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Cyclooxygenase-2-dependent and -independent inhibition of proliferation of colon cancer cells by 5-aminosalicylic acid

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ABSTRACT

The cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) pathway may have a pathogenic role in colorectal cancer (CRC). Recent studies suggest that 5-aminosalycilic acid (5-ASA) reduces the risk of inflammatory bowel disease-related CRC, but the mechanism by which 5-ASA interferes with CRC cell growth remains unknown. In this study, we have examined whether the negative effect of 5-ASA on CRC cells is dependent on COX-2/PGE2 axis inhibition. We show that 5-ASA down-regulates both constitutive and TNF-α or IL-1β-induced COX-2 in HT-115 and HT-29 cells. Inhibition of COX-2 by 5-ASA occurs at the RNA and protein level, and is associated with a significant decrease in PGE2 synthesis, arrest of growth and enhanced death of CRC cells. However, exogenous PGE2 does not revert the 5-ASA-mediated CRC cell proliferation block. 5-ASA also inhibits the growth of DLD-1, a COX-deficient CRC cell line, thus suggesting that the anti-proliferative effect of 5-ASA on CRC cells is not strictly dependent on the inhibition of COX-2/PGE2. Taken together our data indicate that 5-ASA causes both a COX-2-dependent and -independent inhibition of CRC cell growth.

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1. Introduction

Patients with inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) have an increased risk for the development of colorectal cancer (CRC), that is influenced in part by the duration and anatomical extent of the disease, as well as by the severity of the ongoing inflammation [1]. Present CRC management techniques in IBD patients include surveillance colonoscopy and/or colectomy, but such strategies have not yet been proven to reduce mortality [1]. The development of safe and effective chemo-preventive measures for reducing the risk of CRC would thus be of substantial benefit to IBD patients.

Several experimental animal studies, large retrospective and prospective population-based studies indicate that regular intake of non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of developing polyps and sporadic CRC, and may induce the regression of adenomas in familial adenomatous polyposis (FAP) [2–8]. A major molecular target for CRC chemoprevention by these agents is the cyclooxygenase (COX) [2,3]. COX transforms arachidonic acid into prostaglandin (PG)G2, an unstable intermediate which is rapidly converted to PGH2. Subsequently, PGH2 is metabolized into different structurally related PG, including PGE2, PGD2, PGF2, PGI2 and thromboxane A2 [9]. COX-1 is the constitutive isoform of this enzyme, and is expressed in a wide range of mammalian tissues. It functions as a housekeeping gene that controls the production of prostacyclins, PG and tromboxane, which are essential for physiological functions, such as protection of gastric mucosa, platelet aggregation and dynamics of the renal microvasculature. In contrast, COX-2 is constitutively expressed in human kidney and brain, and it is

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inducible by inflammatory cytokines, growth factors, oncogenes, serum and tumour promoters in several cell types [9,10]. COX-1 expression remains unaltered in CRC, whereas increased levels of COX-2 have been seen in 50% of colorectal adenomas and in up to 85% of sporadic CRC [10,11]. Evidence also suggests that COX-2 could play a role in the pathogenesis of CRC complicating the natural history of patients with IBD [12,13].

The clinical relevance of these observations relates to the demonstration that specific pharmacological inhibition or genetic ablation of COX-2 can prevent the development of CRC and can even cause regression of existing adenomatous polyps in both humans and rodents [3,14]. Moreover, blockade of COX-2 associates with a reduced frequency of colitis-driven CRC in mice [13]. It has been postulated that the antitumor effect of NSAIDs, including COX-2 inhibitors, is mediated through reduction in PG production, most notably PGE2, which is frequently overexpressed in CRC tissues [2,3]. Nonetheless, more recent studies suggest that the role of COX-2 in the pathogenesis of CRC may be more complex than that indicated by initial studies. In fact, various COX-independent targets of NSAIDs have been described, as well as there is evidence that some agents that induce COX-2 are chemopreventive [15,16]. Unfortunately, the wide spread use of NSAIDs and COX-2 inhibitors in the chemoprevention of CRC has been limited by their frequent and often severe side effects [17,18]. Moreover, because NSAIDs may aggravate the symptoms of colitis, their sustained use for the purpose of cancer chemoprevention is relatively contraindicated in IBD patients [19]. Thus, there is a need to identify alternative chemopreventive agents that are more appropriate for use in IBD patients.

