**Vessel Cooption, Regression, and Growth in Tumors Mediated by Angiopoietins and VEGF**

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In contrast with the prevailing view that most tumors and metastases begin as avascular masses, evidence is presented here that a subset of tumors instead initially grows by coopting existing host vessels. This coopted host vasculature does not immediately undergo angiogenesis to support the tumor but instead regresses, leading to a secondarily avascular tumor and massive tumor cell loss. Ultimately, however, the remaining tumor is rescued by robust angiogenesis at the tumor margin. The expression patterns of the angiogenic antagonist angiopoietin-2 and of pro-angiogenic vascular endothelial growth factor (VEGF) suggest that these proteins may be critical regulators of this balance between vascular regression and growth.

It is widely accepted that most tumors and metastases originate as small avascular masses that belatedly induce the development of new blood vessels once they grow to a few millimeters in size (1–3). Initial avascular growth would be predicted for tumors that arise in epithelial structures that are separated from the underlying vasculature by a basement membrane and for experimental tumors that are implanted into avascular settings (such as the cornea pocket) or into a virtual space (such as the subcutaneum) (2, 3). However, there is also evidence to suggest that tumors in more natural settings do not always originate avascularly, particularly when they arise within or metastasize to vascularized tissue (4). In such settings, tumor cells may coopt existing blood vessels (4). The interplay between this coopting of existing vessels and subsequent tumor-induced angiogenesis has not been extensively examined nor has the role of angiogenic factors in this process. The pro-angiogenic vascular endothelial growth factors (VEGFs) and the angiopoietins are the only known growth factor families that are specific for the vascular endothelium because expression of their receptors is restricted to these cells (5, 6). The angiopoietins include both receptor activators [angiopoietin-1 (Ang-1)] and receptor antagonists [angiopoietin-2

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**References**

20. P. Heitzler, unpublished observations.
24. Single embryos were squished in 10 μl of 10 mM tris–HCl (pH 8.0), 1 mM EDTA, and 25 mM NaCl containing Proteinase K (200 μg/ml, Boehringer Mannheim) and incubated at 37°C for 30 min, followed by 2 min at 95°C. The PCR was performed with 2.5 U of Taq polymerase and 100 ng of each primer. Two pairs of primers were designed to amplify 634 base pairs (bp) of the cren genomic sequence and 200 bp of the genomic region of the doom gene as an internal control [A. J. Harvey, A. P. Bidwaldi, L. K. Miller, Mol. Cell. Biol. 17, 2835 (1997)]. Cren-specific primers were 5'-GGCCACGATGTTCGACAT-3' and 5'-AGCCGAAATGTCGCTGACCTG-3'. Doom-specific primers were 5'-AGGTTAAGCCCACACAGATG-3' and 5'-CATAGTGTTTCTTGCGC-3'. The PCR cycles were 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for 30 cycles. In embryos with the W88 stock, 20 to 79 were missing the cren-specific band.
25. CRQ immunostaining was used to genotype each embryo. Peroxidase immunostaining detected all hemocytes [R. E. Nelson et al., EMBO J. 13, 3438 (1994)]. The nuclear dye 7-AAD labeled all DNA and allowed for the identification of apoptotic corpses. Unless otherwise specified, stage 11 to 16 embryos were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min and subsequently incubated 4°C overnight with the primary antibodies. After washing three times in PBS, the embryos were incubated for 1 hour at room temperature with the following secondary antibodies: fluorescein isothiocyanate–conjugated goat antibody to mouse and Cy5-conjugated goat antibody to rabbit (Jackson Immunoresearch) used at a 1:1000 dilution. Finally, embryos were washed twice in PBS, mounted in Vectashield (Vector), and viewed by confocal microscopy (Leica TCS NT 4D).
26. The efficiency of engulfment was quantified by counting the number of engulfed corpses per macrophage in at least five fields of four embryos of each genotype. A.P.L., that is, the mean number of engulfed corpses per macrophage, was calculated for each embryo, and the mean P.I. was derived for each genotype.
28. N. Franc and K. White, data not shown.
29. Stage 12 to 14 w, UAS-cr:hs-Gal4+/+ and control w; hs-Gal4 embryos were heat-shocked for 1 hour at 39°C, aged for 2 hours at 25°C, and fixed and embedded in Spurr’s resin (24). Serial 1-μm sections of two embryos, each genotype were stained with a solution of methylene, toluidine blue, and borax (44) and viewed by standard microscopy.
32. Stage 11 embryos were microinjected with a solution of PB and 2 mM NaN3 containing about 6 × 106 tetramethyl rhodamine isothiocyanate (TRITC)-labelled fluorescent E. coli (K-12 strain) or S. aureus (Wood strain) bioparticles (heat-killed bacteria; Molecular Probes) with standard microinjection proce-
37. After CRQ immunostaining, the amount of fluorescence seen in five isolated macropods of each genotype was quantified with a confocal microscope. For each macropod, the fluorescence of serial sections of 0.5 μm was quantified from top to bottom of the cell. After subtracting the background fluorescence, the total amount of fluorescence within each macropod was calculated.
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**Footnotes**

