2.5 objective, $\times$1 Optovar, tissue region 3696 $\times$ 4928 μm or 480 $\times$ 640 pixels) and assembled in Photoshop. Nonviable or necrotic regions were identified by absence of YO-PRO-1 staining and excluded from measurements. Total area of the viable tumor (mm2) was calculated from the number of YO-PRO-1 positive pixels above a threshold of 20 to 30. The area of PECAM immunoreactivity within viable regions was calculated by multiplying the area density of PECAM staining in viable areas by the total area of viable tumor.

Morphometric Measurements

Morphometric measurements of blood vessels were made on images obtained from 60 to 80 μm thick sections. Vessel diameter was determined in sections stained for PECAM using the CoolCam CCD camera attached to a digitizing tablet. Measurements were made on live images of 100 tumor vessels per tumor, 5 to 6 tumors per treatment group. Statistical differences between treatment groups were analyzed by the Kolmogorov-Smirnov test where $P < 0.05$ was considered significant.

The number of endothelial sprouts were determined in samples stained for PECAM as previously described.[34] Sprouts were identified as tapered PECAM-immunoreactive processes that extend away from the main axis of a vessel, which end abruptly. The number of sprouts was counted on 10 vessel segments for each tumor, with 5 to 6 tumors per group. The length of each vessel segment was determined using the digitizing tablet. Results are presented as number of sprouts per vessel segment length (mm).

Scanning Electron Microscopy

Samples examined by scanning electron microscopy were prepared and imaged as previously described.[3,35] Briefly, tissues were fixed by vascular perfusion of 2% glutaraldehyde in 100 mmol/L phosphate buffer. The samples were treated with 30% potassium hydroxide at 60°C for 8 minutes to dissolve the extracellular matrix, stained with 2% tannic acid and 1% OsO_4, dehydrated with ethanol, critical point dried, coated in an osmium plasma coater (OPC60A; Filgen, Japan), and examined with a scanning electron microscope (S-5000; Hitachi, Brisbane, CA).

Quantification of Endothelial Cell Junctions

The linear staining of endothelial cell junctions was quantified by extending an algorithm based on the multiscale curvelet transform[36] adapted for edge detection in microscopy images.[37] The curvelet transformat was first applied to the images of tumor blood vessels stained for PECAM. The information for different scales and their directions and positions was stored into the curvelet coefficients. We performed a lossy image reconstruction by keeping 75% of the coefficients at all levels, except the finest, which were discarded. The elimination of the finest scale during image reconstruction eliminated noise and small impurities from the image. The image reconstruction process was robust with the results largely un-

