Inhibition of Cyclooxygenase-2 Disrupts Tumor Vascular Mural Cell Recruitment and Survival Signaling

Alice Lee,1 Jason Frischer,2 Anna Serur,2 Jianzhong Huang,2 Jae-O Bae,2 Zev Noah Kornfield,1 Lucy Eljuga,2 Carrie J. Shawber,1 Nikki Feirt,1 Mahesh Mansukhani,4 Diana Stempak,2 Sylvain Baruchel,1 Julia Glade Bender,1 Jessica J. Kandel,1 and Darrell J. Yamashiro1,2,4

Departments of Pediatrics, Surgery, OB/GYN, and Pathology, College of Physicians and Surgeons of Columbia University, New York, New York and Divisions of Hematology/Oncology and Clinical Pharmacology and Toxicology, Hospital for Sick Children, Toronto, Ontario, Canada

Abstract

Much evidence supports an important role for the inducible enzyme cyclooxygenase-2 (COX-2) in tumor angiogenesis. Previous studies have focused on the role of COX-2 in stimulating endothelial proliferation, with blockade of this enzyme impairing endothelial homeostasis. However, recent data suggest that COX-2 also regulates molecules implicated in endothelial trafficking with pericytes/vascular mural cells (VMC), an interaction crucial to vessel stability. We investigated the role of COX-2 in vascular assembly by testing the effect of the specific COX-2 inhibitor SC-236 in an orthotopic xenograft model of human Wilms’ tumor. Tumor growth was significantly suppressed by SC-236 (78% at day 28, 55% at day 35). Perfusion studies and immunostaining showed a marked decrease in vasculature, particularly in small vessels. Specifically, SC-236 inhibited participation of VMC in xenograft vessels. SC-236–treated tumors developed segmentally dilated, architecturally erratic tumor vessels with decreased nascent pericytes and scant mature VMC. Although vascular endothelial growth factor expression was unchanged, expression of the chemokine receptor CXCR4 was decreased in tumor vessels, consistent with defective homing of vascular progenitor cells. Vascular expression of phosphorylated platelet-derived growth factor receptor-β was also diminished, indicating impaired VMC-endothelial trafficking. Consistent with the key role of this interaction in vessel homeostasis, vascular cells in SC-236–treated tumors displayed markedly diminished phosphorylated Akt, indicating disrupted survival signaling. These results show that SC-236 causes defective vascular assembly by attenuating incorporation of VMC into tumor vessels, impairing endothelial survival, and raise the possibility that blockade of COX-2 may provide therapeutic synergies with angiogenic molecules that more selectively target endothelial cells. (Cancer Res 2006; 66(8): 4378-84)

Introduction

There has been much recent interest in the role of cyclooxygenase-2 (COX-2), one of the three enzyme isoforms that convert arachidonic acid to prostaglandins, in tumor development and progression (1, 2). COX-2 expression is nearly ubiquitous in human cancers and has been correlated with poor prognosis [e.g., in tumors of the breast (3), cervix (4), colon (5), brain (6), and ovary (7)]. Intriguingly, COX-2 seems to have multiple functions in tumor pathogenesis and thus represents an attractive therapeutic target. Among these, COX-2 seems to play a significant role as a positive regulator of tumor angiogenesis (2, 8). For example, expression of COX-2 correlates with vascular density in a variety of human cancers (9–11). In preclinical studies, selective COX-2 inhibitors, such as SC-236 (12) or its analogue celecoxib, show both marked suppression of tumor growth and inhibition of angiogenesis (11, 13, 14). This effect on angiogenesis has been postulated to result from a direct effect on endothelial cells (13), although indirect effects on the same cells have also been suggested (e.g., by decreasing the expression of vascular endothelial growth factor or VEGF; ref. 15).