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The VEGFs and the angiopoietins seem to play complementary and coordinated roles in vascular development (9, 11). During development, VEGF acts via the Flk1/KDR receptor to promote endothelial cell differentiation, proliferation, and primitive vessel formation (12). Ang-1 subsequently acts via the Tie2 receptor to remodel these primitive vessels and is then thought to help maintain and stabilize the mature vessels by promoting interactions between endothelial cells and surrounding support cells (6–9, 11, 13, 14). In adults, Ang-2 is expressed primarily at sites of vascular remodeling (9, 11), where it is thought to block the constitutive stabilizing action of Ang-1. It has been proposed that destabilization by Ang-2 in the absence of VEGF leads to frank vessel regression, whereas such destabilization in the presence of high VEGF levels facilitates the angiogenic response (9, 11). In tumors, hypoxia-induced VEGF (15) apparently recapitulates its developmental actions by contributing to the onset of tumor-associated angiogenesis, and antagonists of VEGF have been shown to inhibit the growth of many tumors (16).

To explore the possibility that VEGFs and angiopoietins collaborate during tumor angiogenesis, we studied early angiogenic events using the rat C6 glioma model (17). Remarkably, even the smallest C6 gliomas at just 1 week after implantation (<1 mm in diameter) were found to be well vascularized (Fig. 1, A and A’). As previously noted (17), this is attributable to the coopting of existing brain blood vessels by the implanted tumor cells. The vessels within these early tumors were similar to normal brain vessels in caliber and heterogeneity. There was no evidence of angiogenesis, as judged by the lack of vascular sprouts, noncanalized endothelial cell chains, and hyperplastic vessels. By 2 weeks after implantation, the tumors had grown to ≥2 mm in diameter but still showed no obvious angiogenic response. Rather, they exhibited a dramatic decrease in vessel density, presumably due to tumor growth in the absence of compensatory angiogenesis (Fig. 1, B and B’). The vessels within the tumors were distinctly larger and more homogeneous in caliber than the microvasculature of the normal brain. By 4 weeks after implantation, the tumors measured several millimeters in diameter and showed marked changes in comparison with tumors at earlier stages of development (Fig. 1, C and C’). Blood vessels within the core of the tumor had undergone dramatic regression, with no evidence of a local, compensatory angiogenic response. The centers of the tumors were largely bereft of vessels, leading to massive tumor cell death (Fig. 1, C and C’). The remaining cells in the tumor interior were organized in cuffs of pseudopalisading cells around the few surviving internal vessels (Fig. 1, C and C’). In contrast to the tumor interior, the tumor periphery displayed robust angiogenesis (Fig. 1, C and C’).

Regression of coopted blood vessels was a very early event that preceded tumor cell death. Apoptotic cells were predominantly found in blood vessels in early-stage tumors, whereas at later stages there was widespread apoptosis of tumor cells (Fig. 2, A through C). Staining with markers for both endothelial cells and supporting pericytes or smooth muscle cells revealed that vessel regression was associated with progressive disengagement of endothelial cells from surrounding support cells (Fig. 1, D through G).

