Original article

A differential effect of 5-ASA and NSAIDs on colonic epithelial cell proliferation

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SUMMARY

5-Aminosalicylic acid (5-ASA), a weak inhibitor of cyclo-oxygenase (COX), has been extensively used in the treatment of inflammatory bowel diseases (IBD) for induction and maintenance of remission. Furthermore, 5-ASA treatment has been suggested to reduce the related risk for colorectal cancer development in IBD. Although the clinical safety and efficacy of 5-ASA has been well known for many years the related mechanism of action on intestinal epithelia has not been fully elucidated. We examined the effect of 5-ASA on proliferation and apoptosis/necrosis of Caco-2 human colonic epithelial cells. In comparison, we examined the effect of three non-steroid anti-inflammatory drugs-NSAIDs- (indomethacin, aspirin and CAY10404) with different COX-inhibitory specificities. We show a differential effect of 5-ASA on colonic epithelial cells survival compared to NSAIDs that suggests an alternative, COX-independent, mechanism of action of 5-ASA on intestinal epithelia.

Key Words: 5-ASA, NSAIDS, Colorectal cancer, Chemoprevention, Inflammatory Bowel Diseases, COX-2 inhibitors, CAY 10404.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), collectively termed inflammatory bowel diseases are characterized by chronic relapsing and remitting intestinal inflammation associated with an increased risk of colorectal cancer development closely related to the duration of disease, the extent of colonic involvement, early age of onset, and the presence of primary sclerosing cholangitis. Although the risk of colorectal cancer in this specific IBD population varies widely in different studies there is a consensus that long term control of colonic inflammation is negatively associated with colonic cancer development while appropriate endoscopic surveillance contributes to early detection of high grade colonic dysplasia or cancer.[1-5] The ideal agent for IBD treatment should be effective in controlling intestinal and extraintestinal complications of IBD and could be used as a life-time treatment, with minimal side effects, in order to achieve long-time remission, eliminate the need for colectomy and act as a chemopreventive agent of colonic neoplasia development.

The chemopreventive efficacy of NSAIDs against intestinal tumors has been well established. Previous studies have demonstrated that NSAIDs may exert an antiproliferative effect on colonic neoplasia, inducing apoptosis, while it has been reported that indomethacin, piroxicam, and sulindac as well as aspirin significantly reduce tumor incidence.[6-9] It is postulated that this antitumor effect is mostly mediated through reduction of cyclo-oxygenase (COX) production, most notably COX-2, which is frequently overexpressed in intestinal tumors, while an additional COX-2 -independent apoptotic mechanism of action has been suggested.[10-12]

5-Aminosalicylic acid (5-ASA) is a well tolerated anti-inflammatory drug that has been used extensively in the treatment of IBD with limited systemic adverse effects, and gastrointestinal toxicity.[13] Several studies have suggested that the long-term use of 5-ASA in IBD patients may significantly reduce the risk of development of colorectal cancer. [14-16] 5-ASA lacks the side effects of NSAIDs because it is rapidly metabolized to pharmaco-

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logically inactive metabolite N-acteyl-5- aminosalicylate and looks like an attractive candidate for the chemoprevention of colorectal neoplasia.

While the anti-inflammatory and antitumor efficacy of NSAIDs is closely related to inhibition of COX, it seems that 5-ASA exerts its anti-inflammatory effect in a COX-independent manner. Noticeably, previous studies have shown a clear effect of 5-ASA on induction of apoptosis, but there are contradictory reports on the effect of 5-ASA on proliferation of colonic epithelial cells. The purpose of this study was to examine the effect of 5-ASA on proliferation and apoptosis/necrosis of Caco-2 human colonic epithelial cells. In comparison, we examined the effect of three NSAIDs, indomethacin, a traditional COX-1 inhibitor, aspirin, a non selective COX inhibitor and CAY10404, a novel hyperselective COX-2 inhibitor, on proliferation of colonic epithelial cells.

MATERIALS AND METHODS

Materials

5-ASA, aspirin, indomethacin and MTT were from Sigma-Aldrich (Steinheim, Germany) while CAY 10404 was obtained from Cayman Chemical (Michigan, USA). Down fresh 5-ASA in PBS (0.5 mg/ml) was used in culture, while stock solutions in 99.8% ethanol of aspirin (3mg /ml) and indomethacin (25 mg/ml) and a stock solution (17 mg/ml) of CAY 10404 in DMF were used in culture. All cell culture reagents and plastics were from Gibco BRL Life Technologies (Paisley, UK) and Nalge Nunc (Hereford, UK) respectively.

