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

Millie Hughes-Fulford[1] and Bruce Boman[2]

[1]Laboratory of Cell Growth and Differentiation
Department of Medicine, Research Service (Code 151F)
Veterans Affairs Medical Center and
Department of Medicine, University of California
San Francisco, CA 94121
[2]Storz Cancer Center
Clarkson Hospital
Omaha, Nebraska 68105

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-oxygenenases, (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.

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Synthesis of prostaglandins is specifically inhibited by NSAID action on cyclo-oxygenase which is one of the rate limiting enzymes in the eicosanoid synthetic pathway. The link between cyclo-oxygenase enzyme and growth is suggested by the recent discovery of the inducible form of cyclo-oxygenase (*cox-2*) enzyme by Harvey Herschman during his studies of growth responsive genes (7–9). *cox-2* is classified as an immediate early gene (7) placing it into the category of growth regulatory proto-oncogenes such as *c-fos* and *c-jun*. This laboratory has recently found that the cyclo-oxygenase product PGE_2 acts in a similar manner as fetal calf serum by inducing *c-fos* and *c-jun* oncogene expression and cell growth, thus linking prostaglandins and their precursor fatty acids to growth regulation (10). The aim of the present study is to assess the effect of NSAIDs on growth regulation of human familial adenomatous polyposis cells by assessing gene regulation of the rate limiting enzyme cyclo-oxygenase (*cox-2*) in the eicosanoid pathway. We have used the newly characterized colo-rectal carcinoma DiFi cell line derived from a familial adenomatous polyposis patient (11,12). This line is known to overexpress p53 and has allelic losses at loci on chromosomes 17p and 18, making it an ideal model for the familial polyposis patient. We have used the DiFi cell line as a model to test the action of NSAIDs in regulation of cell growth and expression of the growth-associated genes p53 and *cox-2*, and the cyclo-oxygenase product, PGE_2.

MATERIALS AND METHODS

Cell Culture

The DiFi colo-rectal cancer cell line was developed by Bruce Boman at the Creighton University School of Medicine (Omaha, NE). It was derived from a Familial Adenomatous Polyposis patient with Gardner's Syndrome characteristics (12). Cells were grown in an incubator at 37°C with 5% CO_2 in 10% fetal bovine serum (GIBCO BRL, Grand Island, NY) in a 1:1 mix of Leibovitz's L-15 media and Dulbecco's Modified Eagle media (DME H-16, 1g/L Glucose) from the University of California Culture Facility (San Francisco, CA). Media was supplemented with Insulin/Transferrin/Na Selenite and an antibiotic/antimycotic solution, both from Sigma (St. Louis, MO) as well as L-glutamine from the UCSF Cell Culture facility. Flurbiprofen were from Sigma. Dimethyl prostaglandin E2 (dmPGE2) and PGE_2 EIA kit were purchased from Cayman Chemicals (Ann Arbor, MI).

Materials

Moloney murine leukemia virus reverse transcriptase and buffer were purchased from GIBCO BRL. *Thermus aquaticus* DNA polymerase (Amplitaq), RNase Inhibitor, pAW109 control RNA, IL-1α primers and dNTPs were purchased from Perkin Elmer-Applied Biosystems (Norwalk, CT). 100bp DNA Molecular weight markers were purchased from GIBCO BRL. Agarose and low-melting agarose was purchased from Fisher. The human *cox-2* cDNA probe was purchased from Oxford Chemicals (Oxford, MI). *cox-2* primers were designed and tested for specificity by this laboratory based on published Genebank™ human sequences. The *cox-2* primers produced the predicted 724 base pair product relating to bases 597–1352 cDNA from the amplified product was identified by Southern blot with probe purchased from Oxford Scientific (Oxford MI) . Primers for p53 were developed from published Genebank sequence and have been previously described

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 uM flurbiprofen, media with 4mg/ml $dmPGE_2$ or media with both flurbiprofen and $dmPGE_2$ 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 2.5×10^4 copies of pAW109 control RNA template and reverse transcriptase buffer according to the manufactures protocol (GeneAMP RNA PCR kit, Perkin Elmer-Cetus, Norwalk, CT). pAW109 RNA which contains IL-1a 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, $MgCl_2$ 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 1×10^6 cpm/ml of a ^{32}P-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 5×10^3 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-

genase activity. Our strategy was to identify RNA molecules coding for *cox-2* enzyme coding region using an oligonucleotide complementary to its unique region. After reverse transcribing the mRNA of the cells, we amplified this signal using PCR primers designed to hybridize with the *cox-1* and *cox-2* cDNAs in their unique coding regions. In data not shown, electrophoresis of PCR products from exponentially growing DiFi cells yielded bands of a size predicted from the *cox-2* cDNA sequences, however, no *cox-1* message was detected for the same RT sample. Southern blotting of these bands with labeled probes of *cox-1* and *cox-2* confirmed the identify of the RT-PCR signals. The RT-PCR reactions for all genes analyzed were linear. An example of the linear response the RT-PCR assays for *cox-2*, pAW109 and the p53 is seen in Figure 1. The linear response was determined from a log plot of the PCR product area versus the log template input as described previously (16).

