Celecoxib Prevents Tumor Growth in Vivo without Toxicity to Normal Gut: Lack of Correlation between in Vitro and in Vivo Models

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NSAIDs inhibit the activity of the cyclooxygenases, which are key enzymes in the conversion of arachidonate to PGH₂, the immediate substrate for a number of specific prostaglandin synthases. Unfortunately, prolonged use of NSAIDs can result in gastrointestinal ulceration and bleeding. It is widely believed that this ulcerogenic activity of NSAIDs is attributable to the chronic inhibition of prostaglandin production in the gastric mucosa. Accordingly, researchers have tried to identify NSAID derivatives that retain anti-neoplastic activity but do not affect prostaglandin production in gastric mucosa.

There are two isoforms of cyclooxygenase, COX-1 and COX-2, which differ in their expression pattern and function within the organism. COX-1 is constitutively expressed in many tissues and is thought to be responsible for maintaining gastric mucosal integrity. COX-2 is induced by a variety of stimuli and plays an important role in wound healing, ovulation, fertilization, and in mediating inflammation (for review, see Ref. 10). COX-2 expression levels are increased in colorectal cancer tissues (11–14). Overexpression of COX-2 has been shown to mediate cell cycle progression and to contribute to such diverse processes such as apoptosis, angiogenesis (15), and tissue invasion (16). On the basis of these effects, we have investigated the role of specific COX-2 inhibitors in the treatment of advanced colorectal cancer.

We have studied previously the effects of a selective COX-2 inhibitor, SC-58125, on the growth and viability of colorectal carcinoma cells grown in vitro and in vivo (7). The precise mechanism for growth inhibition of tumors is under evaluation, but does not seem to involve the induction of apoptosis in vitro (17). It has been reported that nonselective COX inhibitors induce a G₂-M cell cycle arrest resulting in a corresponding reduction of p34cdc2 levels and activity (18). We have observed a similar effect after SC-58125 treatment of colorectal carcinoma cells. These results suggest that SC-58125 may have therapeutic potential for treatment of colorectal cancer. However, SC-58125 will not be developed for clinical use in humans. Furthermore, although SC-58125 reduces tumor growth, it does not cause tumor regression (7, 9). Therefore, we evaluated other COX-2 inhibitors for their effect on the growth of colorectal carcinoma cells in vitro and in vivo.

Here we report that celecoxib (Celebrex), at concentrations >10 μM, potently induces apoptosis and inhibits cell cycle progression in colorectal carcinoma cells grown in culture by mechanisms independent of COX-2 inhibition. Lower concentrations of the drug have no discernible effect on cells grown in vitro. We also found that when celecoxib was administered to mice by inclusion in the diet (1250 mg/kg of chow), serum concentrations of ~2.3 μM were achieved. Nevertheless, in contrast to the results from cell culture experiments, celecoxib significantly reduced the growth rate of colorectal carcinoma cells grown as xenografts, without toxicity to the normal intestine. These data highlight the point that the biological effects of celecoxib against colorectal cancer in vivo do not predict its effects in...
vitro. However, these findings do support additional investigation for the use of celecoxib as a therapeutic agent against colorectal cancer.

**MATERIALS AND METHODS**

**Reagents.** Celecoxib (SC-58635), SC-58125, and SC-560 were provided as kind gifts from G. D. Searle and Co. (St. Louis, MO). Merck and Co. (Rathway, NJ) provided sulindac sulfide. DMSO (EM Science, Gibbstown, NJ) was used as the solvent. Concentrated drug stocks were diluted in DMEM (Life Technologies, Inc., Grand Island, NY) media before addition to cell cultures. The DMSO concentration in cultures was kept at 0.1%.

**Cell Culture.** HCA-7 cells were a generous gift from Susan Kirkland. LLC and HCT-15 cells and HCT-116 cells were purchased from the American Type Tissue Collection (Manassas, VA).