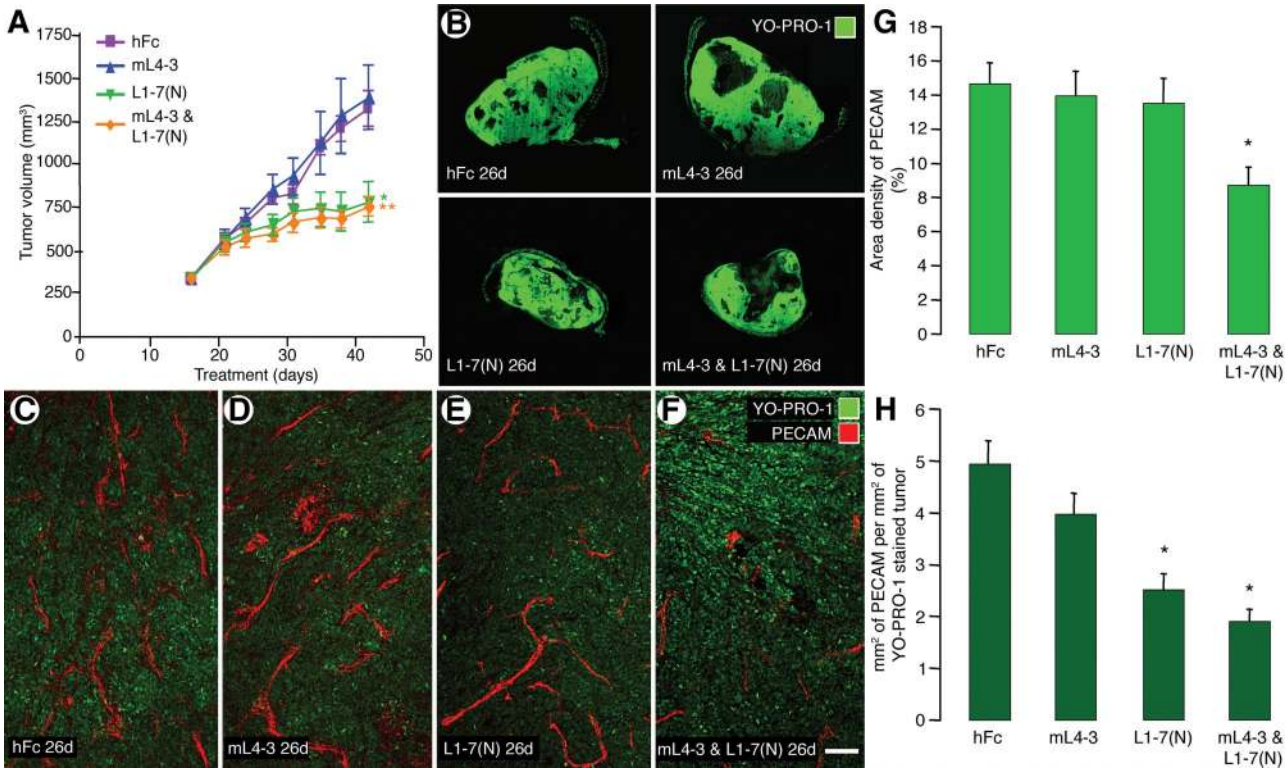

Figure 1. Differences in tumor growth and vascularity after inhibition of Ang1 and/or Ang2. **A:** Growth of Colo205 tumors treated for 26 days with one of four treatment regimens. Growth rates were similar with hFc and mL4-3 (Ang1 inhibitor) but were significantly slower with L1-7(N) (Ang2 inhibitor) or the combination of inhibitors. $^{*}P < 0.01$ vs. hFc, $^{**}P < 0.001$ vs. hFc. **B:** Colo205 tumors stained with the nuclear dye, YO-PRO-1, to show the size of the tumors (green). The tumor treated with the Ang2 inhibitor or the combination of inhibitors was smaller than after the other treatments. **C–F.** Confocal images showing PECAM immunoreactivity (red) of blood vessels surrounded by YO-PRO-1 staining (green) of viable tumor cells in Colo205 tumors treated for 26 days. The vascular density in YO-PRO-1-positive regions of tumors was similar in control tumors (**C**, hFc), after inhibition of Ang1 (**D**, mL4-3), or inhibition of Ang2 (**E**, L1-7[N]), but was less after inhibition of Ang1 and Ang2 together (**F**). The area density of PECAM-positive blood vessels in YO-PRO-1–positive regions was not changed by either inhibitor alone but was significantly less after the combination of inhibitors (**G**). Overall tumor vascular mass, calculated as the product of the fractional area of PECAM staining and tumor size, was significantly less than control after inhibition of Ang2 or after inhibition of Ang1 and Ang2 together (**H**). $^{*}P < 0.05$ compared with hFc. Scale bar: 3.5 mm (**B**); 80 μm (**C–F**).

changed when using between 30% and 95% of the coefficients.

The reconstructed image was then subjected to a morphological opening (an erosion followed by a dilation), using a disk of specified radius, which can be adjusted, as the structural element of the opening operation. This eliminated small, isolated round objects in the image, enhanced the separation between objects, and minimized the effects of local variations. Following this morphological opening, a simple thresholding was applied to identify the objects with the highest intensity in the image. The threshold was chosen between two peaks in the histogram of the curvelet magnitude image. This resulted in a threshold of 90% of the maximum intensity of the image.

After this processing, the boundaries of the objects on the image were identified. The borders of each object were approximated by using straight segment connections between the points on the boundary. The object was fitted with cubic splines, which were in turn used to compute the curvature along the border of the PECAM staining. A size threshold was then applied to eliminate objects that span the full length of the image or were just small isolated blobs.

Statistics

Differences between groups were analyzed by analysis of variance followed by Fisher's posthoc tests. Values are expressed as mean ± SE. Differences with $P < 0.05$ were considered significant.

Results

Differences in Tumor Vascularity after Inhibition of Ang1 and/or Ang2

The four treatments had significantly different effects on tumor growth (Figure 1A). Tumor growth curves were essentially the same in mice treated with the control reagent (hFc) and in mice treated with the Ang1 inhibitor (mL4-3) (Figure 1A). However, the Ang2 inhibitor (L1-7[N]) significantly slowed tumor growth (Figure 1A), as previously reported.[31] Addition of the Ang1 inhibitor did not reverse the effects of the Ang2 inhibitor on tumor growth; instead, the combination of L1-7(N) and mL4-3 resulted in at least as much slowing of tumor growth as the Ang2 inhibitor alone (Figure 1A). Although the aver-

