Cyclooxygenase-2 Is a Target of KRAS^{D12}, Which Facilitates the Outgrowth of Murine C26 Colorectal Liver Metastases

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ABSTRACT

Purpose: Mutational activation of the KRAS oncogene and overexpression of cyclooxygenase-2 (COX-2) contribute to colorectal carcinoma (CRC) development, but the relationship between these two events is unclear. This study was designed to clarify that relationship and to assess the contribution of KRAS-dependent COX-2 to the seeding of CRC cells in the liver and to their outgrowth as liver metastases in an experimental mouse model.

Experimental Design: The effect of RNA interference–mediated KRAS knockdown on COX-2 expression and activity was tested in murine C26 CRC cells. The contribution of KRAS-dependent COX-2 to early metastatic tumor cell seeding (by intravital microscopy) and outgrowth of metastases in the liver (by bioluminescence imaging) was studied by using parecoxib, a novel and highly selective liver-activated COX-2 inhibitor. Intratumoral cell proliferation, apoptosis, and tumor-associated angiogenesis were assessed by immunohistochemistry on liver tissue sections.

Results: Stable knockdown of mutant KRAS^{D12} in murine C26 CRC cells by RNA interference lead to a dramatic reduction of COX-2 synthesis and prostaglandin E2 production. Inhibition of host or tumor cell COX-2 activity had no effect on early metastatic cell seeding in the liver but greatly reduced intrahepatic tumor cell proliferation and the rate of liver metastasis outgrowth. COX-2 inhibition had no effect on early tumor vascularization or on tumor cell apoptosis.

Conclusions: The high levels of COX-2 enzyme and prostaglandin E2 production in C26 CRC cells are primarily caused by the presence of endogenous mutant KRAS^{D12}. Furthermore, COX-2 inhibition affects the tumoral rather than the vascular compartment during the early stages of C26 liver metastasis outgrowth.

INTRODUCTION

Mutations in KRAS are detected at the early stages of adenoma development and contribute to oncogenic transformation of intestinal epithelial cells (1–8). In addition, tumor cells containing mutant KRAS stimulate endothelial cells in nearby vascular beds to grow out and form new blood vessels that supply the developing tumor with nutrients and oxygen (9). The mechanism underlying this phenomenon is complex and involves RAS-stimulated production of several proangiogenic factors, including cyclooxygenase-2 (COX-2; ref. 9). COX-2 is an interesting RAS target, as it is highly expressed during the development of many tumor types, including colorectal carcinoma (CRC; ref. 10). The presence of mutant KRAS and high-level COX-2 expression both correlate with tumor recurrence after surgery, with metastatic spread to the liver and with reduced survival (11–23). Conversely, COX-2 inhibitors reduce the risk of developing CRC, both in FAP patients and in sporadic cases (10).

Whereas the contribution of COX-2 to the early stages of intestinal tumorigenesis is well established, relatively few reports have concentrated on the role of COX-2 during the late metastatic stages of the disease. Clinical trials in which the therapeutic effects of COX-2 inhibitors on metastasized CRC were studied have shown variable results (24, 25). In an experimental model, enforced overexpression of COX-2 in CRC cells promoted the incidence of experimental liver metastasis formation (26), whereas COX-2 inhibitors reduced intrahepatic tumor growth (13, 26–29). However, there seems to be no consensus as to how and on what stage of metastasis formation these inhibitors sort their effect (13, 26–29).

Although overexpression of activated RAS isoforms can stimulate COX-2 expression (30, 31), it remains unclear how KRAS mutation status in CRC cells relates to COX-2 expression and activity. Therefore, we have studied the cause and effect relationship between the presence of mutant KRAS^{D12} and COX-2 expression and activity in C26 CRC cells. In addition, we have investigated whether inhibition of KRAS^{D12}-dependent COX-2 by a novel and highly selective liver-activated COX-2 inhibitor (parecoxib) affected early tumor cell seeding in the liver, intrahepatic tumor cell proliferation, apoptosis, angiogenesis, and overall outgrowth of experimental CRC liver metastases.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. C26, HT-29, and T24 cell lines were all obtained from the American Type Tissue Culture Collection (Rockville, MD). The C26 cell line stably expressing the firefly luciferase gene (C26-Luc) was described previously (32). The C26-KRASkd cells will be described elsewhere.3 Cells were cultured in DMEM (ICN Pharmaceuticals, Costa Mesa, CA) supplemented with 5% (v/v) FCS, 2 mmol/L glutamine, 0.1 mg/mL streptomycin, and 100 units/mL penicillin. All cells were kept at 37°C in a humidified atmosphere containing 5% CO_{2}.