Mesalazine is a 5-aminosalicylic acid (5-ASA) compound largely used for maintaining remission, as well as in the treatment of mildly flare-ups in IBD. 5-ASA is safe and free of serious adverse effects. Recent epidemiological studies suggest that long-term consumption of 5-ASA reduces the risk of CRC developing in patients with IBD [12,20,21]. Additionally, several experimental studies have shown that 5-ASA markedly reduces the growth and survival of CRC cells [22–25]. In this context, we have previously shown that 5-ASA inhibits epidermal growth factor receptor (EGFR) activation, a transmembrane tyrosine kinase which triggers mitogenic signalling in CRC cells [26]. Moreover, Bos et al. showed that 5-ASA affects the Wnt/β-catenin pathway in CRC cells via the inhibition of the phosphatase 2A, and degradation of β-catenin [27]. Finally, Rousseaux et al. demonstrated that 5-ASA interacts with and enhances the expression and activation of PPAR-γ, a negative regulator of colonic inflammation and cancer [28]. Despite these advances, the precise mechanisms by which 5-ASA inhibits CRC cell growth have not been fully established. In this study, we have analyzed if 5-ASA inhibits COX-2/PGE2 axis in CRC cells and evaluated whether the regulatory effects of 5-ASA on CRC cells are strictly dependent on the control of this pathway.

2. Methods and materials

2.1. Cell culture

5-ASA was kindly provided by Giuliani S.p.A. (Milan, Italy) and dissolved as a 100 mM stock solution in culture medium. The pH of the drug solution was adjusted to 7.4 with NaOH, and experiments carried out protected from light. All reagents were from Sigma–Aldrich (Milan, Italy) unless specified. The human CRC cell lines, HT-29 and DLD1, were maintained in McCoy's 5A and RPMI 1640 medium, respectively, both supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 50 μg/ml gentamycin. The human CRC cell line HT-115 was maintained in DMEM medium supplemented with 15% FBS and 1% penicillin/streptomycin. To examine whether 5-ASA affects COX-2 expression, cells were cultured in appropriate media supplemented with FBS and antibiotics in 12-well culture dishes. After reaching 60–70% confluence, cells were starved for 12 h. Non-adherent cells were then removed and fresh medium containing 0.05% bovine serum albumin (BSA) was added to each well. Cultures were performed in the presence or absence of 5-ASA (5–30 mM) or equivalent concentrations of mannitol for 24 h. Additionally, to evaluate whether 5-ASA was able to reduce the expression of COX-2 induced by pro-inflammatory cytokines, serum starved HT-29 cells were pre-incubated with 15 mM 5-ASA for 1 h and then treated with TNF-α or IL-1β (10 ng/ml, R&D Systems, Minneapolis, MN, USA) for 1–5 h. In parallel, cells were pre-incubated with cycloheximide (10 μg/ml, Sigma–Aldrich) for 1 h prior to adding 5-ASA. At the end, cells were harvested and used for extracting total proteins or RNA.

2.2. Cell proliferation

Cell proliferation was assessed by using carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA), which covalently binds cell components to yield a fluorescence that is divided equally between daughter cells at each division. Single-cell suspensions were plated at 2×10^5 cells/ml/well in six-well culture dishes and allowed to adhere overnight. Cells were then labelled with CFSE according to the manufacturer's instruction. After 30 min, the medium was removed and fresh media containing 0.05% BSA, and the desired concentrations of the test compounds were added and incubated for further 24 h. At the end, cells were collected, washed twice with PBS 1×, and then incubated with 5 μg/ml of propidium iodide (PI) for 15 min, at 4 °C in the dark. CFSE and/or PI-positive cells were determined by flow-cytometry (Becton Dickinson, FACSCalibur, Milan, Italy) and the data were analyzed using ModFit LT 2.0 (Verity Software House Inc., ME, USA). To examine whether exogenous PGE2 were able to revert the 5-ASA-mediated anti-proliferative effect on HT-115, CFSE-labelled cells were pre-incubated with 1 μg/ml PGE2 (Sigma–Aldrich) for 1 h prior to adding 5–30 mM 5-ASA. After 24 h, the percentage of proliferating cells was evaluated by flow cytometry.

2.3. Analysis and quantification of cell death

To score cell death, cells were either left untreated or treated with increasing doses of 5-ASA (5–30 mM) for 24–48 h. Cells were then collected, washed twice in PBS 1×, stained with FITC-annexin V (AV, 1:100 final dilution) according to the manufacturer's instructions (Becton

Dickinson) and incubated with 5 μg/ml PI for 30 min at 4 °C, and their fluorescence was measured using FL-1 and FL-2 channels of FACSCalibur using Cell Quest Pro software.

2.4. PGE2 assay

HT-115 cells were plated at 4×10^3 cells/well in 96-well culture dishes in DMEM medium containing 0.5% FBS and allowed to adhere overnight. Cells were treated with graded doses of 5-ASA (15–30 mM) or mannitol (30 mM) for 22 h, and PGE2 secretion was then measured by a commercially available ELISA Kit (R&D Systems).

