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# TNF-α modulates the differentiation induced by butyrate in the HT-29 human colon adenocarcinoma cell line

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**Abstract**

The aim of this study was to determine whether and how tumour necrosis factor alpha (TNF-α) modulates butyrate effects. After the treatment of human colon adenocarcinoma HT-29 cells with sodium butyrate (NaBt), TNF-α or with their combinations we detected cell cycle (flow cytometry), cell proliferation (amidoblack and MTT assays), the amount of dead (floating) and apoptotic cells (flow cytometry and fluorescence microscopy), and the level of differentiation by alkaline phosphatase (ALP) activity (spectrophotometry), relative F-actin content (confocal laser scanning microscopy analysis) and E-cadherin expression (Western blot analysis). Both TNF-α and NaBt decreased cell growth in a dose-dependent manner. After combined treatment of the cells with both agents used, either none or additive effects were observed as compared with NaBt treatment alone. The level of dead and apoptotic cells was dose-dependently increased after this combined treatment. In contrast, TNF-α suppressed ALP activity and F-actin accumulation induced by NaBt. The results suggest that TNF-α does not influence significantly the antiproliferative effects of NaBt but, contrary to its potentiation of apoptosis, it markedly reduces NaBt-induced differentiation of HT-29 colon adenocarcinoma cells. 

*Keywords:* Tumour necrosis factor alpha; Sodium butyrate; Colon cancer; Differentiation markers; Inflammatory bowel diseases

## 1. Introduction

Tumour necrosis factor-alpha (TNF-α) has a crucial role in immune and inflammatory processes, as well as in the pathogenesis of many human and animal diseases [1,2]. It is synthesised by macrophages and other cells in response to bacterial toxins, inflammatory products and other invasive stimuli [3]. Prolonged production of TNF-α is associated with cancer and chronic infections. It has been suggested that gut with active injury (e.g. in Crohn's disease or ulcerative colitis) contains an increased number of TNF-α-secreting cells [4]. In extracts of colorectal tumour tissues resected from human patients Numata and associates [5] have detected endogenous TNF-α at levels higher than those in the corresponding normal colorectal tissues. Thus, TNF-α may play a significant role in the modulation of differentiation and proliferation of colonocytes during cancer progression. In addition, TNF-α is a drug under investigation for the treatment of cancer [6].

An important factor which maintains the balance between proliferation, differentiation and apoptosis of intestinal epithelial cells in the crypt is butyrate, a 4-carbon fatty acid, which is formed in the gastrointestinal tract of mammals as a result of anaerobic bacterial fermentation of fibre [7]. It has been shown to inhibit the growth and stimulate the differentiation of normal and carcinoma colonic cells, both *in vivo* and *in vitro* [8,9] at concentrations of approximately 5 mM [10].

In this study, we used an *in vitro* model — the HT-29 cell line, which is particularly attractive since it is one of the cell lines of intestinal origin which reversibly displays structural and functional features of mature intestinal epithelial cells [11]. HT-29 cells under normal culture conditions display an undifferentiated phenotype, but they can express an 'enterocyte-like' differentiated phenotype in response to various inducers of

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differentiation, including sodium butyrate (NaBt) [12]. NaBt-induced differentiation is associated with an increase in the alkaline phosphatase (ALP) protein level and activity [12,13], increased expression of E-cadherin protein [9] and changes in morphology combined with a progressive flattening of cells with the assembly of cytoplasmic microfilaments (F-actin accumulation) and microtubules [10].

It has been reported previously that in HT-29 cells, relatively resistant to TNF-α or NaBt-induced apoptosis, the level of apoptosis after combined treatment of these two drugs was increased [14]. Because of the possibility of a TNF-α and NaBt interaction in the gut we were interested in how TNF-α could modulate other NaBt-mediated effects on cytokinetic parameters in HT-29 cells, i.e. cell cycle distribution, proliferation and differentiation.

