Research article

CELECOXIB INCREASES RETINOID SENSITIVITY IN HUMAN COLON CANCER CELL LINES

JIAN-PEI LIU, HONG-BO WEI*, ZONG-HENG ZHENG, WEI-PING GUO
and JIA-FENG FANG
Department of Gastrointestinopancreatic Surgery, The Third Affiliated Hospital
of Sun Yat-Sen University, Tian He Road 600, Guangzhou 510630, P.R. China

Abstract: Retinoid resistance has limited the clinical application of retinoids as differentiation-inducing and apoptosis-inducing drugs. This study was designed to investigate whether celecoxib, a selective COX-2 inhibitor, has effects on retinoid sensitivity in human colon cancer cell lines, and to determine the possible mechanism of said effects. Cell viability was measured using the MTT assay. Apoptosis was detected via Annexin-V/PI staining and the flow cytometry assay. PGE_2 production was measured with the ELISA assay. The expression of RARβ was assayed via western blotting. The results showed that celecoxib enhanced the inhibitory effect of ATRA in both COX-2 high-expressing HT-29 and COX-2 low-expressing SW480 cell lines. Further study showed the ATRA and celecoxib combination induced greater apoptosis, but that the addition of PGE_2 did not affect the enhanced growth-inhibitory and apoptosis-inducing effects of the combination. Moreover, NS398 (another selective COX-2 inhibitor) did not affect the inhibitory effects of ATRA in the two cell lines. Western blotting showed that the expression of RARβ in HT-29 cell lines was increased by celecoxib, but not by NS398, and that the addition of PGE_2 did not affect the celecoxib-induced expression of the retinoic acid receptor beta. In conclusion, celecoxib increased the expression of RARβ and the level of cellular ATRA sensitivity through COX-2-independent mechanisms. This finding may provide a potential strategy for combination therapy.

Key words: Colonic neoplasm, Retinoic acid, Celecoxib, Sensitivity

* Author for correspondence. e-mail: drwhb@21cn.com, tel: +86-13060620467

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant diseases worldwide [1]. Approximately half of the patients with colorectal cancer can be cured via surgical resection of the primary tumor, while the remainder eventually succumb to the disease, predominantly due to metastases. Metastasis may already have occurred before the detection of the primary tumor [1]. This characteristic of CRC has prevented any marked improvement in the patient recovery rates despite advances in surgical techniques. In order to improve the prognosis, chemotherapy has become one of the common strategies for CRC treatment, especially for patients with unresectable tumours. However, the conventional chemotherapeutic drugs used in CRC treatment often have severe side effects that limit their efficacy [2]. Therefore, combined treatments with several chemotherapy regimens or even chemopreventive medicines are often used, not only to enhance the treatment effect, but also to reduce the toxicity of these medicines.

Retinoids, a group of structural and functional analogs of vitamin A, regulate several essential biological processes. Pharmacologically, they have been recognized as important in modulating cell differentiation and apoptosis and in suppressing carcinogenesis in a broad range of tissue types, including CRC *in vitro*, in animal models and in clinical trials [3, 4]. In contrast to the reported beneficial clinical effects, there is evidence that intrinsic or acquired resistance can limit retinoid clinical activity. Some colon cancer cell lines, such as HT-29 and HCT-116, are intrinsically resistant to retinoids [5]. Large-scale randomized trials conducted in Europe and the United States showed that moderate doses of natural or synthetic vitamin A compounds were ineffective in reversing premalignancy or in suppressing the recurrence of primary tumors [6, 7]. In order to be able to use retinoids in a clinical setting, it is critical to identify the mechanisms of intrinsic or acquired resistance to retinoids, and to find potential strategies to overcome or reduce the clinical impact of this resistance.