**MEF Derivation.** Primary MEFs were derived by passing day 13.5 C57BL/6J mouse embryos through an 18-gauge needle. The cells were expanded over several days in DMEM supplemented with 10% FBS, 1% P/S/1% t-glutamine. The cok-2 genotype of the MEF cell lines was verified as described previously (19).

**Cell Counts.** LLC cells were seeded into six-well plates at 2.5 x 10^4 cells/well. Cells were treated in triplicate with DMSO or 25, 50, or 100 μM celecoxib for 12 h and then harvested and counted using a Coulter counter model Z1 (Coulter, Fullerton, CA).

**In Vitro Viability Assay.** The MTT assay was used to determine cell viability/proliferation. This assay measures mitochondrial activity. MTT is a yellow-colored tetrazolium salt that is taken up and cleaved only by metabolically active cells, reducing it to a colored, water-insoluble formazan salt. The solubilized formazan product can be quantified via absorbance at 570 nm measured using a 96-well-format spectrophotometer, and the absorbance correlates directly with cell number. Cells were plated at 1.5 x 10^5 cells/well in a 100-μl volume in 96-well plates and grown for 24 h in DMEM supplemented with 10% FBS. The indicated amount of test drug or DMSO in 1% FBS containing OptiMEM media was then added to the cells. At the indicated times, 10 μl of MTT (5 mg/ml) was added, and the cells were incubated at 37°C for 4 h. The tetrazolium crystals were solubilized by the addition of 10% SDS in 0.01 N HCl. After overnight incubation at 37°C, the absorbance was measured at 570 nm using a 96-well-spectrophotometric plate reader (Packard Instruments, Meriden, CT). Results are expressed as the mean ± SD of six wells.

**Prostaglandin Measurement.** Subconfluent cell cultures were treated with either celecoxib or SC-58125 for the indicated time. Thirty min before harvesting media, arachidonate was added to the media to a final concentration of 10 μM. PGE2 was quantified as described previously (20).

**Apoptosis Determination.** The APO-Direct kit (PharMingen, San Diego, CA) was used for quantitative evaluation of apoptosis in response to celecoxib treatment. This assay relies on the characteristic fragmentation of DNA during the apoptotic process. Terminal nucleotide transferase enzyme is used to terminal deoxynucleotidyl transferase enzyme is used to label the free 3′-OH of fragmented DNA using fluorescein-conjugated dUTP as the nucleotide for the exchange reaction. Flow cytometric detection of fluorescein-labeled fragmented DNA was conducted to quantitatively evaluate apoptosis in response to celecoxib treatment. Cells were seeded in 100-mm plates and, when 80% confluent cells were treated with DMSO, or 12.5, 25, or 50 μM celecoxib in 1% FBS-supplemented OptiMEM media. Negative control cells were not treated; vehicle-treated cells had an amount of DMSO equivalent to the celecoxib-treated cells added. Cells were harvested after 12 h of treatment, washed in PBS twice then fixed in 1% paraformaldehyde for 15 min and permeabilized by the addition of ice-cold 70% ethanol. The labeling reaction was performed according to the manufacturer’s recommendations, with the exception that 2 x 10^6 cells/condition were used. After labeling, cells were washed three times in PBS, then resuspended in 1 ml of propidium iodide staining solution (5 μg/ml propidium iodide, 40 μg/ml RNase A, in 1 x PBS). Cells were then filtered through 50-μm mesh immediately before analysis on a Becton Dickinson FACScan flow cytometer. Gating on FL2-width was used to exclude aggregates, and 10^4 gated events were collected and analyzed.

**Xenograft Model of Tumor Biology.** HCA-7 cells were grown on plastic culture dishes according to standard cell culture techniques (7). The cells were trypsinized and resuspended in sterile PBS, then pelleted by brief centrifugation at 1500 rpm. The supernatant was aspirated, and cells were resuspended in PBS and counted using a hemocytometer. A final concentration of 5 x 10^7 cells/ml was made, and 100 μl of cell suspension was injected s.c. using a tuberculin syringe and a 27-gauge needle. Celecoxib was administered in the diet (1250 mg/kg of chow) and the animals were fed ad libitum. The size of the tumor was determined by direct measurement of tumor dimensions as described previously (7).