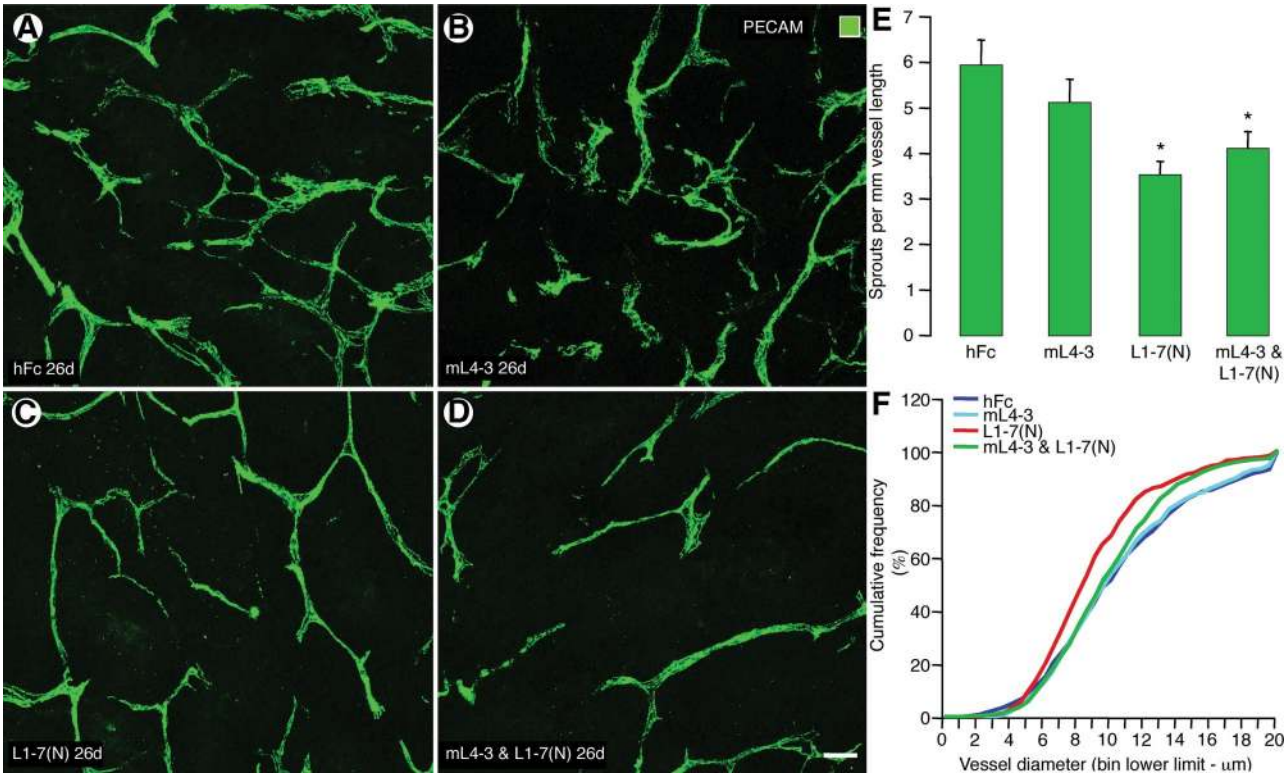

Figure 2. Tumor vessel phenotype after inhibition of Ang1 and/or Ang2. Confocal microscopic images of endothelial cells (PECAM; green) in Colo205 tumors after treatment for 26 days. Blood vessels are tortuous and sprouting in a control tumor (**A**, hFc) and after inhibition of Ang1 (**B**, mL4-3), but are more uniform in size and have less sprouting after inhibition of Ang2 (**C**, L1-7[N]). Tumor vessels are less numerous after treatment with both inhibitors (**D**). Endothelial sprouts were significantly less numerous after inhibition of Ang2 (**E**). This reduction in sprouts by the Ang2 inhibitor was not blocked by co-administration of the Ang1 inhibitor (**E**). Graph of the size distributions of tumor vessels in the four groups shows that the average size of tumor vessels was significantly less after inhibition of Ang2. Inhibition of Ang1 reduced this effect (**F**). $^*P < 0.05$ compared with hFc. Scale bar: 50 μm.

age tumor volume was smaller in the combination treatment group than in the L1-7(N) treatment group, this difference did not reach statistical significance.

To obtain an overview of the effects on tumor vessels by inhibiting Ang1 or Ang2, alone or together, we identified viable regions of tumor cells with the nuclear marker YO-PRO-1 and examined tumor vascularity after staining blood vessels for PECAM immunoreactivity. Histological sections made at the end of the experiment confirmed the smaller tumor size after treatment with the Ang2 inhibitor alone or the combination of inhibitors (Figure 1B). The fractional area (area density) of PECAM immunoreactivity in viable regions of tumors treated with mL4-3 or L1-7(N) for 26 days was similar to that of hFc-treated (control) tumors (Figure 1, C–E, G). Only when mice were treated with both inhibitors together was the fractional area of tumor vessels significantly less (30%) than the control (Figure 1, F and G).

To take changes in overall tumor size into account, total vessel area was calculated. When proportional reductions in tumor size and vessel density are similar, the fractional area of tumor vessels does not reflect changes in overall abundance of tumor vessels. Such changes are reflected by calculations of total tumor vascularity (total tumor vessel area) from the fractional area of tumor vessels and tumor size. Measurements of fractional area of tumor vessels in viable regions of the tumor scaled to tumor size (area density of PECAM staining $\times$ mm^2 of viable tumor per section) were, in comparison with hFc controls, 50% less after the Ang2 inhibitor, and 62% less after the combination of inhibitors (Figure 1H). These values indicate that tumors treated with the Ang2 inhibitor or the drug combination had only half as many blood vessels, or fewer, than control tumors.

Differences in Tumor Vessel Phenotype after Inhibition of Ang1 and/or Ang2

Blood vessels of hFc-treated Colo205 tumors had multiple abnormalities, including variability in size, tortuosity, and presence of sprouts (Figure 2A). After treatment with the Ang1 inhibitor for 26 days, tumor vessels were similar to those after hFc (Figure 2B). However, after inhibition of Ang2, tumor vessels were straighter, more uniform in caliber, and had fewer sprouts (Figure 2C). When the Ang1 inhibitor was combined with the Ang2 inhibitor, tumor vessels were less abundant, had a simpler architecture, and fewer sprouts compared with control (Figure 2D). After 26 days of treatment, tumor vessels had 14% fewer sprouts after the Ang1 inhibitor and 40% fewer sprouts after the Ang2 inhibitor. The two inhibitors in combination did not reduce the number of sprouts as much as the Ang2 inhibitor alone, but sprouts were still significantly (23%) less numerous than in the control (Figure 2E).

