Celecoxib But Not Rofecoxib Inhibits the Growth of Transformed Cells in Vitro

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ABSTRACT

Purpose: Nonsteroidal anti-inflammatory drugs reduce the risk of colorectal cancer. The cyclooxygenase (COX) pathway of arachidonic acid metabolism is an important target for nonsteroidal anti-inflammatory drugs. Increased expression of COX-2 was recently shown to be an important step in the multistep process of colorectal cancer carcinogenesis. The new COX-2-specific inhibitors offer the benefit of cancer protection without the gastrointestinal toxicity reported for the old drugs. The purpose of this study was to compare the growth effects of two specific COX-2 inhibitors, celecoxib (Pfizer, Inc., New York, NY), and rofecoxib (Merck, White House Station, NJ) in normal and transformed enterocytes.

Experimental Design: Cultures of normal rat intestinal epithelial cell line, IEC-18, vector control cells, c-K-ras, c-K-ras-bak, and antisense-bak derivatives were treated with different dosages of celecoxib (0 –60 \( \mu \text{M} \)) and rofecoxib (0–20 \( \mu \text{M} \)). Cell cycle analysis and apoptosis were assessed by fluorescence-activated cell sorting analysis. Protein expression was assessed by Western blot analysis and caspases 3 and 8 activities by ELISA.

Results: Celecoxib inhibited cell growth and induced apoptosis in a time- and dose-dependent manner. IEC18 parental cells were two to four times more resistant to celecoxib than ras, ras-bak, and antisense bak transformed cells that overexpress the COX-2 protein. The induction of apoptosis by celecoxib involved the caspase pathways. Rofecoxib, up to its maximal concentration of 20 \( \mu \text{M} \), did not inhibit cell growth or induce apoptosis.

Conclusions: Celecoxib may prove to be a very efficient component in the prevention and treatment of gastrointestinal tumors because it inhibits the growth of cancerous cells without affecting the growth of normal cells.

INTRODUCTION

Colorectal cancer (CRC), with an estimated lifetime risk of 5–6%, is a major health concern in the Western world (1, 2). The goal of achieving effective cancer prevention is driven by the prediction that cancer will become the leading cause of death (surpassing heart disease) during this decade, with an estimated 1,000,000 new cases and >500,000 deaths/year, worldwide (1–3). Despite continuing advances in diagnosis and therapy, long-term survival has not improved significantly over the last four decades. Nearly 50% of all CRC patients will eventually die of their disease (1, 2).

The association between nonsteroidal anti-inflammatory drugs (NSAIDs) and CRC is an intriguing one and has been studied extensively. There are several lines of evidence suggesting that NSAIDs reduce CRC incidence and mortality (for review, see Refs. 4–6). However, long-term usage of NSAIDs is limited because of the high incidence of side effects and the significant cost. In 1997, 107,000 patients were hospitalized and 16,500 patients died, in the United States alone, as a direct consequence of NSAID usage (7). This mortality rate, of 50 patients/day, is equal to the mortality rate from AIDS or leukemia (7).

There are at least two isoforms of the cyclooxygenase (COX) enzyme. COX-1 is found in the normal gastrointestinal mucosa and is usually constitutively expressed. It serves as the housekeeping protein. The COX-2 gene was discovered about a decade ago (8). Although it is usually undetectable in the normal gastrointestinal mucosa, its expression can be induced by inflammatory and neoplastic stimuli (9). Up-regulation of COX-2 expression occurs in 40–50% of colorectal polyps and in up to 85% of CRC (9). The lack of COX-2 expression in the normal colonic mucosa, together with its increased expression in colonic neoplasm, constitute the rationale for the selective action of COX-2 inhibitors on neoplastic colonic mucosa, without major biological effects on the normal colonic mucosa.