**Scoring Apoptosis and Mitosis in Normal Intestinal Epithelium.** In brief, mice were fed celecoxib (1250 mg/kg of chow) and the small and large intestines were removed separately and prepared by methods described in detail elsewhere (21, 22). Apoptotic bodies were identified by their characteristic morphological appearance in sections stained with H&E. To score the sections, each cell along the long axis of the crypt was numbered sequentially from the base of the crypt; the cell at the crypt base was designated as number 1. Each cell position was scored as containing either a normal, mitotic, or apoptotic cell. For each cell position, apoptotic and mitotic percentages were calculated. We have already shown this methodology to be more accurate when examining tissues than when identifying apoptotic bodies by TUNEL staining (23).

**Gut Morphometry.** Villus height and crypt depth was measured on an Axioshome microscope on 20 crypts and 20 villi from at least six animals/experimental group.

**RESULTS**

**Treatment with Celecoxib Induces Concentration-dependent Apoptosis in Vitro.** We have observed previously that a compound related to celecoxib, SC-58125, effectively inhibits the growth of LLC and colorectal cancer cells by 40% after 3 days of treatment in vitro. When celecoxib became available, we sought to test its effect on the growth of LLC cells. SC-58125-treated LLC cells remain morphologically unchanged; however, when LLC cells were treated with 50 μM celecoxib for 12 h, they became rounded and detached from the culture dish (Fig. 1A, left panel). As with SC-58125, we found that celecoxib inhibited the growth of LLC cells but was much more potent. After only 12 h of treatment, the IC50 for celecoxib inhibition of cell number was 40 μM (Fig. 1A, right panel). The cytotoxicity of celecoxib against LLC cells was also quantified using the MTT assay. When celecoxib concentrations of <10 μM were used, no effect on cell viability or growth was observed. However, treatment for 12 h with >20 μM celecoxib resulted in a dramatic decrease in cell viability (Fig. 1B). We next determined whether celecoxib also had potent cytotoxicity against two human colorectal carcinoma cell lines (HCT-15 and HCA-7). Celecoxib had similar cytotoxic effects against these cells with significant loss of viability at concentrations of >20 μM (Fig. 1B). Once again, treatment of these cells with celecoxib for 3 days at concentrations of <10 μM had no effect on cell viability. To determine whether the cytotoxic effects of celecoxib were the result of induction of apoptosis, LLC, HCA-7, and HCT-15 cells were treated with 25 or 50 μM celecoxib. Apoptotic cells were identified by TUNEL staining and the results analyzed by dual parameter flow cytometry using DNA content to identify the cell cycle position of cells undergoing apoptosis. Treatment with 50 μM celecoxib caused significant apoptosis in all three cell lines with 88%, 37%, and 15% of LLC, HCA-7, and HCT-15 cells, respectively, undergoing apoptosis (Fig. 2). We found that LLC and HCA-7 cells undergoing apoptosis contained 4 N DNA content, suggesting that arrest at the G2-M stage of the cell cycle was occurring. In contrast, apoptotic HCT-15 cells contained a subdiploid DNA content after celecoxib treatment, suggesting that apoptosis was occurring when the cells contained 2 N DNA. The induction of apoptosis was confirmed by the detection of oligonucleosomal cleavage of DNA in LLC and HCA-7 cells after treatment with 50 μM celecoxib for 6 h.

