Molecular Cancer Therapeutics

Celecoxib Can Induce Vascular Endothelial Growth Factor Expression and Tumor Angiogenesis

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Abstract

Increased COX-2 expression has been linked to increased angiogenesis and a worse prognosis in patients with malignant gliomas and other tumor types. This led to our interest in assessing the response of glioma cell lines to treatment with celecoxib, a selective COX-2 inhibitor. However, contrary to its reported antiangiogenic effects, treatment with celecoxib actually induced the expression of VEGF in multiple glioma as well as other cancer cell lines. This induction of VEGF was comparable to, if not greater than, that found after exposure of cells to hypoxia. Pharmacologic inhibition and siRNA silencing of p38-mitogen-activated protein kinase and the Sp1 transcription factor revealed their involvement in this celecoxib-induced VEGF expression. Consistent with the documented role of Sp1 in this effect, VEGF induction was found to involve transcriptional activation and not to change the stability of VEGF mRNA. The biological significance of this effect was confirmed *in vivo* by showing both induction of VEGF expression and microvessel density in tumor xenografts and increased angiogenesis in a matrigel plug assay in nude mice that were administered celecoxib. We speculate that treatment with celecoxib may, in some instances, enhance tumor cell expression of VEGF as well as angiogenesis and, consequently, may have detrimental effects on the response of tumors to this drug. *Mol Cancer Ther; 10(1); 138–47. ©2011 AACR.*

Introduction

COX-2 is aberrantly overexpressed in a variety of cancers and numerous experimental studies have implicated COX-2 in the genesis and progression of different tumor types (for review, see ref. 1). In line with these studies, COX-2 appears to enhance resistance to cytotoxic therapies, including chemotherapy and radiation, making it more difficult to treat cancers that overexpress it (2–5). COX-2 may also contribute to cancer development by promoting tumor angiogenesis making it a potentially attractive target for antiangiogenic therapy (for review, see ref. 6). On the basis of promising preclinical results, COX-2 inhibitors are being evaluated in clinical trials for various malignancies. Early results have been mixed with some trials (especially chemoprevention ones) showing promise whereas others have been disappointing including ones for malignant gliomas (7–10).

Specific COX-2 inhibitors were initially developed as a nonsteroidal anti-inflammatory drug (NSAID) that could relieve pain without the gastrointestinal toxicities associated with nonselective NSAIDs (for review, see ref. 11). Because of the apparent role of COX-2 in various tumors, these inhibitors, including celecoxib, have been evaluated as potential cancer therapeutic agents with antiangiogenic and proapoptotic activities (for review, see ref. 12, structure shown in Supplementary Fig. 1). However, growing evidence suggests that celecoxib has multiple cellular effects independent of its COX-2 inhibitory activity. For example, this drug can promote leakage of calcium from the endoplasmic reticulum (ER) resulting in an unfolded protein response (UPR; ref. 13). Others have shown that it can modulate the activity of various mitogen-activated protein kinases (MAPK) leading to gene expression changes (14, 15). Some of these effects may be unfavorable for cancer treatment. In one instance, celecoxib upregulated ER chaperones and the 150-kDa, oxygen-regulated protein resulting in the inhibition of celecoxib-induced apoptosis in human gastric cancer cells and potentially reducing this drug's antitumor activity (16). Recently, investigators also found that circulating VEGF levels were increased during celecoxib treatment in breast cancer patients although the underlying mechanism was unclear (17). Taken together, these studies suggest that celecoxib may have contradictory effects on tumor responsiveness and angiogenesis.

To gain a better understanding of cellular responses to celecoxib treatment in malignant gliomas, we assessed

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VEGF expression after drug treatment in multiple glioma cell lines and found that celecoxib induced VEGF expression at both the mRNA and protein levels. Multiple other tumor cell lines also displayed a similar response. The extent of celecoxib-induced VEGF expression was on the order of that achievable by hypoxia and appears to be biologically significant using *in vivo* models. In dissecting the underlying mechanism for this effect, p38-MAPK and Sp1 were found to play important roles in transcriptional regulation of VEGF by celecoxib.

Materials and Methods

Cell culture conditions and reagents

Glioma cell lines (LN229, SF767, SF763) as well as breast (SK-BR3, MCF7, MDA-MB-453), colon (DLD1, HCT116), head and neck squamous (SQ20B) and epithelial (A431) carcinoma cell lines were cultured in high-glucose DME medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma). The prostate carcinoma cell line (C4-2) was cultured in RPMI 1640 (Sigma) supplemented with 10% FBS. LN229, SF767, and SF763 were obtained from Mark Israel (previously at University of California; currently at Dartmouth Medical School) in 1999. SK-BR3, MCF7, MDA-MB-453, and SQ20B were obtained from Amit Maity (University of Pennsylvania School of Medicine) in 2004. DLD1 was obtained from Shi-Yong Sun (Emory University School of Medicine), HCT116 was obtained from Vincent Yang (Emory), and C4-2 was obtained from Yoke Wah Kow (Emory) in 2009. A431 was obtained from the American Type Culture Collection in November, 2005. Standard aseptic culture techniques were used in propagating these cells. All cell lines were periodically checked for mycoplasma infection by PCR (last tested in 2009). No other authentication tests have been done on these cell lines since they were acquired in the lab. For hypoxia experiments, a gas mixture (1% O_2/ 5% CO_2/94% N_2) was used to replace ambient air in a sealed chamber. Celecoxib (LC Laboratories), SB203580, U0126, PP2, LY294002 (Calbiochem), and mithramycin A (Sigma) were made as stock solutions in DMSO and used at working concentrations, as indicated. For SB203580, U0126, PP2, and LY294002, inhibition of phosphorylation of p38, ERK1/2, Src, and AKT, respectively were confirmed in LN229 and SF767 at the working concentrations used here (data not shown). Epidermal growth factor (EGF, Roche Diagnostics) was used at 100 ng/mL.