## 2. Materials and methods

### 2.1. Cell culture

The human colon adenocarcinoma HT-29 cells, kindly donated by Dr Šloncová (IMG, Prague, Czech Republic), were cultured in 25 $cm^2$ flasks in Dulbecco's modified Eagle's medium (glucose — 1000 mg/l; Sigma, Germany), supplemented with gentamycin (50 μg/ml; Sigma) and 10% fetal calf serum (PAN Systems, Germany). Cultures were maintained at 37°C in 5% $CO_2$ and 95% humidity. They were passaged twice a week after exposure to trypsin/EDTA (0.05/0.02%; PAN Systems, Germany) with a plating density of 1:3 ($5–6\times10^5$ cells/ml). For the experiments, cells were seeded in 40×10 mm dishes ($2\times10^5$ cells per dish), 24-well ($10^5$ cells per well) or 96-well plates ($2\times10^4$ cells per well) at a concentration of $2\times10^5$ cells/ml. Twenty-four hours after seeding the cells were treated with NaBt (3 or 5 mM; Sigma), TNF-α (0.5, 5, 10, 15 or 30 ng/ml; PAN Systems), or both, as indicated in the respective experiments. Culture medium was not replaced during experiments.

### 2.2. Amidoblack assay

HT-29 cells growing on 96-well plates were treated with NaBt (3 or 5 mM), TNF-α (0.5, 15 or 30 ng/ml) or their combinations for 24 or 48 h. Untreated cells served as a control. Amidoblack assay was performed according to Schultz and colleagues [15]. The cells were fixed by adding 100 μl of 10% formaldehyde in each well. After 15 min, the medium was removed and 100 μl of amidoblack (0.1% solution in 0.1 M sodium acetate) was added. The cells were stained for 30 min, then washed in acidified water (pH 3.5–5) and gently shaken with 50 mM NaOH (150 μl/well) for 15 min. The optical densities at 620 nm were measured using a DigiScan Reader (ASYS, Austria).

### 2.3. MTT assay

HT-29 cells growing on 96-well plates were treated with NaBt (3 or 5 mM), TNF-α (0.5, 15 or 30 ng/ml) or their combinations for 24 or 48 h. Untreated cells (appropriate amount of buffer solution added) served as a control. Ten μl of the 2.5 mg/ml stock solution of 3-[4,5-dimethylthiaolyl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) was added to each well. After 1 h of incubation at 37°C, the medium was removed, 50 μl of the extraction buffer (10% Triton-X100; 0.1 M HCl) was added, and plates were gently shaken for 30 min at room temperature [16]. The optical densities were measured at 570 nm (DigiScan Reader).

### 2.4. Analysis of the cell cycle

Cells grown on 40×10 mm dishes were treated with NaBt (3 or 5 mM), TNF-α (0.5 or 15 ng/ml) or their combinations for 24 or 48 h. The cells were harvested by trypsinisation, washed twice in phosphate-buffered saline (PBS), and fixed in 70% ethanol. DNA was stained by incubation (37°C; 30 min) with Vindelov's solution (10 mM Tris buffer, pH 8; 0.7 mg/ml RNase; 50 μg/ml propidium iodide; 0.1% Triton-X100; 10 mM NaCl). The DNA content was analysed using flow cytometry (argon ion laser, 488 nm for excitation, FACSCalibur, Becton Dickinson, San Jose, CA, USA). $15\times10^3$ cells were acquired for each sample and the percentages of cells in the individual cell cycle phases were analysed using ModFit 2.0 software (Verify Software House, ME, USA). Single cells were identified and gated by pulse-code processing of the area and the width of the signal. Cell debris was excluded by appropriate raising of the forward scatter threshold.

### 2.5. Floating cell quantification

The cells grown on 24-well plates were treated with NaBt (3 or 5 mM), TNF-α (0.5, 15 or 30 ng/ml) or their combinations for 24, 48 or 72 h. Untreated cells served as a control. Dead HT-29 cells were shed to the media and accumulated there as floating cells. Floating and adherent cells were counted separately using a Coulter Counter ZM, and the amount of floating (dead) cells was expressed as a percentage of the whole cell number.

