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Relationship between endogenous colony stimulating factors and apoptosis in human colon cancer cells: role of cyclo-oxygenase inhibitors

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1 Nonsteroidal anti-inflammatory drug (NSAID) usage is associated with gastrointestinal inflammatory damage and aggravation of gut inflammatory conditions. NSAIDs also exert a preventive effect against colon cancer that seems to be due to increased colon cell apoptosis. NSAIDs have been shown to modulate the release of colony stimulating factors (CSFs) in some cells. In the present study we analysed the effect of these drugs on secretion of CSFs and apoptosis in human colon epithelial cells (HT-29).

2 HT-29 cells secreted bioactive levels of GM-CSF, G-CSF and M-CSF when stimulated with IL-1ß and TNF-α, and diclofenac ($10^{-7}-10^{-4}$ M), indomethacin ($10^{-7}-10^{-4}$ M) and sodium salicylate ($10^{-5}-10^{-2}$ M) induced concentration-dependent increases in GM-CSF secretion.

3 Reduced secretion of G-CSF and M-CSF and increased cell apoptosis were observed with the highest concentrations of these non-selective NSAIDs.

4 No changes in any CSF release or HT-29 cell apoptosis were detected in the presence of the COX-2 selective inhibitor DFP ($10^{-7}-10^{-4}$ M).

5 Neither the exogenous addition of CSFs nor the blockade of secreted CSFs modified apoptosis in HT-29 cells stimulated with cytokines and/or NSAIDs.

6 These results suggest that colon epithelial cells can contribute to local inflammatory responses by releasing CSFs and thus extend the life span of local leukocytes. Modulation of CSF levels by non-selective NSAIDs may be involved in the pro-inflammatory effects of these agents in the gut.
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Abbreviations: CSFs, colony stimulating factors; G-CSF, granulocyte-CSF; M-CSF, macrophage-CSF; GM-CSF, granulocyte-macrophage-CSF; NSAIDs, nonsteroidal anti-inflammatory drugs

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly self-prescribed drugs world-wide. However, in addition to their therapeutic benefits NSAIDs induce inflammatory damage in the gastrointestinal tract. These drugs can induce direct injury of the gastrointestinal mucosa as well as exacerbate pre-existing gastrointestinal diseases, such as ulcerative colitis. Most of the beneficial and deleterious effects of the NSAIDs have been linked to their ability to suppress prostaglandin synthesis through inhibition of the activity of the enzyme cyclo-oxygenase (COX) (Vane, 1971). Two isoforms of this enzyme, COX-1 and COX-2, have been characterized. COX-1 is constitutively expressed in the gastrointestinal tract. Prostaglandins generated by this isoenzyme are mainly involved in homeostatic functions and its inhibition seems to account for some of the injurious actions of the NSAIDs (Langenbach *et al*., 1995). COX-2 is mainly induced at sites of inflammation (Mitchell & Evans, 1998). This isoenzyme is not present in healthy gastrointestinal mucosa, but significant amounts of COX-2 have been detected in pathological gut conditions such as peptic ulcer and *H. pylori* gastritis (Fu *et al*., 1999), inflammatory bowel disease (Singer *et al*., 1998) and colon cancer (Eberhart *et al*., 1994).

More recently, NSAIDs have been discovered to have antineoplasic influences in the gastrointestinal tract, especially in the colon. In particular, retrospective clinical analysis has shown regular aspirin consumption to be associated with a reduced incidence of colon cancer (Thun *et al*., 1991). Moreover, NSAIDs reduce the development of colon tumours in susceptible rats (Rao *et al*., 1995). The retardation by NSAIDs of the development of colon cancer appears linked to their ability to stimulate colon epithelial cell apoptosis, as has been demonstrated *in vitro* in several colon cancer cell lines (Shiff & Rigas, 1997). However, the mechanisms responsible for NSAID-induced cell apoptosis are far from clear at the moment since conflicting results about the relative importance in this effect of inhibition of COX-1 *vs* COX-2 have been reported.

