GROWTH REGULATION OF GARDNER'S SYNDROME COLORECTAL CANCER CELLS BY NSAIDS

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INTRODUCTION

The use of NSAIDs (non-steroidal anti-inflammatory drugs) has been shown to reduce risk of mortality from colorectal cancer. It is not known how NSAIDs inhibit the growth of colorectal cancer, and whether this inhibition of growth is mediated through its action on the enzymatic activity of the cyclo-oxygenases, (COX) which are responsible for prostaglandin synthesis. NSAIDs have been shown to reduce the size and number of colorectal cancer lesions in familial adenomatous polyposis patients. We present data that suggest that the COX product, prostaglandin E\(_2\), plays a key role in regulation which supports cell proliferation in cancer. In this study, we analyzed gene expression of COX-2 in concert with cell growth in order to study the mechanism of NSAID inhibition of growth of colorectal carcinoma cells derived from familial adenomatous polyposis patients.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to provide chemoprotection from colorectal cancer in animal and man (1,2,3). A recent retrospective study of 662,424 patients (4) reported that aspirin use decreased death rates from colon cancer by approximately 40 percent (at a 95 percent confidence interval). There was no association between the use of acetaminophen and reduced risk of colon cancer. The NSAID sulindac (which inhibits the cyclo-oxygenase synthesis of prostaglandin), caused regression of tumor growth (5,6) in clinical studies suggesting a direct link between NSAID inhibition of prostaglandin synthesis and tumor growth regulation. Taken together, these studies suggest that low doses of NSAIDs reduce the risk of fatal colon cancer, but whether this is due to a direct effect of the NSAIDs on cyclo-oxygenase or to other factors is unclear.

Eicosanoids and Other Bioactive Lipids in Cancer Inflammation and Radiation Injury
Materials

...are developed from published genetic sequences and have been previously described... Een Klue... from the cDNA libraries... from... from the NIH Cell Culture Facility. The fibroblast lines were grown in... Enzymes: RNase, DNase, DNase, RNase, and protease... The immortalized C3H-10T1/2 cells were purchased from the American Type Culture Collection. The immortalized C3H-10T1/2 cells were grown in Dulbecco's modification of Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 units/ml each of penicillin and streptomycin.

Cell Culture

Materials and Methods

and the cyclooxygenase product, PGE2, are produced by the growth-associated genes and cyclooxygenase product PGE2...

Similar results were obtained using the cyclooxygenase product PGE2... in that experiment recently found that the cyclooxygenase product PGE2... has a significant effect on the cyclooxygenase activity...
and identified (13). The p53 PCR product is composed of 220 base pairs covering the sequences from the 622–842 base segment of the gene. The IL-1α primers produced a PCR product of 308 bp as described (14). The oligonucleotides were synthesized at the University of California Biomolecular Resource Center (San Francisco, CA).

RNA Isolation and cDNA Synthesis

For RT-PCR experiments, an equal number of cells were grown in either control media with no treatment, media with 3.5 μM flurbiprofen, media with 4mg/ml dmPGE₂ or media with both flurbiprofen and dmPGE₂ at the specified time points. Total RNA was collected at 2 and 24 hr timepoints. Total RNA was isolated using STAT-60 from Tel-Test (Friendsworth, TX). An RNA formaldehyde gel (1% agarose) was run with 1.5 μg total RNA and ethidium bromide for each sample to check mRNA purity and to confirm RNA concentration calculations.

Reverse Transcription Reactions

(RT’s) were run with 1.5mg total RNA, with 2.5 x 10⁴ copies of pAW109 control RNA template and reverse transcriptase buffer according to the manufacturers protocol (GeneAmp RNA PCR kit, Perkin Elmer-Cetus, Norwalk, CT). pAW109 RNA which contains IL-1α template, was added as a control for the reverse transcriptase and PCR reactions. All PCR reactions were run with the RT cDNA, AmpliTaq, PCR buffer, primers, MgCl₂ and dNTP’s according to the manufactures protocol (GeneAmp RNA PCR kit, Perkin Elmer-Cetus Norwalk, CT).

Southern Hybridizations

COX-2 bands were verified by Southern analysis. PCR samples were run on a 2% agarose gel, visualized by ethidium bromide staining and blotted to nitrocellulose membranes by overnight capillary action transfer in 1M Sodium Phosphate buffer. Hybridizations were performed at 42°C overnight in a solution containing 0.1g bovine serum albumin (essentially fatty-acid free), 2mM EDTA, 0.2M sodium phosphate buffer, 6% SDS, 35% formamide, and 1x10⁶ cpm/ml of a 32P-labeled probe. The blots were washed 3 times in a high salt solution containing 2X SSC and 0.2% SDS and 3 times at 42°C in a low salt solution containing 0.2X SSC and 0.2% SDS. They were then exposed to KODAK XAR-5 film for 1–5 hours at -74°C.

Cell Growth

Cells were plated at 5 x 10⁴ cells per well in a 6 well plate in 1% FCS media and were examined for viability by microscopy with a Nikon inverted scope and cell number was estimated using the Alamar blue method.

RESULTS

Given the pronounced clinical effect of the NSAID sulindac on reduction of polyp number and size in FAP patients, (5,6) we employed a quantitative assay using RT-PCR to determine if the NSAID was acting on cell growth through its influence on cyclo-oxy-
expression remained unchanged.

of Cox-2 mRNA significantly reduced in the cells treated with other NSAIDs while PGE2
of Cox-2 and control is shown after 24 hours of treatment. 24 hours where the expression
cox-2 mRNA was markedly decreased by NSAI3D after 24 hours. The relative expression
NSAI3D caused no striking changes in PGE2 mRNA expression. In contrast the expression of
NSAI3D and control in the other condition was increased in the cells treated with PGE2

NSAI3D Does Not Affect Gene Expression of Control RNA and P.G

previously (16). However, the RT-PCR products only versus the 5' labeled primers
assay for Cox-2, PGE2 and the PGE2 is seen in Figure 1. The linear response was linear.

