Sustained VEGF Blockade Results in Microenvironmental Sequestration of VEGF by Tumors and Persistent VEGF Receptor-2 Activation

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Abstract

Vascular endothelial growth factor (VEGF) blockade has been validated clinically as a treatment for human cancers, yet virtually all patients eventually develop progressive disease during therapy. In order to dissect this phenomenon, we examined the effect of sustained VEGF blockade in a model of advanced pediatric cancer. Treatment of late-stage hepatoblastoma xenografts resulted in the initial collapse of the vasculature and significant tumor regression. However, during sustained treatment, vessels recovered, concurrent with a striking increase in tumor expression of perlecan, a heparan sulfate proteoglycan. Whereas VEGF mRNA was expressed at the periphery of surviving clusters of tumor cells, both secreted VEGF and perlecan accumulated circumferentially to central vessels. Vascular expression of heparanase, VEGF receptor-2 ligand binding, and receptor activation were concurrently maintained despite circulating unbound VEGF Trap. Endothelial survival signaling via Akt persisted. These findings provide a novel mechanism for vascular survival during sustained VEGF blockade and indicate a role for extracellular matrix molecules that sequester and release biologically active VEGF.

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Introduction

Existing antiangiogenic paradigms do not fully explain tumor recurrence after an initial response to vascular endothelial growth factor (VEGF) blockade, although remodeling of persistent vessels accompanied by changes in vascular-specific gene expression has been described in experimental tumors in which VEGF signaling is disrupted (1-4). Such changes in vessel structure have been linked to paradoxical early increases in the perfusion of surviving tumor cells (5, 6). These observations raise the possibility that vascular remodeling, over the long-term, might ultimately result in the recovery of perfusion and tumor recurrence. Previous work has largely focused on the changes in growth factor secretion by tumors subjected to VEGF blockade, and consequent mural cell recruitment (1, 4). However, the tumor matrix, including the vascular basement membrane, a self-assembling layer of insoluble glycoproteins that acts as a scaffold, has been shown to play a significant role in angiogenesis (7-9).

Tumor vascular basement membrane typically includes collagen IV and the heparan sulfate proteoglycan perlecan, fosters the stable assembly of vascular cells, and sequesters heparin-binding growth factors perivascularly (7, 10). New capillary sprouts form within an envelope of these matrix molecules, and conversely, VEGF withdrawal could cause the apoptosis of vascular cells but leave an intact vascular basement membrane sleeve (10, 11). The bioavailability of these locally bound heparin-binding growth factors critically influences interactions with endothelial cell surface receptors, and thus, tumor angiogenesis. The endoglycosidase heparanase promotes tumor angiogenesis in part by releasing these heparan sulfate proteoglycan–sequestered growth factors, and (conversely), the reduction of heparanase activity suppresses tumor vessel formation (12, 13). Hypoxia may enhance both of these microenvironmental processes. For example, the expression of matrix proteoglycans could be altered during chronic microenvironmental stress (14, 15), whereas hypoxia increases the activity of secreted heparanase in tumors (16).

Collectively, the plasticity of this perivascular tissue, its persistence during vascular regression, and its ability to support vascular survival and proliferation suggested to us that tumor vascular basement membrane might function to support vasculature during prolonged VEGF blockade. Reasoning that patients with aggressive neoplasms are the most likely to require novel VEGF-targeting drugs, we explored this possibility by testing the effect of sustained potent VEGF blockade in a model analogous to an advanced human cancer. Hepatoblastoma is a pediatric cancer frequently presenting with...
significant tumor burden and chemoresistance, which develops around a mature, hierarchical vasculature. We therefore used this model to investigate the role of the perivascular basement membrane in the response of tumor vasculature to sustained VEGF blockade.