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In other, non-gastrointestinal cells NSAIDs have been shown to modulate the release of colony stimulating factors (CSFs) such as granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF) and granulocyte-macrophage-CSF (GM-CSF) (Saunders *et al*., 1997; Stanford *et al*., 2000). These CSFs stimulate the proliferation and differentiation of cells in the bone marrow and also promote the activation and survival of mature leukocytes at inflammatory sites (Lopez *et al*., 1986; Metcalf, 1986). CSFs extend the life span of mature leukocytes by inhibiting their apoptotic death (Branch *et al*., 1992) and we have recently observed that these cytokines are also able to modulate apoptosis in colon cancer epithelial cells (Calatayud *et al*. submitted). Thus, we have evaluated (i) the ability of human colon cancer epithelial (HT-29) cells to secrete GM-CSF, G-CSF and M-CSF, (ii) the effects of non-selective and COX-2 selective NSAIDs in the secretion of CSFs and apoptosis in these cells, as well as (iii) the involvement of endogenous CSFs in NSAID-induced apoptosis.

Methods

Cell culture

HT-29 cells (Human Caucasian Colon adenocarcinoma Grade II) were obtained from the European Collection of Animal Cell Culture. Cells were seeded at a density of 2×10^4 per well in 96 well plates, cultured in McCoy's medium with 2 mM glutamine and 10% foetal calf serum (37°C, 5% CO_2) and grown in monolayers to confluency. Cells were serum starved for 24 h prior to use. Cells were then incubated with the cytokines interleukin-1ß (IL-1ß, 10 ng ml^{-1}) and tumour necrosis factor α (TNF-α, 10 ng ml^{-1}) in the presence or absence of different concentrations of the non-selective NSAIDs indomethacin, diclofenac or sodium salicylate or the COX-2 selective inhibitor DFP (5,5-dimethyl-3-(2-propoxy)-4-methanesulphonylphenyl)-2(5H)-furanone) (Leblanc *et al*., 1999).

Measurement of CSFs

The concentration of GM-CSF, G-CSF or M-CSF was measured in cell culture supernatant at 4 h or 24 h after treatment using specific ELISAs constructed from commercially available components. In brief, 96 well plates were coated with the correspondent capture antibodies (rat anti-human GM-CSF, G-CSF or M-CSF, R&D Systems). Following 1 h blocking (1% BSA, 5% sucrose and 0.05% NaN_3 in PBS, 100 μl per well), 100 μl of standard or undiluted cell culture supernatant was added and incubation continued for a further 2 h. The wells were subsequently incubated with the respective detection antibodies (rat anti-human GM-CSF, G-CSF or M-CSF, R&D Systems, 2 h) and streptavidin peroxidase (Sigma, 30 min). Finally, 100 μl of substrate solution (1 : 1 mixture of tetramethylbenzidine and hydrogen peroxide) was added and the reaction stopped 30 min later by addition of 50 μl of 1N H_2SO_4. Absorbance was then read at 450 nm (λ correction 550 nm) and the concentrations of CSFs calculated from the standard curves.

Measurement of apoptosis

At the end of each treatment the level of apoptosis was measured. Apoptosis was measured by the degree of cytoplasmic histone/associated DNA fragments (mono- and oligonucleosomes) by ELISA (Programmed Cell Death Detection ELISA, Roche) according to the manufacturers recommendations. Briefly, the medium was removed and the cells lysed in 200 μl of the lysis buffer provided. The lysate was centrifuged and 20 μl of supernatant added to a streptavidin-coated microtiter plate with a mixture of mouse biotin-labelled anti-histone and mouse peroxidase-conjugated anti-DNA. After 2 h-incubation period, the plate was washed and the retained peroxidase determined photometrically with ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) as a substrate. Optical density was measured at 405 nm (λ correction 492 nm).

Measurement of cell death

Cellular respiration was assessed by mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan. At the end of the experiment, medium was removed from the cells and replaced with 100 μl of warm (37°C) medium containing 0.2 mg ml^{-1} MTT. After incubation for a further 30 min at 37°C the medium containing the MTT was removed and the cells dissolved in 100 μl dimethyl sulphoxide (DMSO). The extent of MTT conversion to formazan was quantified by measurement of optical density at 550 nm with a wavelength correction of 650 nm.

Experimental protocols

Drugs or vehicle (0.1% DMSO) were added in 10 μl aliquots to wells of cells containing 90 μl of culture medium. The COX-1/COX-2 'non-selective' NSAIDS used were diclofenac ($10^{-7}-10^{-4}$ M), indomethacin ($10^{-7}-10^{-4}$ M), sodium salicylate ($10^{-5}-10^{-2}$ M) and the COX-2 selective inhibitor used was DFP ($10^{-7}-10^{-4}$ M, IC_{50}(COX-1)$>10^{-4}$, IC_{80}(COX-2)$=5\times10^{-6}$) (Warner *et al*., 1999). After 4 or 24 h medium was removed for the measurement of CSFs. The effects of endogenously produced CSFs on apoptosis was determined by adding neutralizing antibodies raised specifically to human GM-CSF, G-CSF or M-CSF (10 μg ml^{-1} each, R&D Systems). Using neutrophil apoptosis as a bioassay of the activity of the CSFs, this level of antibody was sufficient to bind the levels of CSF released in by HT-29 cells in this study (Stanford *et al*., 2000). In other experiments, human recombinant GM-CSF, G-CSF or M-CSF (10 ng ml^{-1} each) were exogenously added to the culture medium. Each treatment was tested in triplicate, on a minimum of three separate occasions.