Cycloxygenase-2 (COX-2) expression is upregulated by approximately 50% in colon adenomas and 85-90% in colorectal cancer [8]. The selective COX-2 inhibitor celecoxib (Celebrex®) has been reported to reduce the incidence of colonic adenomas in clinical trials [9]. It has also been reported that COX-2 can suppress the inhibitory and apoptotic effects of N-(4-Hydroxyphenyl) retinamide (4HPR, a functional analog of retinoic acid) in breast cancer cells [10, 11], and that 4HPR had an enhanced anti-cancer effect in combination with celecoxib in lung cancer cells [12]. All of this evidence led us to hypothesize that celecoxib-induced suppression of COX-2 could improve the effectiveness of retinoids. In addition, the level of COX-2 has been reported to negatively correlate with retinoic acid receptor beta (RARβ) [13-15], the level of which has been proved to strongly connect to retinoid sensitivity [4]. However, a study conducted by Mernitz failed to confirm this correlation [16]. We conducted this study to determine the combined effects of celecoxib and all-trans retinoic acid (ATRA)

on human colon cancer cell lines and the role of COX-2 in mechanisms of retinoid resistance.

MATERIALS AND METHODS

Cell culture and reagents
The human colorectal cancer cell lines HT-29 and SW480 were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, CHINA). HT-29 was maintained in McCoy's 5A media (Sigma, St. Louis, USA), and SW480 in RPMI 1640 media (Gibco, New York, USA). Both cell lines were supplemented with 10% fetal bovine serum (FBS) and incubated at 37ºC in a humid atmosphere with 5% CO_2. Cells grown in 75-cm^2 culture flasks were trypsinized every week, and the medium was changed daily from the third day of culture. ATRA and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma Co. (St. Louis, MO, USA). Celecoxib was obtained from Pfizer (Shanghai, CHINA). NS398, Prostaglandin E_2 (PGE_2) and a PGE_2 Elisa kit were purchased from Cayman Chemical (Michigan, USA). ATRA, NS398 and PGE_2 were all dissolved in DMSO as a stock solution. Celecoxib was also dissolved in DMSO as a 20 mM stock solution, and diluted in the culture media before being added to the cell cultures. Anti-COX-2 polyclonal antibody was obtained from Lab Vision Corporation (Fremont, USA). Anti-RARβ polyclonal antibody and anti-GAPDH polyclonal antibody were both purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

Growth inhibition study
Cells were cultured in 96-well plates at a density of 5×10^3 cells/well overnight. Then, the cells were treated with various concentrations of ATRA, celecoxib, NS398 and PGE_2 alone or in combination. After 72 h of drug exposure, 5 mg/ml MTT solution was added to the plates for another 4 h. Formazine was dissolved in 150 μL/well DMSO, and the absorbance was detected at 490 nm using an ELx800 Strip reader (Bio-Tek, USA).

Apoptosis analysis
The effects of the drugs on colon cancer cells were evaluated for apoptosis induction with the Annexin-V/PI assay. HT-29 cells were cultured in 6-well plates at a density of 2×10^4 cells/well overnight. Then, the cells were treated with 1 μM of ATRA, 5 μM of celecoxib or 10 μM of PGE_2 alone or in combination. After 48 h of drug exposure, the supernatant in each culture was gently removed, and the adherent cells were detached by trypsinization. 0.5 to 1×10^6 cells were collected, washed twice with cold phosphate-buffered saline (PBS) and resuspended in 200 μl of binding buffer (2.5 mmol/l $CaCl_2$, 20 mmol/l HEPES, pH 7.4, and 140 mmol/l NaCl). 10 μl of Annexin V-FITC and 5 μl propidium iodide were added and the solution was left for 15 min in dark. The levels of Annexin-V and PI staining were determined via flow cytometry on a FACScan (Becton Dickinson, San Jose, CA). The apoptosis-

induction effect was quantified using the Annexin-V/PI assay. The Annexin-V+/PI- (early apoptotic) population represents apoptotic cells, while the Annexin-V+/PI+ population represents necrotic cells.

Measurement of PGE$_2$ levels

HT-29 cells were cultured in 6-well plates at a density of 2×10^5 cells/well overnight. Then, the cells were treated with 5 μM of celecoxib, 10 μM of celecoxib, 10 μM of NS398 or 20 μM of NS398. After 24 h of drug exposure, the PGE$_2$ level in the culture medium was measured using a commercial ELISA kit (Cayman) according to the manufacturer's instructions.