The apparent association of tumor vessel regression, apoptosis, and disruption of endothelial cell interactions with support cells raised the possibility that blockade of the stabilizing action of Ang-1 might be contributing to tumor vessel regression. Consistent with this possibil-

Fig. 1. Sections from rat C6 gliomas (28) showing progressive vessel regression, accompanied by dissociation of endothelial and smooth muscle cells. (A and A’) Small 1-week tumors that measure a fraction of a millimeter in width are well vascularized as determined by RECA immunostaining (29), apparently because they coopt and grow around existing vessels. (T, tumor; scale bar in (A), 1 mm for (A) and (A’)) Within large 4-week tumors, internal vessels regress with accompanying loss of surrounding tumor (necrotic tumor areas are unstained). Surviving internal vessels are sparse and uniform, are centrally located with respect to surrounding cuffs of well-stained viable tumor cells, and exhibit no evidence of compensatory angiogenesis; although robust angiogenesis is apparent at the margin of the tumor, where increased density of ectatic vessels is noted. Arrowheads in (C) depict a patent (top) and a regressed (bottom) vessel, each surrounded by either a surviving or regressed cuff of tumor. (D through G) Immunostaining with antibodies to SMA (black) and RECA (brown) (29) shows that pericytes and smooth muscle cells detach from the vessel wall in tumors. (D) shows a vessel wall in normal brain tissue in which RECA and SMA staining are essentially superimposed, whereas (E) through (G) depict vessels within tumors with progressive detachment of SMA-positive cells and vessel regression. Scale bar in (D) indicates 50 μm for (D) through (G).
ity, Ang-1 was found to be anti-apoptotic for cultured endothelial cells (25, 29) is evident in early tumors (A and B), and this is followed by widespread apoptosis of tumor cells at later stages (C). Arrowheads denote vessel-specific apoptotic figures (stained black) in panels. Scale bar in (A), 10 μm. Flow cytometry experiments (E through H) indicate that Ang-1 can be as effective as VEGF in preventing apoptosis of serum-starved endothelial cells, as judged by a decreased percentage of endothelial cells with hypodiploid DNA content (see percentages over the sub-G1/G0 peak delineated by brackets). Cell number is shown on the y axis. PI, propidium iodide. However, in contrast to VEGF, Ang-1 cannot promote DNA synthesis in these cells (D) (30). Similar data have just been reported (31).

Fig. 2. Detection of apoptosis in rat C6 gliomas. Vessel-specific apoptosis (25, 29) is evident in early tumors (A and B), and this is followed by widespread apoptosis of tumor cells at later stages (C). Arrowheads denote vessel-specific apoptotic figures (stained black) in panels. Scale bar in (A), 10 μm. Flow cytometry experiments (E through H) indicate that Ang-1 can be as effective as VEGF in preventing apoptosis of serum-starved endothelial cells, as judged by a decreased percentage of endothelial cells with hypodiploid DNA content (see percentages over the sub-G1/G0 peak delineated by brackets). Cell number is shown on the y axis. PI, propidium iodide. However, in contrast to VEGF, Ang-1 cannot promote DNA synthesis in these cells (D) (30). Similar data have just been reported (31).

Fig. 3. In situ hybridization analysis of Ang-2, VEGF, and Tie mRNA in two different 2-week rat gliomas (small and large) and a large 4-week rat glioma (29, 32). At 2 weeks, the vessels within both a small tumor (A through D) and a larger tumor (E through H) consistently express high levels of Ang-2 mRNA (A and E). In contrast, up-regulation of Tie mRNA (C and K) is restricted to the larger tumor. Induction of VEGF is minimal in small tumors (B) and is still modest and patchy in larger tumors (F). In very large 4-week tumors, the tumor is secondarily avascular because of massive vessel regression and thus has few internal vessels, but has a hypervascular plexus at the tumor border. The few internal and the many rim vessels are now marked by both Ang-2 and Tie (I and K), although expression of Ang-2 is more punctate than that of Tie. The remaining live tumor cuffs around vessels show dramatically up-regulated VEGF expression (J). This VEGF expression is highest in palisading, presumably hypoxic, tumor cells that are furthest from vessels; large areas within the tumor, between palisading cells, are necrotic. (D), (H), and (L) outline the boundaries of the tumor within the brain and indicate the relative levels of expression of Ang-2, VEGF, and Tie. Scale bar in (G) indicates 500 μm for (A) through (H); scale bar in (K) indicates 1 mm for (I) through (L).