Drugs

Diclofenac, indomethacin, sodium salicylate and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. The COX-2 selective inhibitor 5,5-dimethyl-3-(2-propoxy)-4-methanesulphonylphenyl)-2(5H)-furanone (DFP) was a gift from Merck-Frosst Labs. IL-1ß and TNF-α were purchased from Roche and human recombinant GM-CSF, G-CSF and M-CSF from R&D Systems.

Results

CSF production by HT-29 cells

In the absence of cytokines, HT-29 cells released low or undetectable levels of GM-CSF, G-CSF or M-CSF. When the cells were incubated with IL-1ß and TNF-α (10 ng ml^{-1} both) for 4 h the levels of GM-CSF, but not G-CSF and M-CSF were increased. However, the levels of all three CSFs were significantly increased when cells were incubated with IL-1ß plus TNF-α for 24 h (Figure 1).

Effects of NSAIDs on CSF release by HT-29 cells

Diclofenac, indomethacin and sodium salicylate induced concentration-dependent increases in GM-CSF secretion by cytokine-treated HT-29 cells (Figure 2). In contrast, the release of G-CSF and M-CSF was reduced by diclofenac and sodium salicylate. Indomethacin also reduced the release of G-CSF, but had no effect on that of M-CSF (Figure 2). DFP had no effect on the release of any of the CSFs tested (Figure 2). None of these NSAIDs modified CSF secretion by non-cytokine treated HT-29 cells.

Effects of cytokines and NSAIDs on HT-29 cell apoptosis and viability

When measured at 4 h, the basal level of HT-29 cell apoptosis was unaffected by treatment with IL-1β plus TNF-α (10 ng ml^{-1}) either in the presence or absence of indomethacin (10^{-7} to 10^{-4} M), diclofenac (10^{-7} to 10^{-4} M) or DFP (10^{-7} to 10^{-4} M) (Table 1). However, at 4 h, sodium salicylate at the highest concentration tested (10^{-2} M) induced a significant increase in apoptosis in the presence, but not in the absence, of cytokines (Table 1). By contrast after 24 h of incubation, apoptosis of HT-29 cells was significantly increased by IL-1ß plus TNF-α (Table 2; Figure 3). At the highest concentrations tested diclofenac (10^{-4} M), indomethacin (10^{-4} M) or sodium salicylate (10^{-2} M) also increased apoptosis of HT-29 over 24 h (Figure 3), an effect that was found to be additive to that of IL-1β plus TNF-α (Figure 3). By contrast to observations made with non-selective inhibitors of COX, the selective COX-2 inhibitor DFP did not influence HT-29 cell apoptosis in any of the protocols used (Table 1, Figure 3).

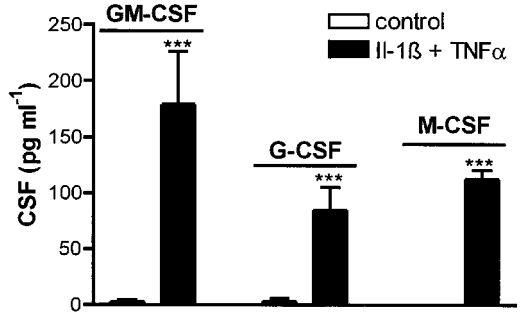

Figure 1 Secretion of GM-CSF, G-CSF and M-CSF by HT-29 cells incubated with IL-1ß and TNF-α (10 ng ml^{-1} both) for 24 h. Results expressed as mean±s.e.mean. ***$P<0.001$ *vs* respective control value (paired Student's *t*-test).