Retroviral infection and transient transfection

MKK3be (constitutively active MKK3 provided by Dr. Jiahuai Han, the Scripps Research Institute) was inserted into the pRetroX-tight-puro retroviral vector (Clontech Laboratories) and transiently transfected into Phoenix cells by $CaPO_4$ precipitation. Cells were infected (×3) with virus-containing supernatants (from transfected Phoenix cells) at 12-hour intervals and subsequently selected with puromycin (2 μg/mL). Stably infected pools (LN229/tMKK3 and SF767/tMKK3) displayed inducible expression of MKK3 when treated with doxycycline (2 μg/mL). siRNA against p38-MAPK and Sp1 (Santa Cruz Biotechnology) was transfected using Lipofectamine 2000 (Invitrogen). The Stealth RNAi medium GC duplex-negative control (Invitrogen) was used to control for sequence-independent effects of short RNA duplexes.

RNase protection assay

RNase protection assay (RPA) was done as previously described (18). Fragments of VEGF (329 bases) and luciferase (351 bases) cDNA were cloned into pCR2.1 (Invitrogen) to form pCR/ribo/VEGF and pCR/ribo/LUC. VEGF and luciferase riboprobes produced as run-off transcript sizes of 317 and 461 bases yielded protected sizes of 232 and 351 bases, respectively. For normalization, cyclophilin riboprobe template (Ambion) was used. Quantitation was accomplished by using PhosphorImager.

Luciferase reporter assay

The human VEGF promoter DNA (approximately 1.29 kb) was amplified by PCR and cloned into pGL3-luciferase vector (termed pGL-VPr). This reporter plasmid was transiently transfected into LN229/tMKK3 and SF767/tMKK3 lines using Lipofectamine (Invitrogen) for VEGF promoter analysis. SB203580 or mithramycin A was added for 1 hour prior to addition of either doxycycline (2 μg/mL) or celecoxib (30 μmol/L) for 6 hours. Luciferase activity was measured as previously described (19) and mRNA level was quantitated by RPA.

Western blotting and ELISA

Western blots were done by standard procedures. Blots were probed with antibodies against Sp1 (Santa Cruz), p38-MAPK/phosphorylated p38-MAPK (Cell Signaling Technology), and EIF5 (loading control; Santa Cruz). Blots were detected using a horseradish peroxidase-conjugated secondary antibody (either anti-rabbit or anti-mouse IgG) and chemiluminescent substrate. Secreted VEGF protein was quantitated by VEGF ELISA (R&D Systems).

Nuclear run-on and RNA stability assays

Nuclear run-on assay was done as previously described (20). Briefly, nuclei were harvested after 1 hour of pretreatment with SB203580 (20 μmol/L) or mithramycin A (100 nmol/L) followed by 5 hours celecoxib treatment (30 μmol/L). Run-on transcription proceeded with labeled [α-^{32}P]UTP and unlabeled ribonucleotides. Radioactive RNA was extracted and hybridized overnight to Hybond N slot blots of denatured GAPDH, VEGF, and pBluescript (empty vector control) DNA at 56°C using an RNA hybridization kit (Ambion). Blots were washed (×3) at 66°C with increasing stringency (2×–0.5×SSC/0.1% SDS) before detection by using PhosphorImager. Relative transcription rates were determined as a ratio of VEGF to GAPDH transcription by volume integration of hybridization signals, which were normalized to the ratio determined within nonstimulated cells. VEGF mRNA stability was measured after stimulation with EGF for 1 hour prior to addition of actinomycin D (5 μg/mL, RNA pol II inhibitor) ± celecoxib (30 μmol/L) for the indicated times. RPA was done to determine VEGF mRNA expression at each time point.

In vitro endothelial cell migration assay

The commercially available QCM 3μm Endothelial Cell Migration Assay kit was used (Millipore). Human umbilical vein endothelial cells (HUVEC; kindly provided by Dr. Erwin Van Meir) were used at passage 3. Overall, manufacturer's instructions were used for doing this assay. Briefly, LN229 (2×10^5 cells) were seeded into each lower chamber with endothelial cell growth media (EGM) containing serum and allowed to attach overnight. These cells were then incubated for 6 hours in serum-free EGM containing either vehicle (DMSO) or celecoxib (30 μmol/L) before placement of the upper chamber containing HUVECs (at 2×10^5 cells/200 μL of media) into each well. As controls, EGM containing 2% FBS only (no cells) with or without celecoxib was also used in the lower chamber. HUVECs were allowed to migrate for 16 hours before removal of unmigrated cells from the upper well and colorimetric detection of migrated cells on the undersurface of the upper chamber membrane.

In vivo xenograft model and matrigel plug assay

Animal experiments were approved by the Institutional Animal Care and Use Committee at Emory University. LN229 cells were either grown as subcutaneous tumors or in a matrigel plug in the flank of athymic nude mice (Harlan Laboratories; refs. 21, 22). Vehicle only or celecoxib (150 mg/kg) was administered daily by oral gavage for the duration of experiments. For xenograft tumors, 5×10^6 cells in 200 μL of HBSS containing 10% matrigel (BD Biosciences) were injected subcutaneously (1 site per mouse) ± celecoxib (30 μmol/L). Mice harboring tumor cells were fed vehicle only or celecoxib for the indicated times before tumor harvest and quantitation of either VEGF expression by RPA or microvessel density after immunofluorescent staining with anti-CD31 antibody (MEC13.3 antibody; BD Biosciences) using standard procedures. Angiogenesis was also measured by a matrigel plug assay as previously described (22). Briefly, 2×10^6 cells in a 400 μL of solution containing 80% matrigel were subcutaneously injected (2 sites per mouse) ± celecoxib (30 μmol/L). Plugs were harvested after 4 days, weighed, photographed, and dispersed in 400 μL of PBS (overnight incubation at 4°C) to collect the hemoglobin. Hemoglobin content was measured using Drabkin's solution (Sigma) according to manufacturer's recommendations.

