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Biochimica et Biophysica Acta 1448 (1999) 425–438

BIOCHIMICA ET BIOPHYSICA ACTA

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NSAIDs and butyrate sensitize a human colorectal cancer cell line to TNF-α and Fas ligation: the role of reactive oxygen species

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Received 2 October 1998; accepted 27 October 1998

Abstract

The nonsteroidal antiinflammatory drugs (NSAIDs) indomethacin and salicylic acid and the short chain fatty acid butyrate are effective colon cancer chemopreventive agents that increase reactive oxygen species (ROS) generation in colon cancer cells. Here we demonstrate that these agents sensitize the normally resistant human HT-29 colon cancer cell line to apoptosis induced by TNF-α or a Fas ligating antibody. The role of ROS in this sensitization is supported by the finding that direct exposure of the cells to H_2O_2 is sufficient for sensitization. Neither TNF-α nor Fas ligation alter basal or chemopreventive agent-activated ROS generation, suggesting that the death ligands and chemopreventive agents act in a complementary fashion. The dual chemopreventive agent/death ligand treatments do not increase Fas, TNF receptor 1, Bak or c-myc expression (although salicylic acid moderately induces of Fas expression). Cell death does correlate with alterations in NF-κB activity: the NSAIDs, butyrate and H_2O_2 enhance c-Rel complex formation by TNF-α and provide an overall enhancement of NF-κB activation by Fas. The antioxidant *N*-acetylcysteine (NAC) blocks cell death and NF-κB activation induced by Fas ligation, suggesting a potential role for NF-κB in Fas-induced apoptosis in these cells. The effects of NAC on TNF-α-induced cell death are more complex, with NAC being marginally protective and itself enhancing the formation of c-Rel containing complexes at higher concentrations (25 mM). The influence of NSAIDs and butyrate on ROS generation and death ligand sensitivity may be relevant to their ability to suppress colon carcinogenesis.

Keywords: Reactive oxygen species; Cancer; Nonsteroidal antiinflammatory drug; Butyrate; Apoptosis; Tumor necrosis factor-α; Fas; NF-κB

1. Introduction

The proper functioning of many tissues requires an intricate balance between cell proliferation and programmed cell death (apoptosis). Healthy colonic epithelium is an excellent example of a tissue that maintains such an equilibrium. Cells derived through cell division at the crypt base migrate towards the surface of the lumen where they undergo apoptosis and are shed from the tissue. If the level of cell death is too low, pathological conditions such as colon cancer may occur [1]. Excessive cell death may lead to disease states such as ulcerative colitis [2]. Understanding how cell death in colonic tissue is regulated could lead to improved treatments and prevention strategies for cancer and other diseases.

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0167-4889/99/$ – see front matter
PII: S0167-4889(98)00156-6

The regulation of cell death in the epithelium of the colon and large intestine are likely to involve a number of signaling systems including the loss of cell-substrate interactions [3,4], and autocrine production of the transforming growth factor-β (TGF-β) [5]. The luminal short chain fatty acid butyrate may also play a role in regulating apoptosis [6], although data has also been obtained suggesting that it serves as an energy source for cells under certain culture conditions [7]. The concept of butyrate as a regulator of cell death in the colon is consistent with the finding that it can suppress colon carcinogenesis [8]. The role of TNF-α and Fas ligand in regulating cell turnover in the colonic epithelium under normal conditions is not well understood. Normal colonocytes display the Fas receptor on their basolateral surface membrane [9], and Fas ligand is expressed by a subpopulation of cells of the lamina propria [10]. During pathological conditions, the presence of these cytokines is increased, probably due to the infiltration of immune cells. In ulcerative colitis, Fas ligand-expressing inflammatory cells have been shown to greatly increase in number [2,10]. TNF-α levels in the mucosa can also increase under conditions that induce ulceration [11]. In addition, TNF-α is detected in cancerous tissue, where its presence correlates with a favorable prognosis [12,13]. Understanding how and when different death inducing systems are mobilized in the intestinal mucosa, and how the cellular responses to these signals are regulated, is a complex and important research challenge.

Reactive oxygen species (ROS) can play a central role in regulating cell proliferation and cell death. Evidence has been obtained that reactive oxygen species such as superoxide and hydrogen peroxide can influence cell death triggered by internal cues (p53-mediated) [14], external cues (TGF-β-mediated) [15–17] and immunogenic signals (TNF-α and Fas ligand) [18–22]. It is therefore possible that ROS play a role in regulating apoptosis in colonic epithelium. To begin exploring this possibility, we recently determined the effect of a number of chemical agents implicated in inducing cell death in the colon for their effects on ROS production. Specifically, we determined the effects of nonsteroidal antiinflammatory drugs (NSAIDs) and short chain fatty acids in the moderately well differentiated HT-29 human colon cancer cell line [23,24]. NSAIDs have been demonstrated to reduce the risk of colorectal cancer, potentially by accentuating apoptosis of transformed cells [25]. In addition, NSAIDs exacerbate ulcerations in the colon, probably by accentuating cell death [26]. Short chain fatty acids derived from dietary fiber that may reduce colon cancer risk by accentuating cancer cell apoptosis were also analyzed [24,27,28]. It was found that all these agents could accentuate ROS generation in HT-29 cells [29]. Here we describe how this increased ROS generation influences cell sensitivity to death ligands, and how cell death signal transduction may be affected.

2. Materials and methods

2.1. Chemicals

Indomethacin, *N*-acetylcysteine (NAC; neutralized with NaOH), H_2O_2, and butyric acid (neutralized with NaOH) were purchased from Sigma. Salicylic acid was purchased from Fisher, and dihydrodichlorofluorescein (H_2DCF; diacetate form) from Molecular Probes (Eugene, OR).

2.2. Antibodies and cytokines

The following antibodies were used in the described studies: anti-Fas clone DX2.1 from R&D Systems for immunoblotting, anti-Fas clone from Kamiya for the induction of apoptosis, anti-TNF receptor 1 clone 16803.1 from R&D Systems, anti-NF-κB p50 Ab-1 from Calbiochem, anti-RelA Ab-1 from Calbiochem, anti-c-Rel Ab-1 from Calbiochem, anti-c-myc N-262 from Santa Cruz, and anti-Bak G-23 from Santa Cruz. Recombinant human TNF-α was obtained from R&D Systems.