More recently, however, COX-2 has been shown to interact with a variety of molecules implicated in the participation of pericytes and vascular mural cells (VMC) in developing vasculature, a crucial determinant of vessel stability and function (16). These include platelet-derived growth factor (PDGF-B), a key mediator of endothelial-VMC interaction, and the chemokine receptor CXCR4, which functions in the homing of vascular progenitor cells to sites of active angiogenesis (17, 18). Although PDGF-B is a key modulator of pericyte participation in tumor vasculature, isolated blockade of PDGF-B signaling seems to be only of minimal antiangiogenic efficacy (19). Despite this, targeting of PDGF-B in combination with VEGF blockade provides additional antitumor efficacy in experimental models (20). These findings argue that identification of additional pathways that influence tumor VMC recruitment may be therapeutically valuable.

Suppression of tumor angiogenesis by targeting endothelial cells, most notably by disrupting VEGF signaling, has recently been validated as a therapeutic strategy in some treatment-refractory human cancers. However, it is clear that even those patients with clinical responses ultimately develop progressive disease, although the mechanisms of resistance to antiangiogenic approaches are not clear (21). Recent experimental data suggest that tumor progression during sustained antiangiogenesis is supported by vascular remodeling, which stabilizes vessels and presumably restores tumor perfusion. Enhanced pericycle/VMC recruitment and increased expression of PDGF-B seem to be characteristic features of this process (22–24).

COX-2 is a key mediator of vascular response to chronic microenvironmental stress, an aspect of which is the modification of VMC proliferation and function (25, 26). We have previously extensively investigated the effect of sustained antiendothelial therapy in an orthotopic murine model of Wilms’ tumor (WT; ref. 24). Therefore, we investigated the effects of COX-2 blockade...
using this paradigm. Consistent with the proangiogenic functions of COX-2, the specific COX-2 inhibitor SC-236 markedly perturbed blood vessel development and perfusion in our model, resulting in inhibition of tumor growth. Xenograft vessels were both quantitatively and qualitatively distinct. Fine vessels were nearly absent in SC-236–treated tumors. In addition, the vasculature that did develop in treated tumors was characterized by erratic, segmentally dilated vessels. These abnormal vessels displayed a paucity of periendothelial stromal cells and decreased expression of CXCR4, indicative of a deficiency in important homing and survival mechanisms for nascent VMC. In addition, activation of the PDGF-B pathway in tumor vasculature was suppressed despite preserved expression of PDGF-B. SC-236–treated tumors also displayed decreased phosphorylated Akt (pAkt), a marker for survival signaling, in vascular cells. Taken together, these results indicate that COX-2 blockade by SC-236 disrupts mural cell participation and consequent homeostasis of tumor neovasculature, thereby restricting perfusion and growth of xenografts.

**Materials and Methods**

**Cell line.** The cell line SK-NEP-1 (American Type Culture Collection, Manassas, VA) was maintained in culture in 75-cm² flasks with McCoy’s 5A medium (Mediatech, Fisher Scientific, Springfield, NJ). Medium was supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY). Cells were grown at 37°C in 5% CO₂ until confluent, harvested, counted with trypan blue staining, and resuspended in sterile PBS (Life Technologies) at a concentration of 10⁶ per mL.

**Animal model.** The Institutional Animal Care and Use Committee of Columbia University approved all experiments. Four- to 6-week-old female NCR nude mice (National Cancer Institute at Frederick, Frederick, MD) were housed in a barrier facility and acclimated to 12-hour light/dark cycles for at least 24 hours before experimental use.

**Tumor implantation.** The mice (n = 40) were anesthetized with i.p. ketamine (50 mg/kg) and xylazine (5 mg/kg). The left flank was prepared in a sterile fashion, and an incision was made, leaving the left kidney. An inoculum of 10⁵ SK-NEP-1 tumor cells in 0.1 mL of PBS was injected into the renal parenchyma using a 25-gauge needle. The flank musculature was closed with a single 4-0 Polysorb suture (U.S. Surgical, Norwalk, CT), and the skin incision was closed with staples.

