ACCELERATED PAPER

The nuclear eicosanoid receptor, PPARγ, is aberrantly expressed in colonic cancers

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Introduction

Numerous studies have demonstrated a 40–50% reduction in relative risk for colorectal cancer in individuals taking nonsteroidal anti-inflammatory drugs (NSAIDs)* compared to those not taking these agents (1–6). Individuals with familial adenomatous polyposis (FAP) treated with sulindac have a striking reduction in adenoma size and number (7–11). In azoxymethane (AOM) induced colorectal carcinogenesis, cyclooxygenase (COX) inhibitors exhibit chemoprotective effects as judged by a reduction in the frequency and number of premalignant and malignant lesions (12–14). Reddy et al. have also demonstrated a marked reduction of aberrant crypt formation in rats treated with a highly selective cyclooxygenase-2 (COX–2) inhibitor (15).

We have previously demonstrated increased COX–2 expression in human colorectal adenocarcinomas when compared to normal adjacent colonic mucosa (16); these findings have been confirmed by other investigators who have shown elevated levels of COX–2 protein in colorectal tumors by immunoblotting (17) and immunohistochemical staining (18,19). We have also observed markedly elevated levels of COX–2 messenger RNA (mRNA) and protein in intestinal tumors that develop in rodents following carcinogen treatment (20) and in adenomas taken from Min mice (21). A recent report by Oshima et al. indicates that COX–2 may play an extremely important role in the development of adenomas following loss of APC function (22,23). Adenomas from APCΔ716 mice were found to have elevated COX–2 levels. Additionally, treatment of APCΔ716 mice with highly selective COX–2 inhibitors significantly reduced tumor multiplicity. Also, our group has recently demonstrated an 85–90% inhibition of tumor growth in a mouse xenograft model by treatment with a highly selective COX–2 inhibitor (24). Taken together, these results provide strong genetic and pharmacologic evidence for a role of COX–2 in adenoma formation following loss of APC function (25).

The mechanism by which NSAIDs act to reduce the risk of colorectal neoplasia is unknown. We have previously demonstrated increased COX–2 levels in tumors taken from AOM-treated rats by Western and Northern blot analysis (20). We have also shown that forced expression of COX–2 in rat intestinal epithelial cells leads to phenotypic alterations which increase tumorigenic potential. Since these phenotypic alterations can be reversed by treatment with highly selective COX–2 inhibitors, the eicosanoid products formed by the COX–2 enzyme are likely affecting downstream signaling pathways, ultimately regulating gene transcription. One candidate for eicosanoid mediated transcriptional regulation is the PPARγ nuclear receptor that is activated by prostaglandin D2, prostaglandin J2, and derivatives thereof (26). At least one PGJ2 derivative, 15 deoxy-Δ12,14 PGJ2, is a direct ligand for PPARγ (27,28). In normal tissues, PPARγ expression is predominantly restricted to adipocytes (29,30). Given the aberrant expression of COX–2 in colon tumors, and the potential that this enzyme may serve to produce PPARγ ligands, we undertook these studies to determine if PPARγ was aberrantly expressed in colon tumor cells. Indeed, we found that PPARγ mRNA and protein was expressed not only in intestinal tumors, but in a subset of polyps and colon cancer cell lines. Additionally, we found that 15 deoxy-Δ12,14 PGJ2 does transactivate PPARγ mediated transcription driven off a

*Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; FAP, familial adenomatous polyposis; AOM, azoxymethane; COX, cyclooxygenase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; PPARγ, peroxisome proliferator activated receptor γ; mRNA, messenger RNA.
PPRE-luciferase construct transfected into the CoCa-2 colon cancer cells. This suggests that if COX-2 plays an etiological role in colon carcinogenesis, the mechanism could involve generation of a ligand that activates transcription via PPARγ.

Materials and methods

Carcinogen treatment, tissue procurement and RNA isolation

The experimental design and protocols have been described previously (20,31). Male F344 rats received at weaning were quarantined for 10 days and then randomly distributed by wt into control or experimental groups. Vehicle-treated subgroups and AOM-treated animals were housed (three per cage) in a room maintained under controlled environmental conditions of a 12-h light–dark cycle with 50% humidity at 22°C. Animals received proper care and maintenance according to institutional guidelines. All animals were fed the AIN–76A modified semi-purified diet. All diets were prepared in the laboratory once weekly and were stored at 4°C in airtight containers under nitrogen gas. Starting at 5 weeks of age, animals were allotted to experimental groups. The regimen was continued until termination of the experiment 52 weeks after carcinogen administration. Starting at 7 weeks of age, animals intended for carcinogen treatment were administered AOM s.c. for 2 weeks (15 mg/kg wt once weekly). Colonic tumors and normal tissues were obtained from six different randomly selected AOM-treated rats following the 52 week period. In each case, accompanying normal mucosa from the same animal was collected for comparison. All tissues were placed in cryovials, flash frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated from these samples using previously reported methods (16).