2.3. Cell culture and cell death assay

HT-29 cells were purchased from American Type Culture Collection (Rockville, MD) and were propagated on tissue culture plastic with McCoy's 5A medium supplemented 10% fetal bovine serum, non-essential amino acids, streptomycin (50 μg/ml) and penicillin (50 U/ml) [29]. All medium components were purchased from Life Technologies (Gaithers-

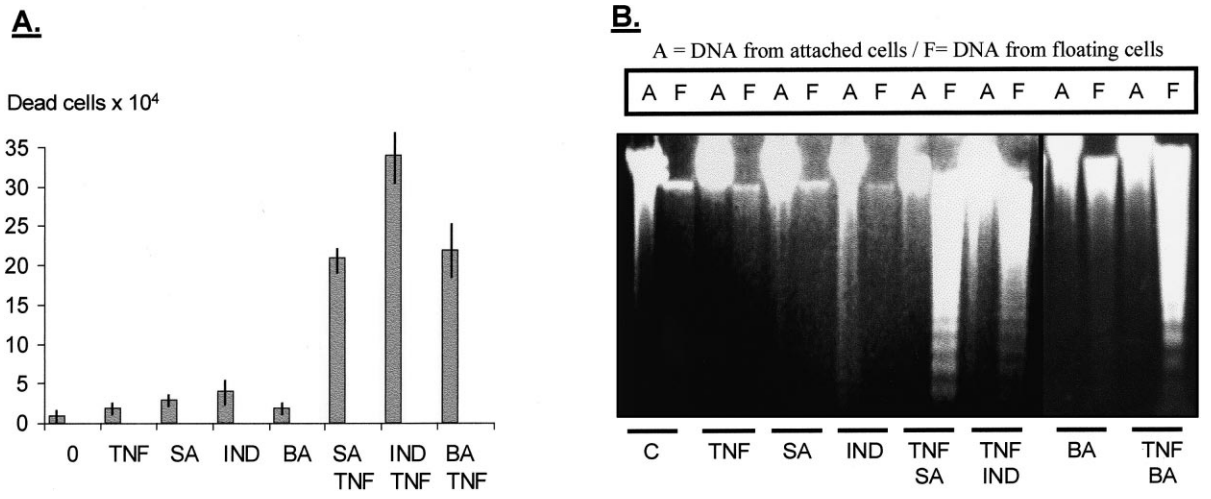

Fig. 1. (A) The NSAIDs salicylic acid and indomethacin, and the short chain fatty acid butyrate sensitize HT-29 cells to TNF-α. HT-29 cells grown to approximately 8×10^4 cells per well on a 96 well plate were treated with salicylic acid (SA; 4 mM), butyrate (BA; 4 mM), or indomethacin (IND; 200 μM) either in the presence or absence of TNF-α (TNF; 100 ng/ml). After 18 h of exposure, the number of dead cells per well was determined. The mean and S.D. of four trials are shown. Cell death in the combination treatments over control is significant with $P<0.01$. (B) Cell death induced by TNF-α in combination with NSAIDs or butyrate is apoptotic. DNA was prepared from dead cells (floating cells) or live cells (attached cells) and analyzed by agarose gel electrophoresis. The appearance of a DNA ladder in the dead cell population indicates that apoptosis has occurred.

burg, MD). For the cell death assays, cells were plated on 96 well plates and grown for 2–4 days to approximately 90% confluency ($\sim5\times10^5$ cells per well). Following treatment, cell death was determined by counting the number of floating cells per well (after gentle mixing) with a hemacytometer. This technique is commonly used for quantifying cell death in HT-29 cells and other cell lines of epithelial origin [6,30,31]. Analysis of DNA fragmentation by gel electrophoresis indicates that the majority of apoptotic cells are in the floating cell population (Fig. 1). This method is, however, semiquantitative since a fraction of the attached cell population stain positively with propidium iodide. For propidium iodide analyses, cells were stained with 50 μg/ml propidium iodide for 30 min, after which the cells were analyzed by fluorescence microscopy. The assay for DNA laddering involved DNA isolation by organic extraction, degradation of RNA by RNase A treatment, and electrophoresis of DNA on 1% agarose/TBE gels.

2.4. H_2DCF oxidation assay for peroxide

For the measurement of H_2DCF oxidation to fluorescent dichlorofluorescein (DCF), cells were grown to near confluency on a 96 well tissue culture plate, and loaded with H_2DCF by adding the diacetate form of this compound to the medium at a final concentration of 50 μM [29]. After 30 min, the medium was completely removed, and 100 μl of fresh medium was added to each well. After addition of test agents, DCF production was detected with a Cytofluor microplate-reading fluorimeter (PerSeptive) with the excitation wavelength set at 475 nm, and the emission wavelength at 525 nm. The fluorimeter was set at a gain of 50 for these measurements.

2.5. Immunoblotting

25 μg of cytoplasmic or nuclear protein (determined by Bio-Rad protein assay) were resolved on 10% SDS polyacrylamide gels, and transferred to nitrocellulose by voltage gradient transfer (see below for cell fractionation protocol). The resulting blots were blocked with 5% nonfat dry milk. Specific proteins were detected with appropriate antibodies using enhanced chemiluminescence for detection, following the manufacturers recommended protocol (Amersham). C-myc and Bak were detected in the particulate, nuclear fraction, while Fas and TNF receptor 1 were present primarily in the cytoplasmic fraction.

Films resulting from the immunoblot analysis, and other assays employed in this paper, were quantified with a PDI scanning densitometer.

2.6. Cell fractionation

The cell fractionation protocol utilized was based on a previously reported protocol [32]. Cells grown on 35 mm plates were washed twice with ice-cold PBS and incubated at 4°C for 8 min in 250 μl of a buffer containing 0.1% NP-40 (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$, 0.5 mM PMSF, 0.5 M sucrose, 2 μg/ml leupeptin). The cells were then scraped into tubes and centrifuged for 10 min at 4°C (14 000 rpm). The resulting supernatant was the 'cytoplasmic extract'. The resulting 'nuclear pellets' were rinsed with the above buffer without NP-40. Nuclear extracts were prepared by resuspending nuclear pellets in 15 μl of a high salt buffer (20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 5% glycerol, 0.5 mM PMSF, 2 μg/ml leupeptin), incubating on ice for 40 min, and then centrifuging for 10 min at 4°C (14 000 rpm). The nuclear extracts were then mixed with 22.5 μl of a low salt buffer (20 mM HEPES-KOH, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 1 mM DTT, 20% glycerol, 0.5 mM PMSF, 2 μg/ml leupeptin). Protein concentrations of the nuclear and cytoplasmic extracts were determined by the Bradford assay (Bio-Rad), and stored at −80°C until used.

2.7. Electrophoretic mobility shift assay

Double-stranded NF-κB consensus oligonucleotide (TGAGGGGACTTTCCCAGGC; Promega) was end-labeled with $\gamma[^{32}P]$ATP (3000 Ci/mmol at 10 mCi/ml; Amersham). Binding reactions were performed by mixing 10 μg of nuclear extract (prepared as described above) in 10 μl with 2 μg poly-dIdC and 1 μg BSA to give a final volume of 19 μl. After a 15 min incubation on ice, 40 fmol of oligonucleotide (1 μl) was added to each reaction, and reactions were transferred to room temperature for an additional 15 min [32]. Reaction products were separated in a 4% polyacrylamide/TBE gel and the resulting gels analyzed by autoradiography. For the supershift experiments, polyclonal rabbit anti-RelA, anti-p50 or anti-c-Rel antibodies (Oncogene Research Products) were included in the binding reaction from the poly-dIdC incubation step, and this incubation on ice was extended to 30 min. In the RelA supershift experiment, the intensity of the supershift band is less than that of the RelA-DNA complex without antibody. This result may be due to dissociation of the antibody during the gel run (leading to smearing), or an inhibition of RelA-DNA complex formation by the antibody. All of the complexes identified as NF-κB-containing by the supershift assay were also specifically competed with the NF-κB binding oligonucleotide (unpublished data).