**Administration of SC-236.** One week after tumor implantation, animals were divided into two cohorts (n = 20 each). SC-236 (Pharmacia, St. Louis, MO) was added to drinking water as previously described (27) at a concentration of 30 g/mL and changed thrice per week. This dose of SC-236 is equivalent to ~6 mg/kg/d (anticipated plasma level of inhibitor of 5 µg/mL, according to Pharmacia). Plasma levels of SC-236 were confirmed by high-performance liquid chromatography (HPLC), using an assay that was previously described for determination of celecoxib levels (28). SC-236 was extracted from 150 µL of plasma using solid-phase extraction, and unknown concentrations were compared with a standard curve spanning the range of 0.5 to 15 µg/mL of SC-236.

**Harvesting of specimens and determination of metastases.** At sacrifice, tumors and contralateral kidneys were removed and weighed and then preserved in 4% paraformaldehyde for immunohistochemistry. Portions of tumor were flash frozen in liquid nitrogen and stored at ~80°C. Both lungs were fixed in 10% formalin for histology. Slides of paraffin-embedded lung tissue were stained by routine H&E methods and examined by a surgical pathologist to determine the presence or absence of metastases, using three levels from each lung.

**Lectin perfusion.** Before euthanasia, selected mice underwent left ventricular injection of fluorescein-labeled *Lycopersicon esculentum* lectin (100 µg in 100 µL PBS; Vector Laboratories, Burlingame, CA). The vasculature was fixed by infusion of 1% paraformaldehyde and then washed by perfusion of PBS. Lectin-perfused tissues were cut into 80-µm sections using a vibratome device and evaluated by fluorescent microscopy. Digital images from the fluorescein-labeled lectin studies were acquired from a Nikon E600 fluorescence microscope (×10 objective) with a Spot RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI) and stored as TIFF files. Quantitative assessment of angiogenesis was done by computer-assisted digital image analysis as described by Wild et al. (29), except that fluorescein-labeled lectin was substituted for phycoerythrin-conjugated monoclonal antibody to CD-31. The fraction of fluorescein-positive pixels per total field was quantified and represents the mean vascular density (MVD). Statistical comparison was done using Kruskal-Wallis analysis.

**Quantification of apoptosis and proliferation.** Tumor proliferation was examined by anti-phospho-histone H3 (Upstate, Inc., Lake Placid, NY). Apoptosis was determined by terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) analysis using the ApopTag Red In situ Apoptosis Detection kit (Intergen Co., Purchase, NY).

**Immunohistochemistry.** Endothelial cell staining was done using a rat anti-mouse anti–platelet-endothelial cell adhesion molecule-1 (PECAM-1) monoclonal antibody (BD-MCD31, Research Diagnostics, Inc., Concord, MA). Vascular mural cells were visualized using a rabbit anti-human α-smooth muscle actin (αSMA) antibody (BB-9010, Lab Vision/Neomarkers, Fremont, CA) and a rabbit anti-human neuronal Glial-2 (NG2) antibody (AB58320, Chemicon, Temecula, CA). Vascular basement membrane was visualized with a rabbit anti-human type IV collagen (1:1,000; LB-1,403, CosmoBio, Tokyo, Japan). Immunostaining was done for phosphorylated PDGF-receptor-β (pPDGFR-β), with a goat anti-phospho-p44/42 MAPK (ERK1/2) antibody (1:25; Santa Cruz Biotechnology, Santa Cruz, CA) and VEGF with a goat anti-human antibody (AF-293-NA, R&D Systems, Minneapolis, MN). pAkt-1 was visualized with a rabbit anti-human pAkt antibody (1:50; Cell Signaling Technology, Danvers, MA). All microscopy was done using a Nikon Eclipse E600 apparatus. CXC4 was visualized with polyclonal goat antibody against a human peptide; due to sequence homology, this would be expected to cross-react with murine CXC4 (1:150; Abcam, Cambridge, MA). Immunostaining for stromal-derived factor 1 (SDF-1) was done using a polyclonal goat antibody raised against the COOH terminus of the human SDF-1 peptide (1:50; Santa Cruz Biotechnology).

In situ hybridization. Tissue was initially preserved in 4% paraformaldehyde overnight, transferred to 17% sucrose, embedded in orinithine carbamyl transferase compound, and frozen at ~80°C. Tissue sections were then probed with ³⁵S-labeled cRNA with probes hybridizing with human VEGF (codons spanning 57–192) and VEGFR2 as described (30).