2.5. Western blotting

Total proteins were extracted using the following lysis buffer: 10 mmol/l HEPES, 1 mmol/l EDTA, 60 mmol/l KCl, 0.2% Igepal CA-630, 1 mmol/l sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l DTT and 1 mmol/l PMSF, separated on an 10% SDS-PAGE gel, and blots were then incubated with a mouse anti-human monoclonal COX-1 or COX-2 antibody (sc-19998 and sc-19999, respectively; 1:500 final dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by a rabbit anti-mouse antibody conjugated to horseradish peroxidase (1:20,000 final dilution). After analysis of COX-1 and COX-2 each blot was stripped and incubated with a mouse-anti-human monoclonal β-actin antibody (1:5000) to ascertain equivalent loading of the lanes. Computer-assisted scanning densitometry (Total Lab, AB.EL Science-Ware Srl, Rome, Italy) was used to analyze the intensity of the immunoreactive bands.

2.6. RNA extraction, cDNA preparation and real-time PCR

Analysis of COX-1 and COX-2 RNA expression was performed by real-time PCR. Total RNA was extracted from cells by using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Milan, Italy). A constant amount of RNA (1 μg/sample) was retro-transcribed into complementary DNA (cDNA) and 1 μl of cDNA/sample was then amplified using the following conditions: denaturation 1 min at 95 °C, annealing 30 s at 62 °C for COX-2 and β-actin or 30 s at 58 °C for COX-1, followed by 30 s of extension at 72 °C. Primers sequence was as follows: COX-2, FWD: 5′-TTC TTT GCC CAG CAC TTC ACG C-3′ and REV: 5′-CTG TCT AGC CAG AGT TTC ACC G-3′; COX-1, FWD: 5′-GTC TCT TGC TCT GGT TCT TG-3′ and REV: 5′-AGG TGG CAT TGA CAA ACT CC-3′. Real-time PCR was performed using the IQ SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy). β-actin (FWD: 5′-AAG ATG ACC CAG ATC ATG TTT GAG ACC-3′ and REV: 5′-AGC CAG TCC AGA CGC AGG AT-3′) was used as an internal control.

2.7. Statistical analysis

Values are expressed as mean ± S.D. or S.E.M. To evaluate the difference in means between groups, the Student *t*-test was used and significance was defined as *P*-values less than 0.05.

3. Results

3.1. 5-ASA dose-dependently inhibits the growth and induces apoptosis in HT-115

COX-2 is over-expressed in the vast majority of CRC, and inhibition of COX-2 in CRC cells results in a decreased growth and survival [2,6–8]. Therefore, COX-2 may represent an important target for CRC chemoprevention. However, there is evidence that various NSAIDs, including selective COX-2 inhibitors, can regulate CRC cell activity by modulating also COX-2-independent pathways [15,29]. As a starting point for our studies, we evaluated whether 5-ASA regulates the proliferation and survival of HT-115, a CRC cell line which expresses COX-2. To this end, we used the intracellular fluorescent label CFSE to tag the proliferating cells, and therefore to determine the proliferation history of specific cell populations. As illustrated in Fig. 1A, 5-ASA significantly and dose-dependently inhibited the percentage of CFSE-labelled cells after 24 h culture. No significant change in cell growth was seen in mannitol-treated cells (not shown). We next

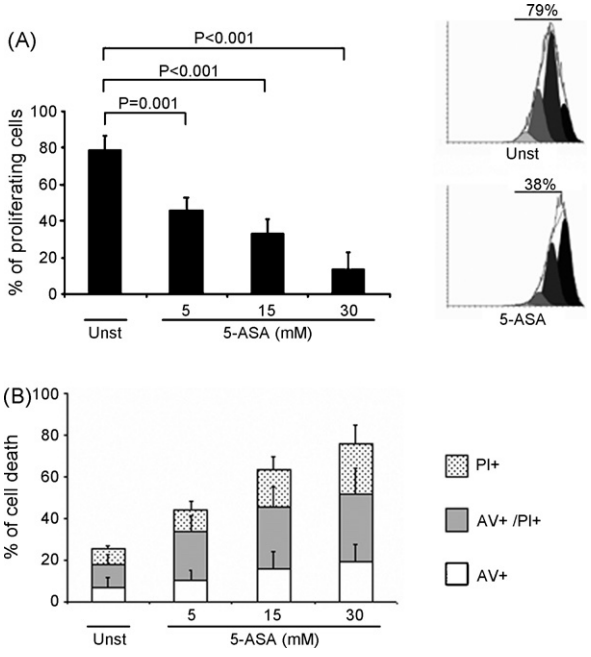

Fig. 1 – (A) 5-ASA dose-dependently inhibits the growth of HT-115 cells. CFSE-labelled cells were either left unstimulated (Unst) or treated with graded doses of 5-ASA. After 24 h, the percentage of proliferating cells was evaluated by flow cytometry. Data indicate mean ± S.D. of five different experiments. (Right insets) Representative histograms of CFSE-labelled HT-115 cells either left unstimulated (Unst) or treated with 15 mM 5-ASA. Numbers above lines indicate the percentages of proliferating cells. (B) 5-ASA significantly enhances HT-115 cell death in a dose-dependent manner. HT-115 cells were either left unstimulated (Unst) or treated with 5-ASA (5–30 mM) for 48 h. Data indicate the percentage of cell death as assessed by FACS analysis of annexin V and/or propidium iodide (PI)-positive cells, and are expressed as mean ± S.D. of three experiments.