### 2.6. Apoptosis quantification

#### 2.6.1. Flow cytometry

The cells were grown and treated as for floating cell quantification and managed as described for cell cycle analysis. The level of apoptosis determined as the sub $G_0/G_1$ population (the floating and adherent cells taken together) using ModFit 2.0 software.

*2.6.2. Fluorescence microscopy*

HT-29 cells growing on 40×10 mm dishes were treated with NaBt (5 mM), TNF-α (0.5 or 30 ng/ml) or their combinations for 24, 48 and 72 h. The cells were trypsinised and 0.5–2×$10^6$ cells (including floating cell population) were incubated with 40 μl of 4,6-diamidino-2-phenyl-indole (DAPI) staining solution (3 μg DAPI/ml of methanol) at room temperature and in the dark for 30 min. They were then mounted in Mowiol, and the apoptotic cells were counted using an OLYMPUS IX70 fluorescence microscope. In each sample, 100 cells were evaluated and the percentage of cells with apoptotic bodies was determined.

*2.7. ALP activity determination*

The cells grown on 40×10 mm dishes were treated with NaBt (3 or 5 mM), TNF-α (0.5 or 15 ng/ml) or their combinations for 24, 48 or 72 h. The cells were trypsinised and counted using a haemocytometer. 5×$10^5$ cells was resuspended in 500 μl of substrate buffer (10% diethanolamine; 5 mM $MgCl_2$; pH 9.7) and lysed by sonication for 5×10 s on a Branson Sonifier B-12 at a power of 30 watts (output 1). The cell lysate and ALP (Sigma) in several concentrations (15.6–1000×$10^{-6}$ U/well) for a calibration curve were incubated at 37°C with ALP substrate (4-p-nitrophenylphosphate; Fluka Chemical Corp., USA) in a 96-well plate (four parallel wells in each group) for 30 min. The reaction was stopped by adding 3 M NaOH (50 μl/well) and the optical densities were measured at 405 nm (DigiScan Reader). The reading values (units ×$10^{-6}$/5×$10^4$ cells) were converted to the percentage of control.

*2.8. F-actin quantification*

The cells were grown on glass coverslips in a 24-well plate and treated with either 1, 3 or 5 mM of NaBt, 0.5, 15 or 30 ng/ml of TNF-α, or with combinations of these two compounds. After 72 h of the treatment, the cells were fixed with 2% paraformaldehyde (pH 7.2) for 30 min in 4°C, permeabilised with 0.1% Triton-X100, washed twice in PBS, and incubated with fluorescein-isothiocyanate (FITC)-conjugated phalloidin (1 μg/ml; Sigma) for 45 min in the dark. The coverslips were then washed five times in PBS, mounted in Mowiol, and examined on a Molecular Dynamics SARASTRO 2000 confocal scanning laser microscope with a 40× objective. The sections were scanned and relative green fluorescence intensities were quantified using Imagespace™ software.

*2.9. Western blot analysis*

Cells grown on 40×10 mm dishes were treated with NaBt (3 or 5 mM), TNF-α (10 or 30 ng/ml) or their combinations for 24 or 48 h. The cells were scraped and lysed in Laemmli sample buffer (100 mM Tris, pH 6.8; 2% sodium dodecyl sulphate (SDS); 10% glycerol) [17]. Extracts of total proteins were assayed with the DC Protein assay kit (Bio-Rad), and equal amounts (10 μg) in 0.01% bromophenol blue and 1% mercaptoethanol were subjected to SDS-PAGE using 10% polyacrylamide gels. The gels were transferred to polyvinylidene fluoride membranes (Immobilon-P, Sigma) electrophoretically in a buffer containing 192 mM glycine, 25 mM Tris and 10% methanol. The membranes were blocked for 1 h in 5% powdered non-fat milk in wash buffer (0.05% Tween-20 in 20 mM Tris; pH 7.6; 140 mM NaCl). Primary monoclonal mouse anti-E-cadherin antibody (at a dilution of 1:5 000; #C20820, Transduction Laboratories, USA) was incubated with the blots for 1 h at room temperature. After washing the membranes in wash buffer, secondary rabbit anti-mouse IgG coupled to horseradish peroxidase (Sigma, A 9044) was added at a dilution of 1:10 000 for 1 h. The membranes were washed and antibody reactivity was visualised using the enhanced chemiluminescence ($ECL^+$) reagent (Amersham, UK) and measuring the signal on X-ray film-CP (AGFA). The equal loading was verified by non-specific amidoblack staining of proteins on the membrane after immunoblotting.

