

Vascular Remodeling Marks Tumors That Recur During Chronic Suppression of Angiogenesis

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Abstract

The potential for avoiding acquired resistance to therapy has been proposed as one compelling theoretical advantage of antiangiogenic therapy based on the normal genetic status of the target vasculature. However, previous work has demonstrated that tumors may resume growth after initial inhibition if antiangiogenic blockade is continued for an extended period. The mechanisms of this recurrent growth are unclear. In these studies, we characterized molecular changes in vasculature during apparent resumption of xenograft growth after initial inhibition by vascular endothelial growth factor blockade, "metronome" topotecan chemotherapy, and combined agents in a xenograft murine model of human Wilms' tumor. Tumors that grew during antiangiogenic blockade developed as viable clusters surrounding strikingly remodeled vessels. These vessels displayed significant increases in diameter and active proliferation of vascular mural cells and expressed platelet-derived growth factor-B, a factor that functions to enhance vascular integrity via stromal cell recruitment. In addition, remodeled vessels were marked by expression of ephrinB2, required for proper assembly of stromal cells into vasculature. Thus, enhanced vascular stability appears to characterize tumor vessel response to chronic antiangiogenesis, features that potentially support increased perfusion and recurrent tumor growth.

Introduction

It has been proposed that a key advantage of antiangiogenic therapy should be the avoidance of acquired tumor resistance because target endothelial cells (EC) are a normal tissue unlike genetically mutable tumor cells (1). However, Klement *et al.* (2) described recurrent tumor growth after an apparent initial

period of suppression during long-term administration of either anti-vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2) antibody or antiangiogenically scheduled vinblastine. The mechanisms contributing to such recurrence are unknown. One possible mechanism is the altered expression of angiogenic factors by tumor vessels or cells during chronic hypoxia, which results in remodeling and increased stability of tumor vasculature, and thereby increases perfusion of surviving tumor tissues. Vascular remodeling during chronic hypoxia has been well characterized in normal tissues and while with maladaptive aspects in the lung (3), appears to augment perfusion to kidney and brain (4, 5). Important features of hypoxic remodeling include the loss of small vessels (rarefaction) and extensive proliferation of vascular mural cells (MC) in surviving vasculature.

Recruited MC are key contributors to the maintenance of tumor neovasculature (6, 7). For example, the presence of MC can selectively determine tumor vessel survival when VEGF is withdrawn (8). Platelet-derived growth factor-B (PDGF-B) signaling via the PDGF receptor- β (PDGFR- β) receptor plays a critical role in MC recruitment. Like VEGF, PDGF-B expression in EC is critically regulated by oxygen tension, and overexpression is associated with abnormal proliferation of MC in normal vasculature subjected to chronic hypoxia (9, 10). Thus, PDGF-B appears to play a significant role in promoting the integrity of vascular networks during conditions of environmental stress.

More recently, members of the ephrin family have been shown to play an important role in regulating assembly of vascular cells into stable networks. In particular, ephrinB2 appears to play a critical role in vessel formation and maturation by mediating endothelial-mesenchymal cell interactions; defective ephrinB2 signaling can disrupt recruitment of vascular smooth muscle cells, resulting in pathologically weak vessels (11, 12). EphrinB2 is normally expressed on arterial vessels, although during tumor angiogenesis it is much more widely detected in new vasculature (13). In contrast, its cognate tyrosine kinase receptor, EphB4, is dominantly expressed on venous EC (13). Thus, we hypothesized that expression of ephrinB2 might contribute to assembling or increasing the MC component of certain tumor vessels, thus enhancing their vascular integrity during antiangiogenic blockade.

Taken together, these data raise the possibility that multiple angiogenic cytokines can cooperate to promote tumor vessel adaptation during prolonged antiangiogenesis (*e.g.*, by enhancing MC recruitment to vasculature). Blockade of VEGF suppresses growth in many cancer models, and recent clinical

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trials lend support to the potential therapeutic importance of this antitumor strategy (14–17). Although recurrent growth of experimental tumors has been observed during anti-VEGF monotherapy, there is evidence that combining VEGF blockade with such cytotoxic agents enhances the duration and efficacy of tumor suppression in some models (2, 18). Previous studies suggest that cytotoxic chemotherapeutic agents delivered frequently on a low-dose regimen (“metronome” schedule) may have antiangiogenic properties (19). Among these, the topoisomerase inhibitors act in part by inhibiting transcriptional activation of hypoxia-inducible factor-1, an important positive regulatory point for many proangiogenic genes (20). In these studies, we examined vascular remodeling and tumor recurrence during prolonged administration of anti-VEGF antibody, “metronome” topotecan, or combined agents using an orthotopic model of human Wilms’ tumor xenografts in nude mice.