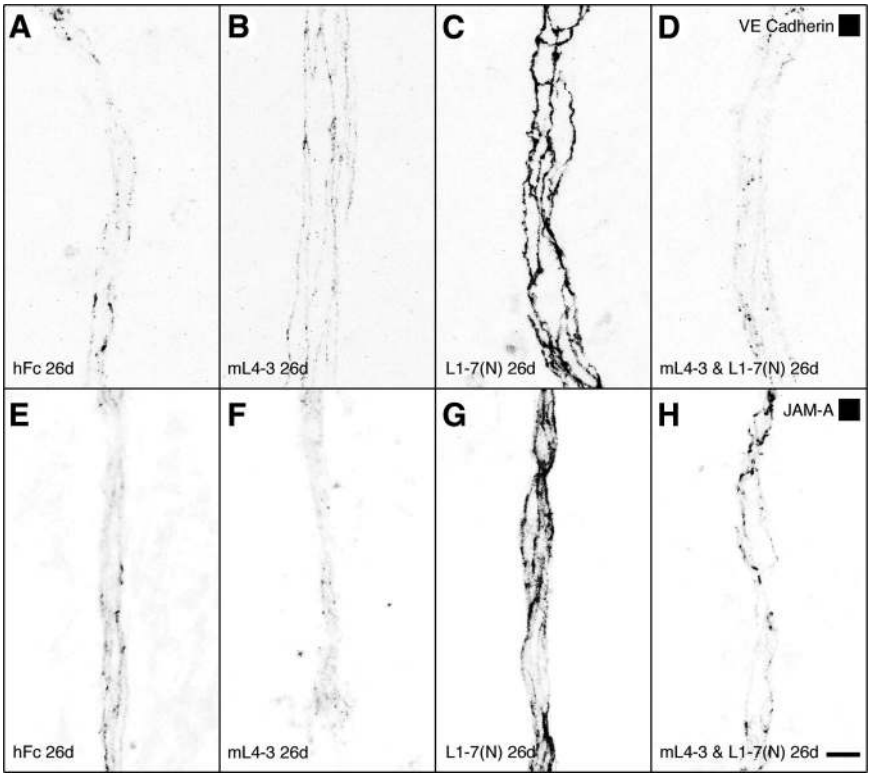

Figure 3. Distribution of VE-cadherin and JAM-A after inhibition of Ang1 and/or Ang2. Fluorescence microscopic images of Colo205 tumors stained for the endothelial adherens junction protein VE-cadherin or the tight junction protein JAM-A after treatment for 26 days. VE-cadherin immunoreactivity was weak in control tumors (**A**, hFc) and after inhibition of Ang1 (**B**, mL4-3) but was strong and linear at endothelial cell borders after inhibition of Ang2 (**C**, L1-7(N)). The linear pattern was not present after inhibition of Ang1 and Ang2 (**D**). Immunoreactivity for the endothelial tight junction protein JAM-A was faint after hFc (**E**), Ang1 inhibition (**F**), or inhibition of Ang1 and Ang2 (**H**), but was strong after inhibition of Ang2 (**G**). Scale bar = 8 μm.

Measurements of vessel diameter revealed that tumor vessels were smaller after inhibition of Ang2 than in the control or after inhibition of Ang1. This difference was evident in a left-shift of the vessel size distribution (Figure 2F). When the Ang1 and Ang2 inhibitors were given together, the distribution of vessel size was significantly greater than with the Ang2 inhibitor alone (Figure 2F).

Differences in Endothelial Cell Junction Proteins after Inhibition of Ang1 and/or Ang2

To determine whether the uniformity of vessel size and reduced sprouting after inhibition of Ang2 were manifestations of tumor vessel normalization, we asked whether the junctions between endothelial cells acquired a more normal pattern, consistent with improved barrier function. We found that staining for the adherens junction protein, VE-cadherin, was diffuse and weak in the vasculature of control tumors treated with hFc for 26 days (Figure 3A) with little or no immunoreactivity located at the intercellular junctions. VE-cadherin had the same appearance in tumors treated with the Ang1 inhibitor (Figure 3B). By striking comparison, after inhibition of Ang2, VE-cadherin staining was more conspicuous because of the linear pattern that was largely associated with endothelial cell junctions (Figure 3C), as in normal blood vessels.[38] When the Ang1 inhibitor was administered with the Ang2 inhibitor, staining for VE-cadherin was diffuse and had little association with endothelial cell borders (Figure 3D).

Like VE-cadherin, the tight junction protein JAM-A was diffuse in the endothelium of tumor vessels after hFc (Figure 3E) and was similarly faint after treatment with the Ang1 inhibitor for 26 days (Figure 3F). Also like VE-cadherin, JAM-A immunoreactivity had strong, linearized staining that localized to endothelial cell borders after inhibition of Ang2 (Figure 3G). When the Ang1 inhibitor was combined with the Ang2 inhibitor, JAM-A was weaker, more diffuse and had less junction-associated staining than after the Ang2 inhibitor alone (Figure 3H). Therefore, the normalization of endothelial cell junctions that is elicited by inhibition of Ang2 is prevented by co-inhibition with Ang1.

Although not a component of intercellular junctions, PECAM is concentrated at cell borders in most normal blood vessels.[39] PECAM immunoreactivity was present in blood vessels of Colo205 tumors treated with hFc for 26 days, but consisted largely of dots that had no obvious association with endothelial cell borders (Figure 4A). PECAM had a similar pattern in the tumor vasculature after treatment with the Ang1 inhibitor (Figure 4B). However, after the Ang2 inhibitor, PECAM immunoreactivity in tumor vessels had a linear pattern at endothelial cell junctions (Figure 4C), which resembled the distribution of VE-cadherin (Figure 3C) and JAM-A (Figure 3G). When the Ang2 inhibitor was combined with the Ang1 inhibitor, the pattern of PECAM was in the form of dots with no apparent association with junctions (Figure 4D), as was found in control tumors (Figure 4A).

The association of PECAM with intercellular junctions was quantified using an algorithm, based on the multiscale curvelet transformat and adapted for edge detection (see Methods). The algorithm estimated the amount of linear staining (mm of staining/mm vessel length) at endothelial cell borders. In tumors treated with hFc (Fig-