Western blotting

HT-29 cells were cultured in 6-well plates at a density of 2×10^4 cells/well overnight. Then, the cells were treated with a placebo (0.1% DMSO), 1 μM of ATRA, 5 μM of celecoxib or 10 μM of PGE$_2$ alone or in combination. After 72 h of drug exposure, total cell lysates were prepared by lysing the cells with the buffer (1% SDS, 10 mM Tris-Cl, pH 7.6, 20 mg/ml aprotenin, 20 mg/ml leupeptin and 1 mM PMSF). The protein concentrations were determined using the Bradford method. About 80 μg of protein were separated on 10% SDS-PAGE gels, and transferred to PVDF membranes. After being blocked with 10% non-fat milk, the membranes were incubated with anti-RARβ polyclonal antibody (1:200 dilution; Santa Cruz, CA, USA) or anti-COX-2 polyclonal antibody (1:200 dilution; Lab Vision Corporation, Fremont, USA) at 4ºC overnight. After three 10-min washes, the membranes were incubated with goat anti-rabbit IgG (1:5000 dilution) for 1 h at room temperature. The signals were developed with the ECL kit (Amersham Pharmacia Biotechnology Inc, Milpitas, CA), using anti-GAPDH antibody (Santa Cruz, CA, USA) as an internal control.

Statistical analysis

The results were expressed as the mean ± SD of at least three determinations for each group, repeated at least three times on different occasions. Biostatistical analyses were done using the SAS 8.1 software package. One-way analysis of variance (ANOVA) was used to evaluate the differences between the means of three or more groups. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Differences in the COX-2 protein expression between HT-29 and SW480 cell lines

Western Blotting showed the intrinsic difference in the COX-2 protein expression between HT-29 and SW480 cell lines. HT-29 cells expressed high levels of the COX-2 protein, while SW480 cells expressed low levels (Fig. 1). Therefore HT-29 cell line was used throughout this study.
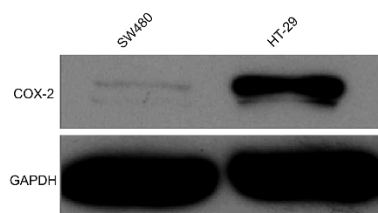
Fig. 1. The expression of COX-2 protein in the SW480 and HT-29 cell lines was detected by Western blotting. Both cell lines (2×10^4 cells/well) were cultured in 6-well plates without drug treatment for 96 h before being lysed. The samples used for electrophoresis consisted of 80 µg of protein from pooled cell extracts. GAPDH was used as an internal control.

Celecoxib, but not NS398, enhanced the inhibitory effects of ATRA in the HT-29 and SW480 cell lines

MTT assays showed that the HT-29 and SW480 cell lines were both resistant to ATRA at the concentrations used, and that 5 µM and 10 µM of celecoxib had slight inhibitory effects in both cell lines. Celecoxib combined with either 1 or 10 µM of ATRA resulted in a dose-dependent increase in growth inhibition in the HT-29 and SW480 cell lines ($p < 0.05$). NS398, unlike celecoxib, did not enhance the inhibitory effects of ATRA in the two cell lines. 10 µM of PGE_2 showed growth-stimulatory effects on HT-29, but not on SW480 cell lines. However, the addition of PGE_2 did not affect the enhanced inhibitory effects of the ATRA and celecoxib combination (Fig. 2).

Celecoxib enhanced ATRA-induced apoptosis in HT-29 cell line

The proportion of apoptotic cells induced by 1 µM of ATRA alone and 5 µM of celecoxib alone were both less than 5%, but the proportion of apoptotic cells induced by ATRA was significantly increased in the presence of celecoxib ($p < 0.01$). The addition of PGE_2 did not affect the number of apoptotic cells induced by the ATRA and celecoxib combination in the HT-29 cell line ($p < 0.01$; Tab. 1).

Tab. 1. Celecoxib enhanced ATRA-induced apoptosis in the HT-29 cell line.