**Microarrays and probes.** HG-U133A GeneChips (Affymetrix, Santa Clara, CA) were used to investigate gene expression in xenograft tumors. The cRNA probes were synthesized as recommended by Affymetrix. Briefly, total RNA was isolated in two steps using ToTALLY RNA Total RNA isolation kit (Ambion, Austin, TX) followed by RNeasy (Qiagen, Valencia, CA) purification. Double-stranded cDNA was generated from 5 µg of total RNA using a polystol oligonucleotide that contained a T7 RNA polymerase initiation site and the Superscript Choice System kit (Invitrogen, Carlsbad, CA). Biotinylated cRNA was generated by in vitro transcription using the Bio Array High Yield RNA Transcription Labeling System (Enzo, Farmingdale, NY). The cRNA was purified using RNeasy and fragmented according to the Affymetrix protocol, and 15 µg of biotinylated cRNA were hybridized to U133A microarrays (Affymetrix). After scanning, expression values for each gene were determined using Affymetrix GeneChip software version 4.0. GeneSpring (Silicon Genetics, Redwood City, CA) software was used to analyze GeneChip data.

**Methodology for semiquantitative reverse transcription-PCR.** Semiquantitative reverse transcription–PCR (RT-PCR) for VEGF was determined as previously described (31). First-strand syntheses were done using random hexamers and Superscript II reverse transcriptase (Invitrogen). A PCR amplicon for human VEGF was subcloned into a cloning vector for use as PCR reference standards. Reactions were removed at five cycle intervals and separated on nondenaturing bis-acrylamide gels and stained with Syber Green (Invitrogen). Band intensities were determined using Kodak Digital Science (1D Image Analysis Software). A standard curve for each gene was generated from the reference standard dilution series, and values for
unknown samples were extrapolated. To correct for sample variations in RT-PCR efficiency and errors in quantitation, analysis of β-actin expression was used to normalize the RNA samples.

**Immunohistochemistry of COX-2 in primary WT specimens.** Paraffin-embedded sections from 26 WT resected at the Children’s Hospital of New York-Presbyterian were obtained from the Columbia University Cancer Center Tissue Bank. There were 23 favorable histology and three unfavorable (diffusely anaplastic) WTs of different clinical stages. Antigen retrieval was done with steam and an antigen retrieval solution (DAKO, Carpinteria, CA) for 20 minutes. The sections were incubated with the rabbit polyclonal anti-human COX-2 antibody (1:600; Cayman Chemical, Ann Arbor, MI) overnight at 4°C. Negative controls were done with the antibody incubated with the corresponding blocking peptide. Biotinylated goat anti-rabbit IgG secondary antibody followed by an AEC substrate (Invitrogen) was used to visualize the reaction product. Expression of COX-2 was independently evaluated and scored by two surgical pathologists (N.F. and M.M).

**Statistical analysis.** Tumor weights were expressed as mean ± SE and compared by Kruskal-Wallis analysis. The presence or absence of lung metastases was evaluated by Fisher’s exact test.

## Results

**SC-236 inhibits growth of WT xenografts.** To determine the role of COX-2 in tumor vessel assembly, we used our well-characterized xenograft model (24, 32). The anaplastic WT cell line SK-NEP-1 was implanted into the kidney of nude mice, and treatment with the selective COX-2 inhibitor SC-236 (6 mg/kg, given in the drinking water) began 1 week after implantation until day 28 (n = 10 each, control and treated) or day 35 (n = 10 each, control and treated). We found that SC-236 significantly inhibited tumor growth by 78% at day 28 (mean tumor weight ± SE: control, 4.3 ± 0.3 g; SC-236, 0.9 ± 0.3 g; P = 0.0004) and 55% at day 35 (mean tumor weight ± SE: control, 7.2 ± 0.3 g; SC-236, 3.3 ± 0.3 g; P = 0.0007). Lung metastases tended to be reduced in treated tumors at each time point. At day 28, 4 of 10 treated mice had metastases compared with 8 of 10 mice in the control group; at day 35, 4 of 10 treated mice had metastases versus 7 of 10 mice in controls, but these differences did not reach statistical significance.