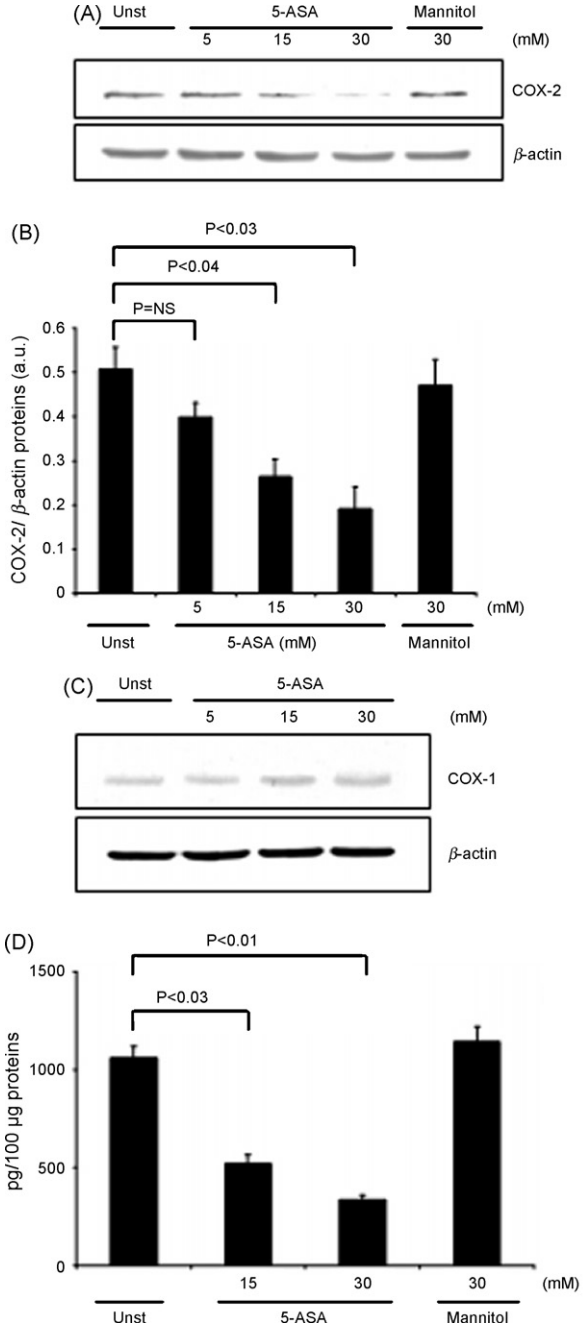

Fig. 2 – (A) 5-ASA down-regulates COX-2 in a dose-dependent fashion. HT-115 cells were either left unstimulated (Unst) or treated with graded doses of 5-ASA or mannitol (30 mM) for 24 h. COX-2 and β-actin were analyzed by Western blotting. One of three representative Western blots is shown. (B) Quantitative analysis of COX-2/β-actin protein ratio in total extracts of HT-115 cells either left unstimulated (Unst) or treated with graded doses of 5-ASA or mannitol (30 mM) for 24 h, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± S.E.M. of three experiments. (C) 5-ASA does not affect COX-1 protein expression in HT-115 cells. HT-115 cells were either left unstimulated (Unst) or treated with graded doses of 5-ASA (5–30 mM) for 24 h. COX-1 and β-actin were analyzed by Western blotting. One of three representative Western blots is shown. (D) 5-ASA inhibits PGE2 secretion in HT-115 cells. Cells were treated with 5-ASA (15–30 mM) or mannitol (30 mM) for 24 h, and PGE2 secretion was then measured by ELISA. Data are expressed as pg per 100 μg total protein of cell supernatants, and indicate the mean ± S.D. of three separate experiments.

assessed whether the 5-ASA-induced CRC cell growth arrest was accompanied by any change on cell death. A time-course analysis of 5-ASA effect on the percentage of AV and/or PI-positive cells showed that 24 h treatment with 5-ASA resulted in no increase in cell death compared with untreated cells (data not shown). Analysis at later time points (i.e. 48 h) revealed however that 5-ASA dose-dependently reduced the survival of HT-115 cells (Fig. 1B). In particular, by flow-cytometry it was shown that 5-ASA significantly enhanced the fraction of AV-positive/PI-negative cells (16.3 ± 7.6 and $19.5 \pm 8.1\%$ in cells treated with 15 and 30 mM 5-ASA, respectively, versus $7.15 \pm 4.7\%$ in untreated cells; $P < 0.04$ and $P = 0.01$, respectively), while no significant increase was seen in cells treated with 5 mM 5-ASA ($10.7 \pm 4.6\%$). Moreover, 5-ASA significantly enhanced the percentage of AV-positive and PI-positive cells (23.2 ± 7, 29 ± 10 and $32.1 \pm 12.2\%$ in cells treated with 5, 15 and 30 mM 5-ASA versus $10.7 \pm 4.6\%$ in untreated cells, $P < 0.02$). Finally, a significant increase in AV-negative/PI-positive cells was seen in cultures added with 30 mM ($24.4 \pm 8.6\%$, $P < 0.04$) but not 15 mM ($18.4 \pm 6.3\%$) or 5 mM ($10.5 \pm 3.5\%$) 5-ASA compared to untreated cultures ($7.65 \pm 1.5\%$).