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Chemicals. Indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid] and NS-398 (N-[2-cyclohexyloxy]-4-nitrophenyl)-methanesulfonamide) were purchased from Cayman Chemical (Ann Arbor, MI). Parecoxib was purchased from Pfizer (New York, NY).

Antibodies. Primary antibodies were anti-COX-2 (#33, BD Biosciences PharMingen, Alphen aan den Rijn, the Netherlands); anti-KRAS, anti-NRAS, and anti-actin (F234, F155, and C11, Santa Cruz Biotechnology, Heidelberg, Germany); and anti–von Willebrand factor (DAKO, Amsterdam, the Netherlands); anti-Ki-67 (MM1, Novocastra, Newcastle upon Tyne, United Kingdom); and anti-active caspase-3 (C92-605, BD Biosciences PharMingen). Secondary antibodies were rabbit anti-mouse horseradish peroxidase (Pierce, Rockford, IL) or swine anti-rabbit horseradish peroxidase (DAKO, Glostrup, Denmark).

RAS Assay. The RAS activity assay was done by using the RAS-binding domain of Raf fused to glutathione S-transferase immobilized on glutathione-Sepharose as an affinity matrix for activated RAS exactly as described (33).

Prostaglandin E2 Assay. Prostaglandin E2 (PGE2) levels were measured in the culture medium using a commercial enzyme immunoassay kit (Cayman Chemical) according to the manufacturer’s instructions. The corresponding cells were lysed and protein concentration was determined to normalize the PGE2 levels measured in the medium samples.

Immunohistochemistry and Image Analysis. Immunohistochemistry on paraffin-embedded tissue sections was done as described (34). Randomly chosen tumors were scored as angiogenic, perivascular, intravascular, or nonvascularized. The percentage of Ki-67 positivity was determined by counting all positive and negative tumor cells in five randomly chosen tumors in each specimen. Microvessel densities were determined in all angiogenic tumors. The percentage of tumor area that was occupied by von Willebrand factor–positive cells was determined by analyzing digital tumor images using Leica QWIN software. All analyses were done in a blinded manner. The percentage of apoptotic cells was determined by counting all activated caspase-3-positive and caspase-3-negative tumor cells in five randomly chosen tumors in each specimen. At least 600 cells were counted in each specimen. In all cases, the observer was blinded to treatment.

Animals and Surgery. Male BALB/c mice ages 8 to 10 weeks (Harlan, Leicestershire, United Kingdom) were housed under standard conditions and received food and water ad libitum. Liver metastases were induced exactly as described (35). All experiments were done in accordance with the guidelines of the Animal Experimental Committee, University Medical Center Utrecht (Utrecht, the Netherlands).

Hepatic Replacement Area. Intrahepatic tumor load was scored as the percentage of hepatic tissue replaced by metastatic tumor cells as described (35).

Analysis of Tumor Cell Seeding in the Liver. C26 cells were labeled (15 minutes, 37°C) with 4% carboxyfluorescein succinyl ester (Molecular Probes, Leiden, the Netherlands) and resuspended in PBS to a concentration of 105 cells per 100 μL. Red fluorescent polystyrene microspheres (10 μm, Molecular Probes) were included at a 5:1 cell/bead ratio. Following anesthesia with fentanyl citrate/fluanisone (0.3 mg per mouse; Janssen-Cilag, Brussels, Belgium) and midazolamchloride (12.5 mg per mouse, Roche, Brussels, Belgium), the cell/bead mixture (100 μL) was injected into the spleen parenchyma. Mice were then placed in heated cages until microscopy.