3. Results

3.1. NSAIDs and butyrate sensitize cells to TNF-α and Fas: contribution of ROS

Our previous analysis indicated that the NSAIDs indomethacin, aspirin, salicylate and ibuprofen stimulate ROS generation in the human HT-29 cell line, as determined by the H_2DCF oxidation assay [29]. In addition, we demonstrated a similar effect of the short chain fatty acids butyrate and propionate [29]. Since these agents have been implicated in regulating apoptosis in the colon, we wished to determine how ROS generated by these agents affect apoptosis of HT-29 cells. We focused our efforts on three compounds. The NSAIDs indomethacin and salicylic acid were chosen for further analysis because indomethacin is a cyclooxygenase inhibitor whereas salicylic acid is not. The short chain fatty acid butyrate was chosen because it was found to be a particularly potent ROS-generating short chain fatty acid.

Initial efforts to determine the influence of NSAID and butyrate on apoptosis of HT-29 cells were hampered by the fact that these agents induced only low levels of apoptosis. Of the three compounds under study, only butyrate consistently triggered apoptotic DNA fragmentation (after a 3–4 day exposure; unpublished data). We then determined whether these agents could enhance cell death induced by receptor-mediated routes. We first determined the influence of these agents on cell killing by TNF-α. TNF-α has been directly implicated causing tissue damage in ulcerative colitis [11] and studies have also implicated

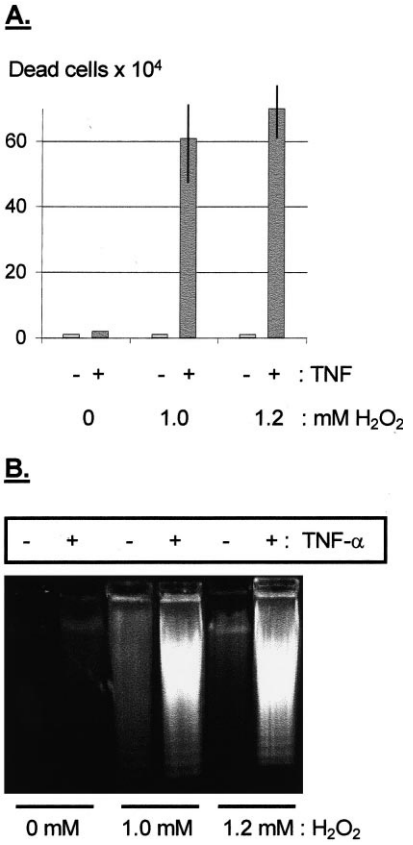

Fig. 2. (A) H_2O_2 sensitizes HT-29 cells to TNF-α. Cells were incubated for 18 h in the presence or absence of 100 ng/ml TNF-α (TNF), in the presence or absence of the indicated concentration of H_2O_2. After 18 h, cell death was determined as in Fig. 1A. The mean and S.D. of 4 trials are shown, with significant increases in death observed ($P < 0.01$). (B) Cell death by the TNF-α/H_2O_2 combination treatment is apoptotic. DNA from dead cells obtained after an 18 h incubation with TNF-α and/or H_2O_2 as indicated was analyzed by agarose gel electrophoresis. The characteristic DNA fragmentation indicates apoptotic cell death is occurring in the culture.

this cytokine in suppressing tumor progression in the colon [12,13]. As shown in Fig. 1A, HT-29 cells are largely resistant to killing by TNF-α. This finding is in agreement with published reports from other laboratories [30]. However, inclusion of indomethacin, salicylic acid or butyrate in the incubation greatly enhances cellular sensitivity to TNF-α (Fig. 1A). The level of cell death in Fig. 1A was determined after 18 h treatment and represented 30–40% of the cultured cells exposed to the combination treatments. To obtain evidence that cell death under these conditions was apoptotic, DNA was isolated from the floating, dead cell population and the live attached cells, and analyzed by agarose gel electrophoresis. The results of this agarose gel are shown in Fig. 1B. The DNA isolated from the floating cells displays the DNA fragmentation pattern characteristic of an apoptotic cell death. Additional conformation of apoptosis in the floating cell population was obtained by propidium iodide staining: for the combination treatments greater than 95% of the floating cells stained positively with propidium iodide. At-

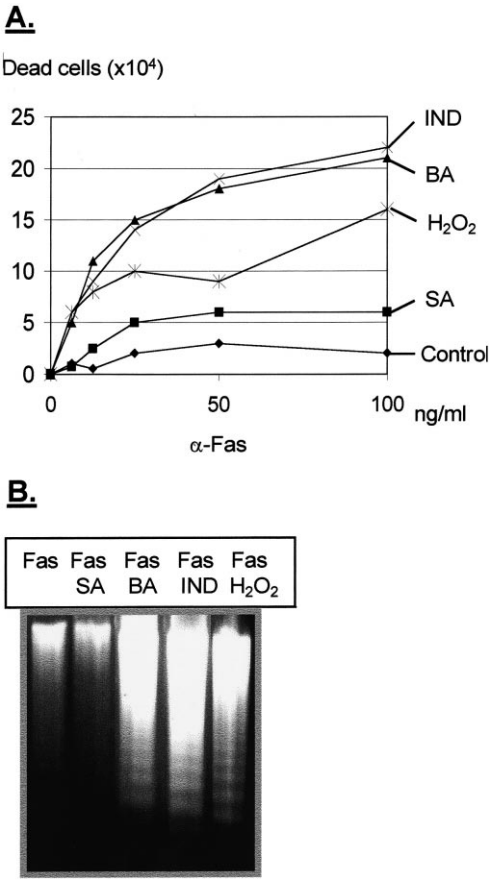

Fig. 3. (A) NSAIDs, butyrate and H_2O_2 enhance cell death induced by a Fas ligating antibody. The HT-29 cell death assay was performed as described in Fig. 1A, after an 18 h incubation with a Fas ligating antibody (at the indicated concentrations) in the presence of the following agents: Control, no additions; SA, 4 mM salicylic acid; H_2O_2, 1.2 mM H_2O_2; BA, 4 mM butyrate; or IND, 200 μM indomethacin. (B) Cell death induced by the anti-Fas combination treatments is apoptotic. DNA from dead cells obtained after an 18 h incubation with anti-Fas alone or anti-Fas in combination with the indicated agents was analyzed by agarose gel electrophoresis (anti-Fas was at 50 ng/ml; NSAIDs, butyrate and H_2O_2 were at concentrations used in (A)). The characteristic DNA fragmentation indicates apoptotic cell death is occurring in the culture.

tached cells were also analyzed by propidium iodide staining. The dual treatments also increased the fraction of attached cells that were stained with propidium iodide, with the staining cells presumably representing cells at earlier stages of apoptosis. The fraction of attached cells staining with propidium iodide in untreated cultures averaged between 0.6 and 1.3%, with TNF-α treatment alone increasing this number to 5–8%. Inclusion of the NSAIDs or butyrate with TNF-α further increased attached cell staining to 16–22%.

