Celecoxib Inhibits Vascular Endothelial Growth Factor Expression in and Reduces Angiogenesis and Metastasis of Human Pancreatic Cancer via Suppression of Sp1 Transcription Factor Activity

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ABSTRACT

The aggressive biology of human pancreatic adenocarcinoma has been linked with overexpression of vascular endothelial growth factor (VEGF). Constitutive activation of the transcription factor Sp1 plays a critical role in VEGF overexpression. Recent studies indicated that celecoxib, a selective cyclooxygenase-2 inhibitor, exhibits potent antitumor activity. However, the underlying molecular mechanisms of this activity remain unclear. In the present study, we used a pancreatic cancer model to determine the role of Sp1 in the antitumor activity of celecoxib. Treatment of various pancreatic cancer cells with celecoxib suppressed VEGF expression at both the mRNA and protein level in a dose-dependent manner. VEGF promoter deletion and point mutation analyses indicated that a region between nucleotide −109 and −61 and its intact Sp1-binding sites were required for the inhibition of VEGF promoter activity by celecoxib. Also, celecoxib treatment reduced both Sp1 DNA binding activity and transactivating activity. This decreased activity correlated with reduced Sp1 protein and its phosphorylation as determined using Western blot analysis. Furthermore, in an orthotopic pancreatic cancer animal model, celecoxib treatment inhibited tumor growth and metastasis. The antitumor activity was consistent with inhibition of angiogenesis as determined by evaluating tumor microvessel formation, which correlated with decreased Sp1 activity and VEGF expression. Collectively, our data provide a novel molecular mechanism for the antitumor activity of celecoxib and may help further improve its effectiveness in controlling pancreatic cancer growth and metastasis.

INTRODUCTION

Pancreatic cancer is currently the fourth leading cause of cancer-related death, with an estimated 30,300 new cases and 29,700 deaths occurring annually in the United States (1). More than 80% of pancreatic cancers metastasize or extend locally beyond the pancreas at the time of diagnosis, and only about 10% of patients with pancreatic cancer are able to undergo curative resection. The average survival duration from diagnosis to death is about 4–6 months, and the overall 5-year survival rate is <5% (2–4). These findings underscore that potential new targets must be identified for development of novel effective treatment modalities for primary cancer and metastatic lesions as well as prevention of this deadly disease.

As with other solid tumors, growth and metastasis of pancreatic adenocarcinoma depend on angiogenesis, which is the formation of new blood vessels from a pre-existing network of capillaries (5). Of the numerous angiogenic factors discovered thus far, vascular endothelial growth factor [VEGF (6)] has been identified as a key mediator of tumor angiogenesis. Elevated expression of VEGF in human tumor biopsy specimens has been reported in cases of various cancers, including pancreatic adenocarcinoma (7, 8). Also, increasing evidence suggests that VEGF expression is regulated by a plethora of external factors. Major stimulators of VEGF expression include hypoxia and acidosis (9, 10), which occur frequently within diverse types of expanding tumors, particularly in regions surrounding necrotic areas (9). Moreover, loss or inactivation of tumor suppressor genes and activation of oncopgenes are associated with VEGF overexpression (11, 12). VEGF promoter analyses have revealed several potential transcription factor-binding sites, such as hypoxia-inducible factor-1 (HIF-1), activator protein (AP)-1, AP-2, early growth response-1 (Egr-1), Sp1 (13), and many others (14–17). Our previous results indicate that Sp1 in particular plays an important role in tumor angiogenesis and contributes to the aggressive nature of human pancreatic adenocarcinoma (10).

Cyclooxygenases (COXs) are responsible for the conversion of arachidonic acid to prostaglandins, and their metabolites play a pivotal role in multiple physiological and pathophysiologic processes. Specifically, COX-1 is constitutively expressed in most tissues and is responsible for maintaining physiological processes such as gastric and renal protection and platelet function. In contrast, COX-2 is induced in response to growth factors, cytokines, and tumor promoters (18–21). Mounting evidence suggests that COX-2 is chronically overexpressed in many premalignant, malignant, and metastatic human cancers, including pancreatic cancer (22–27), and that the level of overexpression is significantly correlated with invasiveness, prognosis, and survival in some cancers (28–36). In comparison, evidence from epidemiological and animal studies and in vitro cell culture experiments indicates that nonsteroidal anti-inflammatory drugs can significantly reduce the risk of colorectal, gastric, and, to a lesser extent, pancreatic, breast, and other cancers (37, 38). Furthermore, selective COX-2 inhibitors have been shown to inhibit the growth and metastasis of established tumors (39–45). The possible molecular mechanisms of the antitumor effects of COX-2 inhibitors may be due to inhibition of angiogenesis (35, 43, 46–50). However, these mechanisms have not been fully elucidated. It remains unknown whether and, if so, how COX-2 inhibitors regulate VEGF expression in pancreatic cancer and growth and metastasis of pancreatic cancer.

