Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells

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The γ isofrom of the peroxisome proliferator-activated receptor (PPARγ) is a nuclear receptor that regulates adipocyte differentiation. Recently it has been shown to be expressed in human colonic mucosa and cancer, but its role in colon carcinogenesis and progression is still unclear. We demonstrate that activation of PPARγ by ciglitazone (cig), a selective PPARγ ligand, induces HT-29 human colon cancer cells to undergo apoptosis. Treatment with cig also down-regulates expression of cyclooxygenase-2 (COX-2) protein. Simultaneous exposure of cells to cig and 9-cis-retinoic acid (9-cis-RA), a ligand for retinoid X receptor, results in increased apoptotic effect and increased inhibition of COX-2 expression, compared with cells treated with either cig or 9-cis-RA alone. As COX-2 is overexpressed in human colon cancer and has been implicated in augmenting invasiveness and tumorigenicity, the ability of PPARγ activation to decrease COX-2 expression and induce apoptosis suggests that the PPARγ pathway may be considered as a therapeutic target for colon cancer.

Introduction

Peroxisome proliferator-activated receptor (PPAR) γ, a member of the steroid/thyroid/retnoid hormone receptor superfamily (1), is known to play a critical physiological role in adipose tissue. It triggers adipocyte differentiation and lipid storage by regulating the expression of genes critical for adipogenesis (2). PPARγ functions as a ligand-dependent transcription factor (3), which, upon heterodimerization with the retinoid X receptor (RXR) (4), binds to specific response elements termed peroxisome proliferator response elements (PPRE). This in turn regulates the expression of target genes (5,6). Activation of this receptor has been implicated in tumor promotion, cellular differentiation and apoptosis (7). Compounds that activate PPARγ comprise a heterogeneous group that includes polyunsaturated fatty acids (8), the thiazolidinedione class of anti-diabetic drugs such as troglitazone, pioglitazone, BRL49653 and ciglitazone (9,10), and the nuclear prostanoid 15-deoxy-Δ12,14-prostaglandin (PG) J2 (11).

In addition to adipose tissue, recent reports indicate that PPARγ is expressed at significant levels in human liposarcoma and breast adenocarcinoma. Treatment with PPARγ ligands reduces the growth rate and induces terminal differentiation of these malignant cells (12,13). The role of PPARγ activation in colon cancer, however, is controversial. It is expressed at high levels in colon adenocarcinoma, human colonic mucosa and cultured colon cancer cells (14,15). In colon cancer cells ligand activation of the receptor inhibits cell growth, induces a differentiation response and reverses the malignant phenotype (14–16). Paradoxically, activation of this receptor in min mice, an animal model for familial adenomatous polyposis, leads to increased polyposis (17,18). It therefore appears that the action of PPARγ depends on the cell type and/or the contingency of mutational events that predispose tissues to cancer development.

The ability of the PPARγ pathway to modulate growth and differentiation of colon cancer cells prompted us to hypothesize that activation of this receptor would trigger colon cancer cells to undergo apoptosis, the terminal point of cell differentiation and growth inhibition. To test this hypothesis, we used cig, a selective PPARγ ligand (19), to determine whether receptor activation would induce apoptosis of HT-29 human colon adenocarcinoma cells. As shown by DNA fragmentation assay, we demonstrate that cig induces apoptosis in HT-29 cells. Simultaneous exposure of cells to cig and 9-cis-retinoic acid (9-cis-RA) results in a potentiated apoptotic effect. In addition, PPARγ-induced apoptosis in HT-29 cells is associated with down-regulation of cyclooxygenase (COX)-2 protein expression, which is a known promoter of gastrointestinal cancer. Results from the current study suggest that compounds that affect the PPARγ pathway may be useful for colon cancer therapy.

Materials and methods

Tissue culture

Colon cancer cell line HT-29 was obtained from the American Type Culture Collection and cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 95% air/5% CO2 at 37°C. Prior to treatments, the medium was replaced with fresh medium (10% FBS) containing either vehicle control (ethanol) or experimental compounds for various time frames as indicated.