Previous studies indicated that all three of the chemopreventive agents employed in Fig. 1 increase cellular peroxide generation (without increasing superoxide generation as determined by lucigenin-enhanced chemiluminescence) [29]. We therefore determined whether direct treatment of cells with H_2O_2 was sufficient to sensitize them to TNF-α. As shown in Fig. 2A, HT-29 cells are largely resistant to H_2O_2 at 1.0 and 1.2 mM concentrations. However, these H_2O_2 concentrations sensitized the cells to TNF-α. Cell death observed in the co-treated cells was apoptotic, as demonstrated by the appearance of a DNA ladder in the dead cells. ROS levels provided by 1.0 and 1.2 mM H_2O_2 are similar to those triggered by the NSAID and butyrate concentrations employed in Fig. 1 ([29] and Fig. 4 below).

To determine if the NSAIDs and butyrate could sensitize HT-29 cells to other physiological death ligands, it was determined how these agents influence cell killing by the Fas pathway. As shown in Fig. 3A, HT-29 cells are largely resistant to the Fas ligating antibody after an 18 h incubation. This finding is consistent with published reports from other laboratories [30], although we have noted that extended incubation periods can result in more extensive cell death. Inclusion of indomethacin, butyrate or salicylic acid in the incubation sensitized cells to killing by the Fas ligating antibody (Fig. 3A). Salicylic acid is, however, consistently less effective than the other agents in sensitizing cells to Fas-mediated cell death. The potential role of NSAID- and butyrate-induced ROS generation in this sensitization is supported by the finding that direct treatment of the cells with H_2O_2 is sufficient for sensitization to Fas ligation (Fig. 3A). Cell death induced by the Fas combination treatments is apoptotic, as revealed by the appearance of the characteristic DNA fragmentation

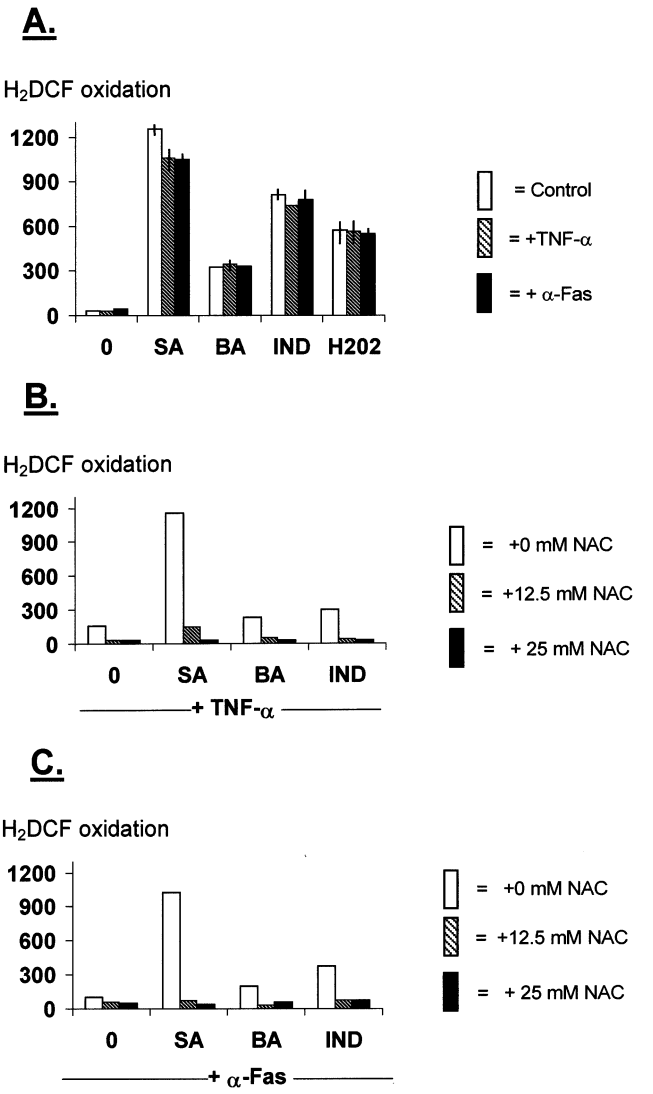

Fig. 4. (A) TNF-α and anti-Fas do not alter ROS generation induced by salicylic acid, butyrate or indomethacin. HT-29 cells were loaded with H_2DCF as described in Section 2. Cells were then exposed to TNF-α or anti-Fas, in the presence or absence of 4 mM salicylic acid (SA), 4 mM butyrate (BA), 200 μM indomethacin (IND) or 1.2 mM H_2O_2 (H2O2), as indicated. The rate of H_2DCF oxidation to DCF from 60 to 75 min after exposure to the indicated treatments was determined by reading DCF fluorescence on a fluorescent plate reader. Similar results were obtained with pro-longed exposure to these treatments. The average of two trials is shown. (B, C) NAC suppresses H_2DCF oxidation in cells treated with TNF-α (B) or α-Fas (C) alone, or in combination with salicylic acid (SA), butyrate (BA) or indomethacin (IND). Treatments were performed as in (A), except NAC was present at 0, 12.5 or 25 mM concentrations where indicated.

pattern in the dead cell population (the floating cells; Fig. 3B). Propidium iodide staining confirmed that greater than 95% of the cells in floating cell population were dead. Analysis of the attached cells also indicated an enhancement of cell death by indomethacin, butyrate and H_2O_2: in cultures treated with Fas alone, 6–18% of the cells stained with propidium iodide, while 18–35% of the attached cells stained positively when sensitized with butyrate, indomethacin or H_2O_2.

In some cell types TNF-α and Fas have been found to increase ROS generation [19–22,33]. These death ligands might therefore sensitize cells by contributing to the increased ROS generation triggered by the NSAIDs and butyrate. To determine if TNF-α or the anti-Fas antibody were contributing to cellular ROS generation, an H_2DCF oxidation assay was performed. As shown in Fig. 4A, neither TNF-α nor Fas increases ROS generation by HT-29 cells. Moreover, neither cytokine alters the ROS generation triggered by the NSAIDs or butyrate (Fig. 4A). The results shown in Fig. 4 were derived after a 1 h incubation, but similar results were obtained when longer treatments were performed (up to 6 h; C. Giardina, unpublished data). As shown in Fig. 4B and C, the antioxidant NAC suppresses H_2DCF oxidation by the NSAIDs and butyrate, consistent with these agents enhancing ROS production. These data are consistent with a model in which the ROS generated by the NSAIDs and butyrate complement the cell death signal stimulated by TNF-α and Fas.

It should be noted that the H_2O_2 concentrations used in this paper generate a rate of H_2DCF oxidation similar to those obtained by the chemopreventive agents. While this concentration was chosen in part for this similar influence on internal redox state, it should be cautioned that external H_2O_2 application is likely to oxidize media components more extensively.