In the present study, we found that celecoxib, a selective COX-2 inhibitor, suppresses VEGF gene expression and that Sp1-binding sites on the VEGF promoter were responsible for this inhibitory effect. Celecoxib treatment reduced Sp1 protein and its phosphorylation and in turn its DNA binding activity and its transcriptional activity, which led to the inhibition of VEGF gene expression. Consistent with in vitro experiments, in vivo experiments using an orthotopic pancreatic cancer animal model also showed that celecoxib significantly suppressed tumor angiogenesis, growth, and metastasis. The in vivo antitumor activity correlated with suppression of Sp1 activity, lowered VEGF expression, and decreased tumor microvessel formation. Therefore, we provide a novel molecular mechanism for the antitumor activity of celecoxib and may help further improve...
its effectiveness in controlling pancreatic cancer growth and metastasis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. PANC-1 human pancreatic adenocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). FG cells were established by Vezzeridis et al. (51). All of the cell lines were maintained in plastic flasks as adherent monolayers in MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a vitamin solution (Flow Laboratories, Rockville, MD).

Northern Blot Analysis. Total RNA was extracted using TRizol reagent (Invitrogen, San Diego, CA). RNA (12 μg) was separated electrophoretically on a 1% denaturing formaldehyde agarose gel, transferred to a GeneScreen nylon membrane (DuPont, Boston, MA) in 20× standard saline citrate, and UV-cross-linked using a UV-Stratallinker 1800 (Stratagene, La Jolla, CA). Additionally, the VEGF probe was labeled with [32P]dCTP using a random labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Equal loading of RNA samples was monitored by hybridizing the same membrane filter with a human β-actin cDNA probe (10).

VEGF Protein Measurement. The VEGF protein level in the culture supernatants, mouse serum, and ascites was determined using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN), which is a quantitative immunometric sandwich enzyme immunoassay. A curve of the absorbance of VEGF versus its concentration in the standard wells was plotted. By comparing the absorbance of the samples with the standard curve, we determined the VEGF concentration in the unknown samples (10).

Western Blot Analysis and Immunoprecipitation. Whole-cell lysates and nuclear extracts were prepared from human pancreatic cancer cell lines and tissues (10). Standard Western blotting was performed using a polyclonal rabbit antibody against human and mouse Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) and the antirabbit IgG antibody, a horseradish peroxidase-linked F(ab′)2 fragment obtained from a donkey (Amersham, Arlington Heights, IL). Equal protein sample loading was monitored by hybridizing the same membrane filter with an anti-β-actin antibody (10). The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the manufacturer’s instructions. For immunoprecipitation assay, equal amounts of protein extracts were incubated with Sp1 antibody (Santa Cruz Biotechnology) at 4°C for 12 h and then incubated with protein A/G-agarose beads for 1 h. The immunoprecipitates were resolved by SDS-PAGE, and the membranes were probed with anti-phosphoserine antibody (Sigma Chemical Co., St. Louis, MO) to determine the phosphorylation status of Sp1 (phospho-Sp1), and then the membrane was stripped and reprobed with anti-Sp3 antibody to detect total Sp1 protein.

Analysis of VEGF Promoter Activity. The activity of full-length and mutant VEGF promoter constructs was analyzed as described previously (10). In brief, plasmids containing firefly luciferase reporters were cotransfected into tumor cells in triplicate with an internal control plminITK-RL using the LipofectAMINE method (Invitrogen). The plminITK-RL contained a full-length Renilla luciferase gene under the control of a minimal thymidine kinase (TK) promoter (636–757 bp from pTK-RL; Invitrogen). In some of the experiments, the reporters were cotransfected with pCMV4-Sp1, pCMV4-Sp3, or pCMV4 (obtained from Dr. Jonathan M. Horowitz; Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University). After transfection, the cells were washed three times with PBS and refed with fresh medium. The activity of both the firefly and Renilla luciferase reporters was determined 48 h later using the Dual Luciferase Assay Kit (Promega, Madison, WI). Specific VEGF promoter activity was calculated as described previously (10).