Mice were pretreated with parecoxib (5 mg/kg i.p. or 0.9% saline) 38, 26, 14, and 2 hours before tumor cell injection. Alternatively, tumor cells were pretreated in vitro with indomethacin or NS-398 (20 μmol/L) or vehicle (0.05% DMSO) 24 hours before injection. Cell viability was assessed by trypan blue staining.

Intravitral Microscopy. Intravitral microscopy was done as described (36). Using a 20× lens, 30 randomly selected hepatic fields were chosen in each animal. Images were recorded and analyzed offline. The effect on tumor cell seeding was measured as the ratio of tumor cells per beads per high-power field. Data are average counts of two procedures done by independent observers blinded to treatment.

In vivo Bioluminescent Imaging. Intrahepatic tumor growth was assessed by bioluminescent imaging 7, 9, and 11 days after intrasplenic injection of luciferase-expressing C26 cells exactly as described (32). Data are presented as total photon counts as obtained by acquisition (MetaVue) and analysis (MetaMorph) software.

Statistical Analyses. Differences between the groups were statistically evaluated using the Mann-Whitney test. Results are presented as means ± SE. All P values were two tailed. P < 0.05 was considered statistically significant.

RESULTS

Knockdown of Endogenous KRASD12 Reduces COX-2 Expression and PGE2 Secretion in Murine C26 CRC Cells. To assess how KRAS mutation status is related to COX-2 (over)expression, we have used murine C26 CRC cells. We recently found that C26 cells contain constitutively active KRAS due to an activating mutation in codon 12 (G12D).4 Whereas KRAS in C26 cells is constitutively active, it is wild-type in human HT-29 CRC and in T24 bladder carcinoma cells (Fig. 1A). Figure 1B shows that C26, but not HT-29, cells express high levels of COX-2 enzyme and secrete PGE2, one of the major COX-2-produced inflammatory mediators. The human T24 bladder carcinoma cell line (expressing HRASV12) is shown as a positive control for COX-2 expression and activity. To test whether the high levels of COX-2 in C26 cells are related to the presence of mutant KRASD12, we generated a C26-derived cell line in which mutant KRAS, but not NRAS, is suppressed by RNA interference (C26KRASkd; Fig. 1C). KRAS knockdown resulted in strongly reduced COX-2 expression and PGE2 secretion (Fig. 1C). We conclude that activated KRASD12 is, to a large extent (~80%), responsible for the high levels of COX-2 synthesis and activity in C26 cells.

COX-2 Inhibition Reduces C26 Liver Metastasis Formation. We found previously that stable suppression of KRASD12 in C26 cells abrogates intrahepatic C26 tumor growth.5 Because high-level COX-2 expression in C26 cells depends on the presence of KRASD12 (Fig. 1C), we investigated whether COX-2

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contributes to KRAS<sup>D12</sup>-dependent liver metastasis formation. To this end, we used parecoxib, a highly specific water-soluble prodrug that is activated in the liver, the organ in which COX-2 inhibitory activity was required. It is among the most potent (IC<sub>50</sub>COX-2 = 0.005 μmol/L) and selective (IC<sub>50</sub>COX-1= 140 μmol/L) COX-2 inhibitors that have been identified to date (37, 38).

C26 liver metastases were induced by injection of C26 cells into the spleen. Treatment was then started by i.p. injections of parecoxib (5 mg/kg twice daily). Seven days after tumor cell injection, intrahepatic tumor growth was assessed by counting macroscopic tumor nodules on the liver surface as well as by histochemical determination of the area of liver tissue that had been replaced by tumor tissue (hepatic replacement area). Parecoxib reduced the number of macroscopic tumor nodules by ~30% (Fig. 2A) and reduced the hepatic replacement area by a dramatic 63% (Fig. 2B).