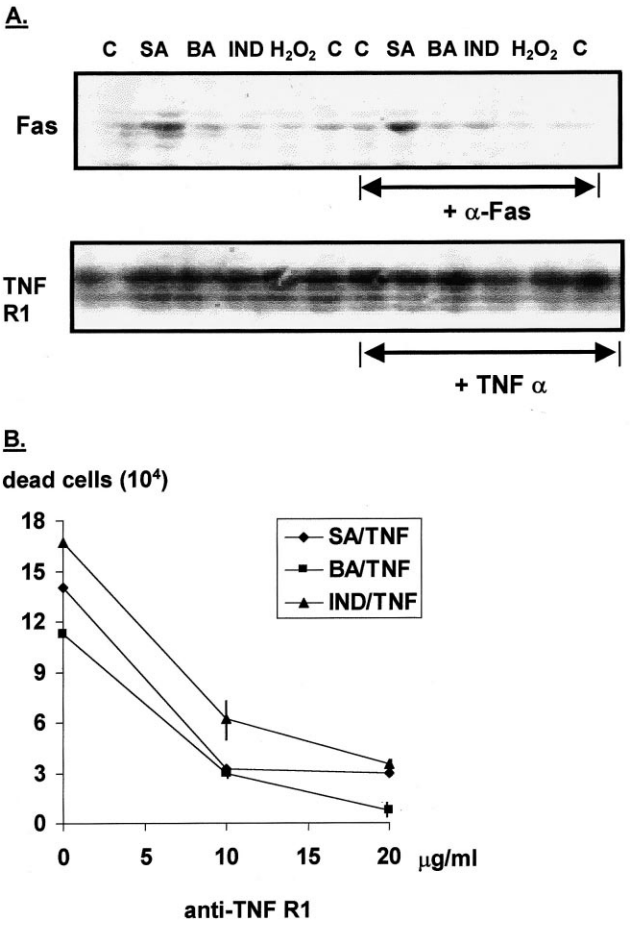

Fig. 5. (A) The effect of NSAIDs, butyrate and H_2O_2 on Fas and TNF receptor 1 expression. HT-29 cells were exposed to 4 mM salicylic acid (SA), 4 mM butyrate (BA), 200 μM indomethacin (IND) or 1.2 mM H_2O_2 (H2O2), as indicated, in the presence or absence of anti-Fas antibody (α-Fas; 50 ng/ml) or TNF-α (100 μg/ml). After 8 h, lysates were prepared and analyzed by immunoblotting for Fas or TNF receptor 1 (TNF R1), as indicated. (B) TNF receptor 1 contributes to HT-29 cell sensitivity to TNF-α. Cells were treated with the salicylic acid/TNF-α (SA/TNF), butyrate/TNF-α (BA/TNF) or indomethacin/TNF-α (IND/TNF) combination treatments described in Fig. 1, in the absence or presence of a neutralizing antibody to TNF receptor 1 (anti-TNF R1). Cell death under these conditions was quantified as described in Fig. 1.

3.2. *NSAIDs, butyrate, H_2O_2 and death gene activation*

We have initiated an analysis to determine how indomethacin, salicylic acid and butyrate complement TNF-α- and Fas-induced cell death signal transduction in HT-29 cells. One mechanism by which cells can be sensitized to these death ligands is through the increased expression of the death ligand receptors. IFN-γ, for example, sensitizes HT-29 cells to Fas ligation and TNF-α by increasing expression of Fas and TNF receptor 1 [30]. As shown in Fig. 5A, neither of the NSAIDs under study nor butyrate enhance TNF receptor 1 expression (this receptor participates in TNF-α-induced apoptosis of HT-29 cells; Fig. 5B). The influence of the chemo-

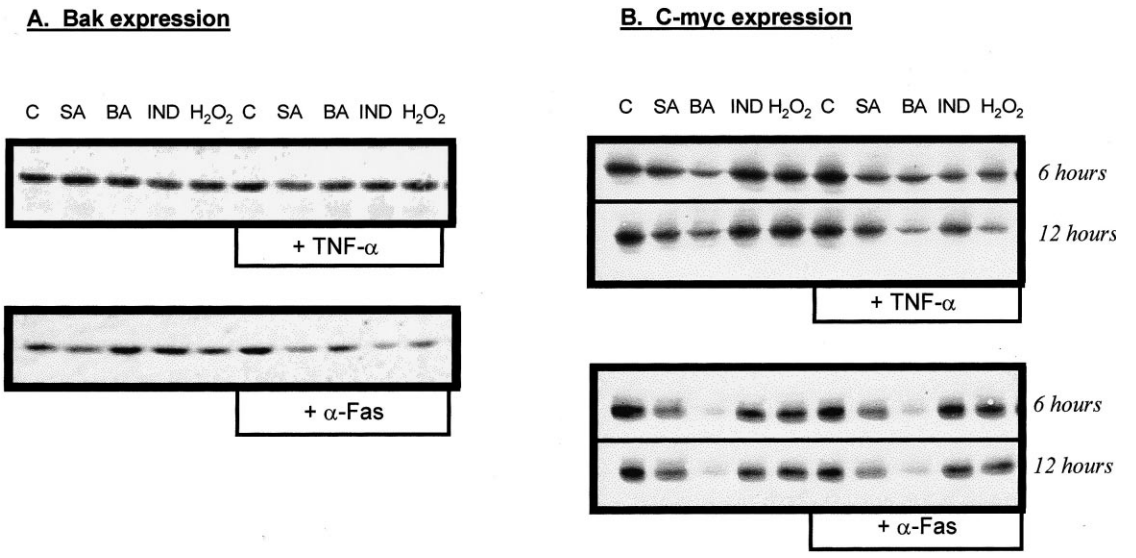

Fig. 6. (A) The effect of NSAIDs, butyrate and H_2O_2 on Bak expression in HT-29 cells. HT-29 cells were treated with salicylic acid (SA; 4 mM), butyrate (BA; 4 mM), indomethacin (IND; 200 μM), or H_2O_2 (1.2 mM) in the presence or absence of TNF-α (100 μg/ml) or anti-Fas antibody (α-Fas) for 12 h. Cell lysates were prepared, immunoblotted and probed using a Bak specific antibody. (B) The effect of NSAIDs, butyrate and H_2O_2 on c-myc expression in HT-29 cells. Cells were treated as in (A), only the resulting immunoblots were probed with a c-myc-specific antibody. A 6 h time point was also generated for this experiment.

preventive agents on Fas expression was also determined (Fig. 5A). Of the agents under study, only salicylic acid increased Fas expression (1.5–2.8-fold, as determined by scanning densitometry). This increased Fas expression is not likely to be ROS-mediated, since H_2O_2 does not have this effect. Since salicylic acid is the least effective Fas sensitizing agent, expression of this receptor is probably not rate-limiting for Fas-induced cell death. These data indicate that the NSAID- and butyrate-induced ROS production does not function by increasing receptor expression.