Results and Discussion

Tumors Resume Growth After Initial Suppression by Antiangiogenic Agents

We have previously shown that both VEGF antagonists and a low, frequently administered dose of the DNA topoisomerase I blocking agent topotecan selectively inhibit angiogenesis and suppress tumor growth for 6 weeks in SK-NEP-1 human Wilms’ tumor xenografts implanted in nude mice (21, 22). To examine longer-term effects of antiangiogenesis using these agents, we implanted SK-NEP-1 xenografts in nude mice ($n = 60$). Beginning 1 week after tumor induction, mice were injected with anti-VEGF antibody (MAbs), topotecan, or vehicle. To examine whether the combination of VEGF antagonist and “metronome” cytotoxic agent would more completely prevent tumor growth during chronic suppression of angiogenesis, we also treated a cohort of tumor-bearing mice with both MAbs and topotecan. Tumors were monitored *in vivo* biweekly with calipers and animals were euthanized when xenografts met size criteria (1.5 cm in greatest diameter).

Control mice developed xenografts meeting criteria prior to day 50 (Fig. 1). As in previous reports, SK-NEP-1 xenograft growth was suppressed by MAbs alone or topotecan alone during the initial 6 weeks (21, 22). However, growing tumors were detected in the majority of animals exposed to either single agent by day 100. A subset of the animals treated with combined agents also developed enlarging tumors, although the appearance of these xenografts was relatively delayed (days 70–110). Time to euthanasia was significantly prolonged in topotecan-only and topotecan + MAbs groups ($P < 0.05$).

Alterations in Vessel Morphology Accompany the Resumption of Tumor Growth During Antiangiogenic Therapy

To examine remodeling of xenograft vasculature, a potential mechanism of increasing tumor blood supply and promoting recurrent growth, we examined vessel architecture by specific immunostaining for EC. CD31-immunopositive vessels were sparse in treated tumors. However, vessel morphology was strikingly altered from controls, with multiple layers of CD31-positive cells apparent in treated tumors, although this

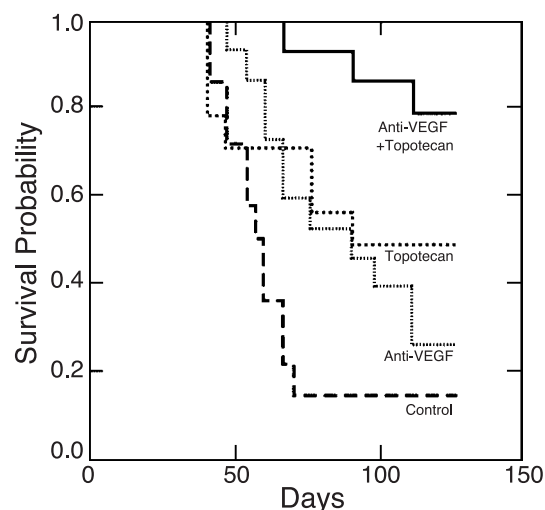


FIGURE 1. SK-NEP-1 xenografts are initially inhibited but resume growth during prolonged administration of anti-VEGF antibody, “metronome” topotecan, or combined agents. Cultured human SK-NEP-1 Wilms’ tumor cells (10^6) were implanted intrarenally in athymic mice ($n = 60$). Animals were divided into four cohorts receiving MAbs, “metronome” topotecan, combined agents, or control vehicle beginning 1 week after implantation. Mice were monitored biweekly with calipers and euthanized when tumors reached 1.5 cm in greatest dimension. Tumor growth was delayed but not abolished by MAbs or topotecan administration, emerging at ~60 days after tumor implantation. Growth in combination-treated tumors was also detected although in a more delayed fashion.

difference was more marked in MAbs-exposed tumors (Fig. 2). In addition, mean diameters increased significantly in treated tumors: 68% in topotecan, 97% in MAbs, and 96% in MAbs + topotecan tumors (diameter expressed as $\mu\text{m} \pm \text{SEM}$: controls 31.1 ± 2.7 ; topotecan alone 52.3 ± 6.9 , $P = 0.0036$ versus controls; MAbs 61.3 ± 6.2 , $P < 0.0001$ versus controls; MAbs + topotecan 60.9 ± 6.2 , $P < 0.0001$ versus controls; Fig. 3).