Cell Culture

Caco-2 colonic epithelial cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, Wiltshire UK). Cells were passaged weekly and grown at 37°C in MEM, which were supplemented with 10% foetal bovine serum (FBS), penicillin/streptomycin (10U/ ml and 10μg/ml) and fungizone (0.5μg/ml), as previously described.[17]

Cell Viability and Growth Assay

Caco-2 cell lines were seeded in 24 well plates at an initial concentration of $2X10^4$ cells/well. This resulted in 60-70% confluence after 6 days of culture confirmed by an initial experiment. The effect of Aspirin, Indomethacin, CAY10404 and 5-ASA was assessed one day after seeding. For incubations lasting 6 days, medium and compounds were renewed every 3 days. Each treatment was done in triplicate. After 6 days of treatment, growth and viability of cells were measured by the tetrazolium salt assay (MTT) as described by Mosmann *et al*.[18]

Measurement of apoptosis-necrosis

Assays were performed using a cell death detection ELISA kit based on the photometric sandwich-enzyme-immunoassay principle and the use of mouse monoclonal antibodies directed against cytosolic DNA fragments and histones. Caco-2 cells were seeded in a 96 well plate at an initial concentration of $1.5X10^4$cells/well, according to manufacturer's instructions. After 24 hours media was replaced with media containing 5-ASA or CAY10404. Cells were treated for 24 hours and ELISA was performed in supernatants and cell lysates in order to detect necrotic and apoptotic cells respectively. Briefly, three incubation steps were performed. First, the antihistone antibody was fixed on the wall of a microtiter plate. Second, the nucleosomes contained in the sample were bound via their histone to the antihistone antibody. Third, anti-DNA-peroxidase was added to react with the DNA part of the nucleosome. The amount of peroxidase retained in the sample was determined photometrically (absorbance at 405/490 nm) with 2, 2-azino-di-(3-ethylbenzthiazoline sulfonate) as a substrate.

Statistical analysis

Unless otherwise indicated values represent mean ± SEM of at least three independent experiments. Comparative statistical evaluation between groups was done by ANOVA. Bonferroni *t* post-hoc analysis and Student's *t* test were used for statistical comparison between individual variables as appropriate. A probability value of $p<0.05$ was taken as the criterion for statistical significance.

RESULTS

5-ASA had no effect on proliferation of Caco-2 cells.

Proliferation of Caco-2 cells was assessed following culture of Caco-2 for 6 days in the presence of various concentrations of 5-ASA (0-100 μM), a preferential COX-1 inhibitor that is Indomethacin (0-800 μM), a non-selective COX-1/2 inhibitor that is Aspirin (0-10 mM) and a new selective COX-2 inhibitor that is CAY10404 (0-100 μM). Proliferation rate was significantly suppressed by indomethacin, aspirin and CAY 10404, in a concentration dependent manner, compared to untreated cells. The maximal antiproliferative effect was observed with concentrations of indomethacin greater than 200 μM, aspirin greater than 2.5 mM and CAY10404 greater than 20 μM. Interestingly, we did not observe a similar antiproliferative effect after treatment with 5-ASA (Figure 1).

5- ASA and CAY10404 induce apoptosis of Caco-2 cells

While concentrations of 5-ASA used in our experiments did not exert any significant dose-dependent anti-proliferative effect on Caco-2 cells we examined whether similar concentrations of 5-ASA (10 and 100 μM) had any effect on apoptosis of Caco-2 cells. We also examined the effect of the hyperelective COX-2 inhibitor CAY10404 (5 and 50 μM) on apoptosis of Caco-2 cells that was hypothesised to induce apoptosis in our cells. Surprisingly, 5-ASA induced apoptosis of Caco-2 cells either used in concentrations of 10 μM or 100 μM ($p<0.05$ and $p<0.01$ respectively). As expected, CAY10404 (50 μM) produced a significant induction of apoptosis of Caco-2 cells ($p<0.05$) (Figure 2).