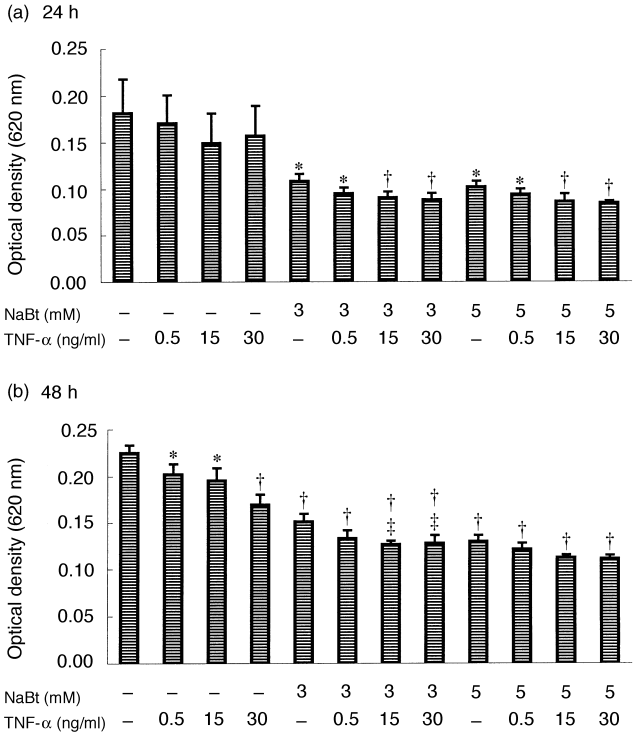


Fig. 1. Total protein staining with amidoblack of HT-29 cells treated for (a) 24 or (b) 48 h with either NaBt (3 or 5 mM), TNF-α (0.5, 15 or 30 ng/ml), or with their combinations. The data represent optical density measurements at 620 nm. The values are means ±SEM of three independent experiments. *$P < 0.05$ (†$P < 0.001$) versus untreated control; ‡$P < 0.05$ versus relevant doses of NaBt alone.

### 2.10. Statistical analysis

All the data are expressed as the means (±SEM) of at least three independent experiments. Comparisons between the groups were calculated using one-way ANOVA. With all statistical analyses, an associated probability ($P$ value) of $\leqslant 5\%$ was considered as significant.

## 3. Results

### 3.1. Growth of HT-29 cells after treatment with TNF-α and NaBt

In comparison with untreated control cells, both TNF-α and NaBt used as single agents inhibited the cell growth measured by amidoblack assay in a dose-dependent manner (Fig. 1). The effect of NaBt was already apparent after 24 h (approximately 40–45% decrease versus control at both concentrations of NaBt). Significant effects of TNF-α were observed after 48 h of the treatment, and the highest degree of growth inhibition was found at a concentration of 30 ng/ml of TNF-α (25% decrease versus control). As compared with NaBt-treated cells, after combined treatment of the cells with both agents either none or small additive effects were observed. Similar results were achieved using the MTT proliferation assay (data not shown).

### 3.2. Effects of TNF-α and NaBt on the HT-29 cell cycle

To find out whether TNF-α would modulate NaBt-mediated arrest of HT-29 cells in the $G_0/G_1$-phase of the cell cycle, the cell cycle parameters after the exposure to NaBt, TNF-α or their combinations were analysed (Fig. 2; because of the absence of any effects of TNF-α on cell cycle parameters, these data were not included in the figure). In comparison with the control, NaBt arrested cells in the $G_0/G_1$-phase of the cell cycle and simultaneously decreased the proportion of cells in the S-phase. Enhancement of the proportion of cells in the $G_0/G_1$-phase was most profound after 48 h of the treatment (37% and 56% increase following the addition of 3 mM or 5 mM of NaBt, respectively, in comparison with untreated control). The results of combined treatment with NaBt and TNF-α (except the values of the S-phase in the NaBt 5 mM/TNF-α 15 ng/ml group after 24 h of treatment) were not significantly different from those achieved after the treatment with NaBt as a single factor.