Vascular MC Proliferate in Tumors Resuming Growth During Chronic Antiangiogenic Therapy

Previous work suggests that tumor blood vessels lacking vascular smooth muscle or pericytes are relatively susceptible to withdrawal of VEGF, while vessels, which are stabilized by adjacent vascular MC, may preferentially survive VEGF depletion (8, 23). Because resumption of tumor growth during antiangiogenic therapy may be supported by the recruitment of a new blood supply, remodeling of the surviving vessels may augment perfusion and thus contribute to this process. To test for this possibility, we first compared the relative distribution of recruited MC in tumors that resumed growth and controls. Control xenografts displayed relatively scant, discontinuous α -smooth muscle actin (α SMA) staining in comparison with the dense network of CD31-immunopositive vessels, indicating that many tumor vessels lacked a smooth muscle element (Fig. 4). In comparison, α SMA-positive cells were more abundant, constituting a thick, continuous layer in vessels in tumors recurring during MAbs and MAbs + topotecan administration. While topotecan-treated tumors also displayed an increase in α SMA-positive cell recruitment, this was again less marked. Next, we examined proliferation in recruited MC

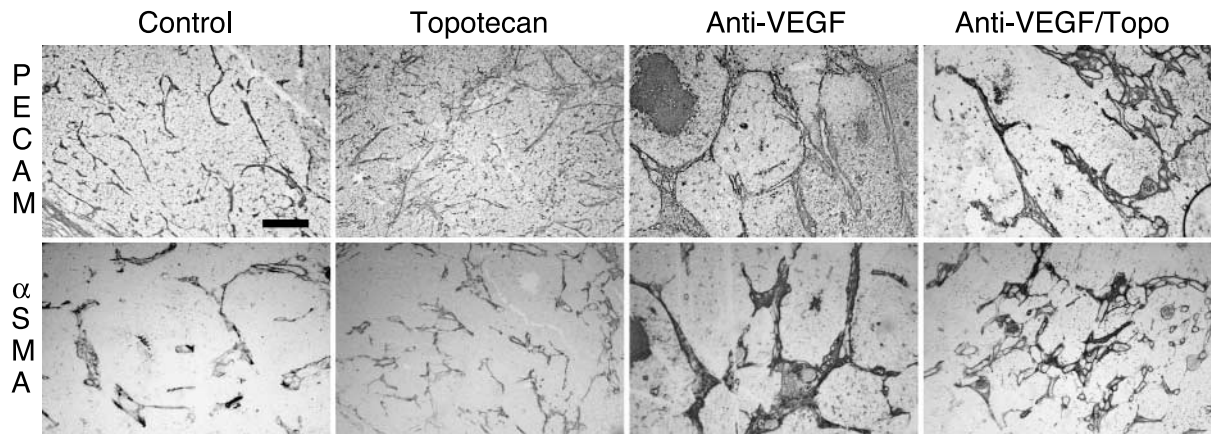


FIGURE 2. Remodeling of endothelial and vascular MC compartments marks the tumors growing during antiangiogenic therapy. Xenografts, which grew during topotecan, MAb, or MAb + topotecan administration, displayed sparse but strikingly remodeled vasculature with irregular, large-caliber lumens and multiple layers of adhering vascular MC (most markedly in MAb and MAb + topotecan-treated tumors) in comparison with controls. *Size bar = 200 μ m.*

by performing double-label immunostaining for α SMA and the proliferation marker phosphohistone H3. MC in remodeled vessels of recurrent tumors displayed brisk proliferation, whereas H3-positive nuclei were scant in control tumor MC. In contrast, in a separate experiment, we evaluated the rare surviving vessels in xenografts that were suppressed by VEGF antagonism at 6 weeks. Vasculature in these tumors displayed neither proliferation nor apoptosis, indicating relative quiescence (data not shown). These results indicate that active MC proliferation in remodeled vessels is concurrent with growth of tumors.