Results

Celecoxib induces VEGF expression in glioma cells and multiple other cancer cell lines

Because COX-2 has been reported to regulate VEGF expression (23) and elevated COX-2 expression correlates with poor prognosis in glioma patients (24), we sought to explore the influence of celecoxib treatment on VEGF expression in glioma cells. When LN229 and SF767 glioma cells were treated with 30 μmol/L of celecoxib, significant VEGF mRNA induction was observed as early as 2 hours with maximal induction achieved 16 to 24 hours posttreatment (Fig. 1A). Next, to determine whether this response was restricted to glioma cells, a panel of other cancer cell lines were tested including those derived from colon carcinoma (DLD1 and HCT116), head and neck squamous carcinoma (SQ20B), breast carcinoma (SKBR3, MCF1, MDA-MB-453), vulvar epidermoid carcinoma (A431), and prostate carcinoma (C4-2). In each case, celecoxib (30 μmol/L) was sufficient to induce VEGF mRNA expression from 3- to 12-fold (Fig. 1B). Thus, induction of VEGF by celecoxib is widely seen among different cancer cells. Evaluation of dose–response relationships for this effect revealed that 5 μmol/L of drug was sufficient to induce VEGF expression in the C4-2 prostate carcinoma line, whereas other assessed lines (LN229, SF767, A431) required at least 15 μmol/L of drug to significantly induce VEGF (Fig. 1C).

Celecoxib induce VEGF mRNA expression at levels comparable to that induced by hypoxia

Hypoxia is a crucial regulator of VEGF expression through its induction of hypoxia-inducible factor 1α (HIF-1α; for review see ref. 25). Because this response to hypoxia is believed to be an important determinant of prognosis, and potentially response to therapy, we compared VEGF induction by celecoxib with its induction by hypoxia. Both celecoxib and hypoxia significantly increased VEGF mRNA expression in the glioma lines tested (Fig. 2A). Furthermore, combining celecoxib and hypoxia resulted in additive, if not greater, levels of VEGF induction (Fig. 2A). Overall, celecoxib induced VEGF mRNA at levels that rival those seen with hypoxia. Correspondingly, VEGF protein also increased following celecoxib treatment. VEGF ELISA analyses on conditioned media from celecoxib-treated glioma cells revealed significant induction of VEGF protein (Fig. 2B). Again, VEGF protein induction achieved with celecoxib (30 μmol/L) was on the order of, if not greater than, that achieved with hypoxia (Fig. 2B).

Celecoxib-induced VEGF expression requires Sp1 transcription factor and p38-MAPK

Sp1 can transcriptionally regulate VEGF through tandem Sp1-binding sites located in the proximal VEGF promoter (26, 27). To determine whether Sp1 has a role in the induction of VEGF by celecoxib, LN229 and SF767 cells were pretreated with the Sp1 inhibitor mithramycin

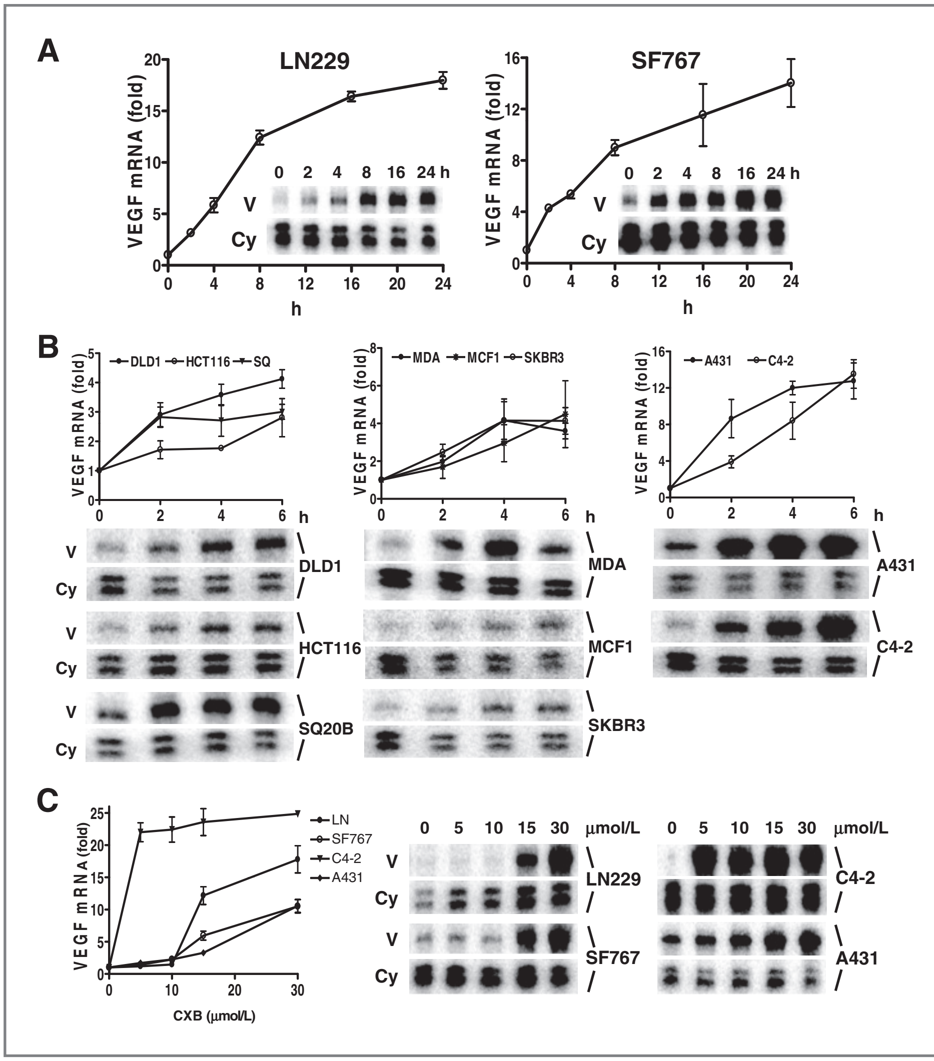

Figure 1. VEGF is induced by celecoxib. VEGF mRNA (V) was quantitatively assessed by RPA with cyclophilin (Cy) used as the normalization control. A, LN229 and SF767 glioma lines were treated with celecoxib (30 μmol/L) for the indicated times. Representative PhosphorImager images are inset on the graph. B, colon (DLD1, HCT116), head and neck (SQ20B), breast (MDA-MB-453, MCF1, SKBR3), epithelial (A431), and prostate (C4-2) carcinoma cell lines were treated for the indicated times with celecoxib (30 μmol/L). Representative PhosphorImager images are shown below each graph. C, indicated cell lines were treated with increasing levels of celecoxib (CXB) for 16 hours. Representative PhosphorImager images are shown beside the graph. Points on graphs represent mean fold induction of VEGF mRNA (from 3 independent experiments) relative to the value at either 0 hour (A and B) or 0 μmol/L (C). Error bars are ±1 SEM.