Results
VEGF Trap Causes Tumor Regression in Established, Late-Stage Hepatoblastoma Xenografts
In order to test the effect of sustained potent VEGF blockade in advanced experimental cancers, we allowed intrarenal implants of 10^6 cultured human HUH-6 hepatoblastoma cells to grow for 5 weeks. As previously described, intrarenal implantation results in tumors which replicate the characteristic architecture of the human disease, in which tumor trabeculae form around vascular cores (Supplemental Fig. S1A; ref. 17). Intraperitoneal injection with 500 ng of the soluble VEGF receptor 1 and 2 (VEGFR1 and 2) construct VEGF Trap (18) was begun after a cohort was euthanized (day 0 controls). We did this experiment twice, with similar results. In experiment no. 2, a larger number of mice were treated with VEGF Trap [experiment no. 1 (n = 12) versus experiment no. 2 (n = 47)], allowing us to perform a more robust analysis of vasculature at day 15, and to maintain an additional cohort of treated mice after day 44 to monitor for further vessel and tumor remodeling. Therefore, experiment no. 2 was selected for further analysis (results from experiment no. 1 are presented in Supplemental Fig. S1B). Animals were killed after day 0 (n = 19), day 15 (n = 21), and day 44 (n = 5). Twenty-one mice were monitored between days 44 and 60, of which three were euthanized for progressive tumor burden; all remaining animals were euthanized at day 60. After 15 days, treated tumors had regressed by 54% (Fig. 1, day 0 controls, 3.48 ± 0.68 g, versus day 15 treated tumors, 1.52 ± 0.39 g; P < 0.01). Mean tumor weight in VEGF Trap–treated animals did not change significantly at day 44 (1.78 ± 0.79 g) or in surviving animals at day 60 (1.22 ± 0.22 g). In contrast, untreated controls grew progressively and were euthanized at day 15 (mean tumor weight, 4.07 ± 0.39 g). Unbound VEGF Trap was present in the treated animals as determined by serum levels measured by ELISA (33,422 ± 10,077 ng/mL; mean ± SE).

Sustained VEGF Trap Administration Results in Initial Regression of Vasculature in Hepatoblastoma Xenografts, Followed by Progressive Remodeling and Vessel Recovery
We found the collapse of vasculature and tumor necrosis at day 15, concurrent with significant tumor regression. Tumor weights did not change significantly in the next 29 days of treatment (day 44; mean weight, 1.78 ± 0.79 g, P = not significant as compared with day 15). However, vessel length increased statistically significantly between days 15 and 44, together with recovery of organized tumor trabeculae (Fig. 2).

To analyze these alterations in tumor vasculature, we did lectin perfusion angiography and specific immunostaining for vascular components. Lectin perfusion studies confirmed the initial marked loss of perfused vessels during VEGF Trap treatment, with lengthening of vessels at day 44 (Supplemental Fig. S2A).

Immunohistochemistry showed branched, hierarchical vasculature at day 0, which was largely ablated by day 15 of treatment, concurrent with widespread tumor necrosis (Fig. 2). By day 44, vessels had reappeared and lengthened, although branching was still relatively suppressed. The vasculature did not change significantly between days 44 and 60. To quantify vascular recovery, we compared mean vessel length using computer-assisted image analysis (16) at days 0, 15, and 44 (Fig. 2B). At day 15, mean vessel length had decreased to 43% of untreated controls at the same time point (P < 0.0001). However, by day 44, vessel length had recovered to 70% of day 15 controls, a significant increase from treated tumor vessel length values at day 15 (P = 0.0035).

Because mural cell recruitment has been implicated as a critical survival mechanism for endothelium when VEGF is withdrawn (19, 20), we examined the expression of neural-glial 2, a proteoglycan expressed by nascent pericytes (21, 22), and of α-smooth muscle actin, a marker for differentiated vascular mural cells (Fig. 2). Both neural-glial 2– and α-smooth muscle actin–immunopositive cells formed a closely apposed periendothelial layer in day 0 control tumors. At day 15, this layer of neural-glial 2– and α-smooth muscle actin–immunopositive cells became irregular in persisting vessels, forming a loose, reticular envelope. At day 44, this irregular pattern of neural-glial 2(+) and α-smooth muscle actin(+) covering persisted, although vessel length increased significantly. The percentage of endothelial vessels covered by mural cells was high (>90%) and did not change during the course of the experiment (Supplemental Fig. S2B and C). These data suggest that, in this system, VEGF blockade did not simply cause the pruning of endothelial vessels lacking mural cells, as has been described in other tumor systems after VEGF withdrawal (19), but induced
both loss of covered vessels and disrupted apposition of mural and endothelial cells. These findings raised the possibility that another mechanism was responsible for remodeling and survival of the vasculature in these tumors.