These results collectively suggest that the 5-ASA-mediated block in cell growth precedes the induction of death.

3.2. 5-ASA inhibits COX-2 expression and PGE2 synthesis in HT-115 cells

In subsequent experiments, we evaluated whether the ability of 5-ASA to regulate HT-115 cell growth and survival was associated with changes in the expression/activity of COX-2. For this purpose, we treated HT-115 cells with graded doses of 5-ASA for 24 h, and then total extracts were analyzed for COX-2 expression by Western blotting. COX-2 was detectable in unstimulated cells and was down-regulated by 5-ASA (Fig. 2A and B). COX-2 expression quantitated by densitometry and normalized by β-actin expression was significantly reduced in HT-115 cells treated with 15 and 30 mM 5-ASA (Fig. 2B, $P < 0.04$ and $P < 0.03$, respectively) but not in cells treated with 5 mM 5-ASA (Fig. 2B). Mannitol (30 mM) did not alter COX-2 protein expression (Fig. 2A and B). By contrast, 5-ASA did not alter the expression of COX-1 in HT-115, and this was evident at RNA (not showed) and protein level (Fig. 2C).

COX-2 enzyme catalyzes the synthesis of PGE2 [9,10]. To examine whether the 5-ASA-mediated down-regulation of COX-2 was biologically active, we measured the level of PGE2 in the culture supernatants of cells either left untreated or treated with 15 and 30 mM 5-ASA. As shown in Fig. 2D, both doses of 5-ASA significantly reduced the PGE2 secretion by HT-115 cells. No reduction in PGE2 secretion was seen in mannitol-treated cells, indicating that the effect of 5-ASA