Reddy et al. (10) showed that celecoxib had chemopreventive activity in the rat aberrant crypt focus model induced by azoxymethane. In a landmark study, Oshima et al. (11) demonstrated that crossing COX-2 knockout mice with APC mutant Min-mice resulted in a marked reduction in the number of intestinal adenomas. Both celecoxib and rofecoxib have successfully been shown, in this model, to inhibit polyp number and multiplicity in a dose-dependent manner (12, 13). Indeed, a controlled trial of Celebrex (Celecoxib, Pfizer, Inc., New York, NY) in familial polyposis patients demonstrated a 30% reduction in tumor burden (14).
Our group, in an open labeled study, demonstrated that rofecoxib (25 mg qd) prevented the growth of 80% of adenomas in familial polyposis patients for up to 30 months (15).

In recent years, our group has shown that transfection of normal enterocytes (IEC18 cells) by a variety of oncogenes resulted in malignant cell transformation (16–18). These cells proliferate faster, form colonies in soft agar, and have higher saturation density and plating efficiency. Most importantly, these cells form tumors when injected s.c. in nude mice (16–18). These sets of normal and transformed cell lines can serve as a unique in vitro model to assess the effects of drugs on cell lines that differ in only one oncogene.

In the present study, the growth inhibition of the two most important coxibs, celecoxib and rofecoxib, was evaluated in normal and transformed intestinal cells. The growth inhibitory effects produced two unanticipated findings. First, celecoxib and rofecoxib, having similar COX-2-selectivity and clinical efficacy for inflammatory indications, but differed significantly in their in vitro antiproliferative effects on cancer cell lines. Second, the antiproliferative effect of celecoxib was noted to particularly inhibit the growth of the transformed cells but not the growth of the normal cells.

We conclude that in this in vitro model, the antitumor effect of rofecoxib was much lower than the antitumor effect of celecoxib, an equally powerful COX-2 inhibitor. This difference implies that the antitumor effects of these drugs may be distinct from their effects on COX-2 inhibition.

MATERIALS AND METHODS

Reagents and Chemicals. Celecoxib was provided by Pfizer, Inc. Merck Research and Development (White House Station, NJ) supplied rofecoxib. All other reagents, with the highest purity, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Growth. The growth inhibition of rofecoxib was tested on the following cell lines: normal enterocytes derived from the rat ileum; IEC-18 cells (19); normal enterocytes transformed by c-K-ras (IEC-18-ras; Ref. 17); IEC-18-ras cells overexpressing bak (IEC-18-ras-bak; Ref. 16); and IEC-18 cells transformed by antisense-bak (IEC-18-AS-bak; Ref. 18). The different cell lines were grown and maintained in DMEM (Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 10% FCS, penicillin, and streptomycin at 37°C in an atmosphere of 95% oxygen and 5% CO2. A total of 200 μg/ml G418 (Haemek) served as the selectable marker for IEC-18-ras and IEC-18-AS-bak cells. Hygromycin served as the selectable marker for IEC-18-ras-bak cells.

Assays for Growth Inhibition. Cells were plated at a density of 7 × 104/10-cm dish in complete medium. The adherent and nonadherent cells were collected during exponential growth of the cells and counted. A total of 1–2 × 106 cells was washed in PBS, and the pellet was fixed in 3 ml of ethanol for 1 h at 4°C. Cells were pelleted and resuspended in 1 ml of PBS and incubated for 30 min with 0.64 mg/ml RNase at 37°C. Cells were stained with 45 μg/ml propidium iodide at least 1 h before analysis by flow cytometry, using a standard protocol for cell cycle distribution and cell size (17). Necrotic cells were counted using trypan blue before fixation. All experiments were repeated three times with similar results. Data acquisition was performed on a FACS calibur and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). All fluorescence and laser light scatter measurements were made with linear signal processing electronics. Data for 20,000 cells were collected for each data file.

Protein Extraction and Western Blotting. Exponentially growing cells were collected with a rubber policeman and washed three times in ice-cold PBS. Cell pellets were resuspended in lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 6 mM 6-mercaptoethanol, 1% NP40, 0.1% SDS and 10 mM NaF, plus the protease inhibitors 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). For Western blotting, samples containing 50 μg of total cell lystate were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were transferred to Hybond-C membranes (Amersham, Arlington Heights, IL) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol), using a Trans Blot transfer apparatus at 70 mA for 12–18 h at room temperature. Membranes were blocked with blocking buffer (PBS/0.2% Tween 20/0.5% gelatin) for 1 h at room temperature and were subsequently washed three times for 5 min in a washing buffer (PBS/0.05% Tween 20). The membranes were incubated with a 1:1000 diluted monoclonal human anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 18 h at room temperature. Membranes were washed and re-incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) for 1 h at room temperature. Additional washes were carried out as described previously and immune detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham). All experiments were repeated at least three times and yielded similar results.