Preparation and labeling of cDNA probes

The rat COX–2 cDNA was isolated and characterized as previously reported (32). The cDNA probes were α-[32P]-dCTP labeled using a commercially available random primer labeling kit (Stratagene, La Jolla, CA) and purified using NICK® column chromatography (Pharmacia LKB, Piscataway, NJ) before use (33).

Northern hybridization analysis

RNA samples were electrophoresed in denaturing agarose gels and transferred to nitrocellulose. Nitrocellulose blots were hybridized using standard conditions followed by 0.1% SSC/0.1% SDS post-hybridization washes at 42°C. Blots were exposed for various lengths of time prior to development. Paired carcinoma and normal mucosa were evaluated for PPARγ expression in paired normal (N) and tumor (T) from the same rats (upper panel). The degree of elevation of PPARγ mRNA as determined by densitometry ranged from 1.5- to 6-fold in the cancer sample compared to the paired normal mucosa. The tumor sample on the far right lane appears to be under-loaded, so the fold difference in this case is probably an underestimate (lower panel Figure 1). These results demonstrate that PPARγ RNA levels are increased in most colonic tumors which develop in rodents following carcinogen treatment. We have previously reported that COX–2 is elevated in AOM-induced rat tumors (20) and demonstrate this again in Figure 1 (lower panel).

PPARγ protein expression is increased in intestinal tumors

We next proceeded to investigate PPARγ protein levels in colon tumors to determine if elevation of PPARγ RNA levels results in increased translation of PPARγ protein. In one of the colonic tumor samples there was no detectable difference in PPARγ protein levels between the tumor and normal mucosa by Western blotting (Figure 2A). However, in the remaining four samples there was a variable increase in PPARγ protein in the tumor sample compared with the paired normal mucosa. These results clearly demonstrate an increase in PPARγ protein levels in 4 out of 5 of the tumor specimens. There are two isoforms of PPARγ, called PPARγ1 and PPARγ2 (30), that are easily distinguished in the Western assay (34). PPARγ1 is identical to PPARγ2 except that it lacks the first 31 amino acids. PPARγ2 is derived from a different mRNA than PPARγ1, however, the PPARγ1 protein can be produced from the PPARγ2 mRNA by initiation of translation at an internal AUG codon. Since both PPARγ1 and PPARγ2 are expressed in adipocytes, an extract from mouse 3T3-L1 adipocytes is shown in Figure 2A as a control. Interestingly, the colon tumors specifically express PPARγ1 in contrast to the adipocytes which express both PPARγ1 and PPARγ2. The biological significance of this observation is unclear since, to date, no functional differences between the two PPARγ isoforms have been established. Interestingly, other non-adipocyte tissues that specifically express PPARγ also contain predominantly PPARγ1 (35–37).

Fig. 1. PPARγ and COX–2 gene expression is increased in intestinal tumor samples from azoxymethane-treated rats. Northern analysis of PPARγ expression in paired normal (N) and tumor (T) from the same rats (upper panel). Each lane contains 30 µg of total RNA. Equal loading was observed by equivalent intensity of 28S ribosomal bands of an ethidium bromide-stained gel (lower panel).
Since PPARγ is expressed at very high levels in adipocytes, it is important to control for fat contamination of the samples. The near absence of PPARγ mRNA or protein expression in the normal samples (Figures 1 and 2A) is reassuring, since peri-intestinal fat contamination might be expected to be more of a concern in the normal tissues. To confirm that the tumor samples were not contaminated by adipose tissue, we performed Western analysis on the four tumors with the highest expression of PPARγ1, as confirmed in Figure 2B. The blot was then reprobed with antibody to adipsin, a fat-specific marker. Figure 2B shows that the extract from adipocytes indeed contained adipsin, as expected, whereas none of the tumor extracts contained detectable levels of adipsin protein, consistent with the lack of adipose contamination. The Western blot was also reprobed with antiserum to COX–2. The results, shown in Figure 2B, show that the tumors which expressed PPARγ1 also expressed COX–2, although there was not a direct correlation between the two suggesting that the expression of each was independent of the other.