A for 1 hour prior to celecoxib addition. In this experiment, mithramycin A substantially reduced celecoxib-induced VEGF expression (Fig. 3A). Similar results were obtained with the SF763, C4-2, and A431 cell lines (data not shown). Because pharmacologic inhibition may have off-target effects, we confirmed a role for Sp1 in celecoxib-induced VEGF expression by inhibiting Sp1 expression in LN229 and SF767 cells with siRNA (data not shown). As a result, celecoxib-induced VEGF mRNA expression was attenuated (Fig. 3B). These results show that Sp1 is involved in celecoxib-induced VEGF expression.

We previously found that EGF-induced COX-2 expression in glioma cells required p38-MAPK–dependent activation of the Sp1 transcription factor (18, 28). VEGF expression is similarly induced by EGF in various systems (29–31). Thus, on the basis of our findings that Sp1 has a role in celecoxib-induced VEGF expression, we speculated that p38-MAPK is also involved in this process. First, we found that p38-MAPK is activated (as evidenced by phosphorylation) within 10 minutes after celecoxib treatment in both the LN229 and SF767 glioma cells (data not shown). Next, we tested whether inhibition of p38-MAPK would suppress celecoxib-induced VEGF expression. Because other signaling kinases, including extracellular-regulated kinase, phosphatidylinositol-3 kinase (PI-3K), and Src, have also been shown to modulate VEGF expression, we simultaneously assessed their role in celecoxib-induced VEGF expression. Each signaling kinase was pharmacologically inhibited (p38-MAPK by SB203580, MEK1 by U0126, PI-3K by LY294002, and c-Src by PP2) prior to treatment with celecoxib. Only inhibition of p38-MAPK with SB203580 was consistently able to suppress celecoxib-dependent induction of VEGF mRNA (Fig. 3C). Similar results were obtained in SF763 and A431 cells (data not shown). Again, because of potential off-target effects of SB203580, p38-MAPK was

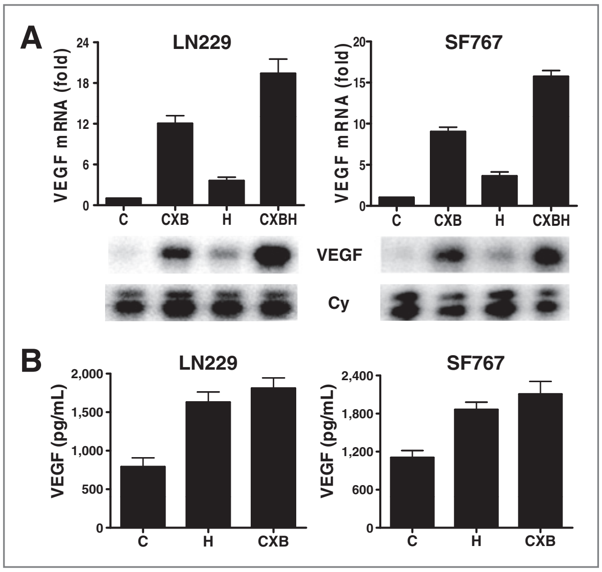

Figure 2. VEGF induction by celecoxib and hypoxia are comparable. A, VEGF mRNA expression in LN229 and SF767 glioma cells was assessed by RPA. Cells were treated with no drug (C, control), celecoxib (CXB, 30 μmol/L), hypoxia (H, 1% O_2), and both (CXBH) for 16 hours. Graphs show mean fold induction of VEGF mRNA relative to control (from 3 independent experiments). Representative PhosphorImager images are shown below each graph. Cyclophilin (Cy) was used as the normalization control. B, VEGF protein secreted into media was quantitatively assessed by ELISA. LN229 and SF767 cells were cultured with fresh media under the following conditions [no drug (C, control), CXB (30 μmol/L), and H (1% O_2)] for 16 hours before harvesting. Graphs show mean level of VEGF protein within conditioned media (from 3 independent wells). Error bars are ±1 SEM in each case.

genetically suppressed with siRNA (Supplementary Fig. S1B). As expected, following siRNA-mediated knockdown of p38-MAPK, celecoxib-induced VEGF mRNA expression was significantly reduced (Fig. 3D). Thus, celecoxib-induced VEGF expression also requires p38-MAPK.

Activation of p38-MAPK is sufficient to induce VEGF expression

As we showed that p38-MAPK is required for celecoxib-induced VEGF expression, we next sought to test whether activation of p38-MAPK alone is sufficient for VEGF induction. To this end, LN229 and SF767 were engineered to express a constitutively active form of mitogen-activated protein kinase kinase 3 (MKK3), an upstream activator of p38-MAPK, under control of a tetracycline-inducible promoter (LN229/tMMK3 and SF767/tMKK3). MKK3 induction with doxycycline (evidenced by p38-MAPK phosphorylation) resulted in concomitant induction of VEGF mRNA expression (Fig. 4A). Next, we showed that doxycycline was no longer able to increase VEGF expression after inhibition of Sp1 with mithramycin A or siRNA-targeting p38-MAPK (Fig. 4B and C). Thus, p38-MAPK activity is sufficient for induction of VEGF in a process that still requires Sp1.