Reasoning that other stromal elements might contribute to these alterations, we examined the expression of collagen IV, a principal element of tumor vascular basement membrane (10). Collagen IV formed typical thin vessel sleeves in day 0 tumors, and seemed to be slightly thickened in the rare surviving day 15 vessels, but accumulated strikingly perivascularly in the day 44 tumors (Fig. 3A). Trichrome staining similarly showed a marked deposition of collagen-surrounding vessels (Fig. 3A). We quantified this increase using computer-assisted image analysis (16). At day 15, collagen deposition in VEGF Trap–treated tumors was 2.0 times that in controls at the same time point, and by day 44, it was 2.6 times greater in treated tumors as compared with day 15 controls (\( P < 0.0001 \), both; Fig. 3B).

Thus, vascular lengthening seemed to be concurrent with a striking and quantitatively highly significant increase in vascular basement membrane.

**FIGURE 2.** VEGF Trap causes an initial regression of the vasculature, but is followed by progressive vessel recovery. A. Tumor vasculature was analyzed by immunohistochemistry for endothelial cells (PECAM), early (NG2), and more differentiated (\( \alpha \)-SMA) pericytes (bar, 100 \( \mu \)m). Vessels were present at day 0 (arrows) and markedly diminished with near-total loss of branching at day 15 of treatment. In addition, the normally smooth apposition of pericytes to the endothelial cells was perturbed, becoming loose and reticular. Vessels increased in length and recovered some branching at day 44, although the changes in mural cell patterning persisted (arrows). B. Quantification of vascular changes by computer-assisted image analysis of platelet-endothelial cell adhesion molecule signals showed decreased length at day 15, with significant recovery at day 44 (day 15 treated versus control (\( P < 0.0001 \)), day 44 versus day 15 control (\( P = 0.0035 \))].

**Recovering Tumors Display Striking Perivascular Accumulation of Perlecan and VEGF**

Perlecan is another key participant in vascular basement membrane, binding to collagen IV via its heparan sulfate moieties. We examined the expression of both murine (host-derived) and human (tumor-derived) perlecan (Fig. 4). Expression of murine perlecan was restricted to the vasculature and increased slightly by day 44; in contrast, perivascular accumulation of human perlecan increased dramatically. Because heparan sulfate proteoglycans can sequester heparin-binding growth factors, we reasoned that VEGF might be bound in the immediate perivascular environment, potentially allowing tumor vessels to largely regain their pretreatment appearance. We found markedly increased VEGF protein deposited in the same perivascular pattern as tumor-derived perlecan, and that these colocalized when examined by double immunofluorescence (Fig. 4). Intriguingly, fibroblast growth factor-2, another heparin-binding endothelial mitogen recently implicated in tumor progression during VEGF blockade (3), was predominantly intracellular and not perivascular.
We found modest increases in the expression of platelet-derived growth factor B and angiopoietin-1 (Supplemental Fig. S3), also previously reported to increase in tumors subjected to VEGF blockade (1-3, 6). We investigated the expression of both perlecan and VEGF mRNA by in situ hybridization (Fig. 4). Perlecan expression was broadly increased in day 15 and day 44 tumors. In contrast, consistent with its known regulation by hypoxia, VEGF mRNA expression was seen at the periphery of tumor trabeculae. Taken together, these data indicate that tumor-derived perlecan and VEGF mRNA are expressed in relatively avascular zones, but that the secreted products are subsequently localized to the vasculature.

In order to determine whether these alterations could be detected in a different tumor system during VEGF blockade, we studied SKNEP-1 xenografts, derived from a Ewing sarcoma cell line. As in the above studies, established SKNEP-1 tumors were treated with VEGF Trap after 5 weeks of growth; significant regression (79%) occurred by day 36 (as previously reported in ref. 23). A subset of tumors was maintained after this time point until day 72. Similar to our findings in the HUH-6 model, collagen IV and perlecan were detected as thin lines in day 0 tumor vasculature, with little VEGF protein. In contrast, in late-stage VEGF Trap–treated SKNEP-1 tumors, perivascular collagen IV, perlecan, and VEGF deposition increased markedly (Supplemental Fig. S4). Although changes were less striking than in the HUH-6 tumors, these results suggest that this phenomenon is not limited to the hepatoblastoma model.