ATRA (1 µM)	Celecoxib (5 µM)	PGE_2 (10 µM)	Apoptosis rate (%)
-	-	-	2.29 ± 1.18
+	-	-	3.07 ± 1.33
-	+	-	2.45 ± 1.22
+	+	-	15.51 ± 2.37 [a,b,c]
-	-	+	2.19 ± 1.09
+	+	+	15.73 ± 2.65 [a,b,c]

a – P < 0.05 compared with the control group, b – P < 0.05 compared with the ATRA group, c – P < 0.05 compared with the celecoxib group
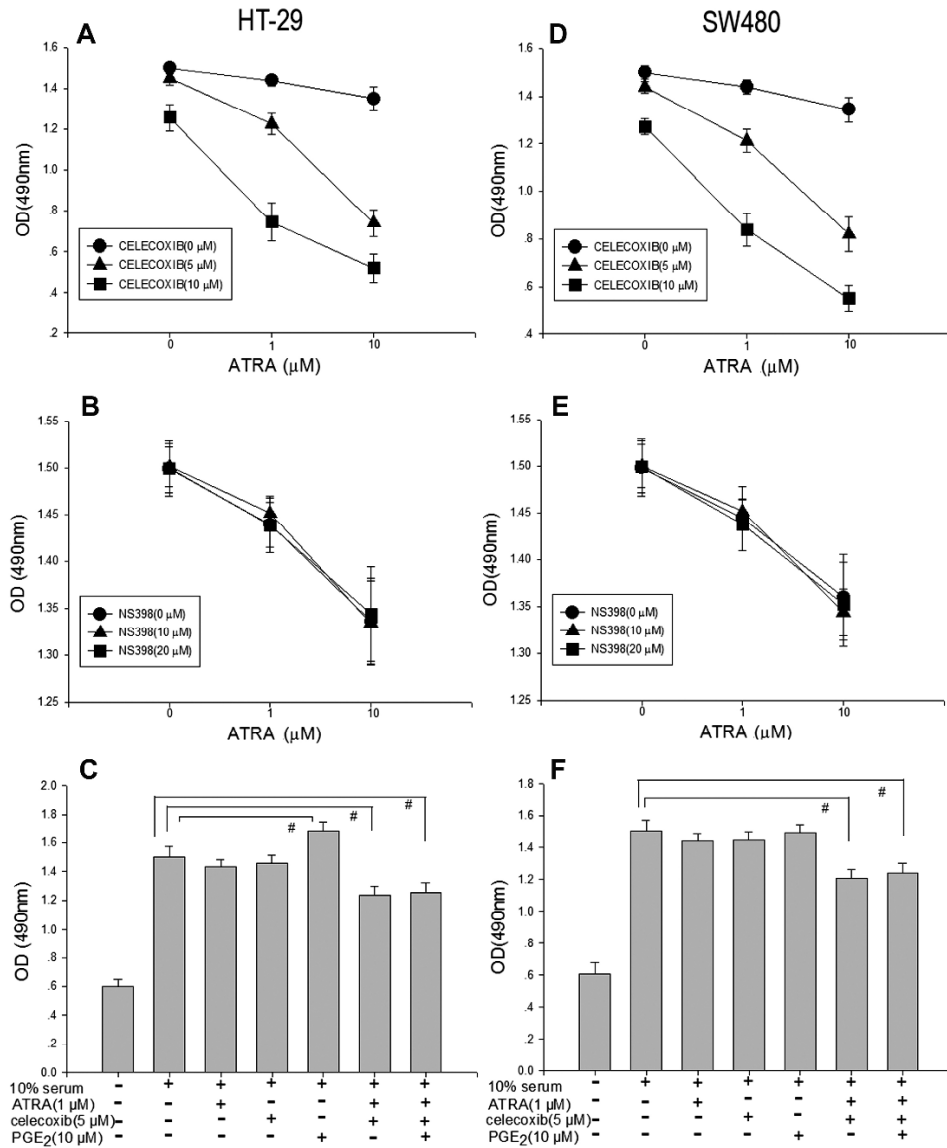
Fig. 2. The inhibitory effects of ATRA, celecoxib and NS398 on the HT-29 (A, B, C) and SW480 (D, E, F) cell lines. Both cell lines were cultured in 96-well plates at a density of 5×10^3 cells/well overnight. Then the cells were treated with 5 or 10 μM of celecoxib, 10 or 20 μM of NS398, 1 or 10 μM of ATRA and 10 μM of PGE_2 alone or in combination for 72 h. Viable cell counts were measured using the MTT method. Each point or bar represents the mean ± standard deviation of at least three determinations. # = $p < 0.05$.