Western blotting

Cells were lysed with RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris, pH 7.2, 100 μM sodium orthovanadate, 50 mM sodium fluoride) containing protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN). Lysates were frozen at –80°C for 15 min and centrifuged at 10 000 g for 10 min to remove the particulate sediment. Protein concentration in the supernatant was measured using the BCA protein assay (Pierce). Total cellular protein (50–60 μg) was separated by electrophoresis on a 4–20% gradient SDS–polyacrylamide gel (Bio-Rad). Membranes were then soaked in Ponceau S solution (Sigma, St Louis, MO) for 2 min and washed five times with distilled water. Membranes with equal amounts of protein in each lane were incubated with the primary antibody indicated as follows: COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI), bcl-2 monoclonal antibody (PharMingen, San Diego, CA), p21 monoclonal antibody, cyclin D1 monoclonal antibody, c-myc monoclonal antibody, TGFβ-1 monoclonal antibody, β-catenin polyclonal antibody and vascular endothelial growth factor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then probed with horseradish
peroxidase-labeled secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The signal was revealed using the chemiluminescence detection system (Kirkegaard & Perry). Quantitation of the western blots was performed by scanning the blots with Adobe Photoshop and performing densitometry with NIH Image.

**Qualitative and quantitative DNA fragmentation analysis**

Cells grown at 70% confluency were treated with either cig (Biomol, Plymouth Meeting, PA), 9-cis-RA (Biomol, Plymouth Meeting, PA) or both or vehicle at the concentrations and for the times indicated in each experiment. For qualitative assay both floating and attached cells were collected, washed with cold phosphate-buffered saline and lysed in lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA and 1% Triton X-100) for 20 min on ice at the end of treatment. Microcentrifugation was performed at 11,000 g for 20 min to separate the nuclear DNA from the fragmented DNA present in the supernatant. The supernatant was treated with 50 µg RNase A at 37°C for 30 min and then proteinase K was added at 0.1 mg/ml for a further 1 h. After phenol/chloroform extraction, DNA was precipitated by ethanol. The pellet was rinsed with 70% ethanol, air dried and dissolved in TE buffer (10 mM Tris·HCl, 1 mM EDTA). The DNA (10 µg) was fractionated in a 1.8% agarose gel and visualized by ethidium bromide staining. Quantitative measurement of apoptotic cell death was performed using a commercial kit (Cell Death Detection ELISA; Boehringer Mannheim) according to the manufacturer’s instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis.

**Results**

**Activation of the PPARγ pathway induces cell death by apoptosis**

Previous studies have shown that HT-29 cells express functional PPARγ and RXRα (14,16). Activation of the PPARγ pathway promotes cellular differentiation and inhibits growth in culture by means of G1 cell cycle arrest (16). We therefore hypothesized that the increased differentiation and reduction in colon cancer cell number induced by PPARγ activation is associated with an increase in apoptosis. To test this hypothesis, we investigated the effect of PPARγ activation on apoptosis using DNA fragmentation analysis.

**DNA fragmentation**

Internucleosomal degradation of genomic DNA, due to activation of endogenous endonuclease, is one feature that occurs during apoptosis. Release of oligonucleosome-associated DNA fragments in this process results in a DNA ladder when analyzed by agarose gel electrophoresis. Such DNA laddering was negligible within 48 h of cig treatment (Figure 1A). However, a distinct oligosomal ladder was observed 3 days following treatment with either cig (10 µM) alone or cig (10 µM) in combination with 9-cis-RA (1 µM). This effect was negligible in cells treated with vehicle (ethanol) or 9-cis-RA alone (Figure 1A). These results demonstrate that activation of the PPARγ pathway induces apoptosis. The effect of cig on apoptosis was potentiated when administered in combination with 9-cis-RA.

**Cell death detection by ELISA**

PPARγ pathway-induced apoptosis was further confirmed using the Cell Death Detection ELISA kit, which uses a photometric enzyme immunoassay for qualitative and quantitative measurement of cytoplasmic histone-associated DNA fragments. As shown in Figure 1B, cig alone at a concentration of 1 µM is able to induce a 2-fold increase in DNA fragmentation after 3 days treatment. Higher doses of cig, up to 10 µM, did not significantly increase the amount of cellular DNA fragmentation. This suggests that intracellular ligands for RXR, a heterodimerization partner of PPAR, are limited. This notion is supported by evidence showing that at any given dose of cig a significant increase in DNA fragmentation is noted in the presence of 9-cis-RA (1 µM) when compared with cells treated with cig alone (Figure 1B). In addition, cig at concentrations of 1–10 µM induced a dose-dependent increase in cellular DNA fragmentation in the presence of 9-cis-RA (1 µM) (Figure 1B).