Localization of VEGF and VEGFR-2 Expression in Tumors Resuming Growth During Antiangiogenic Therapy

To examine the distribution of VEGF after extended exposure to anti-VEGF antibody contributed to tumor growth after initial inhibition, we studied expression of VEGF and its VEGFR-2 receptor by *in situ* hybridization in control and MAb-, topotecan-, and MAb + topotecan-exposed tumors (Fig. 5). Coexpression of VEGF and its VEGFR-2 receptor was

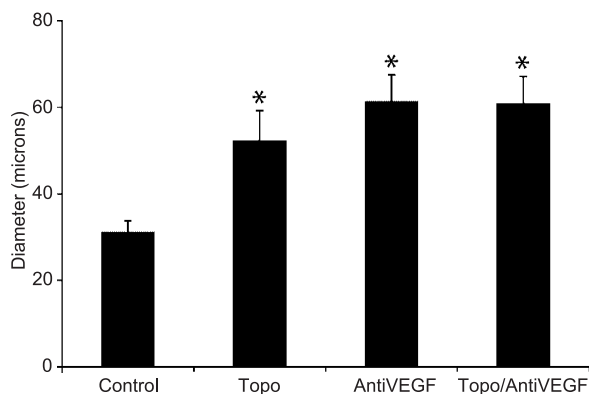


FIGURE 3. Diameters of remodeled vessels increased significantly in the tumors growing during antiangiogenic therapy. Mean diameters, measured using fluorescent immunostaining for α SMA, increased significantly in treated xenografts *versus* controls: 68% in topotecan, 97% in MAb, and 96% in MAb + topotecan tumors. *Columns*, mean diameter (μ m); *bars*, SEM.

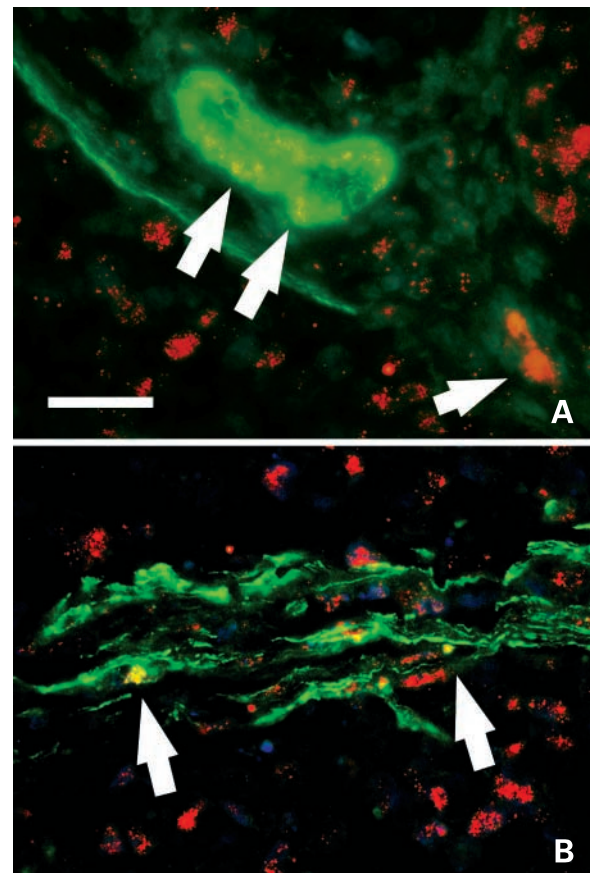


FIGURE 4. Proliferation of vascular MC in vessels of tumors recurring during antiangiogenic treatment. Double-label immunostaining, indicated by yellow areas (arrows) where α SMA (green fluorescent signal) and the proliferation marker phosphohistone H3 (red fluorescent signal) coincide, demonstrates active proliferation in the MC of a vessel supplying a tumor that resumed growth during anti-VEGF blockade. Cross section of MAb tumor (A) and longitudinal view of vessels in combination-treated tumor (B) demonstrating thick layer of vascular MC with frequent proliferating nuclei. In comparison, surviving vessels in tumors inhibited by VEGF antagonism at 6 weeks were quiescent (data not shown). *Size bar = 50 μ m.*