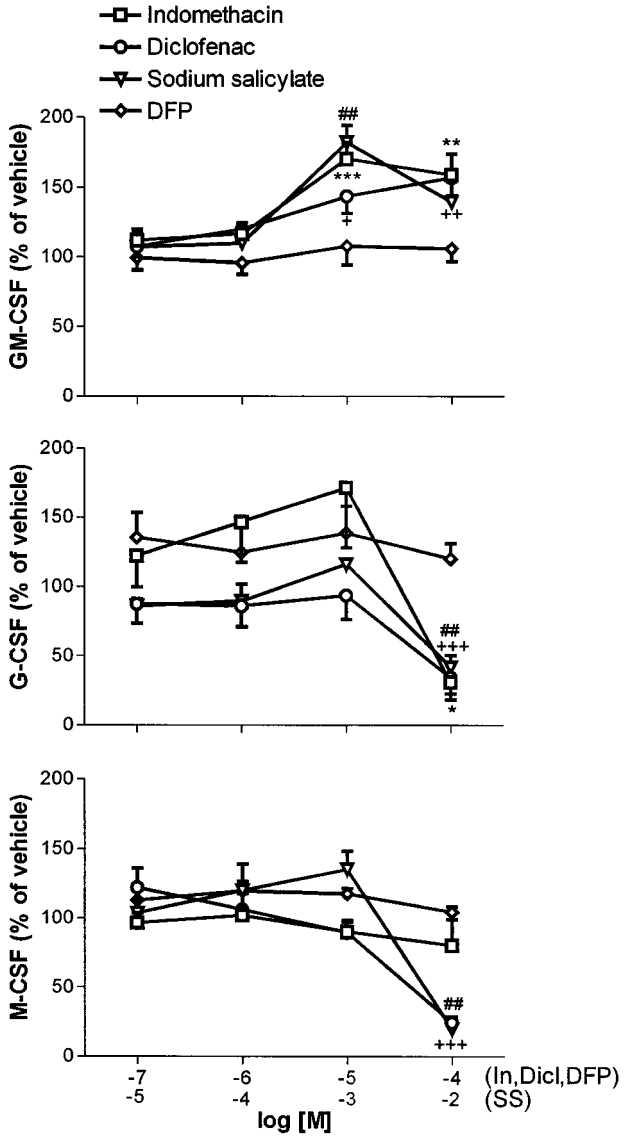

Figure 2 Effect of non-selective COX inhibitors (diclofenac, indomethacin and sodium salicylate) and the COX-2 selective inhibitor (DFP) on GM-CSF, G-CSF and M-CSF secretion by HT-29 cells co-incubated with IL-1ß and TNF-α (10 ng ml^{-1} both) for 24 h. Results expressed as mean±s.e.mean. $+P<0.05$, $++P<0.01$ and $+++P<0.001$ *vs* control value for diclofenac treated cells, $*P<0.05$, $**P<0.01$ and $***P<0.001$ *vs* control value for indomethacin treated cells, $\#\#P<0.01$ *vs* control value for sodium salicylate treated cells (ANOVA followed by Dunnet's test).

The increase in apoptosis of HT-29 cells observed after incubation with cytokines or NSAIDs was accompanied by reductions in cell viability (Figure 4).

Effect of endogenous and exogenous CSFs on apoptosis of HT-29 cells

Spontaneous apoptosis observed in HT-29 cells over 24 h was unaffected by the addition of exogenous GM-CSF, G-CSF or M-CSF (10 ng ml^{-1} each; Table 2). Furthermore, neither GM-CSF, G-CSF nor M-CSF had any effect on apoptosis induced by diclofenac (10^{-4} M) or IL-1ß plus TNF-α (10 ng ml^{-1} both) or a combination of diclofenac plus

Table 1 Effect of NSAIDs on apoptosis in HT-29 cells

Diclofenac	–	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Medium	0.77±0.12	0.84±0.14	0.80±0.11	0.77±0.14	0.70±0.11
II-1β+TNF-α	0.79±0.14	0.87±0.16	0.81±0.14	0.82±0.18	0.83±0.14
Indomethacin	–	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Medium	0.86±0.13	0.78±0.10	0.79±0.10	0.79±0.10	0.91±0.11
II-1β+TNF-α	0.91±0.13	0.91±0.12	0.90±0.10	0.94±0.15	0.98±0.15
Sodium Salicylate	–	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
Medium	0.58±0.12	0.53±0.07	0.56±0.06	0.62±0.07	0.79±0.08
II-1β+TNF-α	0.51±0.05	0.55±0.11	0.54±0.09	0.59±0.06	1.33±0.22***,###
DFP	–	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Medium	0.56±0.08	0.50±0.08	0.55±0.09	0.58±0.08	0.62±0.05
II-1β+TNF-α	0.56±0.08	0.55±0.07	0.61±0.06	0.56±0.08	0.55±0.07

HT-29 cells were incubated with non-selective COX inhibitors (diclofenac, indomethacin and sodium salicylate) and the COX-2 selective inhibitor (DFP) in the presence or absence of IL-1β and TNF-α (10 ng ml^{-1} both) for 4 h. Results (optical density) expressed as mean±s.e.mean. ***$P<0.001$ *vs* value in the corresponding IL-1β plus TNF-α treated cells, ### *vs* value in cells treated with 10^{-2} M of sodium salicylate (ANOVA followed by Tukey's test).