Recurrent Tumors Express Heparanase-1 and Display Binding of VEGF to VEGFR2, Phosphorylated VEGFR2, and Activation of the Akt Survival Pathway in Endothelial Cells

These findings raised the question of whether this sequestered VEGF was biologically active. The endoglycosidase heparanase-1 has a well-established role in angiogenesis, functioning to release molecules bound to heparan sulfate moieties of matrix proteoglycans (13). Consistent with this function, we detected increased expression of murine heparanase-1 perivascularly in day 15 and day 44 tumors, indicating that this process was localized to the immediate vicinity of tumor vessels (Supplemental Fig. S5). Expression of heparanase-1 was also detected in tumor cells using antihuman heparanase-1 antibody (data not shown).

In order to determine whether VEGF was physically associated with its VEGFR2 receptor, we used the GV39M antibody, which selectively recognizes the ligand/VEGFR2 receptor complex and is thus an indicator of bioavailability (24). Immunofluorescently visualized ligand/receptor complexes were found in control tumors, and persisted in the scant vessels of the largely necrotic day 15 tumors, and were apparent throughout day 44 tumors (Fig. 5). We also investigated the activation of the VEGFR2 receptor by ELISA for its phosphorylated form (pVEGFR2); pVEGFR2 was increased by 30% in VEGF Trap–treated tumors versus controls at day 15 (P < 0.05; Fig. 5). To determine the status of the phosphoinositide-3-kinase-Akt pathway, a target of VEGF/VEGFR2 signaling and a critical mediator of survival in
endothelial cells, we examined the expression of the phosphorylated form of Akt. Phosphorylated Akt was detected in endothelial cells of day 0, day 15, and day 44 tumors, indicating preserved survival signaling despite ongoing VEGF blockade (Fig. 5). Treatment of tissue sections with lambda protein phosphatase abolished staining for both pVEGFR2 and phosphorylated Akt, indicating that staining was specific for the phosphorylated form of VEGFR2 and AKT (Supplemental Fig. S6).

Discussion
Tumor progression during VEGF blockade is likely to reflect interactions in the microenvironment that promote vascular survival. Here, we provide the evidence for a novel mechanism contributing to vascular recovery during VEGF blockade based on increased expression of perivascular extracellular matrix elements. The target ligand VEGF is sequestered in the vessel microenvironment via increased tumoral expression of the heparan sulfate proteoglycan.

FIGURE 4. Perlecan and VEGF accumulate perivascularly. Immunohistochemistry was done for murine perlecan (mPERL; U.S. Biochemical antibody), human perlecan (hPERL; Zymed antibody), and VEGF (bar, 100 μm). Immunofluorescence was done for VEGF (red) and human perlecan (green), with a 4′,6-diamidino-2-phenylindole nuclear stain (blue; bars, 100 μm). Expression of murine perlecan increased and localized to the perivascular envelope. However, much more striking was the increase in perivascular deposition of human perlecan and VEGF, which (similar to collagen IV) formed a thick layer encasing the vasculature at day 44. Immunofluorescence showed the colocalization of these signals. To compare the localization of mRNA, we did an in situ hybridization for perlecan (bar, 100 μm) and VEGF (bar, 400 μm). Perlecan mRNA expression increased diffusely across tumors, whereas VEGF mRNA was detected at the edges of viable tumor islands, consistent with the greatest distance from perfusing vasculature. These data indicate that whereas perlecan and VEGF may have been synthesized at a distance from the vessels, each protein was sequestered by the perivascular envelope.
perlecan, itself localized to the vasculature by interaction with collagen IV. Consistent with this mechanism, we observed the increased perivascular expression of heparanase in day 44 tumors. Thus, these results lead us to propose a novel model for support of the vasculature during treatment with the VEGF-binding drug VEGF Trap (Fig. 6), based on an increase in bioavailable VEGF in the immediate vessel microenvironment.

Altered expression of vascular-specific growth factors has been found to contribute to vascular integrity in the setting of VEGF blockade. For example, recent reports implicate angiopoietin-1 in the early recovery of tumor perfusion, and fibroblast growth factor-2 in tumor progression when VEGF signaling is disrupted (3, 6). Fibroblast growth factor-2 is known to bind perlecan, raising the possibility that fibroblast growth factor-2 contributed to the vascular recovery we observed. However, in these experiments, fibroblast growth factor-2 was found intracellularly, and did not colocalize with perlecan, indicating that it may not participate in this matrix-mediated form of vascular support in our xenograft model.