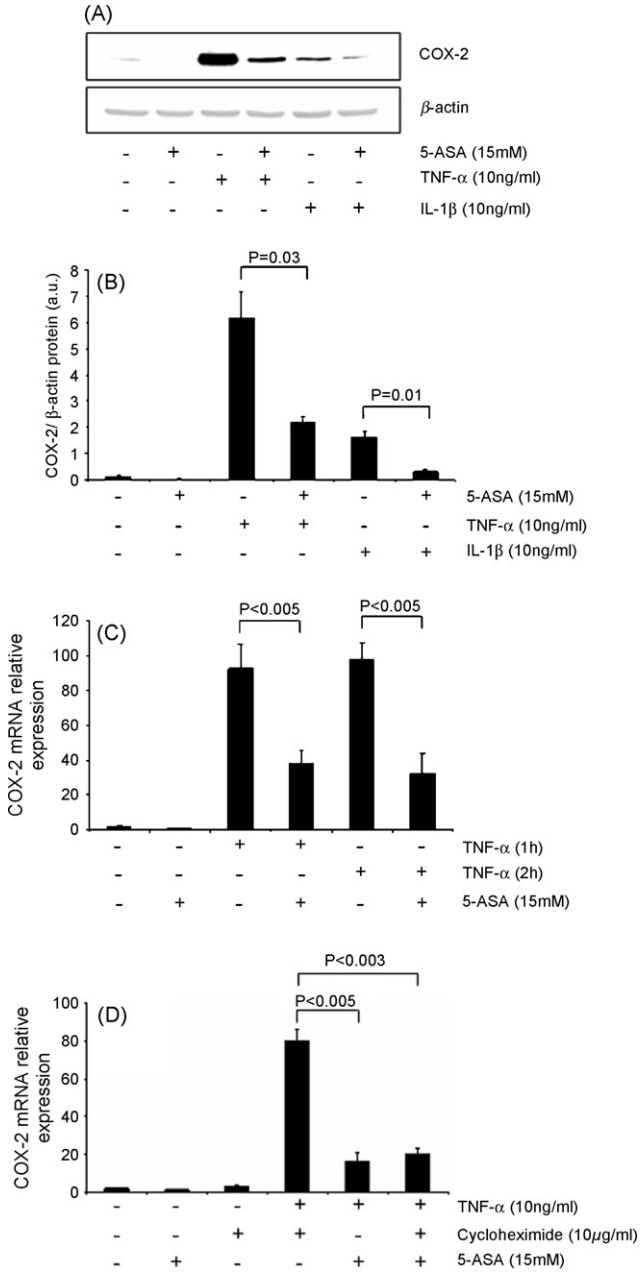

Fig. 3 – 5-ASA reduces COX-2 mRNA and protein expression induced by proinflammatory cytokines in HT-29 cells. (A) Representative Western blots showing COX-2 and β-actin in HT-29 cells either left untreated or treated with TNF-α or IL-1β in the presence or absence of 15 mM 5-ASA for 5 h. (B) Quantitative analysis of COX-2/β-actin protein ratio in total extracts of HT-29 cells either left untreated or treated with TNF-α or IL-1β in the presence or absence of 15 mM 5-ASA for 5 h, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.) and are the mean ± S.E.M. of three experiments. (C) Analysis of COX-2 RNA expression in HT-29 cells either left untreated or treated with TNF-α for 1–2 h in the presence or absence of 5-ASA 15 mM. Levels are normalized to β-actin. Values are mean ± S.D. of three experiments. (D) Analysis of COX-2 RNA expression in HT-29 cells cultured in the presence or absence of cycloheximide for 30 min, followed by stimulation with or without 5-ASA for 1 h and then TNF-α for further 2 h. Levels are normalized to β-actin. Values are mean ± S.D. of three experiments.

on PGE2 secretion was not due to osmolarity shifts into the culture medium.

3.3. 5-ASA negatively regulates the expression of COX-2 induced by TNF-α and IL-1β in HT-29 cells

Since COX-2 expression is positively regulated by inflammatory cytokines, such as TNF-α and interleukin (IL)-1β that are supposed to play a pathogenic role in IBD [30–32], we next examined if 5-ASA was able to inhibit the TNF-α and IL-1β-mediated induction of COX-2. To this end, COX-2 analysis was performed in HT-29 cells, since these cells lines are known to functionally respond to TNF-α and IL-1β with enhanced induction of COX-2 [30,31]. HT-29 cells were pre-incubated with 15 mM 5-ASA for 1 h and then treated with TNF-α and IL-1β for 5 h. Western blotting analysis of total extracts showed that COX-2 expression was increased by TNF-α and IL-1β (Fig. 3A and B). Importantly, 5-ASA significantly reduced the expression of COX-2 induced by both TNF-α and IL-1β. To confirm further these results, we assessed whether 5-ASA also inhibited the cytokine-induced COX-2 RNA expression. By real-time PCR it was shown that COX-2 mRNA was increased in HT-29 cells stimulated by TNF-α (Fig. 3C) or IL-1β (not shown). Induction of COX-2 RNA was maximal at 1 h and remained elevated at 2 h. Notably, treatment of cells with 5-ASA markedly reduced the COX-2 RNA expression induced by TNF-α (Fig. 3C) or IL-1β (not shown), and this was evident at each time point analyzed. Moreover, pre-incubation of HT-29 cells with cycloheximide did not revert the 5-ASA-mediated inhibition of TNF-α-induced COX-2 RNA expression (Fig. 3D).

3.4. The anti-mitogenic effect of 5-ASA on HT-115 is not reverted by exogenous PGE2

To examine if the 5-ASA-induced anti-proliferative effect on HT-115 was strictly dependent on the inhibition of COX-2/PGE2 axis, cells were treated with graded doses of 5-ASA in the presence or absence of exogenous PGE2. The addition of PGE2 to HT-115 cells cultured in the absence of 5-ASA slightly but not significantly increased the fraction of proliferating cells. Notably PGE2 reverted neither the block in growth (Fig. 4) nor the induction of death (not shown) in 5-ASA-treated HT-115 cells. However, PGE2 enhances the phosphorylation of EGFR in HT-115 cells thus indicating that it was active in our systems.

3.5. 5-ASA inhibits the growth but does not enhance the death of DLD-1, a COX-2-deficient CRC cell line

Overall the above results suggest that 5-ASA exerts its anti-proliferative effects through a mechanism that is not entirely dependent on the inhibition of COX-2/PGE2 pathway. To test further this hypothesis, we analyzed the effect of 5-ASA on growth and death of DLD-1, a COX-2-deficient CRC cell line [33]. For this purposes, CFSE-labelled DLD1 cells were treated

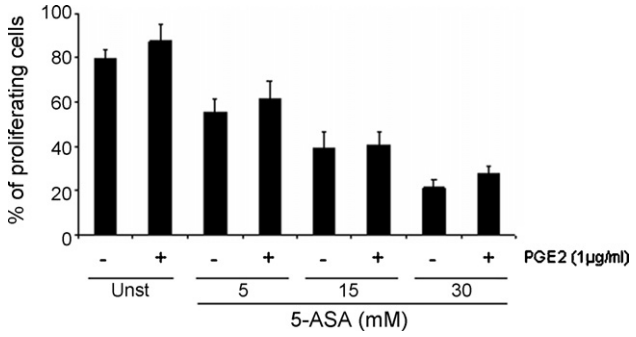

Fig. 4 – Exogenous PGE2 does not revert the 5-ASA-mediated anti-proliferative effect on HT-115 cells. Cells were pre-incubated with PGE2 for 1 h prior to adding 5–30 mM 5-ASA. After 24 h, the percentage of proliferating cells was evaluated by flow cytometry. Data indicate mean ± S.D. of five different experiments.