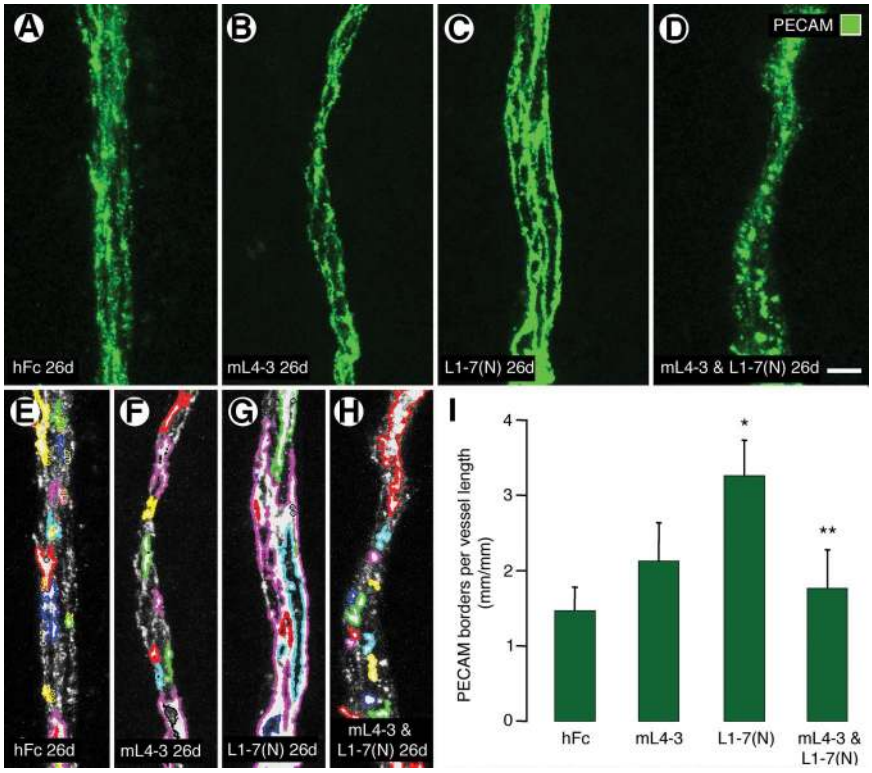

Figure 4. Distribution of PECAM after inhibition of Ang1 and/or Ang2. Confocal microscopic images showing the distribution of PECAM immunoreactivity in blood vessels of Colo205 tumors treated for 26 days. PECAM staining was strong in all groups (**A**–**D**), but was patchy in control tumors (**A**, hFc), after inhibition of Ang1 (**B**, mL4-3), or after inhibition of Ang1 and Ang2 (**D**). By comparison, PECAM staining was largely localized to endothelial cell junctions after inhibition of Ang2 (**C**, L1-7(N)). Contiguous linear regions of PECAM staining, identified by the algorithm used to measure junctional normalization, are marked by colored lines (**E–H**). Linear PECAM staining were less in control tumors (**E**) and after inhibition of Ang1 (**F**) or inhibition of Ang1 and Ang2 (**H**) than after inhibition of Ang2 alone (**G**). Measurements of contiguous linear PECAM staining revealed significantly larger values after inhibition of Ang2 alone than after inhibition of Ang1 and Ang2 (**I**). $^{*}P < 0.05$ compared with hFc. $^{**}P < 0.05$ compared with Ang2 inhibitor. Scale bar = 10 μm.

ure 4E) or with the Ang1 inhibitor (Figure 4F), the identified regions of contiguous PECAM staining were small and limited. In contrast, the algorithm identified larger regions of PECAM staining after treatment with the Ang2 inhibitor (Figure 4G). As was evident visually, only small regions of contiguous PECAM staining were present when the Ang2 inhibitor was combined with the Ang1 inhibitor (Figure 4H). Linear regions of staining were significantly larger after inhibition of Ang2 (Figure 4I). This expansion of linear staining was not present when the two inhibitors were combined (Figure 4I).

Differences in Pericyte Distribution after Inhibition of Ang1 and/or Ang2

Pericytes identified by PDGFR-β immunoreactivity were abundant in tumors treated with hFc for 26 days, but were loosely associated with tumor vessels (Figure 5A). Pericytes in tumors treated with the Ang1 inhibitor had a similar abundance and distribution (Figure 5B). However, after inhibition of Ang2, pericytes were more closely associated with tumor vessels (Figure 5C). When the Ang1 inhibitor was combined with the Ang2 inhibitor, pericytes were less closely associated with tumor vessels (Figure 5D).

Closer examination revealed that few PDGFR-β–positive pericytes were located immediately next to the vessels of control tumors (Figure 5E). Pericytes were similarly sparse near the endothelium after the Ang1 inhibitor (Figure 5F), but not after the Ang2 inhibitor, where close associations between pericytes and endothelial cells were numerous (Figure 5G). Blood vessels of tumors treated with both inhibitors resembled those of tumors treated with hFc or the Ang1 inhibitor alone (Figure 5H). Similar staining patterns were also observed with another pericyte marker, α-smooth muscle actin (data not shown).

Measurements of PDGFR-β–positive pericytes tightly associated with tumor vessels (within 10 μm of the endothelium) were consistent with visual impressions (Figure 5I). The amount of pericytes associated with the tumor vessels was 114% greater after Ang2 inhibition than those for control tumors treated with hFc (Figure 5I). Values for the combination of inhibitors were significantly lower than those for the Ang2 inhibitor alone and resembled Ang1 inhibitor alone (Figure 5I).

Surface views of tumor vessels examined by scanning electron microscopy revealed few pericytes in contact with the endothelium in control tumors (Figure 5J). By comparison, pericytes were close to the endothelium of tumor vessels after treatment with the Ang2 inhibitor for 7 days (Figure 5K).

Discussion

These studies sought to elucidate the actions of Ang1 and Ang2 on Colo205 tumor blood vessels, with a focus on vascular abnormalities and tumor vascularity. The experiments revealed that treatment of Colo205 tumors with the Ang2 inhibitor, L1-7(N), resulted in tumor blood vessels that had more normal features, as reflected by more uniform caliber, redistribution of VE-cadherin, JAM-A, and PECAM to endothelial cell junctions, less sprouting,