It has been reported that butyrate enhances Bak expression in HT-29 cells, a pro-death Bcl-2 family member expressed by colonic epithelium [34]. We determined whether the sensitization of HT-29 cells could be the result of increased Bak expression. As shown in Fig. 6A, neither the individual nor combi-

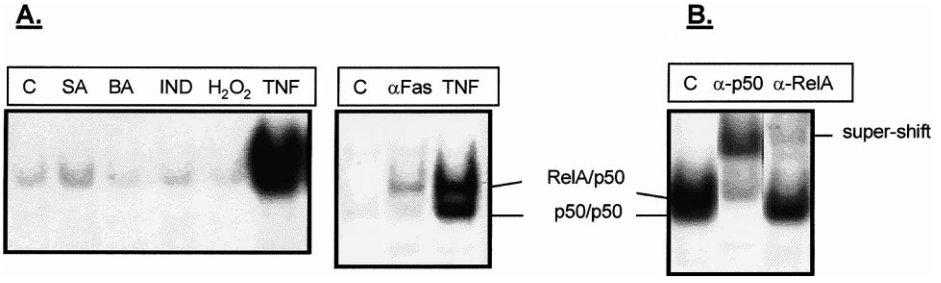

Fig. 7. (A) Salicylic acid, indomethacin, butyrate and H_2O_2 do not activate NF-κB DNA binding in HT-29 cells, while TNF-α and a Fas ligating antibody do. HT-29 cells were exposed to 4 mM salicylic acid (SA), 4 mM butyrate (BA), 200 μM indomethacin (IND) or 1.2 mM H_2O_2, 100 ng/ml TNF-α (TNF) or 50 ng/ml Fas ligating antibody (αFas) as indicated. After a 6 h incubation, nuclear extracts were prepared and analyzed for NF-κB DNA binding activity by EMSA. To enhance resolution of the protein-DNA complexes, the unbound probe was run off the gel. Only the protein-DNA complexes are shown. (B) Supershift analysis of NF-κB activated in HT-29 cells. DNA binding reactions using nuclear extracts from TNF-α treated HT-29 cells were performed in the absence of antibody (lane marked 0), or in the presence of antibody against the p50 (αp50) or RelA (αRelA) subunit of NF-κB. The location of the super-shifted complex is indicated. As in (A), the free probe was run off the gel.

nation treatments increase Bak expression after 12 h of incubation (at which time cell death can be observed in the combination treatments). Longer exposures to butyrate (24 and 36 h) do enhance Bak expression (unpublished data), but this increased expression occurs too late to account for the ability of butyrate and NSAIDs to sensitize cells to TNF-α or Fas ligation. Increased expression of the c-myc gene has also been implicated in NSAID-induced cell death of transformed chick embryo fibroblasts [35]. As shown in Fig. 6B, none of the agents under study increase expression of the c-myc protein in these cells. In fact, as has been reported by others, butyrate is a potent suppressor of c-myc expression in HT-29 cells [24,36]. Increased c-myc expression can therefore not account for the enhanced sensitivity of HT-29 cells to TNF-α or Fas ligation.

3.3. NSAIDs, butyrate, H_2O_2 and NF-κB activation

In an attempt to uncover common cellular responses to the NSAIDs and butyrate that might be mediated by their ability to induce ROS generation, we determined the effect of these agents on the transcription factor NF-κB. NF-κB regulates the expression of genes involved in both the induction of apoptosis (Fas ligand, p53 and possibly ICE) [37–39], as well as its suppression (Mn SOD and A20) [40,41]. While the influence of this transcription factor on Fas-mediated apoptosis is not clear, a body of work indicates that NF-κB activation protects cells from TNF-α-induced apoptosis [44–48]. Our hypothesis therefore was that the chemopreventive agents may sensitize cells to TNF-α by inhibiting NF-κB activation. NF-κB was also of interest because its activity is sensitive to cellular redox state [42,43].

Fig. 7 shows the results of electrophoretic mobility shift assays (EMSAs) used to determine the NF-κB DNA binding activity from HT-29 cells incubated under a number of conditions. In all cases, only the shifted, protein-DNA complexes are shown: the free probe is run off the gel to enhance resolution of the protein-DNA complexes. As shown in Fig. 7A, the NSAIDs under investigation, butyrate and H_2O_2 fail to activate NF-κB in HT-29 cells. In contrast, α-Fas and TNF-α do increase NF-κB binding activity in these cells. Two predominate forms are activated. A supershift assay demonstrates that the bottom band is likely to be the p50/p50 homodimer, while the top band appears to be the RelA/p50 heterodimer (Fig. 7B; the weaker intensity of the RelA-supershift complex may be the result of an instability of the antibody complex leading to dissociation during electrophoresis). The Fas ligating antibody also stimulates NF-κB binding in HT-29 cells, forming predominately the RelA/p50 heterodimer (Fig. 7A).

We then determined if salicylic acid, butyrate, indomethacin or H_2O_2 affect NF-κB activation by TNF-α. These sensitizing agents were found to enhance the formation of a more rapidly migrating band on the EMSA approximately 2.5-fold (Fig. 8A; again, only the protein-DNA complexes are shown). This more rapidly migrating band does not associate with antibodies against the p50 or RelA

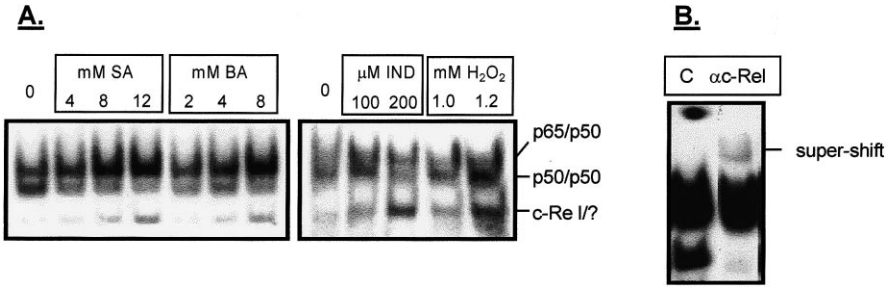

Fig. 8. (A) Salicylic acid, butyrate, indomethacin and H_2O_2 influence the subunit composition of TNF-α activated NF-κB in HT-29 cells. HT-29 cells were treated with TNF-α alone (100 ng/ml) or TNF-α in the presence of the indicated concentrations of salicylic acid (SA), butyrate (BA), indomethacin (IND) or H_2O_2. Treatments were performed for 6 h, after which nuclear extracts were prepared and analyzed for NF-κB DNA binding activity by EMSA. Only protein-DNA complexes are shown. (B) A supershift assay identifies c-Rel as a component of the NF-κB DNA binding activity stimulated by the NSAIDs, butyrate and H_2O_2. A supershift assay was performed using an antibody specific for the c-Rel NF-κB subunit (the αc-Rel lane). The super-shifted complex is indicated.

subunits (as determined by supershift, data not shown), but it is bound by an antibody against the c-Rel subunit (Fig. 8B). Whether this complex is a c-Rel homodimer, or c-Rel associated with another factor (rel family member or otherwise) remains to be determined. The increased formation of the c-Rel complex could be the result of increased cellular ROS production, since H_2O_2 also stimulates formation of this complex. Salicylic acid, butyrate and indomethacin also suppress the formation of the p50/p50 homodimer, and may stimulate p50/RelA complex formation. While our initial prediction was that these agents would suppress NF-κB activation, the finding that they alter the NF-κB subunit composition suggests a somewhat more complicated scenario (although 2 and 4 mM butyrate do reproducibly suppress the total NF-κB DNA activity by up to 50%). It is not presently clear how these different forms of NF-κB function on specific promoters: i.e., whether they are stronger or weaker transcriptional activators, and whether there are different responses on pro- and anti-death genes.

We also determined the effect of the NSAIDs and butyrate on NF-κB activated by Fas ligation. As shown in Fig. 9, all three agents were found to enhance NF-κB activation induced by Fas (with salicylic acid being notably weaker). Direct treatment with H_2O_2 likewise enhances NF-κB activation by Fas, suggesting that increased cellular ROS generation triggered by these agents contributes to this activation. In the case of Fas treatment, all of the NF-κB forms appear to be enhanced to a roughly equal extent. The extent of this enhancement was approximately 6-fold for H_2O_2, 9-fold for indomethacin and 20-fold for butyrate.