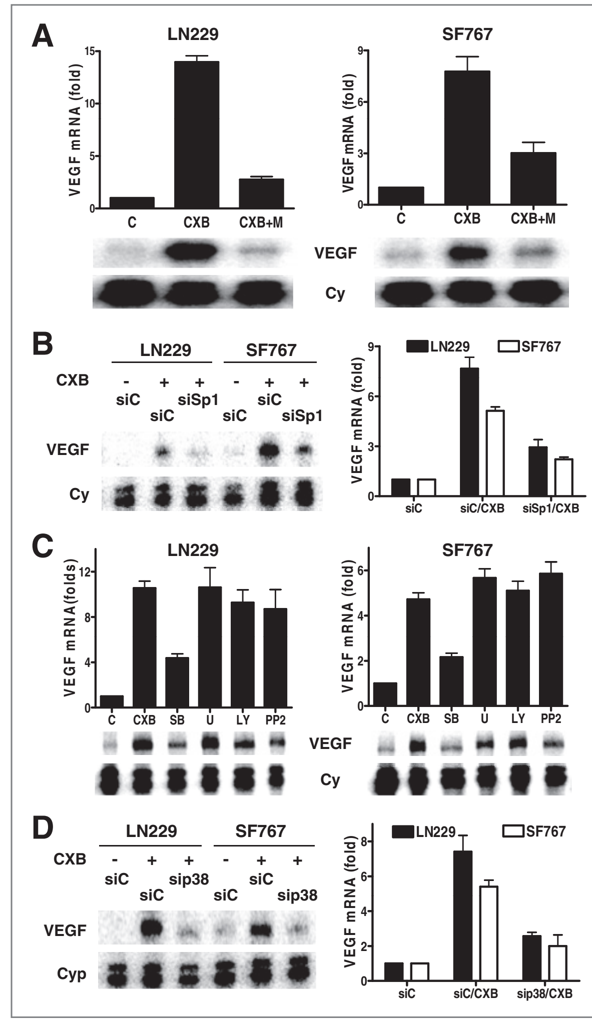

Figure 3. Sp1 and p38-MAPK are involved in celecoxib-induced VEGF expression. A and C, VEGF mRNA expression in LN229 and SF767 cells treated in A with no drug (C, control), celecoxib (CXB, 30 μmol/L), and CXB + mithramycin (M, 100 nmol/L) or in C, with no drug (C, control), CXB (30 μmol/L), CXB + SB203580 (SB, 20 μmol/L), CXB + U0126 (U, 10 μmol/L), CXB + LY294002 (LY, 25 μmol/L), and CXB + PP2 (5 μmol/L) for 16 hours was assessed by RPA. B and D, expression of VEGF mRNA in LN229 and SF767 after transfection in B with control (siC) and Sp1-targeting (siSp1) siRNA or in D, with control (siC) and p38-MAPK–targeting (sip38) siRNA was assessed by RPA after treatment ± CXB (30 μmol/L). Representative PhosphorImager images are shown next to each graph with cyclophilin (Cy) serving as the normalization control. Graphs are the average of 3 independent experiments. Error bars are ±1 SEM.

Celecoxib increases the transcription but not the stability of VEGF mRNA

Because celecoxib-induced VEGF expression requires the Sp1 transcription factor, we hypothesized that transcriptional activation is involved in this process. However, increased mRNA stability may still play a role in this induction. To determine whether celecoxib-induced VEGF expression results from transcriptional activation and/or increased mRNA stability, nuclear run-on analysis was done after treatment of LN229 cells with

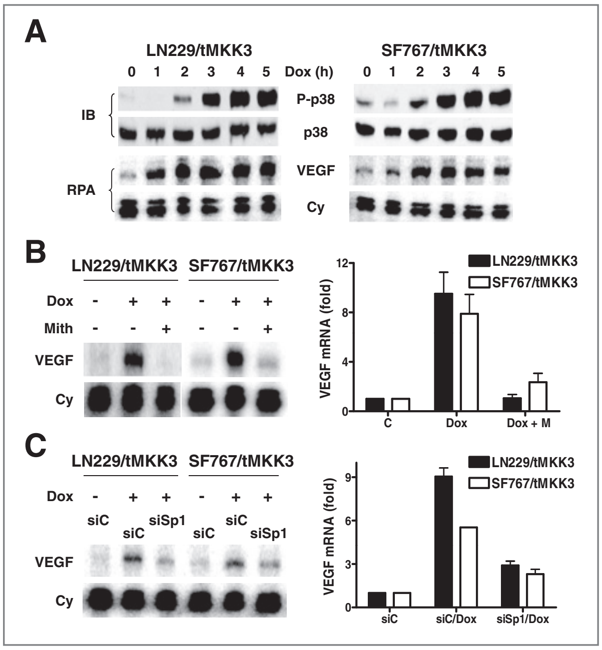

Figure 4. Activation of p38-MAPK is sufficient to induce VEGF. A, LN229 and SF767 were engineered to express MAP kinase kinase 3 (MKK3) under the control of a tetracycline-inducible promoter (LN229/tMKK3 and SF767/tMKK3) and treated with doxycycline (Dox; 2 μg/mL) for the indicated times to induce MKK3. Immunoblots (IB) probed for phosphorylated (P-p38) and total p38-MAPK (p38) show its activation. Induction of VEGF mRNA is shown by RPA with cyclophilin (Cy) serving as the normalization control. B and C, VEGF mRNA expression in LN229/tMKK3 and SF767/tMKK3 was assessed by RPA with Cy as normalization control. Inhibition of Sp1 was accomplished with mithramycin (M, 100 nmol/L; B) or Sp1-targeting siRNA ± MKK3 induction with Dox (2 μg/mL; C) for 5 hours. Representative PhosphorImager images are shown. Graphs are the average of 3 independent experiments. Error bars are ±1 SEM.