with increasing doses of 5-ASA for 24 h, and then the fraction of proliferating cells was evaluated by flow-cytometry. As shown in Fig. 5A, 5-ASA dose-dependently inhibited DLD-1 cell growth. By contrast, 5-ASA did not affect the viability of these cells both at 48 h (Fig. 5B) and later time points (data not shown). DLD-1 cells are known to have undetectable level of COX-1, even though a previously published study showed that these cells can express COX-1 RNA [34]. Therefore, to confirm that the anti-proliferative effect of 5-ASA on DLD-1 was COX independent, we assessed the effect of 5-ASA on COX-1 expression in these cells. COX-1 RNA transcripts were evident in DLD-1 but remained unchanged following 5-ASA treatment (Fig. 5C). By contrast, no COX-1 protein was seen regardless of whether DLD-1 were treated or not with 5-ASA (Fig. 5D).

4. Discussion

This study was undertaken to examine whether the ability of 5-ASA to regulate CRC cell growth and death is strictly dependent on the inhibition of COX-2, the inducible form of COX, whose expression is up-regulated in the inflamed gut of patients with IBD [35] and in the majority of neoplastic lesions of the colon [11]. We first show that 5-ASA inhibits the growth of HT-115, a CRC cell line that expresses a functionally active COX-2, and this anti-mitogenic effect of 5-ASA is associated with a marked down-regulation of COX-2 at the protein and RNA level. Consistently, secretion of PGE2 by HT-115 is significantly reduced by 5-ASA. Second, 5-ASA inhibits the expression of COX-2 induced in HT-29 cells by inflammatory cytokines, such as TNF-α and IL-1β. Such an effect is direct and not mediated by de novo protein synthesis, because pre-incubation of cells with cycloheximide does not prevent the down-regulation of COX-2 induced by 5-ASA in TNF-α-treated cells.

The exact molecular mechanism by which 5-ASA inhibits COX-2 expression remains to be ascertained. Multiple signalling pathways have been reported to modulate COX-2 induction in CRC cells. For example, inflammatory cytokines can enhance COX-2 gene transcription by promoting MAP