Confirmation of adequate levels of SC-236 in plasma from control and treated mice was assessed by HPLC as described previously for celecoxib (28). No control mice had detectable levels of SC-236, whereas all treated mice did. One treated mouse was euthanized 24 hours after treatment began, with a serum level of 1.33 μg/mL, likely reflecting the relatively short period of ingestion of this orally available agent. The other five treated mice were tested at day 21 (n = 1), day 28 (n = 2), and day 35 (n = 2) and had plasma levels exceeding the predicted value of 5 μg/mL (11.7 ± 1.4 μg/mL, mean ± SD).

**SC-236 does not increase tumor apoptosis.** To determine if SC-236 decreased tumor growth by increasing apoptosis, tumors were examined for TUNEL-immunopositive nuclei and quantified by image analysis as previously described (32). There was no difference in the amount of apoptosis in control tumors compared with the SC-236-treated tumors (TUNEL+ cells per field ± SE: control, 33.6 ± 2.1; SC-236, 29.0 ± 1.6; P = 0.15) and significantly decreased by 30% at day 35 (phospho-H3+ cells per field ± SE: control, 28.5 ± 1.4; SC-236, 20.0 ± 0.8; P < 0.0001). These results indicate that SC-236 inhibits tumor growth by slowing proliferation rather than by increasing apoptosis.

**SC-236 inhibits angiogenesis in WT xenografts.** One mechanism by which selective inhibitors of COX-2, such as SC-236, are likely to suppress tumor growth is by disruption of angiogenesis. To perform a detailed examination of the effects of SC-236 on tumor vasculature, we examined vessels by fluorescein-labeled lectin perfusion studies and specific immunostaining for endothelial and VMC (Figs. 1 and 2). Fluorescein angiography showed that the perfused vasculature in the treated cohort was markedly diminished (Fig. 1B). Microvessel density (MVD) was quantitatively determined by use of computer-assisted digital image analysis as described by Wild et al. (29). This showed a marked decrease in perfused vessels at both time points (Fig. 1C).

Consistent with the decrease in lectin-perfused vasculature, PECAM-1 immunostaining showed that control tumors developed abundant endothelial networks, whereas there was a paucity of endothelial vessels in the treated xenografts (Fig. 2). Similar findings were seen by in situ hybridization (ISH) for the endothelial cell specific receptor VEGFR2 (Fig. 2). In addition, the vessels of the SC-236–treated tumors were characterized by areas of irregularly increased diameter.

**SC-236 inhibits recruitment of VMC.** The erratically dilated vessels seen in SC-236 treated tumors by PECAM-1 immunohistochemistry suggested that there might be defective assembly or recruitment of VMC. To examine this possibility, parallel sections were labeled for αSMA, a marker for differentiated VMC, and for collagen type IV, a marker for perivascular basement membrane, both of which are key modulators of vessel stability. In control tumors, both collagen type IV and αSMA were abundant throughout tumor vessel networks (Fig. 3). These networks were generally hierarchical, ramifying from vessels that were large in caliber, regular, and αSMA+. In contrast, SC-236–treated tumors developed vessels that were larger in caliber, which were irregular and displayed scant αSMA+ cells.

To further examine the status of vascular maturation, we examined by immunohistochemistry the expression of NG2, a proteoglycan that is expressed by nascent or early pericytes...
Control tumors displayed a dense periendothelial layer of NG2-immunopositive cells, whereas SC-236–treated tumors had markedly diminished NG2 + vasculature, indicating deficient association of early pericytes with vessels. These results show that SC-236 inhibits both VMC recruitment and appropriate assembly of vasculature, resulting in qualitatively and quantitatively impaired vessel networks and decreased tumor perfusion.