**Involvement of caspases in cig- and 9-cis-RA-induced apoptosis**

In the light of growing appreciation of the importance of caspase activation in the mechanism of apoptosis (20,21), we investigated the involvement of the caspase pathway in cig- and 9-cis-RA-induced apoptosis. HT-29 cells were treated with ZVAD-fmk (5–30 µM), a broad spectrum caspase inhibitor, together with cig (10 µM) and 9-cis-RA (1 µM). The effect of ZVAD-fmk on PPARγ pathway-activated apoptosis was quantitatively measured with the Cell Death Detection ELISA kit. As shown in Figure 2, ZVAD-fmk at concentrations...
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Fig. 2. Caspase inhibition diminishes cig- and 9-cis-RAl-induced apoptosis. Cells were treated with either vehicle as a control or cig in combination with 9-cis-RAl, with or without various concentrations of ZVAD-fmk as indicated. DNA fragmentation was measured 3 days after treatment with a Cell Death Detection ELISA kit according to the manufacturer’s instructions. Results represent means ± SD.

of 5–30 µM significantly, although not completely, diminished cig- and 9-cis-RAl-induced cellular DNA fragmentation. This indicates that caspase activation is involved in PPARγ-induced apoptosis.

Activation of the PPARγ pathway down-regulates COX-2 protein expression

The fact that the COX-2 promoter contains a PPRE (22) indicates that COX-2 might be one of the downstream targets of the PPARγ pathway. We therefore sought to determine the effect of PPARγ activation on COX-2 expression. HT-29 cells grown at 70% confluency were treated with either vehicle control (ethanol), cig (10 µM), 9-cis-RAl (1 µM) or cig + 9-cis-RAl. At 24, 48 and 72 h after treatment cells were harvested to analyze COX-2 protein expression by western blotting. As shown in Figure 3A, no significant change was detected during the first 24 h of treatment when compared with the control. At 2 days after treatment significant inhibition (50%) of COX-2 expression was observed in cells treated with cig alone. Similar to their effect on apoptosis, a greater inhibition of COX-2 protein (70%) was achieved when cells were treated with cig in combination with 9-cis-RAl (Figure 3A). Further inhibition of COX-2 protein expression was detected after 3 days of treatment: 85% inhibition with cig alone; 60% with 9-cis-RAl alone; 90% with the combined treatment (Figure 3A). When cells were exposed to various concentrations of cig (1–10 µM) in the presence or absence of 9-cis-RAl for 3 days a dose-dependent decrease in COX-2 expression was detected with cig alone (Figure 3B). A potentiated effect was again achieved when cells were exposed to the combined treatment (Figure 3B).

To elucidate the causal relationship between COX-2 inhibition and apoptosis in response to PPARγ pathway activation

we sought to determine whether inhibition of apoptosis has an effect on cig/9-cis-RAl-mediated COX-2 inhibition. ZVAD-fmk (5–30 µM) was added to the culture in the presence of cig and 9-cis-RAl. COX-2 protein expression was analyzed after 3 days treatment by western blotting. As shown in Figure 4, ZVAD-fmk in concentrations that inhibit apoptosis had no effect on cig/9-cis-RAl-induced COX-2 inhibition.

Fig. 3. Effect of cig and/or 9-cis-RAl on COX-2 protein expression in HT-29 cells. Cells were grown to 70% confluency. (A) Cells were treated with either vehicle (ethanol) as a control cig (10 µM), 9-cis-RAl (1 µM) or cig in combination with 9-cis-RAl. (B) Cells were treated with either vehicle as a control or various concentrations of cig (1–10 µM) in the absence or presence of 9-cis-RAl (1 µM) as indicated.

Fig. 4. Caspase inhibitor does not have an effect on cig/9-cis-RAl-induced COX-2 inhibition. Cells were treated with either vehicle or ZVAD-fmk alone (10 µM) or cig (10 µM) and 9-cis-RAl (1 mM) in the absence or presence of various concentrations of ZVAD-fmk, as indicated. Western blotting was performed as described in Materials and methods. The data shown are representative of at least three experiments.
Results from this study indicate that cig/9-cis-RA-induced COX-2 inhibition is not a downstream event of apoptosis.

**Effect of PPARγ pathway activation on downstream target genes**

PPARγ is a transcription factor which exerts its biological effect by modulating expression of its target genes. To further elucidate the role of PPARγ in colon cancer development we analysed the effect of its activation on the protein expression pattern of other genes responsible for crucial points in the development and progression of colon cancer. HT-29 cells grown at 70% confluency were incubated with either vehicle control (ethanol), cig (10 μM), 9-cis-RA (1 μM) or cig + 9-cis-RA. At 24, 48 and 72 h after treatment cells were harvested to analyse protein expression. The genes studied were: p16, p21, cyclin D1, c-myc, TGFβ-1 and β-catenin, which are involved in growth and cell cycle regulation; bcl-2, involved in apoptosis; vascular endothelial growth factor, involved in angiogenesis. Among the genes tested, expression of c-myc was found to be suppressed by PPARγ activation (data not shown). However, the expression pattern was not consistent. There was no change in expression of the other gene products analyzed (data not shown).

