Meloxicam inhibits the growth of colorectal cancer cells

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Cyclooxygenase-2 has been reported to play an important role in colorectal carcinogenesis. The effects of meloxicam (a COX-2 inhibitor) on the growth of two colon cancer cell lines that express COX-2 (HCA-7 and Moser-S) and a COX-2 negative cell line (HCT-116) were evaluated. The growth rate of these cells was measured following treatment with meloxicam. HCA-7 and Moser-S colony size were significantly reduced following treatment with meloxicam; however, there was no significant change in HCT-116 colony size with treatment. In vivo studies were performed to evaluate the effect of meloxicam on the growth of HCA-7 cells when xenografted into nude mice. We observed a 51% reduction in tumor size after 4 weeks of treatment. Analysis of COX-1 and COX-2 protein levels in HCA-7 tumor lysates revealed a slight decrease in COX-2 expression levels in tumors taken from mice treated with meloxicam and no detectable COX-1 expression. Here we report that meloxicam significantly inhibited HCA-7 colony and tumor growth but had no effect on the growth of the COX-2 negative HCT-116 cells.

Introduction

Colorectal cancer is the second leading cause of death from cancer in the Western world, claiming ~55 000 lives each year in the USA. Americans have a 1 in 20 lifetime risk of developing this disease and ~1 in 10 have a family member who develops colorectal cancer. Understanding the environmental and molecular events involved in the multi-step progression from normal epithelium to malignant transformation will be helpful in designing useful diagnostic tests and successful therapeutic interventions. Epidemiological studies demonstrate a 40–50% reduction in the risk for colorectal cancer following prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) (1–5). Recent studies show that cyclooxygenase-2 (COX-2) levels are increased in ~85% of colorectal adenocarcinomas (6).

The chemoprotective effects of currently available NSAIDs are counterbalanced by the risk of gastrointestinal toxicity, which can result in gastritis, ulceration and gastrointestinal bleeding. It is believed that this toxicity is due to the inhibition of gastric COX-1 (7). Sulindac sulfide, for example, has a COX-1 IC₅₀ of 80 nM and a COX-2 IC₅₀ of 310 nM, therefore sulindac sulfide is very effective at inhibiting both isozymes (8). Recently, pharmaceutical companies have developed agents that are highly selective inhibitors of COX-2 with the hope that these drugs will yield analgesic benefit without the associated gastrointestinal side effects. For example, meloxicam has a COX-1 IC₅₀ of 3.27 µM and a COX-2 IC₅₀ of 0.25 µM, 13.1 times more preferential for inhibition of COX-2 (9). Evaluation of selective COX-2 inhibitors for effects on colorectal cancer is currently an area of intense investigation and pre-clinical studies have clearly shown potent anti-tumor properties of selective COX-2 inhibitors (10–13).

There is some controversy concerning the mechanism by which NSAIDs inhibit colorectal tumor growth (14,15). It was recently demonstrated both in vitro and in vivo that a highly selective COX-2 inhibitor, SC-58125 [COX-1 IC₅₀ > 50µM, COX-2 IC₅₀ = 190 nM (8)], markedly reduced cell growth and tumor size (10). If inhibiting COX-2 is the mechanism whereby NSAIDs decrease tumor size, then using another NSAID with selective properties should yield similar results. Therefore, the aim of the present study was to determine if another COX-2 inhibitor, meloxicam, has anti-tumor properties. The present study was designed to ascertain whether meloxicam inhibits the growth of COX-2-positive or COX-2-negative colorectal cancer cells. The effects of meloxicam on COX-1 and COX-2 protein expression and inhibition of prostaglandin production in tumor models were also determined. Additionally, we ascertained whether meloxicam was useful in inhibiting tumor formation, utilizing a mouse xenograft model.

Materials and methods

Cell culture

For the cell growth studies, HCA-7 (a generous gift from S.Kirkland), Moser-S and HCT-116 (ATCC #CCL 247) human colorectal cancer cell lines were selected for evaluation due to their different levels of COX-2 expression. Neither of the COX-2-positive cell lines has detectable COX-1 protein by western blot analysis. HCA-7 and Moser-S colony size were significantly reduced following treatment with meloxicam; however, there was no significant change in HCT-116 colony size with treatment. In vivo studies were performed to evaluate the effect of meloxicam on the growth of HCA-7 cells when xenografted into nude mice. We observed a 51% reduction in tumor size after 4 weeks of treatment. Analysis of COX-1 and COX-2 protein levels in HCA-7 tumor lysates revealed a slight decrease in COX-2 expression levels in tumors taken from mice treated with meloxicam and no detectable COX-1 expression. Here we report that meloxicam significantly inhibited HCA-7 colony and tumor growth but had no effect on the growth of the COX-2 negative HCT-116 cells.