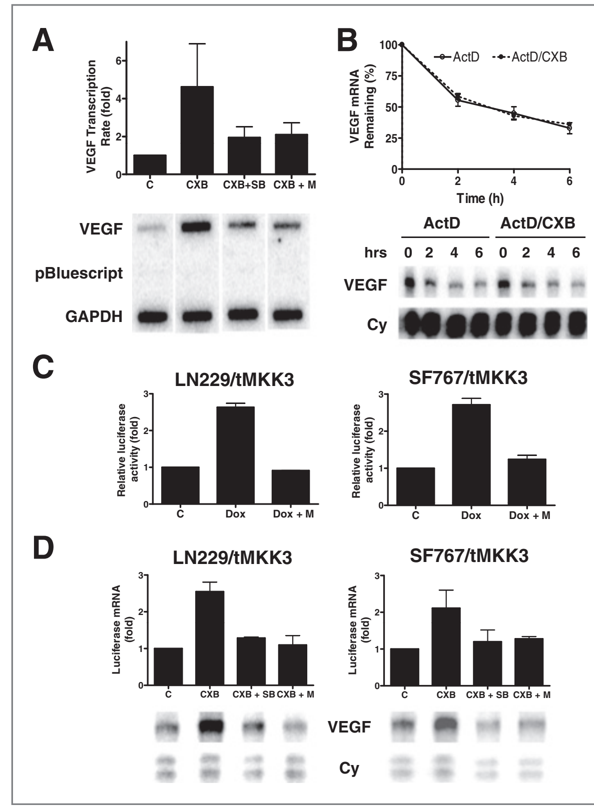

Figure 5. Celecoxib transcriptionally activates the VEGF promoter. A, nuclear run-on was done on nuclei isolated from LN229 cells treated with no drug (C), celecoxib (CXB, 30 μmol/L), CXB + SB203580 (SB, 20 μmol/L), or CXB + mithramycin (M, 100 nmol/L). Labeled transcripts were used to probe a slot blot containing denatured VEGF, pBluescript, and GAPDH. Representative PhosphorImager image is shown. B, VEGF mRNA stability was measured in LN229 cells by inhibiting new transcription with actinomycin D (ActD, 5 μg/mL) and measuring VEGF mRNA levels over time. VEGF mRNA was induced with EGF (100 ng/mL) for 1 hour before addition of ActD ± CXB for the indicated times and assessed by RPA with Cy serving as normalization control. C and D, the VEGF promoter-luciferase construct (pGL-VPr) was transiently transfected in LN229/tMKK3 and SF767/tMKK3. Transfectants were subsequently treated with either no drug (C), doxycycline (Dox, 2 μg/mL), and Dox + mithramycin (M, 100 nmol/L) for 6 hours or in D, with no drug (C), CXB (30 μmol/L), CXB + SB (20 μmol/L) and CXB + M (100 nmol/L) for 16 hours. C, luciferase activity or luciferase mRNA (D) levels were measured. Representative PhosphorImager images are shown in D. Graphs are the results of 3 independent experiments. Error bars are ±1 SEM.

celecoxib. By this assay, celecoxib treatment results in a 4- to 5-fold induction of VEGF transcription (Fig. 5A). This induction was also significantly blocked by pretreatment with the p38-MAPK inhibitor SB203580 or the Sp1 inhibitor mithramycin A providing further evidence supporting the role of p38-MAPK and Sp1 in transcriptional regulation of VEGF by celecoxib (Fig. 5A). Next, we tested whether celecoxib treatment alters VEGF mRNA stability. LN229 cells were first stimulated with EGF to enhance VEGF mRNA level. Then, transcription was blocked with addition of actinomycin D either alone or with celecoxib. Finally, VEGF mRNA was quantified by RPA at different times following celecoxib treatment, as indicated. By this assay, celecoxib treatment had no apparent effect on degradation of VEGF mRNA (Fig. 5B), indicating that its stability remained unchanged. Thus, transcriptional activation is the main mechanism by which celecoxib induces VEGF expression. Similar results were seen in SF767, C4-2, and A431 cells (data not shown).

To further show enhanced VEGF promoter activity with exposure to celecoxib, we first tested whether expression of MKK3 results in elevated luciferase activity. The VEGF promoter-luciferase reporter construct (pGL-VPr) was transfected into LN229/tMKK3 and SF767/tMKK3, doxycycline added 16 hours later and lysates harvested for luciferase assay. Here, induction of MKK3 expression increased luciferase activity by 2- to 3-fold and this increase is blocked by pretreatment with mithramycin A, again indicating that the p38-MAPK/Sp1 pathway activates the VEGF promoter (Fig. 5C). Next, to determine whether celecoxib treatment can similarly activate the VEGF promoter in this reporter gene assay, pGL-VPr was transiently transfected into LN229/tMKK3 and SF767/tMKK3 cells but treated with celecoxib instead of doxycycline. Surprisingly, luciferase activity did not increase after drug treatment (data not shown). Because exposure to celecoxib can produce ER stress or the UPR (13, 32), we postulated that whereas celecoxib can increase transcription from the VEGF promoter, no increase in luciferase protein, and thus luciferase activity, is seen due to a global translational repression that occurs as a part of the UPR (33). In this situation, we predict that luciferase mRNA levels should still rise after celecoxib treatment and this is, in fact, what was observed with a 2- to 3-fold increase in luciferase mRNA (Fig. 5D). As expected, this increase in luciferase mRNA is substantially blocked by either the p38-MAPK inhibitor SB203580 or the Sp1 inhibitor mithramycin A (Fig. 5D). These results further support our idea that celecoxib treatment results in transcriptional activation of VEGF in a process that requires p38-MAPK and Sp1.

Celecoxib-treated glioma cells induce endothelial cell migration *in vitro*

To this point, we can clearly show that celecoxib induces VEGF. However, to determine whether this VEGF induction has functional consequences, we sought to determine whether celecoxib-treated LN229 cells could stimulate the migration of endothelial cells *in vitro*. A transwell assay was done where HUVECs were grown in the upper chamber on fibronectin-coated membrane and LN229 cells treated with celecoxib or vehicle were grown in the lower chamber. HUVECs that migrated through the membrane to the undersurface of the upper chamber were quantitated colorimetrically. In this assay, celecoxib-treated LN229 cells induced HUVEC migration by approximately 1.5-fold (Fig. 6A). EGM supplemented with 2% FBS with or without celecoxib also induced migration of HUVECs at similar levels suggesting that celecoxib itself does not alter endothelial cell migration (Fig. 6A).