**Discussion**

At least three distinct subtypes of the PPAR family are found in vertebrate species: PPARα, PPARδ and PPARγ (5). The biological effects of PPAR activation are diverse and cell type specific. A recent study has demonstrated that PPARα is a target of the adenosomatous polyposis coli (APC) gene product and of non-steroidal anti-inflammatory drugs (NSAIDs) (23), indicating its role in colon cancer inhibition. PPARγ activation has been implicated in tumor promotion, cellular differentiation and apoptosis. Its role in colon cancer formation, however, is not clear. Ligand activation of this receptor causes inhibition of cell growth, increased differentiation and reversal of the malignant phenotype in a cell culture setting. However, receptor activation in the min mouse, which has a mutation in one allele of the apc gene, enhances tumorigenic potential and increases the number of polyps found. The reason for the contrasting effects of PPARγ in cell culture and in min mice is not apparent. As activation of this receptor involves heterodimerization with RXR, the presence of different corepressors and binding to different PPREs (5), it is plausible that the final outcome of PPAR activation is highly dependent on the cell studied and/or the genetic composition, which could modify the events of activation.

We describe, for the first time, that activation of the PPARγ pathway by a specific ligand, cig, induces colon cancer cell line HT-29 to undergo apoptosis. Simultaneous exposure of cells to both cig and 9-cis-RA results in a potentiated apoptotic effect. The exogenous ligand for RXRα (a heterodimerized partner for PPAR) is therefore required for the PPAR pathway to produce an optimal apoptotic effect. As 9-cis-RA has negligible effects on apoptosis, these results suggest that activity is primarily mediated through the PPARγ pathway. As a broad spectrum caspase inhibitor only partially reduced PPARγ-induced apoptosis, it is possible that a part of the apoptotic cascade in HT-29 cells is mediated by a caspase-independent mechanism. Caspase-independent apoptosis has been previously reported by other investigators (24–26).

Meade et al. have demonstrated that COX-2 expression is enhanced by peroxisome proliferators, including some fatty acids, PGs and NSAIDS, as well as the prototypical peroxisome proliferator WY-14,643, in mammary and colonic epithelial cells, presumably through PPARα (23). Lefebvre et al., however, concluded that activation of PPARγ did not affect COX-2 expression in HT-29 cells (17). The discrepancy between our results and theirs is readily reconciled by examining the experimental protocols. In the Lefebvre experiments COX-2 expression was examined 24 h after PPARγ activation. In our study, however, we show that inhibition of COX-2 expression does not occur until 48 h after treatment. A longer time frame (>24 h) is therefore required for a change in COX-2 expression to occur in this cell culture system. This suggests an indirect effect of the PPAR pathway on COX-2 expression.

COXs are rate-limiting enzymes in the synthesis of PGs (27). Our preliminary results show that activation of the PPAR pathway decreases production of PGE2 (data not shown). The effect of PPAR activation on the production of other PGs is currently under investigation.

Results from our current study demonstrate that the decrease in COX-2 expression occurs prior to apoptosis and inhibition of apoptosis by caspase inhibitors does not prevent PPAR-induced COX-2 inhibition, suggesting that inhibition of COX-2 may be an upstream event of cig/9-cis-RA induced apoptosis. This notion was supported by the observation that a selective COX-2 inhibitor (NS398) induces apoptosis in HT-29 cells (27). Taken together, it is possible that PPARγ-induced apoptosis is mediated, in part, through down-regulation of COX-2.

COX-2 is the inducible isozyme of COX which is responsible for much of the PG production in inflammation and is a key factor in colon carcinogenesis (30). Although COX-2 expression is usually suppressed in most tissues, various types of tumors and transformed cells constitutively overexpress COX-2 (31–33). A growing body of evidence suggests that COX-2 expression is associated with colon cancer formation and progression. A null mutation of COX-2 markedly reduces the number and size of intestinal polyps in APCΔ716 knockout mice, a model for human familial adenomatous polyposis (34). Treatment of APCΔ716 mice with a COX-2 inhibitor reduces the number of polyps, predominantly by apoptosis (34). In addition, overexpression of COX-2 not only inhibits apoptosis, as mentioned previously, but also increases the invasive potential of malignant cells (29,35). Studies investigating the role of COX-2 in human disease point to a link between COX-2 overexpression and colon cancer pathology.

As COX-2 is a known promoter of colon cancer, the ability of PPARγ pathway activation to inhibit COX-2 expression and induce apoptosis suggests that PPARγ may be a useful target for therapeutic intervention and chemoprevention of human colon cancer.

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**References**


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