Fluorogenic Assay of Caspases 3 and 8 Activities. The different cells were preincubated in buffer in the presence of 1–20 μM docosahexaenoic acid and exposed to 0–200 μM H2O2 for 0–8 h. After incubation, cells were collected, washed, re-suspended in 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 10 mM EGTA, and lysed by three successive freeze-thaw at dry ice/37°C. Cell lysates were centrifuged at 20,000 × g for 5 min, and the supernatants were stored at –70°C. The protein concentration of each sample was estimated using the Bio-Rad protein assay. For caspase-3 and caspase-8 activities, a total of 50 μg of protein was incubated with 50 μM DEVD-AMC and iEDT-AMP, respectively, at 37°C, for 30 min, and the release of 7-amino-4-methylcoumarine was monitored by a spectrofluorometer using an excitation wavelength of 360 nm.

Flow Cytometric Analysis. Cells were plated at a density of 7 × 104/10-cm dish in complete medium. The adherent and nonadherent cells were collected during exponential growth of the cells and counted. A total of 1–2 × 106 cells was washed in PBS, and the pellet was fixed in 3 ml of ethanol for 1 h at 4°C. Cells were pelleted and resuspended in 1 ml of PBS and incubated for 30 min with 0.64 mg/ml RNase at 37°C. Cells were stained with 45 μg/ml propidium iodide at least 1 h before analysis by flow cytometry, using a standard protocol for cell cycle distribution and cell size (17). Necrotic cells were counted using trypan blue before fixation. All experiments were repeated three times with similar results. Data acquisition was performed on a FACS calibur and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). All fluorescence and laser light scatter measurements were made with linear signal processing electronics. Data for 20,000 cells were collected for each data file.
RESULTS

COX-2 Protein Expression. A low level of COX-2 protein was seen in the parental cells. Increased levels of COX-2 protein (2–5-fold) were seen in IEC 18 cells transformed by ras, ras-bak, and antisense-bak cells.

Effect on Cell Growth. We assessed the effects of celecoxib (0–60 μM) on cell growth for 72 h. Celecoxib inhibited the growth of the transformed cells in a dose-dependent manner. The normal cells were significantly more resistant to the drug than the transformed cells (Fig. 1). At the same time rofecoxib, up to its maximal solubility concentration of 20 μM, did not demonstrate any cell growth inhibition in all of the different clones (Fig. 2).

Cell Cycle Changes and Induction of Apoptosis. Celecoxib induced apoptosis for 72 h, in a dose-dependent manner, in all of the transformed cell lines, (Fig. 3). At the same time, no apoptotic cells were identified in the normal cells (Fig. 3). Exposure of all of the cells to rofecoxib neither altered their cell cycle nor demonstrated an effect on the subdiploid DNA peak; which represents apoptosis (data not shown).

Level of Caspases 3 and 8 after 72 h of Exposure to the Coxibs. The induction of apoptosis after celecoxib treatment was associated with a parallel increase in the level of caspase 3 (Fig. 4) and caspase 8 (Fig. 5) in the ras-transformed cells, with a slight but constant decrease in the level of these caspases in the normal cells (Figs. 4 and 5). Rofecoxib did not alter the level of these caspases in any of the cell types.

DISCUSSION

Celecoxib and rofecoxib belong to a new class of NSAIDs that specifically inhibits COX-2. They have a significant anti-inflammatory and analgesic properties but are far less toxic than traditional NSAIDs, which inhibit both COX-1 and COX-2 (20, 21). However, not all COX-2 inhibitors share the same anticancer effects. The predictive discrepancy between the in vitro growth inhibitions of celecoxib and rofecoxib may be indicative...
of the difference in their mechanism of action. The present study provides the first direct comparison of the in vitro anticancer effects of the two clinically available COX-2 inhibitors.