Celecoxib-induced VEGF expression is detectable and relevant *in vivo*

Although VEGF induction by celecoxib in cell culture is interesting, it is unlikely to be clinically important unless this effect can also be detected *in vivo*. Toward this end, athymic nude mice were treated with celecoxib after establishment of LN229 subcutaneous flank tumors. Celecoxib (150 mg/kg) or vehicle was given by oral gavage on a daily basis for 11 days before tumors were harvested and total RNA was isolated. Relative VEGF mRNA levels, as determined by RPA, was significantly higher in tumors from celecoxib-treated than from vehicle-treated mice ($n = 5$ for each group; P value $= 0.025$; Fig. 6B). Next, nude mice injected subcutaneously with LN229 glioma cells were again treated with celecoxib (150 mg/kg) or vehicle only for a longer period of time (25 days). Tumors were harvested from the mice at that point and subjected to immunofluorescent staining with anti-CD31 antibody to detect microvasculature. Average number of microvessels per high-power field (400×) seen in either celecoxib-treated ($n = 7$) or vehicle-treated ($n = 6$) tumors are shown (Fig. 6C). In this measure of microvessel density, celecoxib-treated tumors had more than 2-fold increase compared with the vehicle-treated controls (P value $=$ 0.043). Finally, a matrigel plug assay was done to further determine whether angiogenesis is increased after celecoxib treatment. Plugs containing LN229 cells were established in nude mice as described in Materials and Methods. Mice were then treated with either celecoxib (150 mg/kg) or vehicle by oral gavage on a daily basis for 4 days before sacrifice of the animal and removal of the plug. Matrigel plugs isolated from celecoxib-treated animals appear to display a gross increase in vasculature compared with those from vehicle-treated controls (Fig. 6D). When quantified, hemoglobin content within the plugs from celecoxib-treated mice were significantly increased ($n = 6$ per group; P value $= 0.004$; Fig. 6D). Thus, celecoxib induces not only VEGF expression but also angiogenesis in tumors.

Discussion

COX-2 expression is linked with poor prognosis in many malignancies including high-grade gliomas (24, 34, 35). This is hypothesized to be due, in part, to its proangiogenic activity through production of prostaglandin E2 and prostaglandin E2-dependent VEGF production (36, 37). Thus, COX-2 inhibitors may be attractive for targeting tumor angiogenesis. However, we now show that treatment of a variety of cancer cell lines with celecoxib can actually enhance VEGF expression and angiogenesis. Mechanistically, this response requires p38-MAPK activity, which enhances Sp1-dependent VEGF mRNA transcription. This VEGF induction is likely to be biologically significant because its magnitude is similar to that seen with hypoxia. Our finding that VEGF expression and angiogenesis is promoted *in vivo* by celecoxib further supports this biological relevance of this effect.

COX-2 inhibitors can suppress VEGF expression in various cancer cells through inhibition of Sp1 activity or promotion of degradation of Sp1 protein (38, 39). However, in contrast, Ueno et al. found that circulating VEGF levels increases during celecoxib treatment in breast cancer patients (17). Eibl et al. also showed the selective COX-2 inhibitors, nimesulide and DuP697, induce VEGF expression in pancreatic cancer cells (40). In this article, the drug concentration required for VEGF induction was higher than that required for COX-2 inhibition indicating that this effect is likely independent of the drug's COX-2 inhibitory activity. Consistent with these reports, we found that celecoxib induces VEGF expression in a concentration-dependent manner (also at higher levels than required for COX-2 inhibition). In addition, the levels of celecoxib required to induce VEGF were achievable in patients taking even moderate doses of this drug (e.g., 400 mg twice a day resulted in an average C_{max} of 7.5 μmol/L and AUC of 52.6 μmol/L.h)