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5 Data not shown.
Induction of Apoptosis in Vitro Is Independent of COX-2 Inhibition. Next we sought to test whether cyclooxygenase inhibition was required for the cytotoxic effect of celecoxib treatment in vitro. If COX-2 is involved in inhibition of cell growth under these experimental conditions, then one would expect that other COX-2 selective inhibitors would have similar effects. We directly tested this hypothesis by treating LLC cells for 12 h with the COX-2 selective inhibitors, celecoxib and SC-58125, the COX-1 selective inhibitor, SC-560, and the nonselective COX inhibitor, sulindac sulfide. SC-58125 did not affect cell viability over the time course of the experiment and sulindac sulfide and SC-560 decreased MTT activity by 15–25%, whereas celecoxib resulted in virtually complete loss of cell viability (Fig. 3A). The lack of correlation between COX-2 inhibitory activity and cytotoxicity suggests that the effect of celecoxib in vitro is independent of its ability to inhibit COX-2. One possible explanation for the different effects of celecoxib and SC-58125 on carcinoma cells might be attributable to variation in inhibition profiles or half-lives of these compounds. We next compared inhibition of PGE2 production by celecoxib and SC-58125. Celecoxib inhibits PGE2 production in LLC cells with an IC50 of 18 nM. Both compounds had similar cyclooxygenase inhibition profiles, decreasing PGE2 levels by 85–95% for at least 12 h after treatment (Fig. 3B, right panel).

Finally, although LLC cells express both COX-1 and COX-2, HCA-7 cells only express COX-2, and HCT-15 cells express neither isoform. Comparison of the cytotoxic effects of celecoxib between these cell lines is not strictly valid because of differences at many genetic loci. Therefore, we tested the effect of celecoxib on MEFs derived from COX-2 (+/+), (+/-), and (−/-) C57BL/6J mice. The cytotoxicity profile and IC50 of celecoxib was similar in all three cell lines regardless of COX-2 status (Fig. 3C). Furthermore, the concentration dependence of cytotoxicity for the cell lines of all three MEFs and LLC cells were similar (Fig. 3C). As with the colon carcinoma cells tested (Fig. 1B), no cell death was observed at celecoxib concentrations of <10 μM. These results strongly indicate that the cytotoxicity of celecoxib in vitro is independent of inhibition of COX-2 and brings up the question of whether this drug is cytotoxic in vivo.

Celecoxib Does Not Cause Apoptosis or Inhibit Cell Division in Normal Gut Epithelium. The COX-2 independent toxicity of celecoxib to MEFs raises the possibility that celecoxib might cause significant apoptosis and/or cell cycle arrest in normal gut epithelium. Therefore, we determined apoptotic and mitotic rates in the small and large intestinal epithelium of nude mice fed celecoxib (1250 mg/kg chow) for 45 days. As described previously, spontaneous rates of apoptosis in the mouse intestine were found to be low (23). Treatment with celecoxib for 45 days did not cause any significant change in apoptotic or mitotic rates in either the small or large intestine (Fig. 4A). Evaluation of intestinal epithelium 4 h after administration of a single dose of celecoxib did not reveal increased rates of apoptosis.5 Furthermore, no significant differences in gut morphology could be discerned. In the small intestine, villus height was 303 ± 695 mm in control and celecoxib-treated animals, and crypt...
depth was 100.4 ± 17 μm in control animals and 100 ± 38 μm treated mice. Similarly, the colon crypt length was unaffected by treatment with celecoxib 150 ± 25 μm in control colon versus 167 ± 31 μm in treated colon.

To relate the in vivo observations to the cell culture studies above, the serum concentration of celecoxib in six mice was measured and found to range from 1.5–3.5 μM (2.3 ± 0.7 μM, Fig. 4B). Thus the peak serum concentration of celecoxib achieved in mice using this dosing regime was 10-fold less than that which led to cytotoxicity in vitro.