Preparation of Nuclear Extracts. For cell cultures, different pancreatic cancer cells were plated into 15-cm culture dishes at 80% confluence. The cells were washed twice with PBS (pH 7.4) and detached by scraping. For in vivo experiments, fresh tumor tissue samples were homogenized as described previously (10). The resulting cells were pelleted via centrifugation at 1200 rpm for 10 min at 4°C. Pellets were resuspended in ice-cold PBS (pH 7.4), and the resulting suspensions were transferred to Eppendorf tubes. Cells were repelleted via spinning in a microcentrifuge at 14,000 rpm for 20 s at 4°C. The cells were then lysed by resuspending the pellet in ice-cold buffer A, which consisted of 10 mM HEPES (pH 8), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 200 mM sucrose, 0.5% NP40, 1 mg/ml leupeptin, and 1 mg/ml aprotinin. The samples were incubated on ice for 5 min and then recentrifuged. The nuclei were lysed in ice-cold buffer C, which consisted of 20 mM HEPES (pH 8), 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin. The cellular debris was removed using centrifugation, and the supernatant containing DNA-binding proteins was combined with an equal volume of ice-cold buffer D [20 mM HEPES (pH 8), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin]. Nuclear extracts were stored at −80°C until they were used.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed using nuclear extracts prepared as described above. The following double-stranded oligonucleotides were used: a wild-type Sp1 consensus sequence (5′-ATTGCATCGGGGGCGCCCAGC-3′) and its mutant Sp1 consensus sequence (5′-ATTGCATCGGGTCGGGGCGCCCAGC-3′); and Sp1 binding sequences corresponding to Sp1-binding sites in the VEGF promoter region (as described in “Results”). The oligonucleotides were annealed and 5′ end-labeled with [32P]ATP (Amersham) plus T4 polynucleotide kinase using standard procedures. A binding reaction was carried out by preincubating 10 μg of nuclear extract protein in 20 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol, 0.1 mM DTT, and 1 μg of poly(deoxyinosinic-deoxycytidylid acid) at room temperature for 15 min followed by addition of the double-stranded 32P-labeled oligonucleotide and a second incubation at room temperature for 15 min. For competition assays, a 50-fold molar (or as indicated) excess of unlabeled oligonucleotides (cold probe) was added to the binding reaction. For supershift reactions, extracts were preincubated with specific anti-Sp1, anti-Sp3, anti-AP-2, or anti-Egr-1 antibodies (Santa Cruz Biotechnology) for 20 min on ice. Samples were then loaded on a 5% polyacrylamide gel, and electrophoresis was performed at room temperature for 3 h at 120 V. The gel was then dried for 2 h at 80°C and exposed to Kodak film (Eastman Kodak Co., Rochester, NY) at −70°C.

Sp1 Transcriptional Activity Assay. Gal4-based plasmid DNA constructs (kindly provided by G. Suske; Institut fur Molekularbiologie und Tumorforschung, Marburg, Germany) were used in this assay as described previously (52). In brief, reporter plasmid Gal4-Luc (five Gal4 binding sites upstream of a minimal promoter linked to luciferase gene) and Gal4-Sp1 or Gal4-Sp3 plasmid (activator constructs containing the Gal4 DNA binding domain linked to the Sp1 or Sp3 transcriptional domain) were cotransfected into tumor cells in triplicate with an internal control pMiniTK-RL using the LipofectAMINE method (Invitrogen). After transfection, the cells were washed three times with PBS and refed with fresh medium. The activity of both the firefly and Renilla luciferase reporters was determined 48 h later using the Dual Luciferase Assay Kit (Promega).