NSAID Does Not Regulate Gene Expression of Control RNA and P-53

The efficiency of the reactions was monitored using an artificial internal standard, pAW109 (14). In data not shown, there are no striking changes in the expression of the internal standard pAW109 RNA over the time periods studied. The results in Table 1 were corrected for both RNA content and RTPCR reaction efficiency. As seen in Table I, NSAID caused no striking changes in p53 mRNA expression. In contrast the expression of *cox-2* mRNA was markedly decreased by NSAID after 24 hours. The relative expression of *cox-2* and control, is shown after 24 hours of treatment 24 hours where the expression of *cox-2* mRNAs significantly reduced in the cells treated with either NSAID while p53 expression remained unchanged.

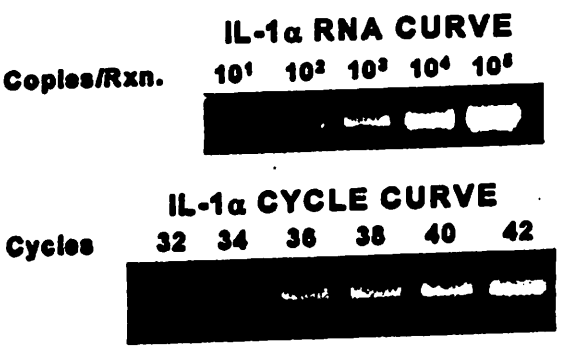

Figure 1. RNA and CYCLE curves for pAW109 RNA using IL1-a primer sets to amplify on PCR. Cycle curves for each of the genes are run to verify that the particular cycles chosen were within the linear range of amplification. PCR product verification included matching appropriate base pair size, Southern analysis with appropriate DNA probe or enzyme digestion using Perkin Elmer's IsoGene Kit for band isolation from low-melting agarose gels and Hae III restriction enzyme digestion. PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide in order to visualize the product bands. A photograph is then taken onto Polaroid 667 film using a Polaroid DS-34 camera. Photographs are then scanned into a digitized image using the Hewlett Packard Scanjet IIc at 600 dpi. Density analysis is performed on an accelerated Macintosh SE/30 computer using the public domain NIH Image 1.52 program written by Wayne Rasband at the U.S. National Institutes of Health

Table I. Analysis of COX-2 and p53 mRNA content corrected for RNA and reaction. Relative abundance of RT-PCR bands with was analyzed by densitrometry. The resulting data was then corrected for the relative density of 28S RNA concentration and for RTPCR efficiency using the pAW109 internal standard at 24 hours. This data is representative of 3 experiments

	Relative pixel density at 24 hours	
Message	Control	NSAID
COX-2	802	44
p53	1428	1351

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

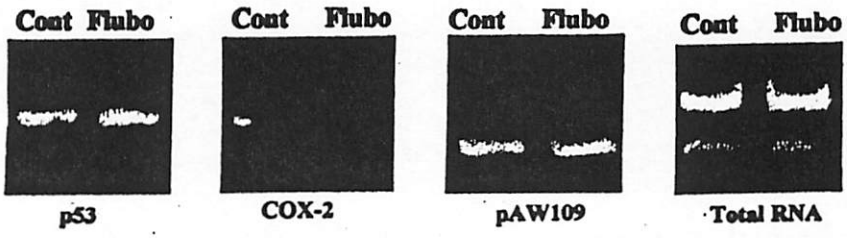

Figure 2. Scanned images of photographed PCR products of p53, COX-2, internal standard pAW109 and total RNA in DIFI cells that were grown 24 hours with and without flubroprofen. Cells were grown as described in materials and methods. Analysis of the genes COX-2 and p53 are seen in Table I.

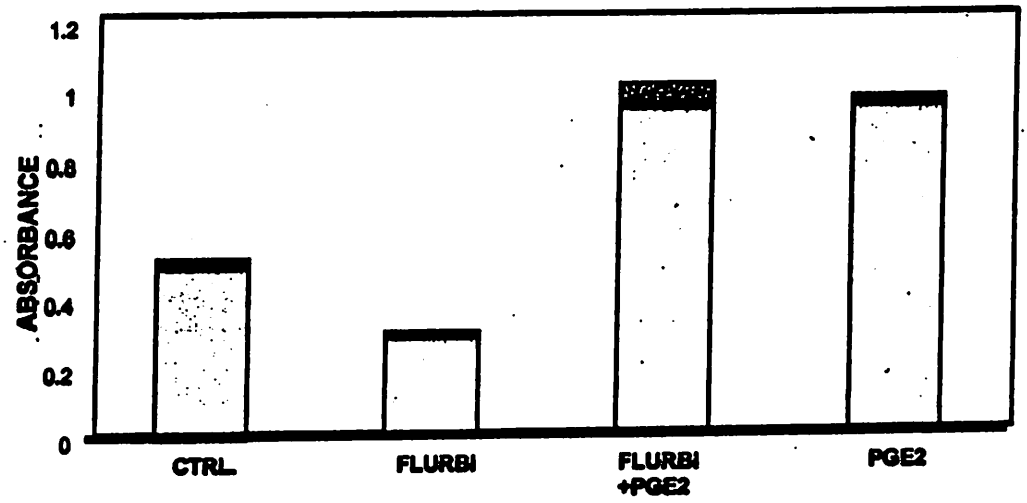

Figure 3. DiFi cells were grown as described in methods with and without flurbiprofen and with and without PGE_2 for 24 hours before cell number measurement with Alamar Blue. Each absorption measurement was taken in triplicate and then converted to total cell number from standards. The dark bar at top of each figure represents standard deviation of the cell number ($x10^6$).