**In Vivo Tumor Growth Is Attenuated by Celecoxib Treatment.**

We have reported previously that celecoxib attenuates the growth of LLC tumors in vivo (17). Given the recent interest in the use of COX-2 inhibitors for chemoprevention of colorectal cancer, we wished to confirm and extend these observations in a colorectal carcinoma cell line. HCA-7 cells were implanted s.c. into athymic mice. Celecoxib was mixed with mouse chow at a concentration of 1250 mg/kg. (The xenograft and normal intestinal studies reported above were performed in the same mice). Whereas data from our in vitro studies may predict that such a low dose of celecoxib (2.3 μM) would be ineffective at inhibiting tumor growth, we found that the growth of HCA-7 xenograft growth was significantly reduced when compared with animals on the control diet (Fig. 5). Delaying treatment for 10, 20, or 30 days postimplantation, by which point the tumors were well established, still resulted in inhibition of tumor growth, indicating that the compound was not affecting tumor implantation (Fig. 5). These data demonstrate that celecoxib prevents the growth of colorectal carcinoma cells in vivo at concentrations that do not cause apoptosis in cells grown in vitro.

**DISCUSSION**

We have shown previously that the specific COX-2 inhibitor, SC-58125, can reduce the growth of COX-2 positive colorectal cancer xenografts (7). We have also observed that SC-58125 primarily affected cell cycle progression via a delay in G2-M. Preliminary studies on celecoxib, another specific COX-2 inhibitor, suggested that it might be even more potent than SC-58125. We studied its effects on the growth of LLC cells and two colon carcinoma cell lines grown in vitro and compared those results with its effect on tumor growth in vivo, as well as evaluating its effect on the normal intestine of treated mice.
At concentrations >20 μM, celecoxib induces cell death as measured by cell counts of LLC cells and the assay of LLC, HCA-7, and HCT-15 cells (Fig. 1). Analysis of TUNEL staining by flow cytometry (Fig. 2) and the demonstration of oligonucleosomal DNA cleavage confirms that the loss of viability is attributable to the induction of apoptosis. In addition, a G2-M block is also induced in LLC and HCA-7 cells by treatment with 50 μM celecoxib. Interestingly, in HCT-15 cells we observed a subdiploid accumulation of fragmented DNA suggesting that apoptosis was occurring during the G1 to early S transition of the cell cycle. These results show that celecoxib, at concentrations >20 μM, strongly induces apoptosis in cells grown in vitro.

Three lines of evidence indicated that induction of apoptosis in vitro by high-dose celecoxib occurs by mechanisms independent of COX-2 inhibition: (a) the cytotoxicity of a variety of NSAIDs did not correlate with their ability to inhibit COX-2 (Fig. 3A); (b) although SC-58125 and celecoxib can both inhibit PGE2 production by >90% at a concentration of 10 μM, SC-58125 at 50 μM has no effect on cell viability, whereas celecoxib reduces viability by 70% when given at that concentration; and (c) MEFs derived from wild-type, cox-2 heterozygous or cox-2 null C57/BL6 mice were treated with the indicated amount of celecoxib for 12 h. Values represent a fraction of control (DMSO)-treated cells. Viability was determined by the MTT assay and cellular morphology. Each data point represents the mean of six replicates, error bars represent +/- SD.

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Fig. 3. Celecoxib inhibits cell growth independent of COX-2 inhibition. A, MTT assay of LLC cells treated for 12 h with 50 μM of SC-58125, NS-398, sulindac sulfide, SC-560, or celecoxib. B, prostaglandin-inhibition profile. B.1, LLC PGE2 levels were determined after 3 h of celecoxib treatment at the indicated dose. B.2, LLC PGE2 levels after 12 h of treatment with either celecoxib (black squares) or SC-58125 (black circles) at 1, 5, or 10 μM. Data are represented as the fold-reduction in PGE2 compared with vehicle-treated cells. C, MEFs derived from cox-2 (+/+), cox-2 (+/-), and cox-2 (-/-) C57/BL6 mice were treated with the indicated amount of celecoxib for 12 h. Values represent a fraction of control (DMSO)-treated cells. Viability was determined by the MTT assay and cellular morphology. Each data point represents the mean of six replicates, error bars represent +/- SD.