### 3.3. Effects of TNF-α and NaBt on the level of floating and apoptotic HT-29 cells

TNF-α and NaBt alone slightly increased the number of floating and apoptotic cells compared with the untreated control (Table 1). Significant results were obtained (for the number of apoptotic cells for TNF-α and for the number of floating cells for NaBt) only after 72 h treatment with the highest doses of both agents. When TNF-α and NaBt were added together, the percentage of floating cells increased significantly particularly for the higher doses. After treatment with 5 mM NaBt and 30 ng/ml TNF-α the ratio between the adherent and floating cells was approximately 1:1 after 48 and 72 h. A certain number of these cells were apoptotic, as confirmed both by the detection of a sub$G_0/G_1$ population by flow cytometry and by the counting of apoptotic cells after DAPI staining. A definite significant increase was observed especially after the treatment with the highest doses used (5 mM NaBt and 30 ng/ml TNF-α).

### 3.4. Effects of TNF-α on NaBt-induced differentiation

The level of differentiation after the treatment of HT-29 cells with NaBt, TNF-α or with their combinations was detected by specific markers, i.e. ALP activity, F-actin polymerisation, and E-cadherin protein expression.

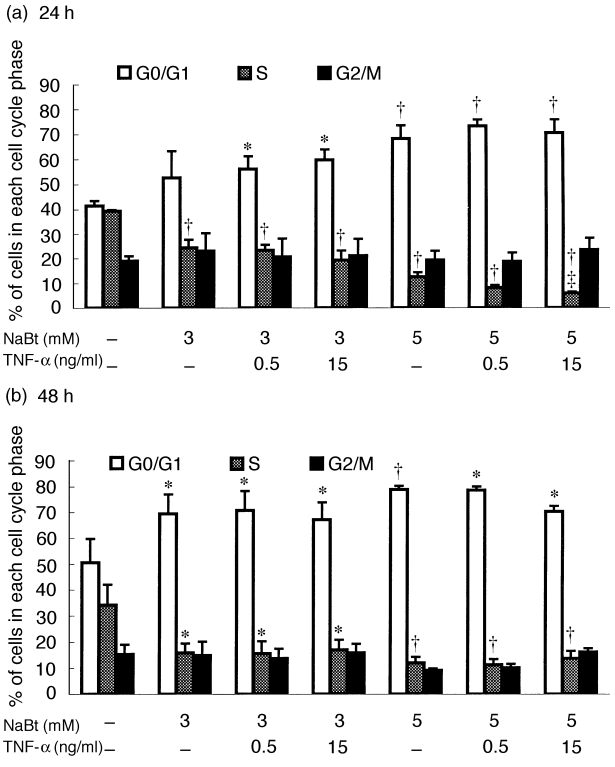


Fig. 2. Cell cycle analysis of HT-29 cells treated for (a) 24 or (b) 48 h with either NaBt (3 or 5 mM) or with combinations of NaBt and TNF-α (0.5 or 15 ng/ml). The values are means ±SEM of three independent experiments. *$P < 0.05$ (†$P < 0.001$) versus untreated control; ‡$P < 0.05$ versus relevant doses of NaBt alone.