Previous data suggests that tumor vessels that persist during VEGF blockade may be supported by increased contact with vascular mural cells. Such remodeled networks often appear pruned, with surviving vessels having relatively large calibers, multiple layers of adherent mural cells, and scant fine branches. Similar to previous reports, we detected moderate increases in the expression of platelet-derived growth factor B and angiopoietin-1 in our system, both of which are implicated in vascular remodeling and mural cell recruitment (1-3). However, our model differed in that a high proportion of initial tumor vasculature was already invested with pericytes. Furthermore, VEGF blockade caused no change in this proportion, but caused the regression of mural cell–covered branches and altered patterning of mural cell layers. Surviving vessels retained these features but lengthened significantly during sustained VEGF inhibition. This pattern of vascular remodeling may reflect the role of perlecan, which restricts vascular mural cell activity and proliferation, and collagen IV, which promotes vessel homeostasis (7, 25, 26). The greatly significantly thickened basement membrane layer sequestered VEGF and supported persistent VEGF signaling, but inhibited the local
breakdown of extracellular matrix required for new branch formation. Vessel growth was manifested as an increase in length, supporting tumor perfusion along existing axes, but not radial tumor growth.

The patients who may experience the most benefit from novel antiangiogenic therapies are those with substantial tumor burdens. Thus, we chose to explore the effect of sustained VEGF blockade in an analogous model of advanced cancer. The mechanism we have uncovered in these studies may be particularly pertinent to large tumors with established vessels, in which regressing vasculature leaves behind "sleeves" of basement membranes that could serve as scaffolding for renascent vessels when proangiogenic signaling resumes (27). Consistent with a role for this vascular scaffolding in tumor vessel recovery, organized tumor architecture reappeared concurrent with increased expression of perivascular collagen IV at day 44, although increased expression of perlecans and activation of VEGFR2 was observed earlier at day 15.

Our results are consistent with previous studies reporting that agents which impair heparanase (e.g., PI-88) or perlecans function effectively restrict tumor angiogenesis in experimental models (28, 29). Furthermore, they suggest that molecules which target VEGFR2 signaling directly, such as small-molecule tyrosine kinase inhibitors, may also evade this compensatory mechanism. Our data provides a rationale for future studies examining the efficacy of combining such approaches with VEGF ligand-binding agents, with the goal of increasing the durability of antiangiogenic blockade in susceptible tumor systems.

In summary, these data suggest a new paradigm for the recovery of tumor vessels, a potential contributor to the tumor progression observed clinically during sustained treatment with drugs that bind VEGF. Tumors subjected to sustained VEGF blockade may partly adapt by increasing the expression of vascular basement membrane collagen IV, heparan sulfate proteoglycan, and heparanases, resulting in the sequestration and release of the ligand in the immediate microenvironment of the tumor vessel. Consequently, VEGFR2 activation persists and endothelial survival signaling is stimulated. Taken together, these findings provide new evidence that even tumors that are initially highly responsive to VEGF blockade could rescue VEGF signaling by increasing the microenvironmental bio-availability of this molecule. Strategies that target this response may enhance the efficacy of anti-VEGF therapy in patients with cancer.

Materials and Methods
Cell Culture, Xenograft Model, and VEGF Trap Administration

Human hepatoblastoma cells (HUH-6; RIKEN BioResource Center) were maintained in DMEM enriched with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen Corporation). Confluent cells were harvested with trypsin, counted, and resuspended in PBS (10⁵ cells/mL). In accordance with protocols approved by the Institutional Review Board and Institutional Animal Care and Use Committee of Columbia University, 4- to 6-week-old female NCR nude mice (Taconic Farms) were anesthetized by intraperitoneal injection of xylazine/ketamine (50 mg/kg and 5 mg/kg, respectively), using a previously reported model (17). Briefly, HUH-6 cells form consistent xenografts which reproduce the histology of the human tumors only after intrarenal implantation (Supplemental Fig. S1A), but not in the liver or subcutaneous tissue. Therefore, the left kidney was exposed via a flank incision, and 10⁶ tumor cells implanted intrarenally. Tumors were allowed to grow for 5 weeks. After confirmation of the presence of a tumor, an initial cohort of control mice was randomly selected for sacrifice. The remaining mice were randomly assigned to treatment and control groups receiving 500 µg of VEGF Trap or Fc protein biweekly, respectively. Tumors were monitored biweekly for regression using calipers, and harvested from each cohort at intervals. We did this experiment twice, with similar results. However, in experiment no. 2, a larger number of mice were treated with VEGF Trap [experiment no. 1 (n = 12) versus experiment no. 2 (n = 47)], and an additional cohort of VEGF Trap–treated mice was maintained after day 44 and monitored until day 60. Experiment no. 2 was selected for further analysis because of this additional data. Experiment no. 1 is presented in Supplemental Fig. S1B. All control mice were killed at the day 15 time point due to progressive tumor burden, whereas treated mice did not begin to develop evidence of significant tumor burden until after day 44. Because intrarenal tumors approaching 2.0 g can be palpated and measured by calipers in the flanks of nude mice, we used this method to monitor tumor burden during the experiment. Gross tumor weight was determined by calculating the difference in weight of the tumor-bearing left and normal right kidney. Tumor specimens were fixed in 4% paraformaldehyde at 4°C for 24 h, then processed by alcohol dehydration and paraffin embedding, or 24-h incubation in 17% sucrose, followed by embedding and freezing in optimal cutting temperature compound.