Animals. Female athymic BALB/c nude mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

Tumor Growth and Metastasis. To prepare tumor cells for inoculation, cells in the exponential growth phase were harvested via brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). Cell viability was determined using trypan blue exclusion, and only single-cell suspensions that were >95% viable were used. Tumor cells (1 × 106 cells/mouse) were injected into the pancreas of nude mice in groups of 10. Celecoxib therapy was initiated in the animals’ diet at doses ranging from 2.5 to 20 mg/kg body weight when the tumors reached a mean volume of 250 mm3 (20 days after tumor cell injection) and maintained for the duration of the experiment as suggested previously (48). The animals were killed 60 days after the tumor cell injection or when they had become moribund. Next, the primary pancreatic tumors were harvested and weighed. In addition, each mouse’s liver was fixed in Bouin’s solution for 24 h to differentiate the neoplastic lesions from the organ parenchyma; metastases on the surface of liver were counted (double-blinded) with the aid of a dissecting microscope as described previously (53).
RESULTS

Suppression of VEGF Expression by Celecoxib in Human Pancreatic Cancer in Vitro. To determine whether COX-2 inhibitor can modulate VEGF expression in human pancreatic cancer, FG and PANC-1 cells were incubated with celecoxib at different concentrations. VEGF expression was analyzed at the mRNA level using Northern blot analysis and at the protein level using ELISA. As shown in Fig. 1, celecoxib inhibited VEGF mRNA (Fig. 1A) and protein (Fig. 1B) expression in both pancreatic cancer cells in a dose-dependent manner. Therefore, the COX-2 inhibitor suppressed VEGF expression in human pancreatic cancer cells.

Identification of Celecoxib-Responsive Elements on the VEGF Promoter. To determine the critical regions on VEGF promoter responsible for the transcriptional inhibition by celecoxib, a series of 5' deletion mutants based on the 2274-bp VEGF promoter (Fig. 2A) were transfected into tumor cells, and the effect of celecoxib on the promoter activity was examined. Loss of the region spanning −2274 to −109 had no substantial influence on VEGF promoter activity. However, further deletion of 5' proximal promoter to −88 reduced 30–40% of the inhibitory effect of celecoxib on VEGF promoter activity. Removal of an additional 27 nucleotides almost completely

Immunohistochemistry and Quantification of Tumor Microvessel Density. For VEGF staining, sections (5-μm thick) of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized in xylene and rehydrated in graded alcohol. For CD31 staining of frozen sections (5-μm thick), the specimens were fixed with acetone. Endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 12 min. The specimens were incubated for 20 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum and then incubated overnight at 4°C in a 1:100 dilution of monoclonal anti-CD31 (PECAM1-M20), polyclonal rabbit anti-Sp1, or polyclonal rabbit anti-VEGF antibodies (Santa Cruz Biotechnology). The specimens were then rinsed and incubated with peroxidase-conjugated goat or antirabbit IgG for 1 h at room temperature. Next, the slides were rinsed with PBS and incubated with diaminobenzidine (Research Genetics, Huntsville, AL) for 5 min. The sections were then washed three times with distilled water, counterstained with Mayer’s hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed once each with distilled water and PBS. The slides were mounted using a Universal mount (Research Genetics) and examined using a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm (CD31 and VEGF) or nuclei (Sp1). For quantification of tumor microvessel density, vessels on each section were counted in five high-power fields (×200 magnification (×20 objective and ×10 ocular)] as described previously (54).

Statistics. Each experiment was performed independently at least twice with similar results; one representative experiment was presented. The significance of the in vitro data was determined using Student’s t test (two-tailed), whereas that of the in vivo data was determined using the two-tailed Mann-Whitney U test. Ps < 0.05 were deemed significant.
abolished the inhibitory effect of celecoxib on VEGF promoter activity (Fig. 2B), suggesting that the −109−61 region contains essential regulatory elements. Moreover, celecoxib produced dose- and time-dependent inhibition of pV109 promoter activity (Fig. 2, C and D).

Considering the crucial role of Sp1 in regulation of VEGF promoter activity and that three potential Sp1-binding sites, GGGGCGGG, are located within this region [nucleotide (nt) −109 to −61], we therefore tested whether mutation of these Sp1-binding sites might affect the responsiveness to celecoxib. As shown in Fig. 2E, mutations in individual Sp1 sites variably reduced the inhibitory effect of celecoxib on the promoter activity, with a more profound reduction by the mutation in the sites 2 or 3, whereas mutations in all Sp1 sites eliminated the inhibitory effect of celecoxib on VEGF promoter activity. In sharp contrast, mutation of either AP-2 or Egr-1 sites did not, suggesting that Sp1-binding sites in the region are responsible for celecoxib-mediated inhibition of VEGF promoter activity.