Celecoxib, but not NS398, increased the expression of retinoic acid receptor beta in the HT-29 cell line

According to the results of the ELISA assays, 10 µM of NS398 and 5 µM of celecoxib had similar effects on PGE$_2$ production in the HT-29 cell line (Fig. 3). Western blotting showed that the expression of retinoic acid receptor beta in HT-29 was increased by celecoxib. The addition of PGE$_2$ did not affect the celecoxib-induced expression of retinoic acid receptor beta. NS398, unlike celecoxib, did not increase the expression of retinoic acid receptor beta in the HT-29 cell line (Fig. 4).
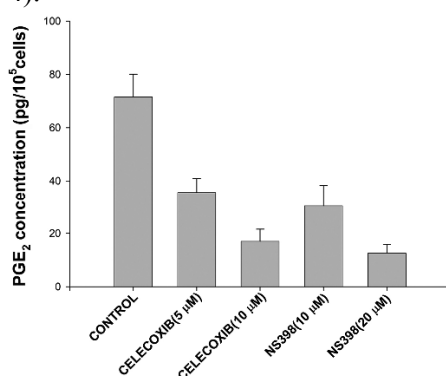

Fig. 3. The PGE$_2$ level in the culture medium of the HT-29 cell line. HT-29 was cultured in 6-well plates at a density of 2×10^5 cells/well overnight. Then, the cells were treated with 0.1% DMSO (control), 5 or 10 µM of celecoxib, or 10 or 20 µM of NS398 for 24 h. The PGE$_2$ level in the culture medium was measured using an ELISA kit. Each bar represents the mean ± standard deviation of at least three determinations.
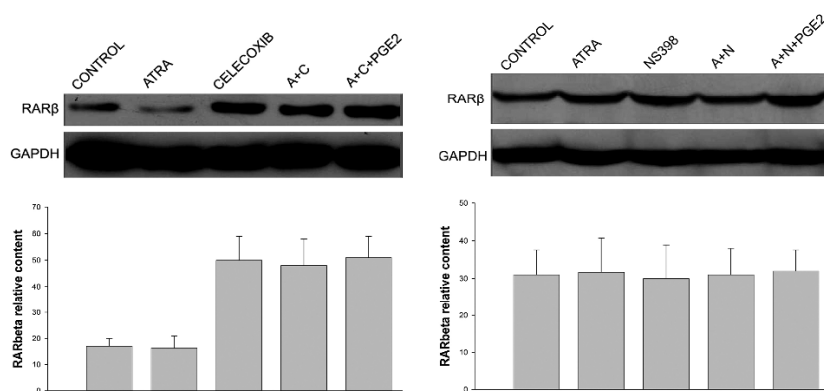

Fig. 4. The expression levels of retinoic acid receptor beta protein in HT-29 cells were determined by Western blotting. HT-29 (2×10^4 cells/well) was cultured in 6-well plates treated with 0.1% DMSO (control), 5 µM of celecoxib, 10 µM of NS398, 1 µM of ATRA and 10 µM of PGE$_2$ alone or in combination for 72 h before being lysed. The samples used for electrophoresis consisted of 80 µg of protein from pooled cell extracts. The detection of GAPDH was carried out in order to provide an internal control. Each column represents the mean ± standard deviation of at least three determinations.