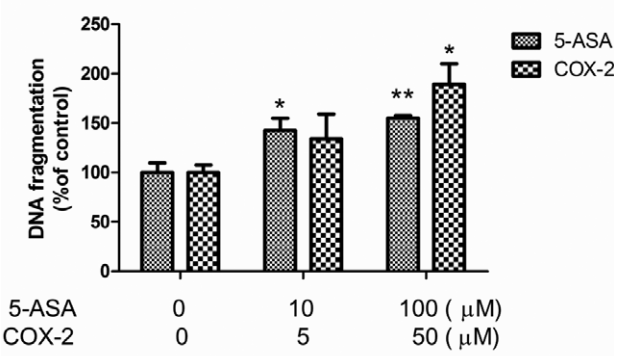

Figure 2. Apoptosis of intestinal epithelial cells in response to 5-ASA and COX-2 inhibitor. Caco-2 cells were seeded in a 96 well plate at an initial concentration of $1.5X10^4$cells/well.After 24 hours media was replaced with media containing 5-ASA or a hyperselective COX-2 inhibitor (CAY10404). DNA fragmentation representing apoptosis was measured in cell lysates (*$p<0.05$, **$p<0.01$ refer to difference from values of control -vehicle).

The effect of 5-ASA and CAY10404 on necrosis of Caco-2 cells

In order to evaluate whether the effect of 5-ASA and CAY10404 on Caco-2 cells is specifically related to apoptosis we further examined the effect of 5-ASA and CAY10404 on necrosis of Caco-2 cells. 5-ASA (100 μM) significantly induced necrosis ($p<0.05$) while 10 μM had no effect on necrosis of Caco-2 cells. Noticeably, we did not observe any significant effect of CAY10404 on necrosis of Caco-2 in both concentrations used (Figure 3).

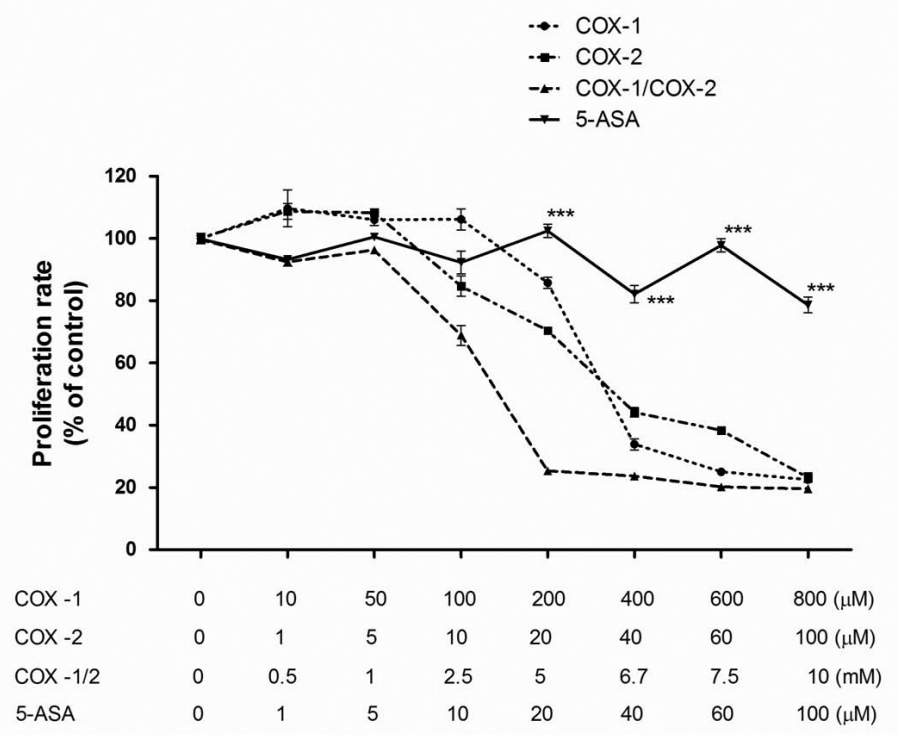

Figure 1. Intestinal epithelial cell proliferation rate in response to NSAIDs and 5-ASA. Caco-2 cells were cultured for 6 days in the presence of various concentrations of COX-inhibitors of different specificities that is a preferential COX-1 inhibitor (Indomethacin), a hyperselective COX-2 inhibitor (CAY10404), and a non-selective COX-1/2 inhibitor (Aspirin) or 5-ASA. *** $P<0.001$ refers to comparison of 5-ASA to COX inhibitors.