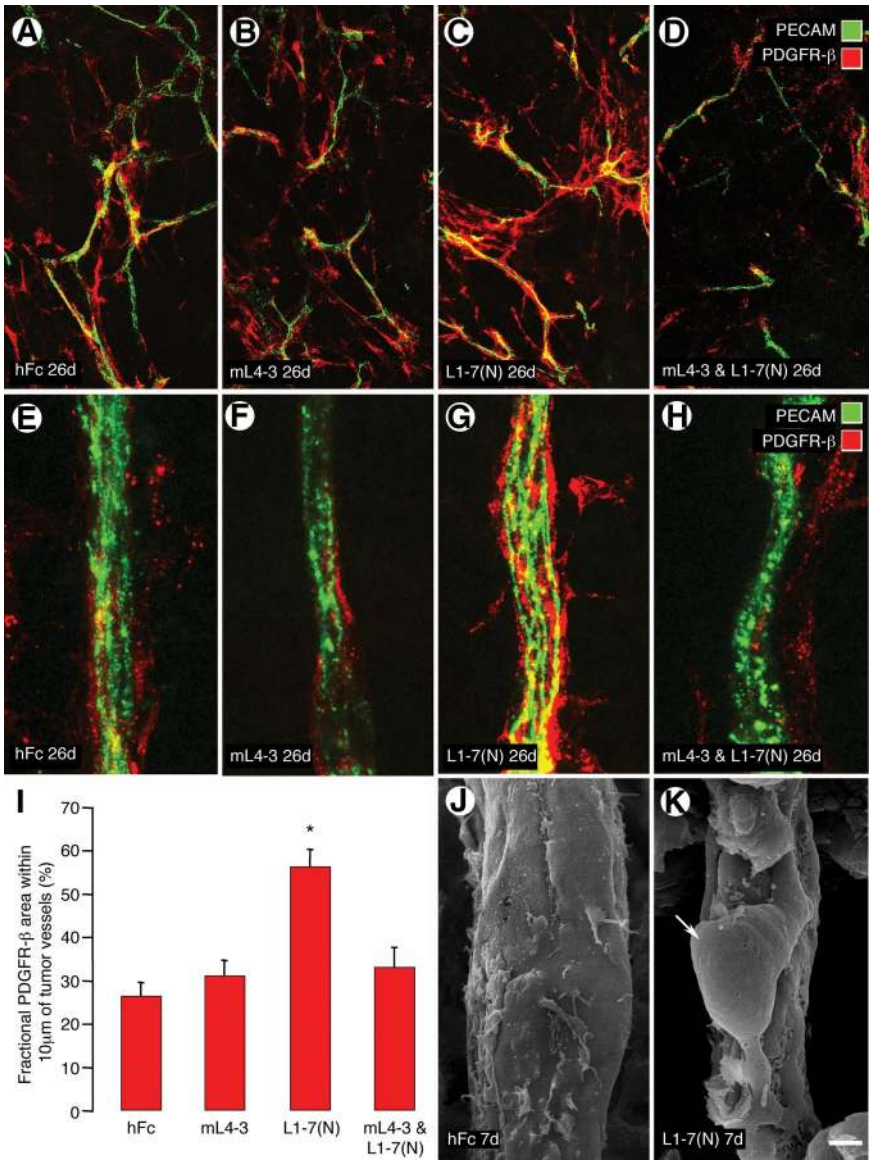

Figure 5. Pericyte distribution after inhibition of Ang1 and/or Ang2. Fluorescence microscopic images of endothelial cells (PECAM; green) and pericytes (PDGFR-β; red) of blood vessels in Colo205 tumors treated for 26 days. Pericytes were loosely associated with blood vessels in control tumors (**A**, hFc), after inhibition of Ang1 (**B**, mL4-3), or after inhibition of Ang1 and Ang2 (**D**) but were more closely associated with endothelial cells after inhibition of Ang2 (**C**, L1-7(N)). Pericytes in contact with tumor vessels were sparse and had faint PDGFR-β immunoreactivity after hFc (**E**), after inhibition of Ang1 (**F**), or after inhibition of Ang1 and Ang2 (**H**) but were abundant and had strong PDGFR-β immunoreactivity after inhibition of Ang2 (**G**). Measurements confirmed the greater abundance of PDGFR-β-positive pericytes within 10 μm of tumor vessels after inhibition of Ang2 than in the other groups (**I**). $^*P < 0.05$ compared with the other groups. Scanning electron microscopic images of the external surface of tumor vessels showing no pericytes in contact with the endothelium of a control tumor (**J**), in comparison with a pericyte closely associated with the endothelium after inhibition of Ang2 (**K**). Scale bar: 65 μm in **A–D**, 10 μm in **E–H**, and 5 μm in **J–K**.

and tighter association of pericytes with the endothelium. These changes were partially or completely blocked by concurrent inhibition of Ang1 by mL4-3. The blockade by mL4-3 is consistent with the normalizing process being largely mediated by unopposed actions of Ang1, and the antagonizing action of Ang2-dominating effects of Ang1 in this system. The reduction in total tumor vascularity after inhibition of Ang2, which were not prevented by inhibition of Ang1, suggest that these changes result from loss of endogenous effects of Ang2 on the tumor vasculature.

Effect of Angiopoietin Inhibitors on Endothelial Junctions

Vessel diameter, pericyte association, endothelial sprouting, and vessel leakiness are all indicators of vascular normalization.[1,2] Here, we also use endothelial junction markers as an indication of normalization. In endothelial cells, junctional complexes are mainly comprised of PECAM, adherens junctions, and tight junctions. PECAM is an endothelial cell adhesive protein concentrated at cell borders, which may suppress both cell activation and cell death.[40] Adherens junctions—specifically VE-cadherin in endothelial cells—are important in contact inhibition of cell growth, while tight junctions such as JAM-A establish a membrane barrier to regulate permeability and cell polarity.[39,41] Ang2 inhibition led to a linear redistribution of PECAM, VE-cadherin, and JAM-A. Collectively, these proteins represent all classes of junctions found in endothelial cells, implying a potential role for Ang2 inhibition in suppressing endothelial cell apoptosis, preventing endothelial cell growth, and reducing tumor vessel permeability or leakiness.

Junctions go through a step-wise maturation process to establish normal cell contacts between adjacent cells.[42] Early cell contacts begin as spot-like junctions. Multiple cellular protrusions interlock and gradually form

cell junctions with a zipper-like appearance. Junctions along the entire cell border form a continuous connection of adjacent cells, which creates the endothelial barrier.[42] Blood vessels in control Colo205 tumors stained for VE-cadherin or PECAM immunoreactivity had spot-like or diffuse junctional staining. Ang2 inhibition changed this irregular staining into continuous, linear staining along the border of endothelial cells. This finding is consistent with Ang2 activity in Colo205 tumors altering the formation, maturation, or maintenance of endothelial cell junctions. Inhibition of Ang1 and Ang2 together prevented this effect of Ang2 inhibition. Thus, unopposed activity of Ang1—when the action of Ang2 is blocked—promotes junctional maturation or normalization.