Table 2 Effects of CSFs on spontaneous apoptosis induced by diclofenac and cytokines

	–	*M-CSF*	*G-CSF*	*GM-CSF*
–	0.58±0.02	0.53±0.03	0.52±0.05	0.55±0.03
Diclofenac	1.06±0.03	0.96±0.07	1.12±0.04	1.14±0.04
II-1β + TNF-α	0.96±0.06	1.00±0.15	0.95±0.11	0.97±0.11
Diclofenac + II-1β + TNF-α	2.02±0.05	2.12±0.09	2.13±0.09	2.09±0.06

HT-29 cells were incubated with diclofenac (10^{-4} M), cytokines II-1β + TNF-α, 10 ng ml^{-1} both) or the combination of diclofenac plus cytokines in the presence or absence of CSFs (10 ng ml^{-1} each). Apoptosis was measured 24 h after treatment. Results (optical density) expressed as mean±s.e.mean.

cytokines (Table 2). Similarly, when binding antibodies to GM-CSF, G-CSF or M-CSF were added alone, or in combination, no effect was seen on apoptosis of HT-29 cells under basal conditions or after induction with cytokines and diclofenac (Table 3).

Discussion

Apoptosis in colonic epithelial cells is part of their normal physiological cycle. When apoptosis of these cells fails hyperplasia occurs which can lead to cancer. Most recently, NSAIDs have been shown both to induce apoptosis of colonic epithelial cells and to reduce the risk of colon cancer in man (Shiff & Rigas, 1997). In leukocytes, which also undergo apoptosis, CSFs are important modulating factors (Branch *et al*., 1992). Here we confirm that some NSAIDs induce apoptosis of HT-29 cells. In addition, we show that HT-29 cells can under the influence of cytokines be stimulated to secrete three important CSFs and that NSAIDs can modulate these CSF releases.

In our study we found that diclofenac, indomethacin and sodium salicylate induced apoptosis in HT-29 cells. In contrast, the COX-2 selective inhibitor, DFP, was without effect. When NSAIDs did induce apoptosis it was only observed at concentrations in excess of those needed to inhibit COX-1 or COX-2 (Warner *et al*., 1999). These observations are in line with others showing that relatively high concentrations of NSAIDs induce apoptosis in human colon cancer cell lines, including HT-29 (Elder *et al*., 1997; Hanif *et al*., 1996; Piazza *et al*., 1997). The issue of high drug concentrations in these effects has brought into question the relevance of COX inhibition in inducing apoptosis (Piazza *et al*., 1997). In addition other studies have shown that metabolites of NSAIDs, devoid of effects on COX activity, can also induce apoptosis (Hanif *et al*., 1996). Furthermore, NSAID induced apoptosis has been observed in cells not expressing COX (Hanif *et al*., 1996). Finally, addition of exogenous prostaglandins does not reverse the apoptosis induced by NSAIDs (Hanif *et al*., 1996; Piazza *et al*., 1997). Thus, it seems likely that the effects of NSAIDs on apoptosis in human colon epithelial cells may be independent of COX inhibition. Activation of the nuclear peroxisome proliferator-activated receptors (PPARs) by NSAIDs has recently been suggested as an alternative pathway *via* which these drugs may induce colon cancer cell apoptosis and reduce the risk of colon cancer in man (Brockman *et al*., 1998). Notably, this idea may fit with the high concentrations of NSAIDs required to produce apoptotic effects since similarly high levels of NSAIDs are required to activate PPARs (Lehmann *et al*., 1997). NSAIDs have also been shown to directly modify mitochondrial function by inhibiting the respiratory chain (Somasundaram *et al*., 1997) and cause cytochrome *c* release which, consequently, activates the caspase cascade (Piqué *et al*., 2000). Moreover, the rapid pro-apoptotic action of sodium salycilate may be related to its ability to bind IKKβ and thus to inactivate the NF-κB pathway (Yin *et al*., 1998).