A crucial observation from these studies is that treatment with 10 μM or less of celecoxib for 3 days has no detectable effect on cell death in vitro. Despite this lack of effect in cultured cells, we found that celecoxib strongly attenuated the growth of xenografted HCA-7 tumors in vivo, although the plasma concentration of celecoxib was ~2.3 μM. This discrepancy highlights the fact that tumor growth in vivo is determined by the interaction between factors intrinsic to tumor cells, the extracellular matrix, stromal cells, and other host factors. These factors are not always present in vitro when cells are grown on plastic culture dishes. Cell culture models are often used to evaluate the therapeutic potential of NSAIDs against cancer, but great caution needs to be taken when extrapolating in-vitro results to the whole organism, particularly with respect to the relative dose of agent used.

These studies do not address the issue of the mechanism by which celecoxib attenuates tumor growth in vivo or evaluate its COX-2 dependency. In vivo, interference with the implantation of carcinoma cells does not seem to play a significant role because growth is still affected even when treatment is started 30 days after inoculation of the host. It has not been well established that COX-2 expression in the...
carcinoma cells in vivo contributes to tumor growth. Previous studies using tumor models lacked the ability to distinguish between tumor-derived COX-2 and extrinsic COX-2. We recently have reported that stromally derived COX-2 is important for tumor growth (17). It is possible that whereas low doses of celecoxib do not affect tumor cells in vitro, stromal or host-derived processes dependent on COX-2 may be sensitive to low-dose NSAID treatment. Angiogenesis, a necessary component of neoplastic growth, is also promoted by COX-2 activity, and its inhibition could play a crucial role in the antineoplastic action of celecoxib (15, 17, 24, 25).

In striking contrast to the antitumor effects of celecoxib, no toxic effects were observed in the normal gut by assessing induction of apoptosis, inhibition of cell division, or reduction of crypt and villus dimensions. The explanation for the differential effect of celecoxib on normal and transformed intestinal epithelial tissue is not known, but it is interesting to note that COX-2 expression and activity is very low to undetectable in normal gut mucosa. Furthermore, gut epithelium is not as dependent on new-vessel growth as tumor xenografts.

In summary, celecoxib can significantly attenuate the growth of colorectal carcinoma xenografts without adverse effects on the normal gut. Although celecoxib causes apoptosis of colorectal cancer cells grown in vitro via a COX-2-independent mechanism, this only occurs at concentrations >10-fold higher than what can be achieved in vivo. Great caution should be taken in interpreting the clinical significance of these in vitro effects.

Fig. 4. Celecoxib does not induce apoptosis or mitosis in gut epithelium. A, nude mice were treated for 45 days with celecoxib. Both small intestine and colon were harvested, fixed, and H&E-stained. Apoptotic bodies and mitotic figures were counted per high-power field, and indices standardized to saline-treated control epithelium were generated. B, serum celecoxib levels. Six animals were treated with 1250 mg/kg celecoxib for 45 days. Serum was harvested and celecoxib levels were measured by mass spectroscopy. Data are presented as the mean (bars) and spread.
of cell culture data, particularly with respect to drug levels that could be safely achieved in humans. Overall, celecoxib seems to have significant therapeutic potential against colorectal cancer, and additional clinical evaluation is warranted.

ACKNOWLEDGMENTS

We thank Penny Ottewell for her technical assistance.

REFERENCES


Fig. 5. Celecoxib inhibits the in-vivo growth of HCA-7 cells. 5 × 10⁶ HCA-7 cells were injected s.c. into the dorsal flank of athymic mice. Mice received either control chow or chow containing 1250 mg/kg of celecoxib. Treatment was initiated either at the time of tumor implantation (white square), or 10 (yellow triangle), 20 (green square), or 30 (purple square) days postimplantation. Tumor dimensions were measured at the indicated intervals, and tumor volume was calculated according to the equation V = (L × W²) × 0.5, where V = volume, L = length, and W = width (26).
Celecoxib Prevents Tumor Growth \textit{in Vivo} without Toxicity to Normal Gut: Lack of Correlation between \textit{in Vitro} and \textit{in Vivo} Models

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