SC-236 causes reduced phosphorylation of PDGFRβ in tumor vasculature, with persistent expression of PDGF-B. A critical mediator of vascular mural cell survival is PDGF-B signaling. We, therefore, examined the expression of PDGF-B and pPDGFRβ in SC-236- and control-treated xenografts. PDGF-B was expressed in both control and treated tumor vasculature, with increased intensity and irregular periendothelial deposition observed in the latter (Fig. 4). In contrast, there was a marked disproportion in pPDGFRβ-immunopositive vasculature. Control vessels displayed continuous, abundant pPDGFRβ, whereas the erratic, segmentally dilated vessels in SC-236–treated tumors contained very few pPDGFRβ-immunopositive cells. These results indicate decreased activation of the PDGF-B/PDGFRβ axis despite the juxtaposition of PDGF-B protein with surviving vessels.

VEGF expression is not significantly altered by SC-236. VEGF is a key survival factor for endothelial cells and can be regulated by COX-2 (11), raising the possibility that altered VEGF expression contributed to the loss of survival signaling in our SC-236–treated xenografts. We examined the status of VEGF protein by immunohistochemistry. In both control and treated xenografts, VEGF was detected in vasculature, with no apparent change in abundance or pattern (data not shown). Similarly, VEGF mRNA expression was not significantly changed by treatment when examined by ISH (data not shown), or semiquantitative RT-PCR (control, 19.5 ± 22.9 fg versus treated, 21.5 ± 13.5 fg, mean ± SD; P = not significant). This indicates that decreased expression of VEGF did not play a role in the vascular defects we observed.

SC-236 alters gene expression in WT xenografts. To more broadly assess alterations in angiogenesis-related gene expression in tumors exposed to SC-236, we analyzed control and treated tumors (n = 8 and 6, respectively), using the Affymetrix HG-U133 microarray chip. The analysis was done by first filtering normalized values for differential gene expression between treated and control tumors by t test, including only genes with a minimum expression of >1.0.

Expression of perlecan, a matrix proteoglycan that can inhibit VMC proliferation and is known to be negatively regulated by prostaglandins (34), was increased 1.8-fold in SC-236-treated tumors. In addition, increased expression was observed in the α3 noncollagenous domain of collagen IV (3.6-fold) and tissue inhibitor of metalloproteinase-1 (TIMP-1; 1.4-fold). Although collagen IV peptides have antiendothelial properties, the intact molecule may be homeostatic for vessels (35). TIMP-1 both suppresses VMC proliferation and stabilizes arteries in vascular injury models (36).
CXCR4 expression is decreased in SC-236–treated tumors. Our results are consistent with previous evidence that CXCR4 is a transcriptional target of prostaglandin E2, with expression consequent suppressed by COX-2 blockade (18). Among the angiogenesis-related genes exhibiting decreased expression in our studies, CXCR4 plays an important role in homing vascular mural precursor cells to actively proliferating vascular beds (37). CXCR4 is known to signal via Akt, raising the possibility that SC-236 specifically contributed to impairment of survival signaling in vasculature (as discussed below) by decreasing vascular expression of this receptor. Therefore, we further investigated the status of CXCR4 in SC-236–treated tumors. Consistent with a defect in vascular mural progenitors, the large erratic vessels of SC-236–treated tumors were largely devoid of CXCR4-positive cells, whereas control tumor vessels were consistently immunopositive for CXCR4 (Fig. 4). We also examined expression of the ligand for CXCR4, SDF-1, which plays a role in homing CXCR4+ progenitor cells to active vascular beds (38). Although SDF-1 was detected throughout xenografts, with focal increases at tumor edges and perivascularly, expression was not altered by SC-236 treatment (data not shown). These results provide additional evidence for direct suppression of vascular expression of CXCR4 by SC-236, rather than diminished chemotaxis of CXCR4+ cells in response to altered SDF-1.

SC-236 causes reduced phosphorylation of Akt in tumor vasculature. A hallmark of effective antiangiogenesis is reduced survival of tumor vascular cells. Endothelial and mural cell survival signaling is mediated by activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (8, 39). Therefore, to document the effects of SC-236 in targeting vascular cells, we examined the expression of pAkt in both tumor and vascular compartments. Striking expression of pAkt in both endothelial and periendothelial cells was present in control tumors (Fig. 5). In contrast, there was scant pAkt detected in vasculature of SC-236–treated tumors, indicating decreased activation of this critical pathway.