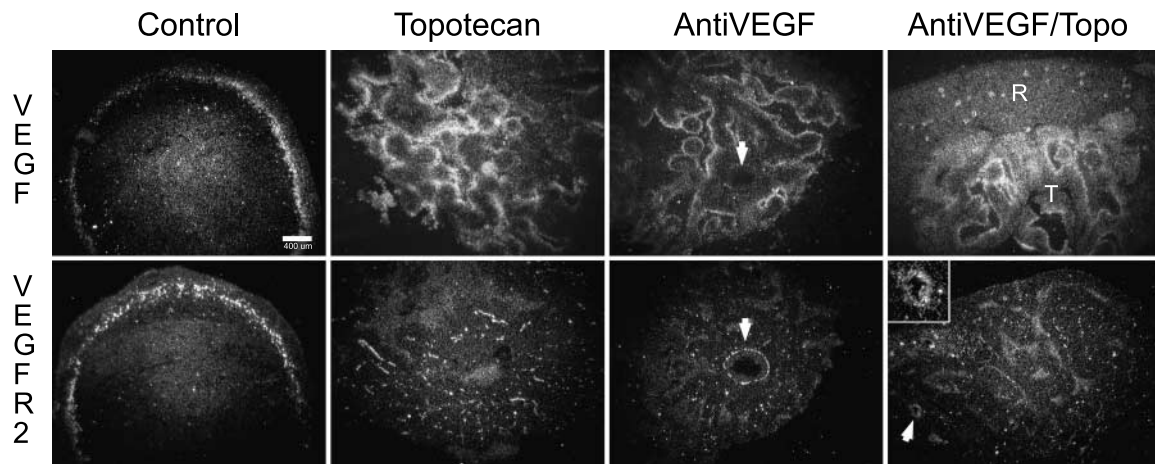


FIGURE 5. Expression of VEGF at the periphery of viable tumor islands contrasts with central expression of VEGFR2. Control tumors express all proangiogenic cytokines at the growing, well-vascularized periphery. Tumors that recur during antiangiogenesis grow as viable clusters or islands around vessels. The peripheral expression of VEGF indicates that the centers of these islands are relatively normoxic; the localization of VEGFR2 indicates the presence of central vessels, which presumably provide perfusion to the viable area of tissue. Size bar = 400 μm .

observed at the growing, vascularized periphery of control SK-NEP-1 xenografts (Fig. 5). In contrast, tumors that resumed growth during prolonged antiangiogenic therapy appeared to form distinct clusters or islands of viable cells separated by areas of necrosis; this pattern was observed both on standard H&E staining (data not shown) and after *in situ* hybridization. Expression of VEGF and VEGFR-2 did not colocalize in these treated tumors. Instead, VEGF was up-regulated at the margin of each viable tumor island, while VEGFR-2 marked vessels at the center of each. The discordance suggests that recurrent tumors were growing circumferentially around surviving vessels, and because VEGF is critically regulated by hypoxia, tumor tissues around such vessels were adequately perfused and thus relatively normoxic (Fig. 5).

PDGF-B, PDGFR- β , and EphrinB2 Expression in the Vessels of Tumors Which Grew During Antiangiogenic Therapy

As previously mentioned, we observed striking proliferation of vascular MC in the vessels of recurrent tumors. PDGF-B is an important mediator of EC-MC interactions (9, 24). Therefore, we tested for altered PDGF-B expression in these tumors by *in situ* hybridization (Fig. 6). Increased expression of PDGF-B was seen only in recurrent xenografts and like VEGFR-2 was localized to the centers of viable tumor islands. PDGFR- β was also found in the large vessels of the recurrent tumors. In addition, because these morphologically altered vessels resembled large arteries, we examined expression of EphB4 and ephrinB2 (Fig. 6). While control tumors displayed both widespread EphB4-positive and ephrinB2-positive vasculature, the remodeled vessels in recurrent tumors appeared to express ephrinB2 with relatively less EphB4 expression (Fig. 6).

Vascular Remodeling and Arterial Specification Mark Tumors That Grow During Long-Term Antiangiogenesis

Our results provide evidence that extended exposure to anti-VEGF antibody, “metronome” topotecan, or combined agents

can result in resumption of growth of initially suppressible nephroblastoma xenografts and that vasculature in these tumors undergoes extensive remodeling concurrent with tumor growth. Despite the up-regulation of VEGF observed in all tumors that resumed growth during prolonged antiangiogenesis, sprouting of new capillaries from existing vessels was not detected in any. Instead, tumors that grew during antiangiogenic therapy developed as islands surrounding large central vessels, a pattern that was distinct from controls. These remodeled central vessels were marked by significant increases in diameter, proliferation of MC, and expression of PDGF-B/PDGFR- β . PDGF-B signaling is implicated in overall vessel stability: PDGF-B can contribute to MC recruitment (24) that protects tumor vasculature when VEGF levels decline (8). In addition, these vessels expressed ephrinB2, suggesting that arterial specification may participate in the vascular stabilization or remodeling stimulated by chronic hypoperfusion.