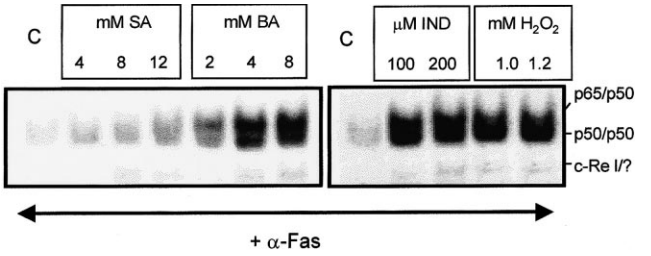

Fig. 9. Salicylic acid, butyrate, indomethacin and H_2O_2 enhance NF-κB activation by Fas ligating antibody. HT-29 cells were treated with Fas ligating antibody alone (50 ng/ml) or Fas ligating antibody in the presence of the indicated concentrations of salicylic acid (SA), butyrate (BA), indomethacin (IND) or H_2O_2. Treatments were performed for 6 h, after which nuclear extracts were prepared and analyzed for NF-κB DNA binding activity by EMSA. Only protein-DNA complexes are shown.

NF-κB plays a complex role in regulating programmed cell death. In many instances the inhibition of NF-κB activity can sensitize cells to death inducers, particularly TNF-α [44–48]. In other instances, however, NF-κB activation has been found to play an important role in the induction of apoptosis [49–51]. To obtain insight into the potential role of NF-κB in HT-29 cell death, we determined the influence of the antioxidant/NF-κB inhibitor NAC on Fas- and TNF-α-induced HT-29 cell death. NAC was chosen because of its demonstrated effectiveness at suppressing ROS generation by the chemopreventive agents under study (Fig. 4 and [29]). As shown in Fig. 10A, NAC suppressed cell death induced by Fas, in the presence or absence of NSAID or butyrate (cells treated with Fas alone were incubated for 24 h instead of 16 h to obtain more extensive cell death). The protection from Fas-induced cell killing correlates with an inhibition of NF-κB activation (Fig. 10C; the inhibition of NF-κB-DNA binding is approximately 10-fold). This finding is consistent with a model in which NF-κB plays a role Fas-induced HT-29 cell death. Additional experimentation however is required to establish the role of this transcription factor in this response.

The effect of NAC on TNF-α-induced cell death was also determined. As shown in Fig. 10B, NAC partially suppresses cell death induced by the TNF-α combination treatments. Interestingly, higher concentrations of NAC also increase TNF-α-induced cell killing, even in the absence of chemopreventive agent. The influence of NAC on TNF-α-induced NF-κB was also determined (Fig. 10C). NAC was found to enhance the formation of the c-Rel complex, the NF-κB complex correlated with TNF-α-induced cell death when cells were exposed to ROS-generating agents (Fig. 8).

Since NAC partially suppresses cell death by the TNF-α combination treatments, a role for ROS in sensitizing HT-29 cells to TNF-α is supported (see also Fig. 2). However, determining the role of NF-κB in TNF-α-induced cell death is complicated by the fact that NAC does not fully protect cells, and can itself sensitize cells to TNF-α at higher concentrations. One possibility is that the TNF-α sensitivity

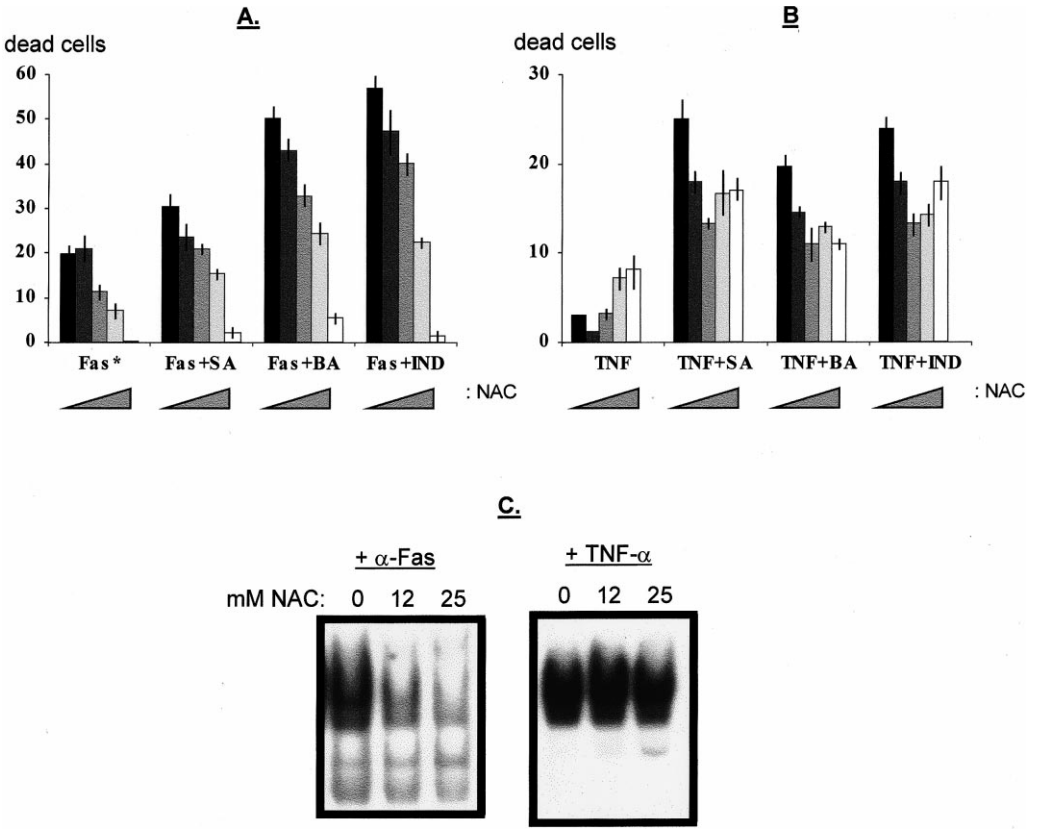

Fig. 10. (A) The effect of NAC on Fas-induced apoptosis of HT-29 cells. Cells were preincubated with increasing NAC concentrations for 4 h, after which time cells were treated with Fas ligating antibody alone (Fas*), or in combination with 8 mM salicylic acid (Fas+SA), 4 mM butyrate (Fas+BA), or 200 μM indomethacin (Fas+IND). Cell death was determined as described in Fig. 1 after 16 h except for cells treated with Fas alone, which were counted after 24 h (to obtain higher levels of cell death). NAC concentrations increase from left to right for each set and were 0, 3, 6, 12 and 24 mM. (B) The effect of NAC on TNF-α induced cell death. Cells were treated as in 10 A, except TNF-α was used instead of the anti-Fas antibody. Cell death was determined after 18 h. (C) The effect of NAC on NF-κB activated by Fas and TNF-α. HT-29 cells were preincubated with 0, 12 and 24 mM NAC for 4 h, after which time anti-Fas antibody (α-Fas; 50 ng/ml) or TNF-α (100 ng/ml) were added to the cultures, as indicated. After a 6 h incubation, NF-κB DNA binding activity was analyzed by EMSA.

of the HT-29 cells is enhanced by increases or decreases in the cellular redox state, and that NF-κB is an important sensor for changes in both directions. Interpretation of this data is complicated by the fact that NAC itself can generate ROS and other free radicals under the proper conditions (i.e., in the presence of transition metals), and that such conditions may be generated in the TNF-α stimulated cell [52]. An NAC-stimulated ROS generation was not however observed in TNF-α-treated cells by the H_2DCF oxidation assay in Fig. 4. Further study will be required to determine the relationship between cellular redox, NF-κB activity and TNF-α-induced apoptosis of HT-29 cells.