Table 1
Quantification of floating and apoptotic cells after treatment of HT-29 cells with TNF-α, NaBt or their combinations

| Treatment | | % of floating cells | | | % of apoptotic cells (flow cytometry) | | | % of apoptotic cells (DAPI staining) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| NaBt (mM) | TNF (ng/ml) | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| – | – | 15.18±3.82 | 6.89±2.13 | 7.44±1.64 | 1.67±0.88 | 1.77±1.07 | 2.50±0.96 | 2.25±0.75 | 2.00±0.50 | 6.00±1.50 |
| – | 0.5 | 11.78±4.80 | 9.50±1.60 | 10.39±5.80 | 1.48±1.02 | 1.04±0.25 | 2.88±1.02 | 3.00±1.00 | 1.50±0.50 | 3.50±1.50 |
| – | 15 | 15.16±5.04 | 11.23±3.95 | 22.35±4.88 | 1.07±0.38 | 3.82±1.14 | 8.70±2.65 | ND | ND | ND |
| – | 30 | 16.38±7.18 | 8.43±0.59 | 22.43±2.79 | 2.34±1.02 | 3.10±1.40 | 11.83±0.68* | 5.50±2.50 | 2.00±0.50 | 7.25±1.25 |
| 3 | – | 18.01±13.32 | 14.09±3.19 | 16.56±4.18 | 2.51±0.19 | 2.78±0.92 | 3.82±2.07 | ND | ND | ND |
| 3 | 0.5 | 25.34±15.04 | 16.40±0.20 | 18.06±2.35 | 6.90±0.40 | 5.26±0.39 | 4.28±1.20 | ND | ND | ND |
| 3 | 15 | 19.33±0.88 | 38.40±8.79† | 27.89±3.67* | 6.96±2.20 | 10.43±0.52* | 9.07±2.84 | ND | ND | ND |
| 3 | 30 | 22.79±8.37 | 24.19±1.81 | 30.01±1.85* | 13.74±1.28† | 8.38±0.38 | 7.74±1.55 | ND | ND | ND |
| 5 | – | 24.92±7.91 | 23.34±6.50 | 29.91±5.53* | 3.85±0.35 | 6.75±2.65 | 7.85±2.47 | 1.50±0.75 | 2.50±0.50 | 5.25±0.88 |
| 5 | 0.5 | 22.98±3.73 | 26.33±3.48 | 30.65±3.52* | 9.86±2.32* | 9.29±4.07* | 9.94±6.72 | 5.25±1.75 | 6.75±0.75† | 6.75±0.75 |
| 5 | 15 | 27.13±4.64 | 38.16±1.16† | 49.14±9.13† | 12.85±4.92† | 15.70±6.96† | 15.04±5.44* | ND | ND | ND |
| 5 | 30 | 28.78±2.00 | 46.85±2.03† | 51.10±6.55† | 14.30±7.15† | 15.87±6.51† | 19.73±5.75† | 4.75±1.25 | 9.50±1.75† | 13.25±1.75† |

The values are means ±SEM of three independent experiments. *$P<0.05$ (†$P<0.001$) versus untreated control. ND, not done; SEM, standard error of the mean.

### 3.4.1. ALP activity

ALP activity is considered as one of the major markers of NaBt-induced differentiation in HT-29 cells. The ALP activity of NaBt-treated cells increased significantly during 24–72 h (both dose- and time-dependently) in comparison with the untreated control (Fig. 3). This increase was the highest after 72 h of treatment (relative ALP activity was enhanced 12-fold and 58-fold in NaBt 3 mM and NaBt 5 mM groups, respectively, in comparison with the control). While TNF-α alone had no effect on cell ALP activity, it significantly suppressed the NaBt-mediated ALP activity induction. Fifteen ng/ml