In summary, our studies indicate that prolonged antiangiogenesis in Wilms’ tumor xenografts significantly alters the expression of angiogenic factors implicated in vascular MC recruitment, causing extensive morphological changes in vessels (Fig. 7). The juxtaposition of remodeled vessels with actively proliferating MC and viable tumor regions suggests that these vessels are contributing to overall tumor progression during chronic antiangiogenesis. Tumors exposed only to topotecan exhibited these changes to a relatively lesser degree than those exposed to MAbs and MAbs + topotecan, consistent with evidence that camptothecin analogues may act to suppress angiogenesis by a different mechanism than VEGF antagonists (20). Monitoring and addressing such progression may be critical to the care of patients who receive this novel type of therapy.

Materials and Methods

Cell Line

The cell line SK-NEP-1 (American Type Culture Collection, Manassas, VA) was maintained in culture in 75 cm^2 flasks with

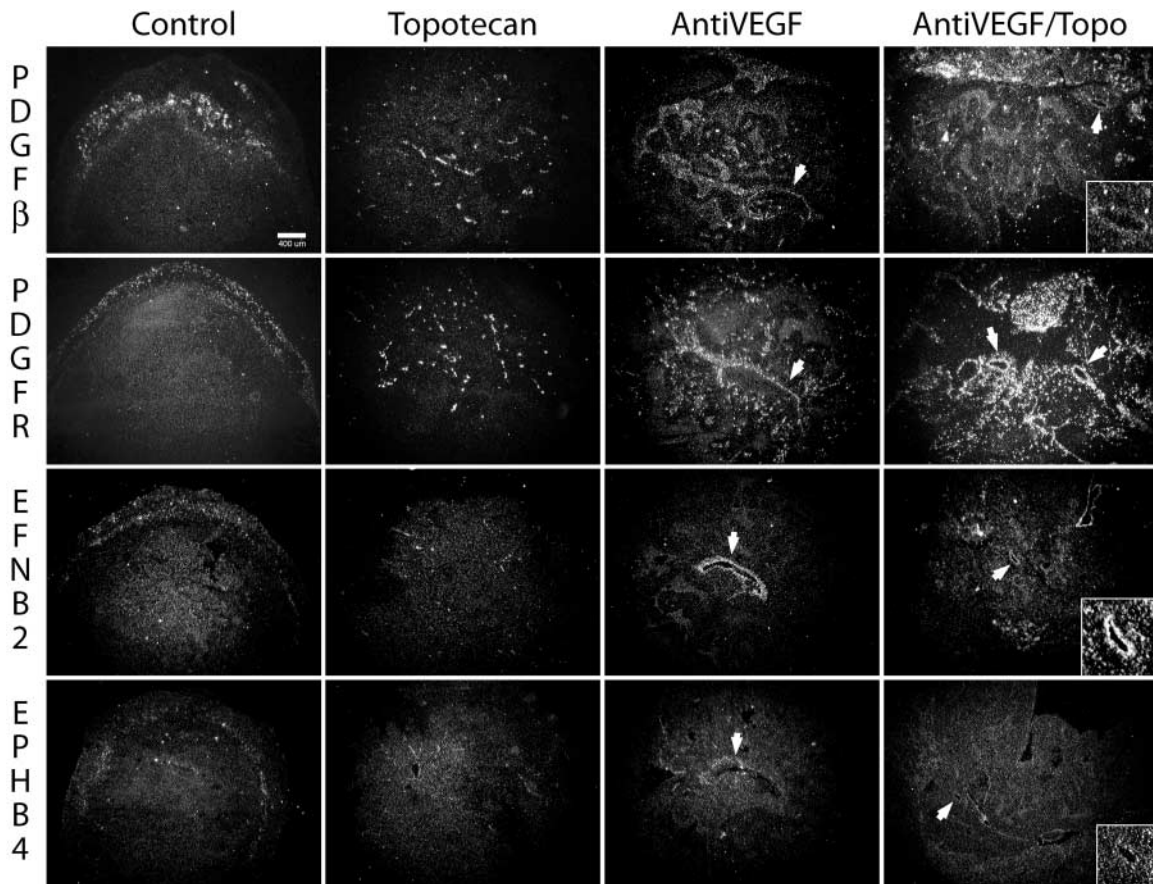


FIGURE 6. Expression of PDGFB, PDGFR β , and EFNB2 are expressed in remodeled vessels of tumor growing during antiangiogenesis. In contrast, expression of PDGFB and PDGFR β appear at the periphery of control tumors. Controls express markers of both venous and arterial specification (EPHB4 and the gene for ephrinB2, EFNB2), whereas EFNB2 was detected in remodeled vessels of treated tumors. Size bar = 400 μ m.