4. Discussion

We reported previously that the NSAIDs indomethacin and salicylic acid (and others) increase ROS generation in HT-29 cells [29]. In addition, a number of short chain fatty acids, including butyrate, also have this effect [29]. Here we show that this increased ROS generation contributes to the sensitization of these cells to apoptosis induced by TNF-α or Fas ligation. Whether this increased ROS generation is the only activity of these compounds relevant to this sensitization remains to be determined. Evidence that ROS do play a role in this sensitization comes from the finding that direct treatment of cell with

H_2O_2 can also sensitize them to Fas or TNF-α, and that cell death by the combination treatments can be suppressed by NAC (albeit partially for TNF-α-induced apoptosis). The role of these cytokines in regulating cancer development is not entirely understood, although their potential role in immune surveillance of colonic tissue has been proposed. The Fas ligand is utilized by T lymphocytes and enhancement of its cell killing activity could facilitate the immunogenic elimination of cancerous cells from the colon [53]. It has also been found that colon cancer cells are generally more resistant to Fas-induced killing, suggesting that enhancing this activity may improve cancer cell clearance [54]. The role of TNF-α in regulating cancer development in the colon is also not well understood, although high levels of this cytokine in human tumors have been correlated with longer patient survival [12]. It is also possible that ROS generation induced by these agents sensitizes cells to other death inducers as well (e.g., TGF-β or other cytokines). Additional studies will be required to determine the generality of this effect.

ROS have been found to play a central role in regulating apoptosis in numerous instances. Given that reduced rates of apoptosis may contribute to carcinogenesis [1], the regulation of cellular ROS production may be an important variable in the development of neoplasias. Other studies have also suggested a potential role for ROS in cancer suppression. For example, the p53 tumor suppressor protein activates the expression of ROS-generating proteins that increase cellular ROS production and eventually trigger apoptosis [14,55]. Increased ROS generation by chemopreventive agents such as NSAIDs and butyrate may serve to compensate the lower levels of ROS generation in p53 null cells. (HT-29 cell are p53 null [30].) Animal studies have also implicated ROS in regulating carcinogenesis. Mice with elevated levels of glutathione peroxidase are more sensitive to skin carcinogenesis than their wild type counterparts [56]. A similar correlation has been made in the colon, where strains with higher levels of glutathione peroxidase activity have a higher cancer risk [57]. The role of ROS in carcinogenesis is however likely to be complex given the potential mutagenicity of ROS. For example, the NSAID inhibition of cyclooxygenase has been proposed to suppress carcinogenesis by suppressing the production of peroxyl radicals and the subsequent formation of mutagenic lipid peroxidation breakdown products [25]. (Although the influence of NSAIDs reported here appears to be distinct from their influence on lipid peroxidation [29].) The role of ROS in carcinogenesis may depend on the relative levels of different ROS generated, and where and when they are present.

In many instances increasing ROS levels in the cell are sufficient to trigger apoptosis. However, to obtain apoptosis of HT-29 cells, TNF-α or a Fas ligating antibody was required in addition to increased ROS levels. Higher concentrations or longer incubation times with the NSAIDs or H_2O_2 did lead to cell death, but in these cases the appearance of the apoptotic DNA ladder was not apparent. With longer incubation times (2–4 days), butyrate was found to enhance apoptosis, suggesting that butyrate may have additional pro-death effects on cells. In cells where ROS rapidly trigger apoptosis, there may be sufficient expression of Fas ligand, TNF-α or related cell death machinery to support apoptosis. In this regard, HT-29 cells may serve as useful model for studying how ROS interact with events triggered by death ligands such as TNF-α or Fas ligating antibodies.

In studying the mechanism by which the NSAIDs under study and butyrate influence cell death in HT-29 cells, common effects on the transcription factor NF-κB were noted. Although none of these agents activated NF-κB on their own, all were found to enhance the formation of c-Rel-containing complexes when applied to cells in combination with TNF-α. It is not yet clear how this alteration in NF-κB subunit composition influences gene expression patterns. In the presence of Fas, all of the agents provided an overall increase in NF-κB DNA binding activity. The role of NF-κB in apoptosis is complex. In a number of cases this transcription factor has been found to suppress apoptosis [44–48], while in other instances it is found to play an important role in inducing apoptosis [49–51]. It has been proposed that the influence of NF-κB on apoptosis may depend on its subunit composition, and other cell-specific variables [58]. Future studies will address the role of specific NF-κB subunits in HT-29 cell death. Since cell death rates may contribute to cancer development, determining the role of NF-κB (in its different subunit compositions) in carcinogenesis is also of

interest. In some cancers, constitutive NF-κB activation has been found to play a central role in generating a transformed phenotype [59,60]. Whether NF-κB plays this role in colon carcinogenesis is not clear. It has been found that activated ras and polyoma middle T oncogenes reduce RelA expression in Caco-2 [61]. In addition, we have observed lower levels of p50 expression in cancerous and pre-cancerous lesions in the mouse colon by immunohistochemical staining (M.S. Inan, D. Wang, D. Rosenberg, C. Giardina, unpublished data). NF-κB's role in the colon may therefore be more akin to its role in skin development, where NF-κB serves primarily as a growth repressor and its reduced activity results in tissue hyperplasia [62]. Determining the role of NF-κB in colon carcinogenesis could help guide the development of improved chemoprevention and treatment strategies.

While it is not clear whether the cellular responses to NSAIDs and butyrate we describe here are operative in the colon, these findings establish a framework through which in vivo experiments that test these responses can be designed. Concerning the concentration of the agents used in our studies, butyrate is present in the colon at concentrations close to those used here [7], while NSAID concentrations employed were several-fold above pharmacological plasma concentrations [63]. These NSAID concentrations were employed to reproduce the in vivo observation that NSAIDs enhance apoptosis in the colon, and are similar to those employed by other labs studying the influence of NSAIDs on apoptosis in vitro [31,35]. Lower concentrations were also found to enhance apoptosis (albeit at lower rates; C. Giardina, unpublished data), and as noted by others, relatively subtle differences in apoptosis rates can have significant consequences on tumor growth when extrapolated over long periods of time [64]. Future experiments will assess the contribution of the events identified in the cell culture model with the ability of these agents to suppress colon carcinogenesis in animals.

Acknowledgements

We thank Daniel Rosenberg and David Wang for many enlightening discussions on carcinogenesis. This work was supported by a grant from the University of Connecticut Research Foundation and a Research Starter Grant from the Pharmaceutical Research and Manufacturers Association of America Foundation.

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