Abbreviations: COX, cyclooxygenase; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; FAP, familial adenomatous polyposis; HRP, horseradish peroxidase; NSAIDs, non-steroidal anti-inflammatory drugs; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PGE₂, prostaglandin endoperoxide E₂; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate.

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random colonies from each slide (30 measurements/well). Means for each dose were calculated and compared with controls.

To determine COX-1 and COX-2 expression levels in vitro, HCA-7 cells were grown in McCoy's media until 90% confluent in 100 mm plates, then treated with 50 μM meloxicam for 2, 4, 8, 12, 24 and 48 h before harvesting and isolating the protein extracts for western blot analysis.

**Prostaglandin production in vitro**

For in vitro cell studies, HCA-7, Moser-S and HCT-116 cells were treated with 1, 5, 10 and 25 μM meloxicam for 23 h at which time the media was replaced with serum-free media containing meloxicam at the concentrations indicated plus 10 μM arachidonic acid for 1 h. Prostaglandin endoperoxide (PGE2) levels in the media taken from the cells were measured (in triplicate) at each meloxicam dose. PGE2 was measured using stable isotope dilution techniques with gas chromatography negative ion chemical ionization-mass spectrometry as previously described (16).

**Immunoblotting**

Following treatment of cultured cells with 50 μM meloxicam for 2, 4, 8, 12, 24 and 48 h, and in nude mice treated with 40 mg/kg meloxicam for 2, 4, 6, 8, 12 and 24 h prior to being killed, immunoblot analyses of HCA-7 cell lysates were performed as previously described (10). Briefly, the cultured HCA-7 cells or tumor homogenates were prepared in RIPA buffer containing 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in water for 30 min. Protein samples (50 μg) were denatured in sample buffer and fractionated by 7.5% SDS-PAGE and following electrophoresis, the proteins were transferred to PVDF membranes. After processing, the membranes were blotted with either anti-human COX-1 or anti-human COX-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The immune complexes were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL (Amersham, Arlington Heights, IL). MC-26 and HCA-7 (untreated) cells were used as a positive control (+) for COX-1 and COX-2, respectively, and the COX-1 and COX-2 null cells, HCT-116, were used as a negative control (−) for western analysis. The blots were washed and incubated with the ECL chemiluminescence system and then autoradiography was performed with Hyperfilm ECL (Amersham).

**Tumor growth in nude mice**

Twelve athymic mice (Harlan Sprague–Dawley, Indianapolis, IN) were housed (12 h light:dark schedule) in autoclaved cages and were provided sterile food and water. Five mice were designated as controls and the remaining seven mice were assigned to the treatment group. Just before HCA-7 cell inoculation, the treatment group was given 40 mg/kg of meloxicam by i.p. injection. Following the meloxicam injection, 5 × 10^6 HCA-7 cells were suspended in 0.1 ml 1× phosphate-buffered saline (PBS) and injected s.c. on the dorsal flank of each mouse. The treatment was continued at a dose of 20 mg/kg per injection twice daily. Length and width measurements of the tumors were made weekly with a caliper according to published methods (13). Tumor volume was calculated based on the equation V = (L × W^2)/2, where V is volume, L is length and W is width. After 8 weeks of treatment, tumors were harvested. Each tumor was divided into three groups, one for routine histological analysis, one for COX-1 and COX-2 immunohistochemistry, and the last for determination of PGE2 levels. Tumor samples for histological analyses were fixed in 10% buffered formalin and routinely processed. Sections (4 μm) were prepared and stained with routine hematoxylin and eosin and examined by a pathologist (L.Lamps) in a blinded fashion. The remaining portions were processed as indicated in Materials and methods.

**Prostaglandin levels in vivo**

Five control mice and seven treatment mice were housed as previously described. Just before HCA-7 cell inoculation, 40 mg/kg meloxicam was given i.p. Following meloxicam treatment, 5 × 10^6 HCA-7 cells were suspended in 0.1 ml of 1× PBS and injected s.c. The control mice were injected with the HCA-7 cell suspension only. Starting on the day following HCA-7 cell implantation, the treated mice were injected with 20 mg/kg meloxicam i.p. twice daily for 8 weeks. Tumor samples were harvested at 0, 2, 4, 6, 8, 12 and 24 h following a final meloxicam injection (40 mg/kg) and PGE2 was quantified using the same procedure as previously described.