McCoy's 5A medium (Mediatech, Fisher Scientific, Springfield, NJ). Medium was supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were grown at 37°C in 5% CO₂ until confluent, counted with trypan blue staining, and washed and resuspended in sterile saline (PBS, Life Technologies) at a concentration of 10⁷ cells/ml.

Xenograft Model

All experiments were approved by the Institutional Animal Care and Use Committee of Columbia University. Female NCR nude mice (4–6 weeks old; Taconic, Germantown, NY) were housed in a barrier facility and acclimated to 12 h light/dark cycles for at least a day prior to use. The left flank was prepped sterilely after anesthetizing the mice with i.p. ketamine (50 mg/kg) and xylazine (5 mg/kg). An incision was made exposing the left kidney and an inoculum of 10⁶ tumor cells was injected with a 25-gauge needle. The flank muscles were closed with a single 4-0 Polysorb suture (US Surgical, Norwalk, CT) and the skin was closed with staples. Mice were divided into four cohorts: control ($n = 15$), topotecan only ($n = 15$), anti-VEGF antibody only (MAbs; $n = 15$), and topotecan + MAbs (combination group; $n = 15$).

Administration of Anti-VEGF Antibody and Topotecan

I.p. injections were initiated 1 week after tumor implantation. Control mice received i.p. injections of PBS (topotecan control) and mouse serum albumin (anti-VEGF control). The humanized monoclonal anti-VEGF antibody A4.6.1 (MAbs; 100 μ g/dose; Genentech, South San Francisco, CA) was given twice per week, and the DNA topoisomerase I inhibitor topotecan (0.36 mg/kg; SmithKline, Philadelphia, PA) was given for 5 days/week. Tumor size was monitored with calipers and animals were euthanized when the greatest tumor axis reached 1.5 cm. Reproducibility of caliper measurements was confirmed by weighing harvested tumors. Mean tumor weight \pm SEM of all tumors harvested was 0.87 \pm 0.1 g, indicating that measured tumor axis correlated with tumor weight within a narrow band of variability. Survival was depicted using Kaplan-Meier graphs.

Fluorescein Angiography

Selected mice underwent fluorescein angiography via left ventricular puncture at the time of euthanasia. Tissues were fixed in 4% paraformaldehyde, and 15 μ m sections were cut using a vibratome device. These slides were then cooled overnight in a 4°C refrigerator and evaluated and photographed the next day by fluorescent microscopy.