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To examine whether these findings are generalizable to other tumor types, we implanted rat RBA mammary adenocarcinoma cells into rat brains. Rather than growing avascularly, the implanted RBA cells rapidly associated with and migrated along cerebral blood vessels in a manner even more striking than that observed with the glioma cells (Fig. 4, A and D). Consistent with the well-vascularized state of these early tumors, there was minimal up-regulation of VEGF (22). However, the coopted vessels displayed striking and specific up-regulation of Ang-2, which was not detectable in the vessels of adjacent brain tissue (Fig. 4B). Preliminary analysis of RBA tumors at a later stage indicated that Ang-2 expression was associated with a pattern of vascular regression (in the absence of VEGF) and angiogenesis (in the presence of VEGF), as was the case with gliomas (22). Ang-1 was not expressed in cultured RBA cells or the tumors themselves (22).

Examination of a model of tumor metastasis, in which the mouse lung is colonized by intravenously injected Lewis lung carcinoma cells, yielded similar results. Tiny tumor metastases (arrowheads, Fig. 4, E and F) as well as moderately sized tumor nodules (arrows, Fig. 4, E and F) were closely associated with pulmonary vessels, and these vessels showed dramatic induction of Ang-2 expression (Fig. 4F). Progressively larger tumor nodules appeared to be characterized by vessel regression as well as neo-angiogenesis, again correlating with Ang-2 and VEGF expression (22).

In summary, our analyses of several different tumor models suggest a modification of the prevailing view that most malignancies and metastases originate as avascular masses that only belatedly induce angiogenic support. Our findings indicate that a subset of tumors rapidly coopts existing host vessels to form an initially well-vascularized tumor mass. Perhaps as part of a host defense mechanism, there is widespread regression of these initially coopted vessels, leading to a secondarily avascular tumor and massive tumor cell loss; however, the remaining tumor is ultimately rescued by robust angiogenesis at the tumor margin.

The expression patterns of VEGF and the natural Tie2 receptor antagonist Ang-2 strongly implicate them in these processes. There is a striking induction of Ang-2 expression in coopted vessels before induction of VEGF expression in the adjacent tumor cells, providing perhaps the earliest marker of tumor vasculature. The intense autocrine expression of Ang-2 by endothelial cells in tumor-associated vessels may counter a paracrine stabilization or survival signal provided by low-level constitutive expression of Ang-1 in normal tissues. We hypothesize that Ang-2 “marks” the coopted vessels for regression by an apoptotic mechanism that may involve disrupted interactions between endothelial cells and the surrounding extracellular matrix and supporting cells. Subsequently, VEGF up-regulation coincident with Ang-2 expression at the tumor periphery is associated with robust angiogenesis. This late expression of tumor-derived VEGF may nullify the regression signal provided by Ang-2, which is consistent with the observation that VEGF is required for tumor vessel survival (23).

The angiogenic properties of tumor-derived VEGF may actually be facilitated when vessels are destabilized by Ang-2. Newly formed tumor vessels are often tenuous, poorly differentiated, and undergo regressive changes even as blood vessel proliferation continues. The failure of many solid tumors to form a well-differentiated and stable vasculature may be attributable to the fact that newly formed tumor vessels continue to overexpress Ang-2. In fact, hyper-vascular hepatomas with aberrant vasculatures show high levels of Ang-2 expression in their endothelium (24). Thus, a persistent blockade of Tie2 signaling, which is otherwise constitutively activated in many normal adult tissues (14), may prevent tumor vessel differentiation and maturation and contribute to their generally tenuous and leaky nature.

In tumors, Ang-2 and VEGF apparently reprise the roles they play during vascular remodeling in normal tissues, acting to regulate the previously underappreciated balance between vascular regression and growth. Our findings bolster the case for anti-VEGF therapies in cancer, not only to prevent further angiogenesis but also perhaps to promote the regression of fragile new tumor vessels. Ang-2 appears to be the earliest marker of blood vessels that have been perturbed by invading tumor cells. As such, Ang-2 may prove to be useful in the imaging of very early angiogenic tumor vessels.
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