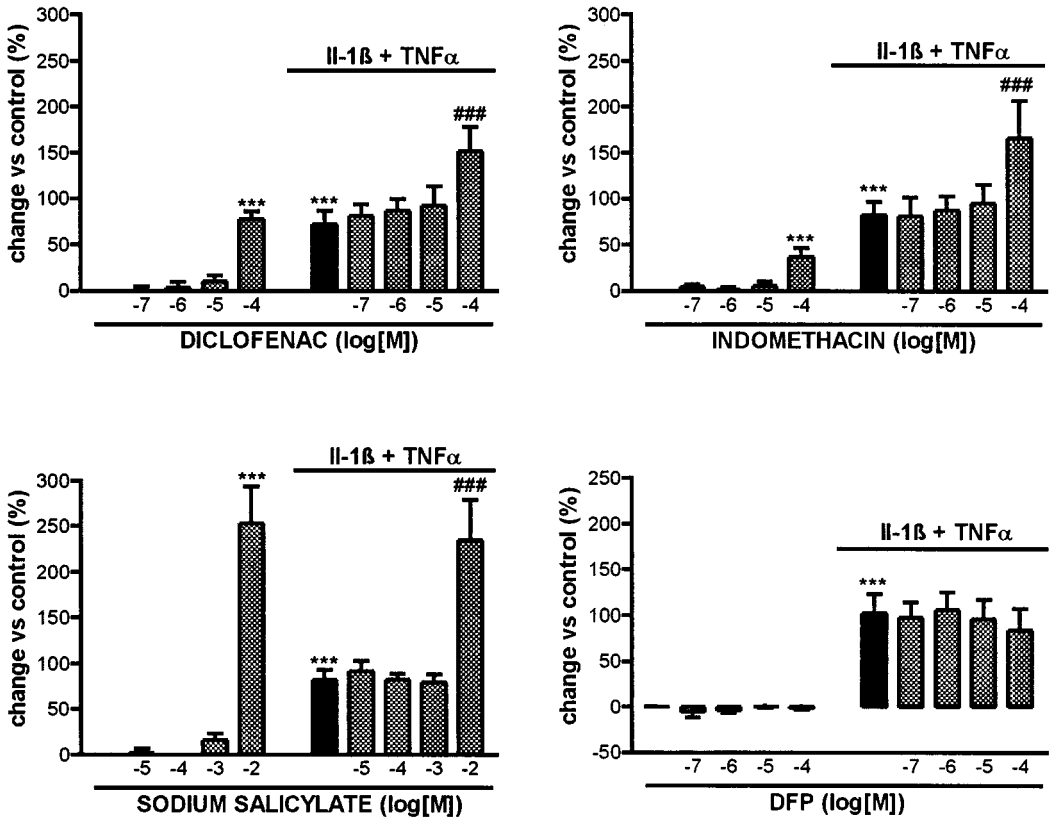

Figure 3 Effect of non-selective COX inhibitors (diclofenac, indomethacin and sodium salicylate) and the COX-2 selective inhibitor (DFP) on apoptosis in HT-29 cells under control conditions or co-incubated with IL-1ß and TNF-α (10 ng ml^{-1} both) for 24 h. Results expressed as mean$\pm$s.e.m. ***$P<0.001$ vs respective control value and ###$P<0.001$ *vs* value in cells treated with IL-1ß and TNF-α (ANOVA followed by Tukey's test).

In addition to the NSAIDs, we found that the combination of TNF-α and IL-1β also induced apoptosis in HT-29 cells. Similar observations recently made by Wright *et al.* (1999) have suggested that cytokine-induced apoptosis of HT-29 cells may involve an IL-13-dependent component. Interestingly, we found that co-treatment of HT-29 cells with NSAIDs and cytokines produced an 'additive' effect on apoptosis. Since the concentrations of cytokines used were maximal for the induction of apoptosis, this suggests that the NSAIDs and cytokines cause apoptosis of these cells by distinct mechanisms.