Importance of Tumor Vessel Normalization

Tumor blood vessels have multiple abnormalities. They are dynamic—naturally undergoing sprouting, proliferation, remodeling, or regression. The vessels are also leaky, irregularly shaped, tortuous, and have fewer, loosely associated pericytes. In this study, vessel diameter, pericyte association, endothelial sprouting, and endothelial cell junctions were all examined as measures of vessel normalization in Colo205 tumors. After Ang2 inhibition, the tumor vessels had a more uniform caliber, there were more pericytes associated with the tumor vessels, there were fewer endothelial sprouts, and the endothelial junctions were more linear. Together these phenotypes indicate that Ang2 inhibition leads to tumor vessel normalization. Combining Ang2 inhibition with Ang1 inhibition prevented the effects of Ang2 inhibition alone, indicating that unopposed Ang1 signaling is responsible for the tumor vessel normalization. These findings are consistent with a previous study by Winkler et al[43] showing increased Ang1 expression associated with tumor vessel normalization after VEGF blockade. Further investigation is needed to determine whether unopposed Ang1 influences vessel diameter, pericyte association, endothelial sprouting, and endothelial junction maturation directly or if some of these changes are consequences of vessel normalization. However, there appears to be a role for PDGF signaling in pericyte recruitment but not junctional normalization after Ang2 inhibition. Blocking Ang2 together with PDGFR-β prevents pericyte recruitment, but the distribution of endothelial junctions is unchanged (H. Hashizume, B. Falcon, and D. McDonald, unpublished observations).

While numerous studies indicate that Ang2 acts as an antagonist of Ang1 signaling, most of these studies have been limited to *in vitro* analysis or transgenic animals.[7,8,15,16] Here, we show in an *in vivo* tumor model that Ang1 and Ang2 have opposing effects on tumor vessel normalization by using Ang1 and Ang2 specific inhibitors. Although these studies focused on the normalization of vascular morphology following inhibition of angiopoietins, previous studies have shown a close association between morphological and functional changes of tumor vessel normalization.[1,17]

Effects of Ang1 and Ang2 Inhibition on Tumor Vascularity

Tumor vessels are dynamic. Growth, remodeling, and regression are common features of tumor blood vessels.[2] Inhibition of Ang2 by L1-7(N) changed this property by normalizing the vessel wall, which decreased endothelial sprouting and tumor vascularity. Tumor vessel normalization did not occur when Ang2 was inhibited in the presence of the Ang1 inhibitor. However, the inhibitor combination resulted in a greater reduction in tumor vascularity than after either inhibitor alone. This augmented loss of tumor vessels was likely to be Ang1-independent, because it occurred in the presence of Ang1 blockade. The reduction in tumor vascularity also may be independent of changes in VEGF expression, as the amount of VEGF immunoreactivity did not change with any of the treatment groups (B. Falcon and D. McDonald, unpublished data).

Tumor vessel area density and total tumor vascularity (total area) are indicators of the dynamic state of angiogenesis and vascular regression. Ang2 inhibition alone did not reduce the area density of PECAM immunoreactivity in tumors, but did decrease the total amount of PECAM immunoreactivity in tumors through the decrease in tumor size. By comparison, inhibition of Ang1 and Ang2 together significantly reduced both tumor vessel area density and total area. Thus, there was a greater reduction in tumor vessels after the combination treatment than after the Ang2 inhibitor alone. This greater antivascular effect of the combination of inhibitors is likely due to the regression of a population of tumor vessels, in addition to the reduction in endothelial sprouting and vessel growth found after Ang2 inhibition alone. Although a number of factors can cause vascular regression, prevention of vessel normalization by inhibition of Ang1 along with Ang2 would be expected to make tumor vessels more susceptible to regression. Treatment with the Ang2 inhibitor significantly decreased the growth rate of Colo205 tumors. Addition of the Ang1 inhibitor to Ang2 blockade did not prevent the reduction in rate of tumor growth, nor did it amplify it to a statistically significant extent. However, the combination of Ang1 and Ang2 inhibitors clearly had effects on tumors different from either inhibitor alone, as revealed by lack of tumor vessel normalization accompanied by a greater reduction in tumor vascularity when the inhibitors were given together. Inhibition of Ang2 alone reduced tumor vessel growth by stabilizing the vessel wall. The addition of the Ang1 inhibitor blocked the normalization and promoted vascular regression without increasing sprouting angiogenesis.

Ang2 as a Partial Agonist

Our data imply that Ang2 prevents Ang1-dependent tumor vessel normalization while influencing total tumor vascularity independent of Ang1 signaling in Colo205 tumors. While Ang2 is described as a naturally occurring antagonist of Ang1 signaling,[7] *in vitro* studies have shown that Ang2 can activate the Tie2 receptor in a concentra-