To dissect the contribution of the individual putative Sp1-binding sites to the VEGF promoter activity, EMSA was performed using oligonucleotides corresponding to the promoter region of nt −104 to −60 of the human VEGF promoter, which contains putative Sp1-binding sites 2, 3, and 4 (Fig. 3A). Three DNA-protein complexes were detected and contain Sp1 and Sp3 protein (Fig. 3B). In contrast, AP-2 and Egr-1 were not detected (data not shown). Furthermore, the interaction of the nuclear proteins with the probes was sequence specific, as demonstrated by probe competition assay (Fig. 3B), because the binding could be competed off by an excess of unlabeled wild-type oligonucleotides (Lane 2), but not by the cold probe with mutations in all three putative Sp1-binding sites (Lane 9). Cold probes with mutation(s) in one or two putative Sp1-binding site(s) variably reduced its competition potential (Lanes 3–8). These data suggest that Sp1 and Sp3 proteins constitutively bound to all of the Sp1 sites located between nt −104 and −60 in the VEGF promoter and contribute to the VEGF promoter activity.

Suppression of Sp1 DNA Binding to the VEGF Promoter by Celecoxib. To determine whether inhibition of VEGF promoter activity by celecoxib is caused by changes in the interaction between nuclear proteins and the promoter, EMSA was performed using the Sp1 consensus sequence (Fig. 3A) and oligonucleotides corresponding to the VEGF promoter region of nt −104 to −72, which contains putative Sp1-binding sites 2 and 3 (Fig. 4B). Three DNA-protein complexes (I, II, and III) were detected (Fig. 4, A and B). Supershift assays identified complex I as Sp1 and complexes II and III as Sp3 (Fig. 4, A and B). We found that celecoxib treatment significantly reduced the binding of Sp1 but not Sp3 proteins to the probes in a dose-dependent manner. Although both Sp1 and Sp3 are important in VEGF expression and regulation (10, 55), our data suggest that Sp1 proteins constitutively bound to the Sp1 site located between nt −104 and −72 in the VEGF promoter and that this binding was suppressed by celecoxib.

We then investigated whether the attenuation of Sp1 binding caused by celecoxib treatment was due to decreased Sp1 protein expression and/or posttranslational modifications. Control and treated cells were harvested, and cellular proteins were extracted for immunoblotting. Our data showed that total protein level of Sp1 was reduced by 20−40% after celecoxib treatment (Fig. 4C). To determine whether celecoxib also regulates Sp1 binding activity through posttranslational modification, we analyzed the phosphorylation status of Sp1, which exhibits apparent molecular masses of 95 and 105 kDa [phosphorylated form (56−59)]. As shown in Fig. 4C, there was a more striking dose-dependent decrease in the relative intensity of the 105-kDa species, i.e., a dose-dependent decrease in phosphorylated Sp1. For example, without celecoxib treatment, the ratio between phosphorylated Sp1 (105 kDa) and unphosphorylated Sp1 (95 kDa) was 1.8, whereas after treatment with 30 μM celecoxib, the ratio was decreased to 0.4. The inhibition of Sp1 phosphorylation was further confirmed by an immunoprecipitation experiment (Fig. 4D). Therefore, celecoxib reduced both Sp1 protein level and Sp1 phosphorylation and decreased its DNA binding and transactivation activity.

Celecoxib Inhibited Sp1 Transactivating Activity. To determine whether celecoxib influenced the transcriptional activation properties of Sp1, we cotransfected tumor cells with Gal4 reporter constructs (Gal4-Sp1 or Gal4-Sp3). Celecoxib treatment resulted in a statistically significant reduction in the transactivation potential of Gal4-Sp1. Although Sp3-mediated transactivation was also decreased by celecoxib, the decrease did not reach statistical significance (Fig. 4E).

Western blot analysis indicated that there was no significant change in the levels of Gal4-Sp1 and Gal4-Sp3 fusion protein with or without celecoxib treatment (Fig. 4F). These data further confirm that celecoxib mainly affects Sp1 transcriptional activity, but not Sp3.

Inhibition of Human Pancreatic Cancer Growth and Metastasis by Celecoxib in Vivo. To determine the effect of celecoxib on tumor growth kinetics, FG cells were s.c. injected into nude mice. Celecoxib treatment started 14 days after tumor injection. As shown in Fig. 5, celecoxib produced dose-dependent antitumor activity. To be more biologically relevant, FG cells were injected into the tail of the pancreas of mice in groups of 10 (an orthotopic pancreatic cancer animal model). Celecoxib or a vehicle was then administered to the mice. The animals were killed 60 days after tumor cell injection or when they had become moribund. Each tumor was resected and weighed, and the tumor weights in all of the groups of mice were
compared. We found that celecoxib-treated mice had much smaller tumors and fewer liver metastases when compared with control mice (Table 1).