Enhanced GM-CSF secretion by HT-29 cells was observed after treatment with non-selective NSAIDs and a similar phenomenon occurred in human gastric biopsies from patients with gastritis (unpublished observations). This cytokine seems to be involved in the pathogenesis of colitis (McCartney *et al.*, 1999) and an increase in its local release could activate and extend the life span of local leukocytes and thus aggravate inflammation. We have previously shown that NSAIDs also increase GM-CSF production in human vascular (Stanford *et al.*, 2000) and airway smooth muscle cells (Saunders *et al.*, 1997) as well as in human synoviocytes (Breesse *et al.*, 2000). In the case of vascular cells and synoviocytes, COX-2 activity was identified as being responsible for inhibiting GM-CSF release (Breesse *et al.*, 2000). However, in the case of airway smooth muscle cells (Saunders *et al.*, 1997) or gastric tissue (unpublished observations), COX-1 activity appeared to be regulating GM-CSF release. The differences observed between these tissues in the relative roles of COX-1 *vs* COX-2 in regulating GM-CSF release, most likely reflects the levels of each enzyme expressed. COX-2 activity seems to be the main cause of prostaglandin synthesis in HT-29 cells stimulated with cytokines (Jobin *et al.*, 1998), however, in our experiments, the COX-2 selective inhibitor DFP did not modify GM-CSF release. This finding and the relatively high concentrations of non-selective NSAIDs needed to increase GM-CSF secretion suggest a COX-independent effect of these drugs.

By contrast to GM-CSF, the release of G-CSF or M-CSF was inhibited by some of the NSAIDs tested. G-CSF release was inhibited by diclofenac, indomethacin or sodium salicylate, whereas M-CSF release was inhibited by diclofenac and sodium salicylate, but not by indomethacin. These effects were only induced by the highest concentration of each NSAID and were, therefore, likely to be independent of COX inhibition. The reduction of G-CSF or M-CSF secretion could, however, be mediated by PPAR receptor activation leading to the induction of inhibitory pathways. Alternatively, the inhibition of M-CSF and G-CSF production by higher concentrations of NSAIDs may well be caused by the induction of apoptosis, which in leukocytes at least, results in an arrest of the release of inflammatory mediators (Haslett, 1999).

The abilities of CSFs to modulate leukocyte apoptosis/differentiation displays some level of selectivity for different cell populations. Indeed, GM-CSF is thought to act on a