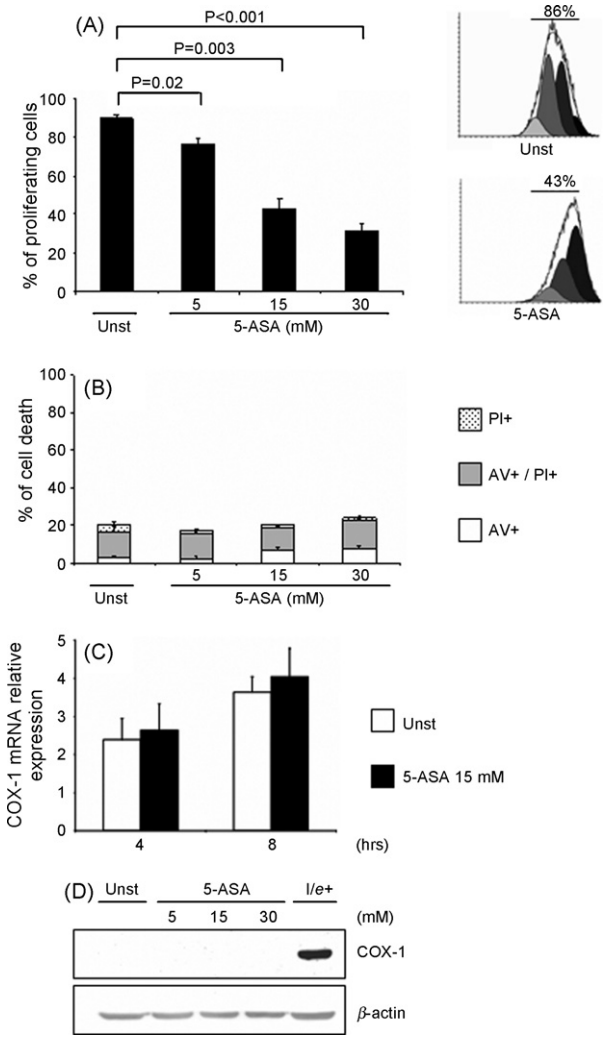

Fig. 5 – (A) 5-ASA dose-dependently inhibits the growth of COX-deficient DLD-1 cells. CFSE-labelled DLD-1 were either left unstimulated (Unst) or treated with graded doses of 5-ASA. After 24 h, the percentage of proliferating cells was evaluated by flow cytometry. Data indicate mean ± S.D. of five different experiments. (Right insets) Representative histograms of CFSE-labelled DLD-1 cells either left untreated (Unst) or treated with 15 mM 5-ASA. Numbers above lines indicate the percentages of proliferating cells. (B) 5-ASA does not affect DLD-1 cell death. DLD-1 cells were either left unstimulated (Unst) or treated with 5-ASA (5–30 mM) for 48 h. Data indicate the percentage of cell death as assessed by FACS analysis of annexin V and/or propidium iodide (PI)-positive cells, and are expressed as mean ± S.D. of three experiments. (C) 5-ASA does not affect COX-1 RNA expression in DLD-1. Cells were treated with or without 15 mM 5-ASA for the indicated time points, and COX-1 transcripts were then evaluated by real-time PCR. Levels are normalized to β-actin. Values are mean ± S.D. of three experiments. (D) Representative Western blots showing COX-1 in DLD-1 cells cultured with or without graded doses of 5-ASA for 24 h. Extracts of HT-115 cells were used as a positive control (ve+).

kinase and NF-kB activation [36]. It is also known that drugs that reduce p38 MAP kinase activity may destabilize COX-2 RNA [37]. Since it is known that 5-ASA inhibit such pathways in epithelial cells [38], it is plausible that the 5-ASA-mediated negative regulation of COX-2 may be in part due to the control of MAP kinase and NF-kB activity. Another possibility is that 5-ASA may suppress COX-2 through the negative regulation of EGFR activation and/or APC/β-catenin pathway [26,27,39]. Finally, it is also possible that 5-ASA interferes with the rate of protein synthesis and/or proteasome-mediated degradation of COX-2 [40].

The fact that 5-ASA-mediated arrest of HT-115 cell proliferation occurs at doses that are lower than those required to suppress COX-2 (5 mM) and that exogenously added PGE2 does not revert the inhibitory effect of 5-ASA on HT-115 cell growth however suggest that the effect of 5-ASA on CRC cell growth is partially independent of COX-2/PGE2 inhibition. This is supported further by the demonstration that 5-ASA also decreases the growth of DLD-1, a CRC cell line that does not express COX protein and therefore does not produce PGE2.

Time-course analysis of AV and PI-positive cells in cultures added with 5-ASA also reveals that HT-115 cells undergo death following 5-ASA exposure. This phenomenon is seen however only after 48 h stimulation with 5-ASA, and therefore it follows and does not precede the 5-ASA-mediated cell growth arrest. These data exclude therefore the possibility that the anti-proliferative action of 5-ASA is due to a toxic effect of the drug. 5-ASA is thus different from other traditional NSAIDs that have been used for chemoprevention in CRC, as these agents inhibit the growth of CRC cells by mostly promoting apoptosis [33]. We also show that 5-ASA does not reduce the survival of DLD-1, raising the possibility that the lack of COX-2 may limit the susceptibility of DLD-1 cells to undergo apoptosis. This finding is not totally unexpected, given that previous studies have shown that SC-58125, a COX-2 antagonist, induces apoptosis in H-ras-transformed RIE cells that express COX-2 but not in the non-transformed parental control cells that do not express COX-2 [41]. A key question arising from these studies is how the inhibition of COX may induce apoptosis. A possibility is that inhibition of COX results in the accumulation of the PG precursor arachidonic acid, which would in turn stimulate the conversion of sphingomyelin to ceramide, thereby triggering apoptotic signals [42]. In this context, it is however noteworthy that other NSAIDs, including sulindac sulfide and piroxicam, are effective in inducing apoptosis and inhibiting proliferation in cells lacking COX-2 [33], thus suggesting that inhibition of COX-2 is not an essential requirement for the induction of CRC cell death by all NSAIDs.

Translation of data generated *in vitro* culture systems is difficult because the interactions of anti-neoplastic drugs with cancer cells *in vivo* are much more complex that those suggested by experimental studies. Moreover, the *in vitro* control of CRC cell activity is usually obtained with concentrations of drugs that could appear not realistic for *in vivo* studies. In the specific case of 5-ASA, we would however like to point out that, in line with data generated *in vitro* systems, administration of 5-ASA to patients with sporadic colonic polyps or CRC reduces the proliferation and increases the apoptotic rate in the tumor samples [43,44]. It is also noteworthy that the anti-mitogenic effect of 5-ASA documented in the present and other *in vitro* studies was obtained with a concentration of drug which is in a range similar to that reached within the gut tissue under standard oral treatment [45].

The demonstration that 5-ASA reduces the growth of both COX-2-positive and -negative CRC cells would imply that chemoprevention of CRC by 5-ASA is not strictly influenced by the expression of COX-2 within the tumour tissue. At the same time however, the fact that 5-ASA enhances the rate of apoptosis only in cells that express COX-2 suggests that COX-2-positive CRC cells are more susceptible than COX-2-negative cells to the anti-neoplastic effect of 5-ASA. Further studies will be necessary to confirm these results, and establish whether data presented herein and generated using CRC cell lines can be extrapolated to IBD-related CRC.

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