DISCUSSION

This study highlights the enhanced growth-inhibitory and apoptosis-inducing effects of ATRA in combination with celecoxib at clinically relevant concentrations compared to single agent treatment in colon cancer cell lines. Retinoic acid resistance has limited the clinical applications of retinoids, which have fallen from their zenith in the 1990s to something of a nadir nowadays. Intense efforts are being mounted to create a new analog of retinoic acid or a combination therapy for retinoid treatment [17]. It had been reported that celecoxib could suppress proliferation and induce apoptosis in colon cancer cell lines at concentrations of only ≥ 10 μM [18, 19]. Our study also showed that 5 μM of celecoxib alone had no growth-inhibitory or apoptosis-inducing effects in colon cancer cells. In fact, pharmacokinetic studies performed in humans revealed that the plasma levels did not exceed 5.6 μM in individuals receiving up to 800 mg of celecoxib per day [20]. Therefore, celecoxib at clinically relevant concentrations restricted anti-cancer effects as a single agent. The result of this study provides a potential strategy for combination therapy.

Celecoxib had been proven to down-regulate the ABC transporters P-glycoprotein (P-gp) and to enhance the accumulation of chemotherapy agents in human colon cancer Caco-2 cell line, which helped overcome multidrug resistance (MDR) [21]. It had also been proven that ATRA was not a P-gp-transportable substance [22]. Thus, the increase in retinoid sensitivity induced by celecoxib could not be attributed to the celecoxib-induced inhibition of P-gp.

Retinoid signals are transduced primarily through the ligand activation of two families of nuclear retinoid receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). In the past two decades, RARβ has attracted much more attention due to findings of frequent loss of RARβ expression in various types of cancer including colon cancer, reduction of cell responsiveness to retinoid-mediated growth inhibition, or increase in the degree of tumorigenicity by knockdown RARβ gene or blockage of its expression using an antisense strategy [4, 23]. Unlike 9-cis retinoic acid, ATRA only activates RARs, which makes it an ideal model for the study of retinoid resistance. The results of this study showed that at clinically relevant concentrations, celecoxib increased the expression of RARβ, which correlated with cell responsiveness to ATRA. This yielded strong evidence of how celecoxib enhanced ATRA-mediated anti-cancer effects.

Celecoxib was reported to act through both COX-2-dependent and COX-2-independent mechanisms. The latter included the inactivation of phosphatidylinositol 3-kinase/Akt, the modulation of NF-κB-dependent gene transcription and peroxisome proliferator-activated receptors (PPARs), alterations in cell-cycle regulatory proteins, and the induction of death receptor expression [18, 24, 25]. Studies proved that COX-2 could suppress 4HPR inhibitory and apoptotic effects in breast cancer cells [10, 11]. However, our results showed that celecoxib acted through COX-2-independent mechanisms to

increase cellular ATRA sensitivity. Firstly, 10 μM of NS398 had similar effects on PGE_2 production to 5 μM of celecoxib in the HT-29 cell line, but 10 μM of NS398 did not affect the inhibitory effects of ATRA. Secondly, celecoxib enhanced the anti-cancer effects of ATRA in both COX-2 high-expressing and COX-2 low-expressing human colon cancer cell lines. Finally, PGE_2 did not suppress the enhanced growth-inhibitory and apoptosis-inducing effects of the ATRA and celecoxib combination. Meanwhile, a study reported by Bruno Lefebvre proved that the phosphoinositide 3-kinase/Akt signaling pathway caused an increased tethering of the corepressor silencing mediator for retinoic and thyroid hormone receptors (SMRT) to the RARβ promoter, down-regulated the RARβ expression, and impaired cellular differentiation in response to the retinoid in P19 and HeLa Cells [26]. A study conducted by Schroeder discovered that even 5 μM of celecoxib could reduce phosphorylation levels of Akt and its direct downstream substrate GSK-3β in lung cancer cell lines, and the effects were augmented by a combination of celecoxib and 4HPR [12]. Consequently, we postulate that the celecoxib and ATRA combination may increase the expression of RARβ and cellular retinoid sensitivity through inactivation of phosphatidylinositol 3-kinase/Akt.

In summary, our study provides evidence for the enhanced growth-inhibitory and apoptosis-inducing effects of ATRA in combination with celecoxib at clinically relevant concentrations in colon cancer cell lines. Celecoxib acts through COX-2-independent mechanisms to increase the expression of RARβ and the cellular ATRA sensitivity. Further testing of this combination regimen in *in vivo* models and potentially also in clinical trials is warranted.

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