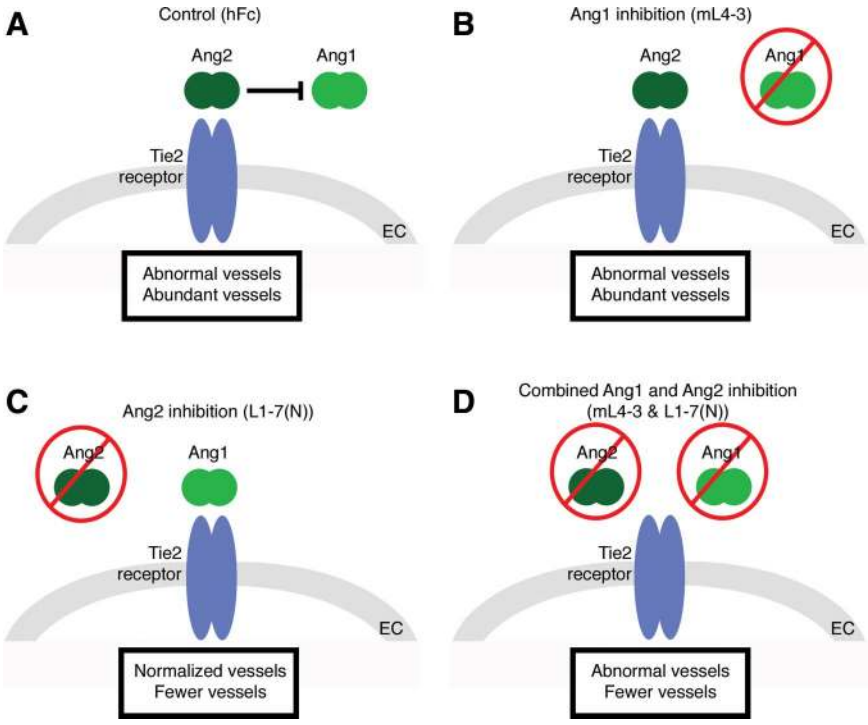

Figure 6. Proposed mechanism of effects of inhibition of Ang1 and/or Ang2 on tumor blood vessels. **A:** In untreated tumors, the actions of Ang2 dominate. Ang1 acts as an agonist, but Ang2 acts as a partial agonist that limits Ang1-induced activation of Tie2 receptors and leads to tumor vessel destabilization, endothelial sprouting, and angiogenesis. **B:** Inhibition of Ang1-induced activation of Tie2 does not change the phenotype of tumor vessels, because Ang2 actions dominate. **C:** Inhibition of Ang2 leads to the unopposed action of Ang1, which promotes tumor vessel normalization, less sprouting, and reduced angiogenesis. **D:** Inhibition of both Ang1 and Ang2 favors tumor vessel abnormalities, because of the absence of the stabilizing action of Ang1, but also reduces sprouting, because of the absence of the angiogenesis promoting action of Ang2.

tion or time-dependent manner.[15,16] While the mechanism of Ang2 acting as both an antagonist and an agonist of the Tie2 receptor has not been elucidated, one explanation is that Ang2 acts as a partial agonist.[15] Ang1 and Ang2 have similar binding sites[44] and affinities for Tie2 receptors.[7] Activation of Tie2 receptors by Ang2 is weaker than by Ang1, consistent with Ang2 acting as a partial agonist[15] and Ang1 acting as a full agonist.

Differences in Ang1 and Ang2 expression in Colo205 tumors may play a role in our observed effects. Expression of Ang2, both human and mouse, was greater than Ang1 in untreated Colo205 tumors. These results are consistent with *in situ* hybridization data that show abundant Ang2 expression[31] but weak and diffuse Ang1 expression (S. Scully, A. Coxon, and J. Oliner; unpublished data). Attempts were made to measure the phosphorylation state of Tie2 receptors and the downstream signaling pathways. However, with the methods used, the baseline phosphorylation state of Tie2 receptors, Akt, and Erk were too low and variable to draw meaningful conclusions. The amount and distribution of VEGF, as determined by immunohistochemistry, were not changed by any of the treatments. Although this suggests that VEGF is not a major factor in tumor vessel normalization or decreased tumor vascularity after Ang2 inhibition, VEGF involvement cannot be excluded because VEGF-dependent actions of angiopoietins are likely to be governed by free VEGF, which is not readily assessed by immunohistochemistry or mRNA measurements.

Based on our results, we propose the following mechanism of Ang1 and Ang2 effects on Colo205 tumors. In control tumors, Ang2 is expressed at much higher levels than Ang1.[31] This difference may prevent Tie2 receptor activation by Ang1, but allow weak activation by Ang2 to reduce vascular stability and promote angiogenesis. Inhibition of Ang1 by mL4-3 does not affect Ang2 binding or the subsequent weak activation of the Tie2 receptor. Ang2 inhibition by L1-7(N) prevents Ang2 binding to Tie2 receptors. This allows binding of Ang1 to Tie2 receptors and induction of tumor vessel normalization (stabilization). Blocking Ang2 also prevents the partial agonist effects of Ang2 and reduces angiogenesis and overall tumor vascularity. Inhibition of both Ang1 and Ang2 prevents Ang1-mediated vessel normalization, but the reduction on tumor vascularity is still observed (Figure 6). Although this model offers a molecular basis for vessel normalization, it is unclear how the differences in Tie2 agonism explain the alterations in tumor vascularity. Specifically, absence of vessel normalization is seen under the three experimental conditions in which Tie2 agonism is predicted to be the lowest (Figure 6, A, B, and D), suggesting that Tie2 activation is linked to vessel normalization. However, it is more difficult to attribute the changes in vascularity to Tie2 activation, as the two conditions in which the fewest vessels are seen (Figure 6, C and D) are also the two conditions that are predicted to have the greatest differences in Tie2 agonism (strong agonism in Figure 6C and weak/absent agonism in Figure 6D). Additional experimental studies are needed to reconcile this apparent disparity.

Therapeutic Implications for Ang1 and Ang2 Inhibition

Tumor growth is dependent on both vessel number and tumor vessel function (normalization).[1,2,18] Vascular normalization may be a consequence of many anti-angiogenic therapies.[1,17,18] In some cases, the number of remaining, normalized tumor vessels is enough to main-

tain tumor growth.[18] Current cancer therapeutics use these normalized tumor vessels to deliver chemotherapeutics or other cancer-cell targeting drugs more efficiently.[1,45,46] An alternative therapeutic objective would be to prevent tumor vessel normalization to keep tumor vessels unstable and subject to regression.

Here, we show that tumor vessel normalization in Colo205 tumors is dependent on unopposed Ang1 signaling. Ang2 expression or inhibition of Ang1 with mL4-3 prevents tumor vessel normalization. Thus, dual inhibition of Ang1 and Ang2 may provide improved therapeutic benefit over selective Ang2 inhibition by reducing tumor vessels while preventing the undesired consequences of tumor vessel normalization.

Growth and maintenance of blood vessels in tumors are dependent on multiple growth factors. Thus angiogenesis inhibitors that block multiple targets are being developed for treatment of solid tumors. A better understanding of the interplay among growth factors is needed to advance growth factor-targeted cancer therapeutics. Here, we show that in the absence of Ang2, Ang1 drives tumor vessels into a more normal phenotype. In evidence of this change, inhibition of Ang2 resulted in normalization of endothelial cell junctions, pericyte coverage, and overall architecture of blood vessels in Colo205 tumors. This normalization was prevented by blocking Ang1. Inhibition of Ang2 also reduced tumor vascularity, and this action was augmented by simultaneous inhibition of Ang1.

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