**Results**

**HCA-7, Moser-S and HCT-116 colony growth in matrigel**

To assess whether meloxicam is effective at inhibiting cell growth in vitro, HCA-7, Moser-S and HCT-116 cells were grown in matrigel in the presence or absence of meloxicam. A dose-dependent decrease in colony size was observed in HCA-7 and Moser-S cells following daily administration of meloxicam (5–25 μM). Meloxicam treatment significantly reduced the size of HCA-7 colonies, with a greater effect seen at higher concentrations. At day 14, HCA-7 colonies treated with 5 μM meloxicam were 62% (P < 0.0001) smaller than controls and those treated with 25 μM showed a 76% (P < 0.0001) decrease in volume (Figure 1). A significant effect, though less dramatic, was observed in the Moser-S cells. Meloxicam did not affect colony volume significantly in HCT-116 cells, which do not express COX-2 protein. A dose-dependent effect was also observed for mean colony number in HCA-7 and Moser-S cells. The mean colony number was reduced by 20% in HCA-7 cells and by 22% in Moser-S cells treated with 25 μM meloxicam (P = 0.003 and P = 0.032, respectively). In contrast, no dose-dependent response in colony number was observed in HCT-116 cells.

**Prostaglandin production in vivo**

The effectiveness of meloxicam to inhibit the synthesis of PGE2 was determined in HCA-7, HCT-116 and Moser-S cells. PGE2 is the predominant prostaglandin product in HCA-7 cells and it has been previously reported that COX-2 inhibitors, such as SC-58125, are effective at decreasing PGE2 levels in these cells (17). HCA-7, Moser-S and HCT-116 were treated with meloxicam (1–25 μM) for 23 h. The media was then replaced with serum-free media containing the indicated concentrations of meloxicam and 10 μM arachidonic acid. One hour later, the media was collected and prostaglandin levels were determined. Compared with the controls, PGE2 levels in the meloxicam-treated HCA-7 cells were decreased by 92% with 1 μM meloxicam. A further reduction to 96% was noted at 5, 10 and 25 μM meloxicam (P < 0.003 for all doses) (Figure 2). Like the HCA-7 cells, secretion of PGE2 by Moser-S cells was decreased by 72% with 1 μM meloxicam and 86% with 25 μM meloxicam (P < 0.0193 for all doses). However, it is important to note that PGE2 levels at baseline were ~90% lower in the Moser-S cells than HCA-7 cells. This may be due to a relatively low level of COX-2 enzyme detected in the Moser-S cells (data not shown).
COX-2 inhibition and colorectal cancer cell growth

**Fig. 2.** Effect of meloxicam on PGE2 levels in HCA-7, Moser-S and HCT-116 cells. HCA-7 (solid bars), Moser-S (hatched bars) and HCT-116 (white bars) were grown to 90% confluence in serum-containing media in either the absence (DMSO) or presence of meloxicam (1–25 µM). At 1 h prior to the analysis of PGE2 levels in the media, the media was changed to serum-free medium containing the indicated meloxicam concentrations and supplemented with 10 µM arachidonic acid. The data are shown as the mean PGE2 levels (ng/ml). All measurements were performed in triplicate and the error bars represent the standard error of the mean.

**Fig. 3.** Western analysis of COX-1 and COX-2 in HCA-7 cells. HCA-7 cells were treated with 50 µM meloxicam for 2 to 48 h in order to evaluate COX-1 (A) and COX-2 (B) expression. Protein extracts (50 µg) were separated by SDS-PAGE, transferred to PVDF, and immunoblotted using either COX-1- or COX-2-specific antibodies. The immune complexes were detected using HRP-conjugated secondary antibodies and ECL. Extracts derived from MC-26 and HCA-7 (untreated) cells were used as a positive control (+) for COX-1 and COX-2, respectively, and protein extracts from the HCT-116 cells were used as a negative control (−).

**COX-1 and COX-2 levels in vitro**

To determine if meloxicam treatment altered the expression of COX-1 or COX-2, western blot analysis was performed on HCA-7 protein extracts. Since the greatest effect of meloxicam, *in vitro*, was observed in HCA-7 cells, COX-1 and COX-2 protein levels in HCA-7 cells were evaluated. Cell lysates were prepared from HCA-7 cells grown in the presence of 50 µM meloxicam for 2 to 48 h and immunoblotted to measure COX-1 and COX-2 levels. COX-1 protein was undetectable in 50 µg of HCA-7 cell lysate at the exposure times indicated (Figure 3A). In contrast, HCA-7 cells expressed high levels of COX-2 and 50 µM meloxicam significantly decreased COX-2 levels after 24 h of treatment and remained depressed at 48 h after treatment (Figure 3B). We have not observed a density or time dependent change in COX-2 expression in untreated HCA-7 cells (data not shown). Therefore, in addition to inhibiting COX enzyme activity, meloxicam treatment reduces COX-2 expression levels in HCA-7 cells.