study evaluated the effect of sulindac, a long acting analogue of indomethacin, on colon polyposis in seven patients with Gardener'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_1 thereby blocking the G_1/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_1 arrest in the cell cycle of lymphoma cells suggesting a role of PGE_2 (and hence cyclo-oxygenases) on cell cycle regulation at the G1/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_1 /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-

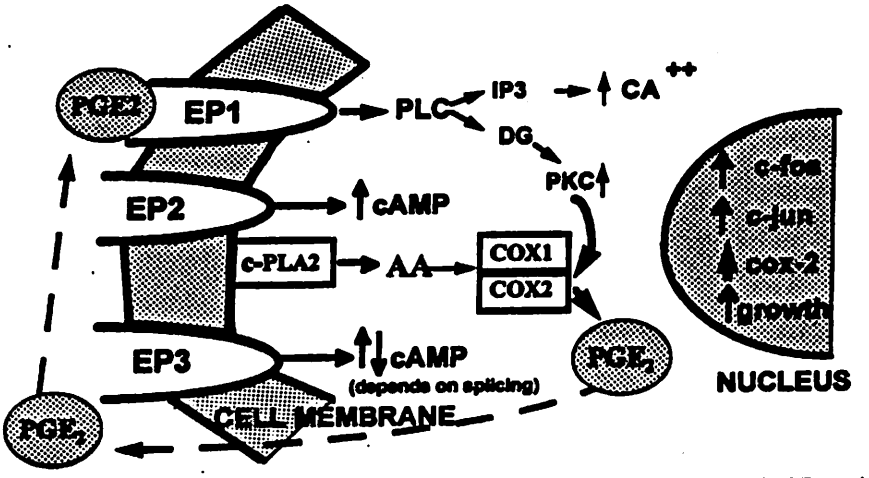

Figure 4. Possible mechanism of growth regulation in colorectal cancer. Increased synthesis of PGE_2 could cause an upregulation of PKC through the EP-1 receptor causing an increase in the inducible form of cyclo-oxygenase, a known immediate early gene. In data not shown here, we have also noted that PGE_2 induces mRNA expression of its own synthetic enzyme, cyclo-oxygenase-2, much like phorbol esters. If PGE_2 is acting on the EP1 receptor, then it would activate PKC through a phospholipase C signaling pathway. Which then would induce an increase in diacyl glycerol which would activate PKC activity and in turn induce *cox-2* mRNA synthesis and cell growth. This self-induced growth stimulation would be blocked by inhibiting cyclo-oxygenase with a NSAID which in turn should decrease PGE_2 synthesis, growth and eventually decrease cox-2 mRNA expression.

tors activate three separate signaling pathways. PGE_2 synthesis is associated with increased cell growth and NSAID decreased PGE_2 synthesis is associated with decreased growth which can be reversed by addition of extracellular PGE_2. Furthermore, in data not shown here, addition of extracellular PGE_2 increases *cox-2* expression, which could be explained by its action through the EP1 receptor. These findings have caused us to examine the signaling pathway and propose a new hypothesis to explain how NSAIDs give chemoprotection.

The inhibition of prostaglandin synthesis by the NSAIDs caused a substantial decrease in growth in these cells, with addition of PGE2 causing a substantial increase in cell growth. These findings correlates well with clinical data on PGE_2 concentration in tissues. In normal mucosa, the PGE_2 concentration is significantly increased in the adenomatous polyp $p<0.018$ and was increased 3 fold in colon cancer to 440±69 pg/mg $p<0.0001$. These data show that *in vivo* large amounts of PGE_2 are produced in neoplasms and may affect progression to cancer by stimulating cell growth (30). Data presented here show that NSAID inhibition of PGE_2 synthesis inhibits proliferation of colorectal cancer cells. Proliferation of the NSAID treated cells was completely restored by the addition of exogenous PGE_2.

NSAIDs decrease PGE_2 synthesis by binding to the active site and decreasing the activity of the cyclo-oxygenase enzyme. In addition to enzyme inhibition, we found the NSAIDs were associated with reduced COX-2 protein and *cox-2* mRNA content in the cells. The reduction of the total *cox-2* protein may be explained by the reduction of its message and by the increased degradation of the enzyme-NSAID complex.

In these studies we have shown the reduction of cell proliferation in a familial adenomatous polyposis patient cell with NSAID that decrease steady state PGE_2 synthesis.

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 PGE_2 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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