**COX-2 Inhibition Does Not Affect Tumor Cells Seeding in the Liver.** Inhibition of COX-2 activity may affect liver metastasis formation at distinct stages of the metastatic process. To discriminate between early and late stages, we studied the effects of COX-2 inhibition on tumor cell seeding and on the rate of tumor outgrowth. First, intravital microscopy was used to assess the seeding of carboxyfluorescein succinyl ester–labeled (green) fluorescent C26 cells in the liver. Red fluorescent polystyrene beads, which are trapped in the liver microvasculature, served as an internal reference for determining the efficiency of cell seeding. PGE<sub>2</sub> is produced by the tumor cells (Fig. 1B) but also by sinusoidal cells in the liver (i.e., Kupffer, endothelial, and stellate cells; refs. 39–41). Therefore, we investigated the effects of inhibiting either host or tumor cell COX-2. Host COX-2 was inhibited by pretreating the mice with i.p. injections of parecoxib 38, 26, 14, and 2 hours before tumor cell injection. Intravital microscopy was then done 1 hour after intrasplenic tumor cell injection. We found no differences in the efficiency of tumor cell seeding in the control versus the parecoxib-treated groups (Fig. 3A). Next, we assessed the effect of inhibiting COX-2 in the tumor cells. Both a selective (NS-398) and a nonselective (indomethacin) COX-2 inhibitor abrogated PGE<sub>2</sub> synthesis as expected without affecting COX-2 expression (Fig. 3B) or cell viability (Fig. 3C). The use of parecoxib in these in vitro experiments was excluded as it is an inactive prodrug. Intravital microscopy analysis 1 hour after intrasplenic injection of C26 cells revealed no differences in the seeding of untreated, indomethacin-treated, or NS-398-treated cells (Fig. 3D). Taken together, the results show that inhibition of either host COX-2 or tumor cell COX-2 has no effect on early C26 tumor cell seeding in the liver.

**COX-2 Inhibition by Parecoxib Slows Down the Outgrowth of Liver Metastases by Reducing Tumor Cell Proliferation.** We next studied whether COX-2 inhibition affected the rate of tumor outgrowth in the liver. Following intrasplenic injection of luciferase-expressing C26 cells (day 0), mice received daily i.p. injections of parecoxib or vehicle. Intrahepatic tumor growth was subsequently measured by bioluminescent imaging on days 7, 9, and 11 postinjection. The results presented in Fig. 4A show that parecoxib treatment greatly reduced the rate of C26 tumor growth in the liver. To get insight into the underlying mechanism, we studied the
effects of parecoxib on tumor cell proliferation and apoptosis and on tumor-associated angiogenesis. To this end, we did immunohistochemistry using Ki-67 as an indicator of cell proliferation, active caspase-3 as an indicator of apoptotic cells, and factor VIII (von Willebrand factor) as an indicator of newly formed blood vessels. We found that the percentage of proliferating C26 cells in the liver metastases was dramatically reduced from a mean 82% in the control group to 54% in the parecoxib-treated group (Fig. 4B), a reduction by 37%. The angiogenic profile of the liver metastases was remarkably heterogeneous in both groups. Many lesions had not yet undergone the angiogenic switch and grew either perivascularly or intravascularly (Fig. 4C). We found no significant differences between angiogenic profiles of liver metastases from parecoxib-treated and control mice (Fig. 4C). In addition, the microvessel densities in the vascularized tumor subgroups were not significantly different (Fig. 4C). Finally, the apoptotic indices in tumors isolated from control and parecoxib-treated mice were not significantly different (Fig. 4D).

DISCUSSION

Activated KRAS affects the proliferation, survival, and invasiveness of tumor cells but also affects the behavior of stromal cells by stimulating the secretion of soluble factors like vascular endothelial growth factor and prostaglandins (9). The ectopic overexpression of mutant HRAS results in elevated expression of COX-2 (30, 31), the major prostaglandin-synthesizing enzyme in neoplastic tissue. Conversely, our results show that the removal of
endogenous mutant KRAS<sup>G12D</sup> from C26 CRC cells was sufficient to reduce COX-2 expression and PGE<sub>2</sub> secretion by ~80%. Thus, the stimulation of COX-2 expression and activity was the direct result from an acquired activating mutation in KRAS.