**Inhibition of VEGF Expression and Angiogenesis by Celecoxib in Human Pancreatic Cancer Growing in Nude Mice.** To further investigate the mechanisms by which celecoxib treatment reduced primary tumor growth and metastasis, we examined the effect of the treatment on tumor angiogenesis. Microvessel formation was identified by immunostaining with anti-CD31 antibody; the number of vessels per high-power field was scored. Representative VEGF expression levels and tumor microvessel densities in both treated and control tumors are shown in Fig. 6. VEGF mRNA expression was further measured using Northern blot analysis, and microvessel formation was further quantified via vessel counting. Celecoxib treatment significantly inhibited VEGF mRNA expression relative to that in the controls (Fig. 7A). Consistently, celecoxib treatment also significantly reduced microvessel formation in the primary tumor relative to that in the controls (Fig. 7B). The results suggest that the altered tumor growth and metastasis were directly correlated with altered VEGF expression and angiogenesis.

**Suppression of Sp1 DNA Binding Activity by Celecoxib in Vivo.** Finally, to determine whether celecoxib also modulates Sp1 activity in tumors growing in nude mice, nuclear proteins were isolated, and both EMSA and Northern blot analysis were performed. As shown in Fig. 7C, celecoxib treatment decreased Sp1 DNA binding activity, which was consistent with decreased VEGF expression (Fig. 7A) and tumor microvessel formation (Fig. 7B).

**DISCUSSION**

There is ample evidence to suggest that COX-2 has an important role in multiple biological events throughout the process of tumor development and progression (60). Lines of evidence have also demonstrated that COX-2 inhibitors exhibit potent antiangiogenic and antimetastatic activity both in vitro and in vivo (35, 37, 46–50). However, the molecular mechanisms of these actions are largely unknown. Considering the prominent role of VEGF in tumor angiogenesis, growth, and metastasis, we examined whether and, if so, how celecoxib regulates VEGF expression. We found that treatment of
various pancreatic cancer cells with celecoxib suppressed VEGF expression at both the mRNA and protein level. A region between nt −109 and −61 of the VEGF promoter and its intact Sp1-binding sites were required for the inhibition of VEGF promoter activity. Furthermore, celecoxib treatment reduced Sp1 DNA binding activity, which closely correlated with reduced Sp1 protein level as well as Sp1 phosphorylation. In an orthotopic pancreatic cancer animal model, celecoxib exhibited dose-dependent antiangiogenic and antitumor activity, which was consistent with decreased Sp1 activity and VEGF expression. Collectively, our data provide a novel molecular mechanism for the antitumor activity of celecoxib and may help further improve its effectiveness in controlling tumor growth and metastasis.

Previous studies have suggested that expression of COX-2 contributes to tumor growth by inducing angiogenesis (48, 61, 62). For example, COX-2 activity was correlated with VEGF mRNA and protein expression in tumor tissue samples (62). COX-2-overexpressing tumor cells secrete VEGF at a very high level (46). Our present study is the first to indicate that celecoxib directly suppresses VEGF gene transcription, which is at least partially responsible for celecoxib-mediated inhibition of human pancreatic cancer angiogenesis and metastasis. Targeting VEGF function mostly through interfering with VEGF and VEGF receptor interaction and signaling has yielded significant therapeutic benefits in both animal models and cancer patients (63–66). Thus, our current findings may suggest that combining celecoxib with the existing anti-VEGF agents produces more effective antitumor activity.