The 5'-3' probes of the RT-PCR products are shown in the upper panel of the RT-PCR
was detected for the same RT products. Solution blocking of these bands with labeled
bands of these products and the cDNA samples. However, when the cDNA bands were

sense activity. Our strategy was to identify RNase molecules coding for Cox-2 enzyme

IL-1alfa RNA Curve

IL-1alfa RNA Curve
Cell Growth

The DiFi cells were grown with and without NSAID (flurbiprofen) and PGE$_2$ for 24 hours before determination of cell number. As seen in Figure 2, the results are shown in the total increase in cell number with each condition. The NSAID inhibited cell growth after the 24 hour. PGE$_2$ added with the NSAID treatment completely restored growth.

DISCUSSION

Over the past twenty years the occurrence of these cancers and the resulting mortality rate have not changed significantly despite intensive attempts at early detection and treatment. Colon and rectal cancer account for 20 percent of all deaths from cancer in the United States. Recently Thun et al, 662,424 adults studied for protective factors of aspirin in colon cancer where he found that death rates from colon cancer were measured from 1982-1988 which showed the death rates from colon cancer decreased with more frequent aspirin use in both men and women. The relative risk of death among persons who used aspirin 16 or more times per month for at least one year was 0.60 (95 percent confidence level) in men and 0.58 (95 percent confidence level) in women. No association was found between the use of acetaminophen and the risk of colon cancer (4).

Their study concluded that regular aspirin use at low doses may reduce the risk of fatal colon cancer. However, the mechanism of action is not yet known. The first report of active treatment of colorectal cancer with NSAIDs was in 1989 by Waddell et al. The

![Figure 2](image-url)

*Figure 2. Scanned images of photographed PCR products of p53, COX-2, internal standard pAW109 and total RNA in DiFi cells that were grown 24 hours with and without flurbiprofen. Cells were grown as described in materials and methods. Analysis of the genes COX-2 and p53 are seen in Table I.*
study evaluated the effect of sulindac, a long acting analogue of indomethacin, on colon polyposis in seven patients with Gardner's syndrome and/or familial polyposis coli. All polyps were eliminated except for a few that arose in the rectal mucosa and the anal canal. No cancers developed in the patients on follow-up (16). A second study on 9 patients with familial adenomatous polyposis, revealed complete, (6 patients) or almost complete, (3 patients) regression of the polyps with NSAID treatment (6). These findings suggest that prostaglandins may play a pivotal role in colon cancer proliferation.

Some believe that the NSAIDs enhance the immune response by lowering prostaglandin synthesis (17–21) while many believe that the NSAIDs directly effect cell proliferation (22–25). Prostaglandins modulate a variety of immunological responses with only PGE$_2$ appearing to cause feed back inhibition for cellular immune processes (17–21). Other studies have shown that the NSAIDs directly inhibit growth of colon tumor cells (22,23). Later analysis showed an arrest in G$_i$, thereby blocking the G$_i$/S progression of the cell cycle, reducing overall DNA synthesis (24). We have also reported that PGE$_2$ increases growth of lymphomas and an analog of PGE$_2$, PGA$_1$, caused a G$_i$ arrest in the cell cycle of lymphoma cells suggesting a role of PGE$_2$ (and hence cyclo-oxygenases) on cell cycle regulation at the G$_i$/S border (25–28). We have also demonstrated in osteoblasts that inhibition of another eicosanoid rate-limiting pathway enzyme, PLA$_2$, with dexamethasone causes inhibition of PGE$_2$ synthesis and inhibition of cell growth at the G$_i$/S border (29).

In this paper we show that in isolated colorectal cancer cells that a NSAID decreases prostaglandin synthesis and cell growth in the absence of the immune system. This inhibition of growth is not due to toxicity, since the addition of exogenous PGE$_2$ to the NSAID treated cells completely restored cell growth. This suggests a direct role of the cyclo-oxygenase product in NSAID growth inhibition. The primary signal transduction of PGE2 is shown below: Prostaglandin E2 is a product of arachidonic acid and is known to act as a local hormone on the cells through its 3 receptors, EP1, EP2 and EP3. These three recep-
Numerous populations of cells within NSCLAD that decrease steadily since PGE _2_ synthetase.

In these studies we have shown the reduction of cell proliferation in a familial order.

NSCLAD were associated with reduced COX-2 protein and mRNA concord in the liver of the oxo-veratrazene etymology. In addition to enzymatic activity, we found the expression of the subunit of COX-1. The decrease of PGE _2_ synthetase by blocking the active site and decreasing the enzyme activity.

NSCLAD decrease PGE _2_ synthetase by blocking the active site and decreasing the enzyme activity.

The inhibition of proliferation functions by the NSCLAD effects shown above.

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This NSAID inhibition of cell growth is accompanied later by inhibition of cox-2 mRNA expression. The NSAID growth inhibition is reversed by the addition of PGE₂, thus demonstrating that the action of the non-steroidal anti-inflammatory drugs is most likely working in the DiFi cell by inhibition of cyclo-oxygenase activity and that this action is not necessarily dependent upon an interaction with the immune system.

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BIBLIOGRAPHY