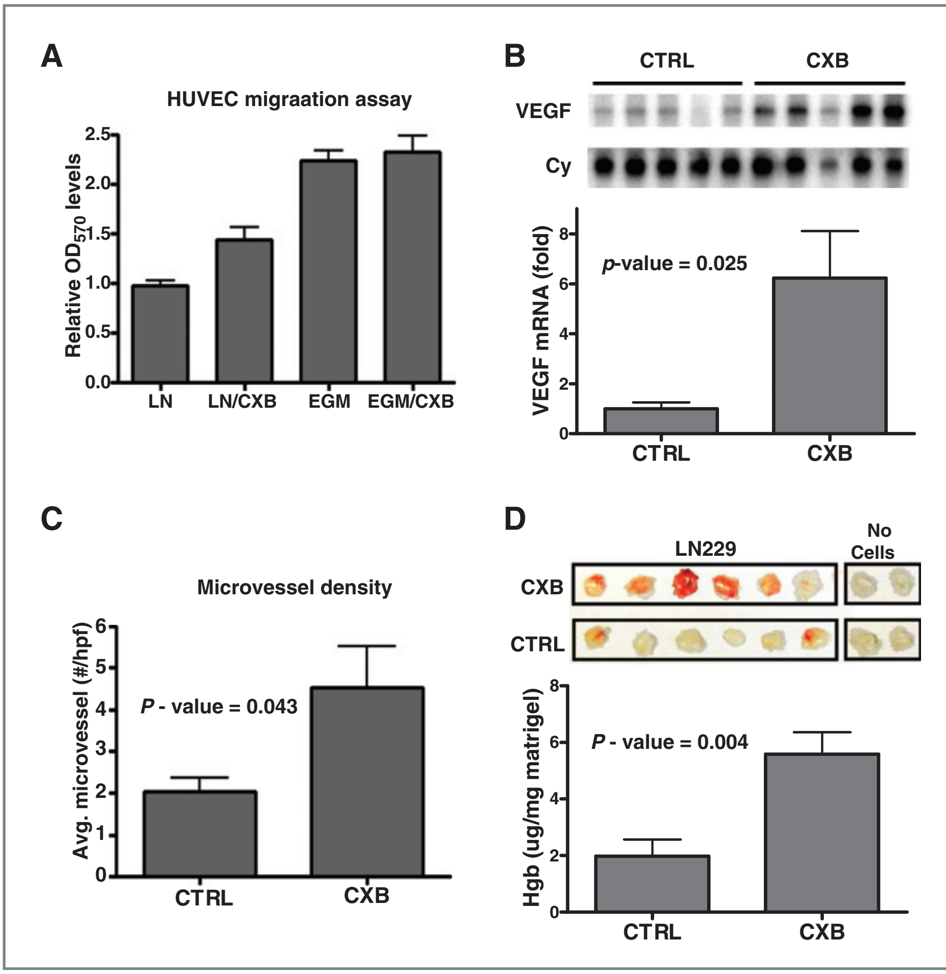

Figure 6. Functional consequence of celecoxib-induced VEGF expression *in vitro* and *in vivo*. A, endothelial cell migration assay was done as described in Materials and Methods. LN229 treated with vehicle (LN) or celecoxib (30 μmol/L; LN/CXB) or EGM supplemented with 2% FBS with vehicle (EGM) or celecoxib (30 μmol/L; EGM/CXB) were used as the attractant in the lower chamber for HUVECs in the upper chamber. Colorimetric assay was used to quantitate HUVECs that have migrated through the membrane. Each graph shows the average relative optical density (OD) at 570 nm of 2 independent wells with the average LN value being arbitrarily set at 1. B, VEGF mRNA expression in LN229 xenograft tumors established in nude mice treated for 11 days by oral gavage with either vehicle alone (CTRL) or celecoxib (CXB, 150 mg/kg) was assessed by RPA. Representative PhosphorImager images show mRNA in tumors from 5 different mice for each group with Cy serving as normalization control. Graph shows relative expression of VEGF with CTRL value set as 1. Each bar is the average of 5 tumors. C, microvessel density in LN229 xenograft tumors established in nude mice was measured. Mice were fed vehicle only (CTRL) or celecoxib (CXB, 150 mg/kg) for 25 days before tumor harvest. Tumors were immunofluorescently stained with anti-CD31 antibody. Quantitation was done by counting microvessels expressing CD31 in high-power fields (hpfs, $\times$ 400). Six hpfs were counted per tumor and the graph is the average count of tumors from vehicle-treated ($n = 6$) or celecoxib-treated ($n = 7$) mice. D, pictures of LN229 matrigel plugs harvested from nude mice are shown. Mice (3 in each group) harboring 2 plugs per animal were treated for 4 days by oral gavage with either CTRL or CXB (150 mg/kg). Plugs containing no cells are also shown on the right. Hemoglobin was extracted from each plug and levels measured as described in Materials and Methods. Graph shows the hemoglobin (Hgb) content (μg) per plug weight (mg). Each bar is the average Hgb content of 6 plugs. In all graphs, error bars are ±1 SEM. Values for the groups in B to D were compared by a 2-tailed, unpaired Student's *t* test with *P* values shown on the graphs.

based on pharmacokinetic studies (41). Furthermore, this induction is likely independent of HIF-1α because celecoxib and hypoxia had additive to supra-additive effects on VEGF levels (Fig. 2A) and HIF-1α was not induced in response to celecoxib treatment (data not shown). Our finding that inhibition of Sp1 significantly blocked celecoxib-induced VEGF expression further supports the idea of a HIF-1α–independent mechanism that requires Sp1.

Sp1 is known to regulate VEGF expression and its activity can be controlled by multiple signaling pathways (for review, see ref. 42). Previously, celecoxib was found to either activate or reduce p38-MAPK activity depending on the cellular context (43–45). In our study, celecoxib activates p38-MAPK and assessment of various signaling inhibitors, including those targeting p38-MAPK, MEK, PI-3K, and Src kinase, suggest that celecoxib-induced VEGF expression is mainly dependent on p38-MAPK acting upstream of Sp1. In fact, blocking this transcription factor with mithramycin A or siRNA significantly inhibits p38-MAPK–dependent VEGF promoter activation and expression. These results are consistent with our previous findings that EGF-dependent activation of the p38-MAPK signaling pathway positively regulates Sp1 activity and further increases COX-2 expression in glioma cells (18, 28). Here, the EGFR/p38-MAPK/Sp1 signaling axis similarly increases VEGF levels, confirming the importance of this pathway in VEGF regulation. Although some suggest that p38-MAPK regulates VEGF expression in an HIF-1α–dependent manner (46, 47), we find that Sp1, and not HIF-1α, is the critical factor downstream of p38-MAPK controlling VEGF transcription in response to celecoxib.

Interestingly, whereas celecoxib increases both VEGF mRNA and protein in glioma cells, luciferase mRNA but not luciferase activity (as a marker of protein) is induced when the VEGF promoter drives this reporter. A possible explanation is that whereas celecoxib-dependent activation of the VEGF promoter leads to accumulation of luciferase mRNA, this message is not efficiently translated due to a global inhibition of translation from celecoxib-induced UPR (33). As previously described, VEGF mRNA is relatively resistant to stress-related translational repression whereas luciferase mRNA should be fully sensitive to this repression (48). This may explain some of the differential regulation of protein expression seen in response to celecoxib treatment. Liu et al. found that celecoxib induces death receptor 5 (DR5), resulting in the induction of apoptosis and enhancement of tumor necrosis factor–related apoptosis-inducing ligand-dependent apoptosis in human lung cancer cells (49). This response may contribute to the antitumor activity of celecoxib. However, in other instances, celecoxib may induce genes that potentially limit its effectiveness as an antitumor agent. Namba et al. reported that celecoxib upregulates the 150-kDa, oxygen-regulated protein resulting in decreased apoptosis (16). Here, we now show that celecoxib can increase VEGF expression and enhance angiogenesis. Clearly, these effects may potentially be detrimental for cancer patients.

In summary, we have defined the mechanism for induction of VEGF by celecoxib identifying p38-MAPK and Sp1 as key players in this process. We have further shown that this induction of VEGF is detectable *in vivo* and can lead to increased angiogenesis. By gaining a greater mechanistic understanding of this effect, combination therapies may be rationally designed to enhance the antitumor activity of these agents. For example, as p38-MAPK and Sp1 are important at mediating celecoxib-induced VEGF expression, combining celecoxib with selective inhibitors of these factors may limit the detrimental consequences of this effect while maintaining celecoxib's antitumor activities. Another potential strategy could involve combining celecoxib with anti-VEGF agents such as bevacizumab. Overall, gaining a greater understanding of the molecular responses to drugs, such as celecoxib, will likely be needed to fully unlock its potential in cancer therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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