COX-2 has been implicated in several processes that affect tumor growth and metastasis formation. These include effects on tumor cell survival, proliferation, and invasiveness as well as on early metastatic seeding and angiogenesis (reviewed in ref. 42). Moreover, COX-2 synthesize inflammatory mediators that induce a state of chronic inflammation and may thereby promote tumor growth (43). Our study shows that parecoxib reduces the proliferation of extremely aggressive CRC cells in the liver by 37% as determined by the Ki-67 proliferation marker. This effect presumably accounts for the reduced outgrowth of CRC liver metastases. NS-398, another highly selective COX-2 inhibitor, reduces C26 cell proliferation in vitro (29). Likewise, the proliferation rate of intestinal adenoma cells in COX-2-deficient mice is suppressed by 50% when compared with control mice (44). Taken together, the data indicate that COX-2 is essential for efficient tumor cell proliferation in early developing intestinal adenomas but also in liver metastases.

An inherent disadvantage of using COX-2 inhibitors is the possibility that enzymes/factors other than COX-2 may be inhibited. COX-independent effects have been reported for several nonsteroidal anti-inflammatory drugs, including sulindac, celecoxib, NS-398, and indomethacin (45). At present, the only parecoxib target known is COX-2. Nevertheless, we cannot exclude the possibility that COX-2-independent effects may contribute to the observed reduction in tumor cell proliferation. Formal proof for the involvement of COX-2 in intrahepatic tumor cell proliferation and outgrowth of liver metastases should come from genetically modified tumors lacking COX-2. Unfortunately, our attempts to generate such cells by RNA interference have failed thus far.

The interaction between human CRC cells and macrovascular human umbilical vein endothelial cells in vitro can be reduced by celecoxib (26). This may implicate COX-2 in the regulation of early metastatic seeding in the liver. Our study, based on the use of intravitral microscopy, does not support a role for COX-2 in the adhesion of C26 tumor cells to the sinusoidal endothelium of the liver in vivo.

A series of elegant studies in genetically modified mice have unequivocally shown that COX-2 and the PGE<sub>2</sub> receptor EP2 contribute to the development of intestinal papillomas through stimulation of angiogenesis (44, 46–48). In addition, COX-2 inhibitors act as antiangiogenic compounds in several angiogenesis assays in vitro, in the chorioallantoic membrane assay, and in the cornea assay (49–53). In marked contrast, it remains unclear whether the antitumorigenic effect of COX-2 inhibitors on tumorigenesis in general and on liver metastasis formation in particular is due to inhibition of angiogenesis. Studies that addressed this issue by analyzing microvessel density in surgically removed tumor specimens of patients that had been treated with COX-2 inhibitors have generated conflicting results (18, 54). In light of the above results, it was perhaps surprising that parecoxib had no discernable effect on angiogenesis of C26 colorectal liver metastases. A possible explanation for this result could be that the majority (~60%) of the liver metastases that we have analyzed had not yet undergone the angiogenic switch due to their still limited size and because many of the tumors grew perivascularly and intravascularly. Therefore, the heterogeneous nature of the angiogenic profile of these tumors may have precluded the detection of antiantiogenic effects in parecoxib-treated mice.

In conclusion, our study shows that the inhibition of KRAS<sup>G12D</sup>-stimulated COX-2 by parecoxib reduces intrahepatic tumor cell proliferation and tumor growth during the early outgrowth of C26 liver metastases.

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REFERENCES


Fig. 4 Parecoxib slows the outgrowth of C26 liver metastases. A. C26-luc cells (10^5) were injected into the spleens of BALB/c mice followed by splenectomy. Mice received i.p. injections of either parecoxib (10 mg/kg/d, n = 3) or vehicle (n = 3). Bioluminescence was measured for 5 minutes on postoperative days 7, 9, and 11. Bars, SE. *, P < 0.05. Inset, representative bioluminescence images on postoperative day 11. Following bioluminescent imaging on day 11, the livers were removed and processed for immunohistochemistry using anti-Ki-67 (B), anti-von Willebrand factor (C), or anti-active caspase-3 (D). Images of five tumors per section (of each individual liver) were recorded and analyzed in a blinded manner. Columns, the number of Ki-67-positive tumor cells, the vascular profile, and the microvessel densities (expressed as % von Willebrand factor-positive tumor area) and the apoptotic indices. ns, nonsignificant differences. Representative anti-Ki-67-stained and anti-active caspase-3-stained sections. Examples of vascularized, avascular, and perivascular growing tumors.
Fig. 4 Continued


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