SC-236 does not alter the status of COX-2–independent targets. In some systems, SC-236 has been found to have COX-2–independent effects in vitro on pathways potentially affecting tumor cell proliferation and survival, including c-Jun N-terminal kinase (JNK)–mediated suppression of activator protein-1 and altered status of peroxisome proliferator-activated receptor-γ (PPAR-γ; refs. 40, 41). To explore this possibility, we examined expression of pJNK in control and SC-236–treated tumors by immunohistochemistry. pJNK-immunopositive tumor cells were readily apparent in control tumors, and no difference was detected in treated xenografts (data not shown). pJNK staining was also seen in a minority of endothelial cells, with no difference in seen in treated tumors (data not shown). Activation of PPAR-γ was assessed by developing a list of 186 validated gene targets (42–44) and then examining patterns of expression by microarray. One hundred seventeen genes were informative, but only four were significantly altered, suggesting that PPAR-γ activation was not broadly present in our system (data not shown).

COX-2 is expressed in primary WTs. The expression of COX-2 was evaluated by immunohistochemistry in 26 primary human WTs. There were 23 favorable histology tumors, three anaplastic, with stage I (n = 6), stage II (n = 4), stage III (n = 11), and stage IV (n = 4). We found that COX-2 was ubiquitously expressed in WT, with immunopositivity most prevalent in the vascular compartment. COX-2 localization varied between individual tumors in blastemal (69%; Fig. 6A), vascular (92%; Fig. 6C and E), and epithelial compartments (73%; Fig. 6F).

Conclusions

COX-2 seems to be nearly ubiquitously expressed in human tumors and their neovasculature, suggesting that agents disrupting
tumor vasculature. SC-236 treatment decreased incorporation of angiogenesis cascade.

that COX-2 blockade affects alternative elements in the tumor selective endothelial target, these findings support the concept were not detected in our model. Because VEGF is a relatively significant from those observed in the same model after treatment with agents targeting endothelial cells, such as VEGF blockade (23, 24). Furthermore, changes in VEGF expression were not detected in our model. Because VEGF is a relatively selective endothelial target, these findings support the concept that COX-2 blockade affects alternative elements in the tumor angiogenesis cascade.

One such potential element is the mural cell component of tumor vasculature. SC-236 treatment decreased incorporation of early pericytes in tumor vessels, as shown by reduced NG2 immunopositivity in our xenograft model. NG2 is a transmembrane proteoglycan expressed in immature pericytes and other precursor cell types, restricted in adult vasculature to sites of active angiogenesis (33). Recruited bone marrow precursors expressing NG2 form periendothelial mural cell layers during tumor angiogenesis (37). A key receptor governing participation of these vascular precursor cells in newly forming vessels is the chemokine receptor, CXCR4 (38). Recent work indicates a significant role for CXCR4 in tumor metastasis and progression (38, 46). Activation of this receptor can stimulate the PI3K/Akt pathway in a number of cell types (47); in endothelium, CXCR4 mediates capillary tube formation stimulated by prostaglandin E, an effect that is disrupted by cyclooxygenase blockade (18). Consistent with these data, significantly lower expression of CXCR4 in SC-236–treated tumors was detected by microarray and confirmed by diminished CXCR4 immunopositivity in the abnormal, segmentally dilated tumor vessels. Taken together, these results indicate that SC-236 disrupted an early phase of VMC incorporation in tumor vessels, potentially by blocking CXCR4-mediated incorporation of early pericytes/vascular mural progenitor cells in xenograft vessels.

As would be predicted from the deficient incorporation of early pericytes, α SMA-immunopositive differentiated VMC were scant in the erratic, segmentally dilated tumor vessels observed in SC-236–treated mice. Intact α SMA function in vascular smooth muscle is requisite for normal vascular autoregulation and blood flow (48). Consistent with this functional requirement, intravascular injection of L. esculentum lectin showed a dramatic decrease in perfused vasculature in SC-236–exposed tumors, which seemed to exceed the decrease in histologically detected vessels. These findings suggest that the altered tumor vessel assembly induced by COX-2 blockade qualitatively impaired blood flow, in addition to quantitatively decreasing vasculature.