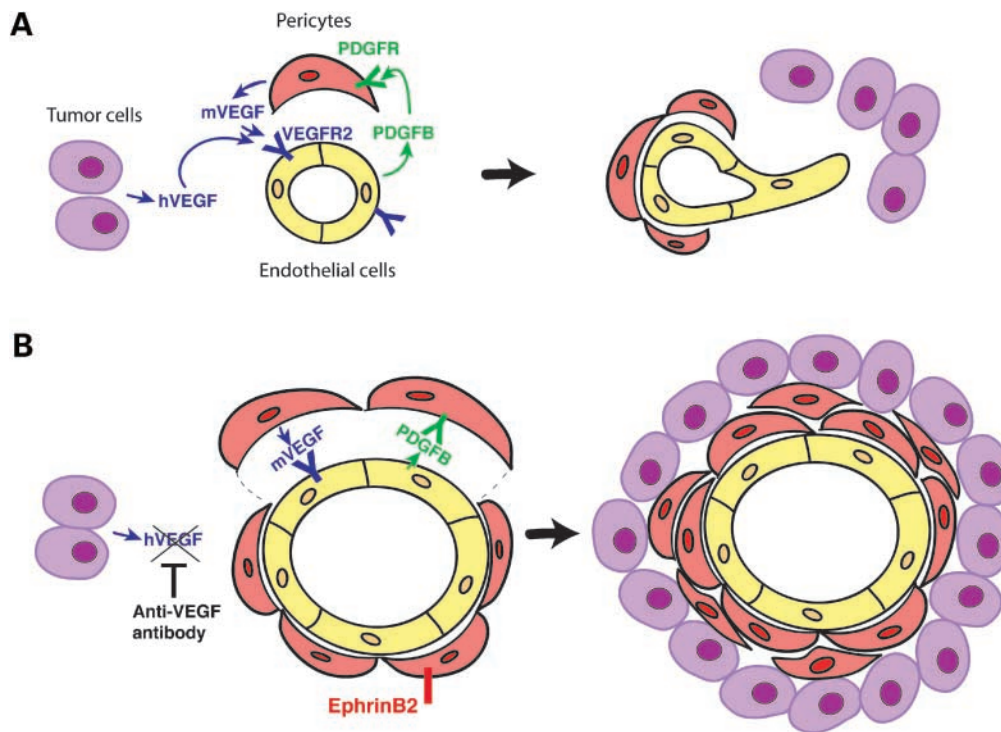


FIGURE 7. Vascular remodeling results from altered expression of angiogenic cytokines during chronic suppression of angiogenesis. **A.** In control tumors, expression of VEGF supports sprouting of new vessel branches, which perfuse growing areas of tumor. PDGFB and VEGF cooperate to recruit MC to endothelial vessels. EphrinB2 and EphB4 are diffusely expressed, consistent with widespread arterial and venous specification in control vessels. **B.** When angiogenesis is chronically blocked, PDGFB expression by surviving vessels increases, which enhances endothelial stability and recruitment of MC. Vessel diameter increases significantly. Tumors resume growth, forming viable layers circumferential to vessels. Vascular expression of EphrinB2 increases, whereas EphB4 expression is relatively less, suggesting that EphrinB2 plays a role in stabilization or remodeling of these vessels.

Immunostaining

Tumors were immunostained for CD31 or α SMA using purified rat anti-mouse CD31 monoclonal antibody (platelet EC adhesion molecule-1; PharMingen, San Diego, CA) or monoclonal anti- α SMA antibody (Sigma Chemical Co., St. Louis, MO), respectively. Biotinylated secondary antibody (Zymed, San Francisco, CA), enhanced horseradish peroxidase-conjugated streptavidin, and a substrate chromogen, 3-amino-9-ethyl carbazole, were used to visualize specific staining (HistoStain-Plus kit, Zymed). Double-label immunostaining for α SMA and histone H3 was performed using an anti-phosphohistone H3 antibody (Upstate Biotechnology, Inc., Lake Placid, NY) and the anti- α SMA antibody indicated above (Sigma Chemical). Slides were incubated with anti-phosphohistone H3 antibody at room temperature for 30 min then with goat anti-rabbit biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA). After PBS washes, specimens were incubated with Alexa Fluor 555 conjugate (generating a red fluorescent signal; Molecular Probes, Eugene, OR) to visualize proliferating cells using fluorescence microscopy. Slides were then incubated with anti- α SMA antibody, biotinylated secondary antibody, and Alexa Fluor 488 conjugate (generating a green fluorescent signal; Molecular Probes). Mounted slides with medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories). Slides were examined and photographed using a Nikon Eclipse E600 microscope (Nikon Inc., Melville, NY) and a SPOT RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI).

Quantification of Vessel Diameters

Vessel diameter was measured from α SMA-immunostained slides using the SPOT RT Software. To confirm that our calculations were indeed of vascular structures, we performed side-by-side comparison of α SMA- and platelet EC adhesion molecule-1-immunostained sections, which indicated that the α SMA-positive cells were perivascular. Diameter increases were also observed by fluorescence angiography. The outside diameter of the largest vessel in a field was measured: 3 tumors/group, 10 fields/tumor (total of 30 fields/group).

In Situ Hybridization

Tissue was initially preserved in 4% paraformaldehyde overnight, transferred to 17% sucrose, and embedded in OCT compound (Miles, Inc., Elkhart, IN) and frozen. Adjacent tissue sections were then probed with 35 S-labeled cRNA with probes hybridizing to human VEGF and murine VEGFR-2, PDGF-B, PDGFR- β , EphB4, and ephrinB2 as previously described (25).

Statistical Analysis

Kaplan-Meier graphs were generated using the MedCalc software package (MedCalc Software, Mariakerke, Belgium). Kruskal-Wallis analysis was used to compare diameters of vessels quantification studies using the Analyse-It + Excel software package (Analyse-It, Leeds, United Kingdom).

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