**Tumor growth in nude mice**

Since meloxicam treatment of HCA-7 cells inhibits PGE2 production and colony growth, we decided to evaluate the effect of meloxicam on growth of HCA-7 cells *in vivo*. Therefore, HCA-7 cells were implanted s.c. on the dorsal flank of nude mice. The mice were then divided into treatment and control groups and tumor size was measured weekly. Examination of the tumors by routine pathological analysis confirmed that the tumors harvested from the mice were adenocarcinomas with well-formed glands and focal mucin production. Treatment with meloxicam significantly reduced tumor size within 3 weeks (*P* = 0.047). This trend continued throughout the remainder of the experiment, which was terminated following 8 weeks of meloxicam administration (Figure 4). At the conclusion of the experiment, the meloxicam-treated tumors were 52% smaller than control tumors. There was no significant difference between the histology of the treated tumors and the control group.

**Eicosanoid levels in vivo**

In an effort to ascertain the effectiveness of meloxicam in blocking prostaglandin synthesis *in vivo*, PGE2 levels were measured in the tumors. After the mice had been continuously treated with meloxicam for 8 weeks, treatment was stopped for 72 h. Then a final injection of meloxicam was given after which the mice were killed at time 0 (no final injection), 2, 4, 6, 8, 12 and 24 h after injection. The PGE2 levels in the tumors were determined and compared with PGE2 levels in tumors from the untreated control mice. The PGE2 levels in the mice treated with meloxicam for 8 weeks were ~62%
lower than in the untreated control mice (76.2 ng/g versus 199.0 ng/g tissue), suggesting that meloxicam inhibits prostaglandin production for at least 72 h following treatment. PGE₂ levels were decreased at 2 h (10.30 ng/g) and 4 h (2.80 ng/g) before gradually increasing to ~67% of the baseline concentration at 24 h (50.86 ng/g) (Figure 5).

**COX-1 and COX-2 levels in vivo**

In order to determine if meloxicam had an effect on COX-1 or COX-2 protein levels in vivo, immunoblot analysis for COX-1 or COX-2 was performed on tumor lysates. Consistent with the COX-1 results in vitro, there was no detectable COX-1 in the tumors (Figure 6A). COX-2 protein levels generally did not vary with the exception of the 4 h time point where a slight increase in COX-2 protein was observed (Figure 6B).

**Discussion**

Epidemiological data suggest that NSAIDs are effective agents in reducing mortality from colorectal cancer (2,18–24). Most of the NSAIDs used in animal studies are non-selective cyclooxygenase inhibitors, thus the precise molecular target of the NSAIDs used in animal studies are non-selective in reducing mortality from colorectal cancer (2,18–24). Most

**Fig. 5.** Relative PGE₂ levels in meloxicam-treated nude mice with HCA-7 xenografts. PGE₂ was measured in tumors harvested from mice killed after 8 weeks of continuous meloxicam treatment (20 mg/kg twice daily). All but one mouse (baseline mouse, no meloxicam was given for at least 72 h) was injected before being killed. The mice were killed at various times following the final injection (0 = baseline, 2, 4, 6, 8, 12 and 24 h). The data are shown as the mean PGE₂ level of the control or the individual PGE₂ level of each mouse/g of tissue.

**Fig. 6.** Western analysis of COX-1 and COX-2 in HCA-7 xenografts. COX-1 (A) and COX-2 (B) expression was evaluated in tumors harvested from untreated control mice (C) and meloxicam-treated (20 mg/kg) mice following 8 weeks of treatment. All but one mouse (baseline mouse, no meloxicam was given for at least 72 h) was injected with meloxicam before being killed and removal of the tumors. The mice were killed at various times following the final injection (0 = baseline, 2, 4, 6, 8, 12 and 24 h). Protein extracts (50 μg) were separated by SDS–PAGE, transferred to PVDF, and immunoblotted using either COX-1- or COX-2-specific antibodies. The immune complexes were detected using HRP-conjugated secondary antibodies and ECL. MC-26 and HCA-7 (untreated) cells were used as a positive control (+) for COX-1 and COX-2, respectively, and the COX-1 and COX-2 null cells, HCT-116, were used as a negative control (–).
of colorectal cancer in individuals chronically using NSAIDs. However, prolonged NSAID use often results in unacceptable gastrointestinal complications, attributable to the sustained inhibition of COX-1. Animal models using COX-2 inhibitors have been effective at reducing tumor burden without gastrointestinal toxicity. Therefore, COX-2 inhibitors may reduce colorectal cancer mortality in humans without undesirable side effects. It will be important to conduct clinical trials with COX-2 inhibitors to determine their effectiveness at decreasing the relative risk of colorectal cancer.

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