Fluorescein Angiography
Selected mice underwent fluorescein angiography prior to sacrifice. After anesthesia with ketamine/xylazine, mice underwent left intraventricular injection of 1 mL of fluorescein-dextran (molecular weight, 1 × 10⁶; Sigma
Chemical Co.). Fluorescein-perfused tumors were fixed in 4% paraformaldehyde overnight, with 100-μm sections (cut by vibrotome) and 5 μm sections visualized by fluorescent microscopy.

Immunohistochemistry

Five-micrometer sections were cut from prepared paraffin blocks. Following deparaffinization and rehydration of specimens using graded alcohol concentrations, endogenous peroxidase activity was quenched with 3% H₂O₂/methanol (v/v). Antigen retrieval, where necessary, was done. After two blocking steps (avadin/biotin; Vector) for 15 min each at room temperature, followed by CAS Block (Zymed Laboratories) for 1 h at room temperature, slides were incubated with primary antibody, optimally diluted in CAS Block, followed by an overnight incubation at 4°C. Appropriate species-specific biotinylated secondary antibodies (Vector Laboratories) were used at a 1:500 dilution, and carried out at room temperature for 30 min. Enhanced horseradish peroxidase–conjugated streptavidin and substrate chromogen signal was detected using the Vector Elite kit according to the manufacturer’s instructions and Nova Red substrate (Vector). All rinses were done in 1× PBS with 0.1% Brij-35 (Fisher Scientific). Slides were examined with a Nikon Eclipse E600 microscope. The quantification of vascular features was done as previously described (16). A list of primary antibodies is included in the Supplemental Data.

Fluorescent Immunohistochemistry

Immunofluorescence was done on frozen specimens. Five-micrometer sections were cut from tumors embedded in optimal cutting temperature compound and stored at —80°C. Slides were brought to room temperature, washed in ice-cold acetone for 10 min, incubated with avidin/biotin, CAS blocking solutions, and primary antibody as described above. A biotinylated secondary antibody was used in combination with fluorescein-labeled avidin to visualize signals. To provide a negative control for each tissue section, the same procedure was followed but primary antibody was omitted. Slides were examined with a Nikon Eclipse E600 microscope and photographed by fluorescent microscopy.

Quantification of pVEGFR2

The amount of pVEGFR2 was quantified by ELISA (DuoSet IC for Phospho-VEGF R2/KDR; R&D Systems). ELISAs were done according to the manufacturer’s instructions.

Masson Trichrome Stain

This stain was done on paraffin-embedded specimens according to the manufacturer’s instruction (Poly Scientific Research & Development Corp.). Briefly, 5 μm sections were cut, deparaffinized, rehydrated, and incubated with Bouin solution for 1 h at 56°C. Slides were cooled, rinsed in distilled water, and stained sequentially with hematoxylin, Biebrich scarlet-acid fuchsin solution, and aniline blue. Slides were mounted and visualized using a Nikon E600 microscope. Quantification of signal was done as described above.

In situ Hybridization

Ten-micrometer sections of parafomaldehyde-fixed, optimal cutting temperature compound–embedded tumor pieces were probed with [35S]-labeled or digoxigenin-labeled cRNA with probes hybridizing to human VEGF and perlecan, as previously described (30).

Statistical Analysis

Mean tumor weights of control and treated cohorts were compared by Kruskal-Wallis analysis, and expressed as weight ± SE. Kruskal-Wallis analysis was also used for comparison of vessel length, trichrome staining, and pVEGFR2 ELISA.

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References

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