PDGF-B signaling via PDGFRβ is a key regulator of survival, proliferation, and migration of tumor VMC yet also may directly promote tumor endothelial survival, activating the PI3K/Akt pathway in both vascular cell types (20, 22, 39, 49). Blockade of COX-2 can modulate PDGF-B signaling (50). Specifically, COX-2 inhibition blocks phosphorylation of Akt in VMC in vitro and in vivo (25). Consistent with these data, in our studies, SC-236–treated xenograft vessels displayed reduced activation of both PDGFRβ, despite marked perivascular deposition of PDGF-B and concurrent with reduced vascular pAkt. Although these findings likely reflect the decreased presence of differentiated VMC expressing PDGFRβ, it is also clear that reduced activation of this receptor may suppress survival signaling in tumor endothelium (39, 49). Taken together, these findings support an additional mechanism by which COX-2 blockade impairs homeostasis in tumor vessels: reduction of PDGFRβ-stimulated activation of the Akt pathway in endothelial and mural cells.

Of note, the alterations in vessel structure we observed in SC-236–treated xenografts differ strikingly from those we have previously reported in this same model during sustained administration of blocking anti-VEGF antibody, either alone or in combination with “metronomic” chemotherapy (24). In such experiments, chronic treatment dramatically stimulated recruitment and proliferation of VMC, with recurrence of viable tumor as perivascular clusters. Much previous data indicate that such periendothelial mural cells serve to protect endothelium during VEGF withdrawal (16). Given this well-established role, the increase in mural cell recruitment seems to be a critical element in adaptive vessel remodeling when endothelial cells are targeted, presumably via the enhanced survival signaling.

Figure 6. COX-2 is expressed in tumor cells and vasculature of primary human WTs. Expression of COX-2 in (A) blastemal cells, (C and E) vascular cells, and (F) epithelial cells. Addition of COX-2 blocking peptide (B and D) shows specificity of staining. Bar, 100 μm (A, B, and E) and 50 μm (C, D, and F).
conferring VMC endothelial contact. Our findings provide new evidence of the mechanisms by which COX-2 attenuates this protective interaction with endothelium. Incorporation of nascent periendothelial cells is inhibited by SC-236, as indicated by decreases in NG2-immunopositive cells, suggesting that COX-2 functions at an early stage of VMC recruitment. Expression of CXCR4 and pPDGFβR, which each mediate recruitment, proliferation, and Akt-mediated survival of mural cells in tumor vasculature, is concurrently suppressed. These studies indicate that the status of COX-2 critically influences the stabilizing interactions between VMCs and endothelium in tumors and suggest that COX-2 blockade may therefore provide important therapeutic synergies with agents targeting other elements in tumor angiogenesis.

Acknowledgments


Grant support: Pediatric Cancer Foundation (J.J. Kandel and D.J. Yamashiro), National Cancer Institute grants 1R01-CA100451 (J.J. Kandel) and 1R01-CA100451 (J.J. Kandel); Children’s Health Research Center (A. Lee); American Society of Clinical Oncology Young Investigator Award (A. Lee) and National Cancer Institute grants 1RO1-CA088951 (D.J. Yamashiro) and 1RO1-CA040531 (J.J. Kandel).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

24. Ozerden U, Graiko KA, Dahlin-Huppe K, Monosov E, Stallcup WB. NG2 proteoglycan is expressed exclusively with 18 U.S.C. Section 1734 solely to indicate this fact.
Inhibition of Cyclooxygenase-2 Disrupts Tumor Vascular Mural Cell Recruitment and Survival Signaling

Alice Lee, Jason Frischer, Anna Serur, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/66/8/4378

Cited articles  This article cites 50 articles, 26 of which you can access for free at: http://cancerres.aacrjournals.org/content/66/8/4378.full#ref-list-1

Citing articles  This article has been cited by 3 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/66/8/4378.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.