The antiproliferative effect of celecoxib was noted to particularly inhibit the growth of the transformed but not the growth of the normal cells. Exposure to 10 μM celecoxib, for 72 h, inhibited transformed cell growth by 50% but had very little effect on the growth of normal cells (Fig. 1). The IC50 of celecoxib ranges between 5 and 20 μM across this entire panel of cell lines. This concentration is very similar to the concentration that can be achieved in the serum of humans with a standard anti-inflammatory dose (200 mg bid) of celecoxib.

Celecoxib induced growth inhibition by the induction of apoptosis (Fig. 3), most probably by the activation of the caspase pathway (Figs. 4 and 5). Exposure to celecoxib induced the characteristic features of apoptosis, including morphological changes, subdiploid DNA pick, and caspases activation. Moreover, the potency of celecoxib in induction of apoptosis is significantly higher than that of rofecoxib.

Thus, celecoxib can be a unique pharmacological tool in the study of apoptosis regulation in colon cancer cells considering its ability to interact with several targets. In addition, evidence is accumulating that the apoptosis-inducing effect of the drug may be different from its COX-2 inhibitory activity. It should be noted that similar results were reported in normal and transformed human prostate epithelial cells (22, 23).

Although there are many studies on the growth inhibition capabilities of rofecoxib in vivo, there are very few reports on its growth inhibition in vitro. Kusuniki et al. (24) recently reported that celecoxib inhibited the proliferation of rheumatoid arthritis synovial fibroblasts, whereas rofecoxib and other COX-2 inhibitors did not have any effect. In another study (25), celecoxib was shown to exhibit the greatest potency among COX inhibitors in inhibiting the growth of hematopoietic and epithelial cell lines. Finally, in the present study, rofecoxib, up to a significantly high dose of 20 μM, did not inhibit the growth of the normal and transformed cell lines (Fig. 2). This concentration is ~10-fold higher than the plasma level achieved in humans with standard clinical antianalgesic dosages of rofecoxib (25 mg once daily), confirming our previous results (26).

Nevertheless, we should not conclude that rofecoxib or any other agent is not a suitable drug for cancer prevention or treatment simply because it does not cause inhibition of cell growth. The efficiency of rofecoxib in vivo, including its significant effectiveness in preventing polyp formation in familial polyposis patients, cannot be ignored. It is suggested that the in vivo growth inhibition of rofecoxib may be attributable to direct inhibition of COX-2 expression in stromal cells, inhibition of the angiogenesis process, or other still unknown targets.

In a recent study, Zhu et al. (27) examined the structural differences between celecoxib and rofecoxib. The structures of both celecoxib and rofecoxib were modified to produce 50 compounds, and the compounds were then tested for their ability to induce apoptosis in human prostate cancer cells. Zhu’s study confirmed that the structural requirements for the induction of apoptosis are distinct from the structural requirements for the mediation of COX-2 inhibition (22, 23).

We have previously shown that sulindac sulfide, which exerts nonspecific activity against COX-1 and COX-2 isoenzymes, inhibits the growth of normal cells significantly more than the growth of neoplastic cells (13, 16, 28). The current study suggests that transformed cells, with oncogenic ras or down-regulation of Bak protein (frequent and important events in the multistep process of CRC carcinogenesis), are more sensitive than normal cells to celecoxib. Hence, the ultimate chemopreventive drug may be a specific COX-2 inhibitor rather than a NSAID that offers the benefits of protection against cancer without the side effects associated with traditional NSAIDs.

In summary, inhibiting the growth of precancerous and cancerous cells without affecting normal cells is the ultimate aim of cancer treatment in general and chemoprevention studies in particular because such studies, which are often long term, involve healthy subjects and have strict adverse event requirements. From a clinical perspective, celecoxib might be the desired drug that we are looking for because it inhibits the growth of malignant cells without affecting the growth of normal cells. The current focus of this laboratory is the separation of the apoptosis-inducing effect of celecoxib from its COX-2 inhibitory activity, which may ultimately lead to the design of a new class of therapeutic agents against colon cancer.

REFERENCES


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