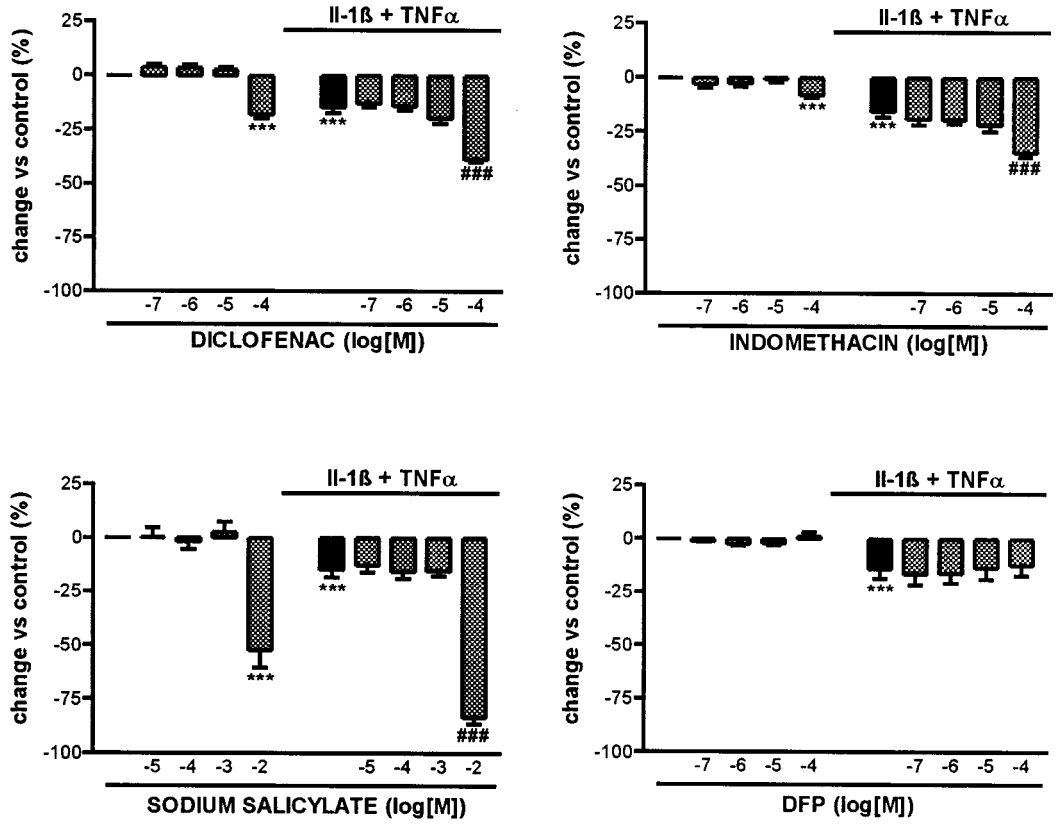

Figure 4 Effect of non-selective COX inhibitors (diclofenac, indomethacin and sodium salicylate) and the COX-2 selective inhibitor (DFP) on cell viability in HT-29 cells under control conditions or co-incubated with IL-1ß and TNF-α (10 ng ml^{-1} both) for 24 h. Results expressed as mean$\pm$s.e.mean. ***$P<0.001$ *vs* respective control value and ###$P<0.001$ *vs* value in cells treated with IL-1β and TNF-α (ANOVA followed by Tukey's test).

Table 3 Effects of CSFs neutralization on spontaneous apoptosis or apoptosis induced by diclofenac and cytokines

	–	*M-CSF-Ab*	*G-CSF-Ab*	*GM-CSFAb*	*M-G-GM-CSF-Abs*
–	0.58$\pm$0.02	0.51$\pm$0.03	0.50$\pm$0.03	0.54$\pm$0.03	0.50$\pm$0.01
Diclofenac	1.06$\pm$0.03	0.86$\pm$0.04	1.13$\pm$0.04	1.07$\pm$0.06	1.20$\pm$0.03
II-1β+TNF-α	0.96$\pm$0.06	0.95$\pm$0.14	0.88$\pm$0.09	0.89$\pm$0.08	0.84$\pm$0.04
Diclofenac+II-1β+TNF-α	2.02$\pm$0.05	2.13$\pm$0.10	2.12$\pm$0.12	1.95$\pm$0.06	2.17$\pm$0.05

HT-29 cells were incubated with diclofenac (10^{-4} M), cytokines II-1β+TNF-α, 10 ng ml^{-1} both) or the combination of diclofenac plus cytokines in the presence or absence of CSFs neutralizing antibodies (10 μg ml^{-1} each). Apoptosis was measured 24 h after treatment. Results (optical density) expressed as mean$\pm$s.e.mean.

wider range of leukocytes including neutrophils, eosinophils and monocytes, whereas G-CSF is thought to be active mainly on neutrophils. M-CSF inhibits apoptosis of monocytes and induces them to differentiate into macrophages. In separate experiments, we have found that GM-CSF and M-CSF modestly (approximately 20%) inhibit spontaneous apoptosis while M-CSF is also able to similarly reduce apoptosis in HT-29 cells stimulated with sulindac sufide. However, in the current study we found no evidence for modulation of HT-29 cells survival by endogenously released GM-CSF, M-CSF or G-CSF. Moreover, when authentic CSF was added to cells, we found no effect on either basal, cytokine-induced or NSAID-induced apoptosis.

It seems, therefore that, in comparison to leukocytes, CSFs have only a minor role in the regulation of HT-29 cell apoptosis. Nevertheless, the finding that colonic epithelial cells release CSFs has implications for our understanding of gastrointestinal diseases. Indeed, these observations illustrate that in addition to providing a metabolic barrier colon epithelia may have secretory and pro-inflammatory functions. This notion is supported by other studies showing that HT-29 cells release ET-1, RANTES, monocyte chemotactic protein-1 and IL-8 (Kolios *et al*., 1999) although, in view of the differences reported by Panja *et al*. (1998) regarding chemokine secretion between cell lines and normal epithelial cells, caution should be taken in extrapolating these results to normal cell function.

Thus, we have shown that human colon epithelial cells can be stimulated to release GM-CSF, G-CSF and M-CSF. Moreover, we have shown that in these cells, the release of GM-CSF is further increased by NSAIDs. However, the release of CSFs by the colonic epithelial cells used in this

study did not have any autocrine function on apoptosis induced by NSAIDs or cytokines. Nevertheless, it is possible for epithelial-derived CSFs to modify tumour progression in other ways. GM-CSF and G-CSF have been shown to stimulate the clonal growth of other human adenocarcinoma cell lines (Berdel *et al*., 1989; Dippold *et al*., 1991). Moreover, colon cancer cells genetically engineered to secrete GM-CSF have been shown to afford specific and long-lasting anti-tumour immunity (Dranoff *et al*., 1993). Thus, we suggest that release of CSFs by colon epithelia could contribute to the inflammatory events in normal and/or neoplastic tissue. Further understanding of how this occurs may well lead to improved therapies for the treatment of some gastrointestinal diseases.

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