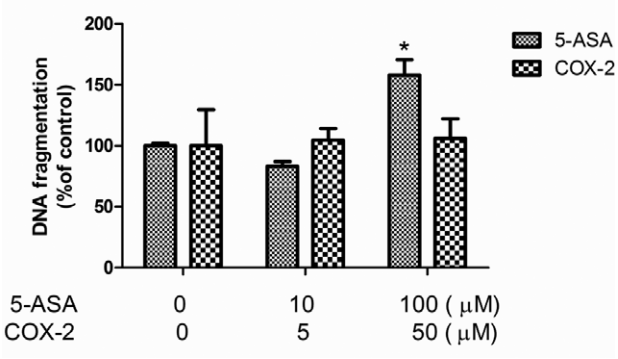

Figure 3. Necrosis of intestinal epithelial cells in response to 5-ASA and COX-2 inhibitor. Caco-2 cells were seeded in a 96 well plate at an initial concentration of $1.5X10^4$cells/well.After 24 hours media was replaced with media containing 5-ASA or a hyperselective COX-2 inhibitor (CAY10404). DNA fragmentation representing necrosis was measured in cell supernatants (*$p<0.05$ refers to difference from values of control -vehicle).

DISCUSSION

In the present study, we demonstrated that all NSAIDs used (aspirin, indomethacin and CAY10404) exhibited a significant dose-dependent effect on proliferation of Caco-2 cells. Overall, the profile of this antiproliferative effect was similar among the three agents indicating that it is not dependent on the COX-2 selectivity of the agent used. COX-2 inhibition is thought to play a basic role in intestinal epithelia proliferation.[19] Results from rodent models of carcinogenesis previously suggested that both non-selective and selective NSAIDs effectively inhibit the early stages of intestinal tumor development, whereas only selective COX-2 inhibitors are effective when treatment is delayed.[20] The clinical relevancy of this result might be the inhibition of intestinal mucosal healing that is observed in patients on NSAIDs' therapy and which could be related with intestinal ulceration, a well-known side effect of COX-inhibitors.[21]

NSAIDs cannot be used in IBD patients, because of their association with the development of ulcerations in the small and large intestine and disease aggravation. However, in non-IBD populations, long-term use of NSAIDs, even in small doses,[22] is associated with significant toxicity primarily related to moderate or severe, life-threatening, gastrointestinal complications. Accordingly, Health organizations and consensus groups have been appropriately cautious by withholding any recommendation regarding the use of NSAIDs for the prevention or treatment of cancer, except for the use of celecoxib or sulindac to suppress the growth of colorectal adenomatous polyps in patients with familial adenomatous polyposis-FAP.[23] Thus, there is a need to identify alternative chemopreventive agents in IBD, as well as in other high-risk groups of patients.

In our experiments 5-ASA, a weak COX inhibitor, used in concentrations achievable in vivo, had no significant effect on proliferation of Caco-2 cells and this might explain its safety profile according to intestinal ulceration in every day clinical practice.[24] Although in previous studies there are contradictory results about the effect of 5-ASA on proliferation of intestinal epithelial cells, our data in Caco-2 cells, suggest that this effect is non-significant.[25-27] On the other hand it seems that either 5-ASA or NSAIDS significantly promote apoptosis of intestinal epithelial cells. This effect has been previously suggested as a preventive mechanism for colorectal neoplasia development. Our data are in accordance with previously published studies outlining the safety and clinical efficacy of 5-ASA in chemoprevention of colorectal neoplasia at least in IBD patients.[15]

The exact mechanism by which 5-ASA acts in colonic epithelia has not yet been fully elucidated. It has been previously suggested that 5-ASA decreases survival of colonic epithelial cells inducing an accumulation of cells in the S phase.[28] Previous studies indicated an antiproliferative effect of 5-ASA even on epithelial cell lines that do not express COX-2.[29] Our experiments showed a differential effect of 5-ASA on survival of Caco-2 intestinal epithelial cell line in comparison with three COX inhibitors. These results indicate an alternative mechanism of action of 5-ASA on intestinal epithelia that, at least in part, might not be COX dependent. Further clinical and experimental research is warranted in order to elucidate the exact mechanism of action of 5-ASA on colonic epithelia that could extend its use in clinical practice.

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