**PPARγ1 is expressed in a subset of carcinogen-induced intestinal polyps**

Much evidence suggests that adenocarcinoma of the colon is a late stage of a multistep process involving numerous changes in gene expression (39). Adenomatous polyps represent an earlier stage, characterized by abnormal proliferation, but lack of metastatic potential. To further characterize the induction of PPARγ during colon carcinogenesis, we examined carcinogen-induced adenomas (polyps) to determine if PPARγ was expressed. Figure 3 shows the results in carcinogen-treated rats killed at a time when the major lesions were colonic as well as small intestinal polyps. Again PPARγ protein was not detectable in normal mucosa. By contrast, PPARγ was expressed in a colonic polyp as well as a small intestinal polyp. The closely spaced doublet (also seen in the other

**Discussion**

Understanding the molecular events involved in the development of colorectal neoplasia has progressed remarkably during
the past decade. There is a wealth of evidence that dietary factors, especially dietary fat and fiber influence the development of colorectal cancer (43). However, there has been little understanding of how these dietary factors and genetic factors interact (44). Clinical and epidemiological studies have shown a relationship between NSAID use and reduction in relative risk for colorectal cancer (1–6). Although the mechanism whereby NSAIDs mediate these effects are unknown, inhibition of cyclooxygenase enzymes leading to a reduction of eicosanoid production remains a possibility. We and others have shown that mitogen-inducible cyclooxygenase (COX–2) expression is upregulated in human colorectal carcinomas (16–19). Recent reports have shown a link between the tumorigenic effect of APC mutations and arachidonic acid metabolism by observation that deletion of the COX–2 (prostaglandin endoperoxide synthase–2) gene reduces the number of tumors in mice heterozygous for an APC mutation by at least 50% by lipid transfection. Cells were treated with or without 15-deoxy-
\( \Delta 12,14 \)-prostaglandin J2 for 12 h. Cells were harvested and the Dual-luciferase assay was performed as described in Materials and methods.

Representative of two independent experiments: the means of normalized relative light units from three independent transfections are presented. Error bars = SEM.

![Graph](image)

**Fig. 5.** Transactivation of PPARγ transcription in CaCo–2 cells. CaCo–2 cells were transiently transfected with PPRE3-luciferase (1 μg) and pRL-TK (0.5 μg) by lipid transfection. Cells were treated with or without 15-deoxy-
\( \Delta 12,14 \)-prostaglandin J2 for 12 h. Cells were harvested and the Dual-luciferase assay was performed as described in Materials and methods.

The results described here demonstrate that PPARγ expression is elevated in AOM-induced rat intestinal tumors, both at the RNA and protein levels. Although PPARγ expression is greatest in adipocytes, expression in other tissues such as liver, muscle, heart, and spleen has also been observed in rodents, rabbits, and humans (35–37). Normal adult rodent intestinal tissues express levels of PPARγ mRNA that are undetectable by standard Northern analysis (29). Thus, the expression of PPARγ in a subset of polyps, cancer cell lines, and adenocarcinomas is aberrant and could play a role in carcinogenesis. Of some interest, we have observed that the highest level of PPARγ expression was found in the CaCo–2 cell line and that the receptor is functional in these cells. We have recently shown that forced expression of COX–2 in CaCo–2 cells leads to a dramatic increase in their metastatic potential (45). It will be important to understand the possible role (if any) of PPARγ in this altered phenotype. It is possible that one of the effects of COX–2 overexpression is to increase local production of a PPARγ ligand, presumably PGJ2 or a related species. This could provide a key link between dietary fatty acid precursors, eicosanoids, COX–2, and transcriptional regulation which could be involved in colorectal carcinogenesis. Work is underway to determine the biological relevance of aberrant co-expression of PPARγ and COX–2 in colon cancer.

**Acknowledgements**

We appreciate the technical assistance of Radhika Armandala. We thank R.Evans for providing us with the PPRE–3–luciferase reporter construct. This work was supported in part by funds from the United States Public Health Services Grants DK 47297 (RND), NIEHS–00267 (RND), Veterans Administration Merit Grant (RND), and DK 09210 (MAL).

**References**