Our recent study showed that Sp1 is a crucial transactivator for VEGF gene expression and regulation (10). The importance of Sp1 in VEGF expression has been further substantiated by several new findings. For example, Sp1 decoy oligonucleotides inhibit VEGF expression in lung cancer and glioblastoma cells (67). Also, mithramycin, an inhibitor of Sp1 DNA binding, blocks the induction of VEGF and its receptors via basic fibroblast growth factor and/or tumor necrosis factor α (68, 69). Interestingly, suppression of VEGF expression by the tumor suppressor genes p53, p75, and von Hippel-Lindau occurs through their formation of complexes with Sp1 and inhibition of its binding to and transcriptional activation of the VEGF promoter (70–72). In the present study, we provided evidence that celecoxib suppresses VEGF expression via targeting Sp1-mediated transcription. Our results are clearly consistent with those of a recent study that demonstrated that NS398 and indomethacin, both COX-2 inhibitors, exert their inhibition of matrix metalloproteinase-2 expression via suppression of Sp1-mediated transcription (73). Therefore, suppression of Sp1 activity by COX-2 inhibitors may at least influence tumor angiogenesis and invasion. Development of more effective and specific molecular inhibitors of Sp1 activity may be important not only for treating human cancer but also for elucidating the molecular mechanisms involved in Sp1 signaling and biological function.

From this initial attempt to define the mechanism by which celecoxib inhibits Sp1 activity, our results show that the Sp1 DNA binding activity was significantly attenuated after celecoxib treatment. The reduced DNA binding activity was correlated with decreased Sp1 protein level and, more strikingly, with Sp1 phosphorylation. Thus, it is possible that celecoxib modulates specific intracellular signaling pathways to affect Sp1 phosphorylation and function. This notion is consistent with a growing body of evidence revealing that the DNA binding and transcription activity of Sp1 may change in response to its posttranslational modification (74). For instance, phosphorylation of Sp1 increases its DNA binding activity and thus enhances Sp1-mediated transcription (75, 76), whereas Sp1 protein dephosphoryl-

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**Table 1 Inhibition of human pancreatic cancer growth and metastasis by celecoxib in a nude mice model**

FG cells (1 × 10⁶ cells/mouse) were injected into the pancreas of groups of nude mice (50 mice). Starting 20 days after tumor injection, mice carrying FG tumors (n = 10) received celecoxib for the duration of the experiment at doses equivalent to 2.5, 5, 10, and 20 mg/kg body weight/day. Mice were killed 60 days after tumor injection, or when they became moribund. The tumor incidence (mice with tumor or metastasis/total mice used), weight (g), and metastases to the liver were determined. *P* < 0.05 were considered statistically significant when comparisons were made between treated groups and untreated groups using the two-tailed Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Median weight (range)</th>
<th>P</th>
<th>Incidence</th>
<th>Median (range)</th>
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<tr>
<td>Vehicle</td>
<td>10/10</td>
<td>0.8 (0.7–1.4)</td>
<td>0.415</td>
<td>10/10</td>
<td>11 (6–34)</td>
<td>0.362</td>
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<td>2.5 mg/kg</td>
<td>9/10</td>
<td>0.7 (1–1.2)</td>
<td>0.046</td>
<td>6/10</td>
<td>6 (0–21)</td>
<td>0.038</td>
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<tr>
<td>5 mg/kg</td>
<td>5/10</td>
<td>0.5 (0–0.6)</td>
<td>0.033</td>
<td>4/10</td>
<td>0 (0–7)</td>
<td>0.038</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>5/10</td>
<td>0.3 (0–0.8)</td>
<td>0.0015</td>
<td>5/10</td>
<td>2 (0–5)</td>
<td>0.002</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>3/10</td>
<td>0 (0–0.5)</td>
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<td>1/10</td>
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**Fig. 6.** Sp1 and vascular endothelial growth factor expression and angiogenesis in human pancreatic cancer growing in nude mice. Tumor tissues were collected from the mice receiving either PBS or 10 mg/kg celecoxib treatment in the experiment summarized in Table 1. Tumor sections were prepared and stained for Sp1, vascular endothelial growth factor, and CD31 using standard methods as described in “Materials and Methods.”
drugs may include the AP-1 (87), PKC-
other identified molecular targets for nonsteroidal anti-inflammatory
coxib to the suppression of Sp1-mediated VEGF expression, other
duction pathways (81–86). However, the signal transduction pathway
and animal experiments have shown that
inhibition of VEGF expression and tumor microvessel formation is one of the potential mechanisms by which celecoxib suppresses the
growth and metastasis of human pancreatic cancer. Additionally, the
suppression of VEGF expression appears to be a consequence of altered Sp1 transactivation. Therefore, our results not only provide a
novel mechanism for the antitumor activity of celecoxib but also
further substantiate the important role of Sp1 in tumor biology and the
biological basis for the development of new Sp1-targeting agents for
cancer treatment.

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