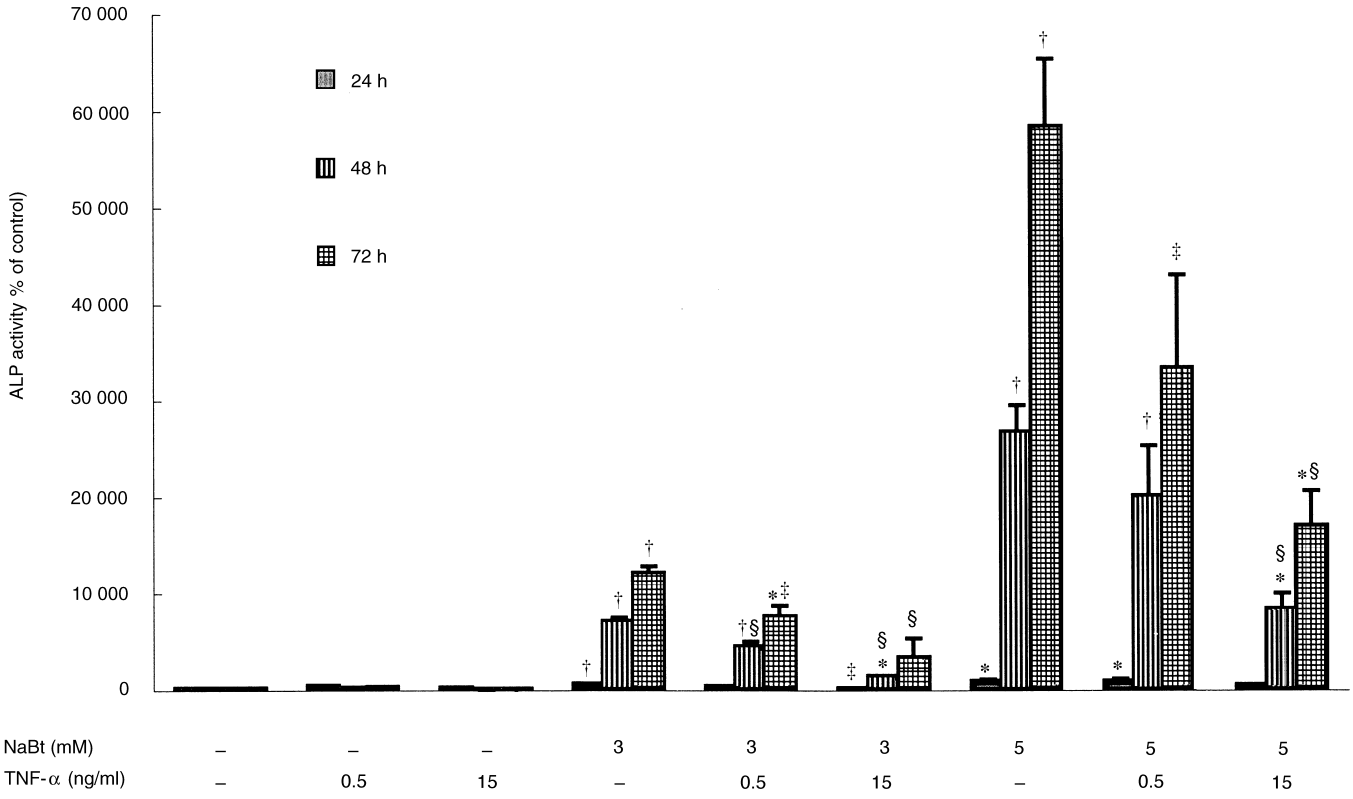


Fig. 3. Relative alkaline phosphatase (ALP) activity of HT-29 cells treated for 24, 48 or 72 h with either NaBt (3 or 5 mM), TNF-α (0.5 or 15 ng/ml) or their combinations. The values are means ±SEM of three independent experiments. *$P<0.05$ (†$P<0.001$) versus untreated control (considered as 100%); ‡$P<0.05$ (§$P<0.001$) versus relevant doses of NaBt alone. Absolute control values expressed as units$\times 10^{-6}/5\times 10^{4}$ cells: 3.84±1.75 (24 h), 2.51±1.06 (48 h) and 1.19±0.85 (72 h).

of TNF-α decreased the relative ALP activity in all combinations with NaBt 3.5-fold, on average, in comparison with the relevant NaBt single treatment, while 0.5 ng/ml of TNF-α did so only 1.5-fold on average. The highest effect (5-fold reduction) was observed in the group of NaBt 3 mM/TNF-α 15 ng/ml after 48 h of treatment.

### 3.4.2. Accumulation of F-actin

NaBt treatment led to morphological changes of HT-29 cells which were accompanied by an accumulation of F-actin (Fig. 4). In comparison with the untreated control, significant effects were observed after the 72 h treatment with NaBt at a concentration of 5 mM (the

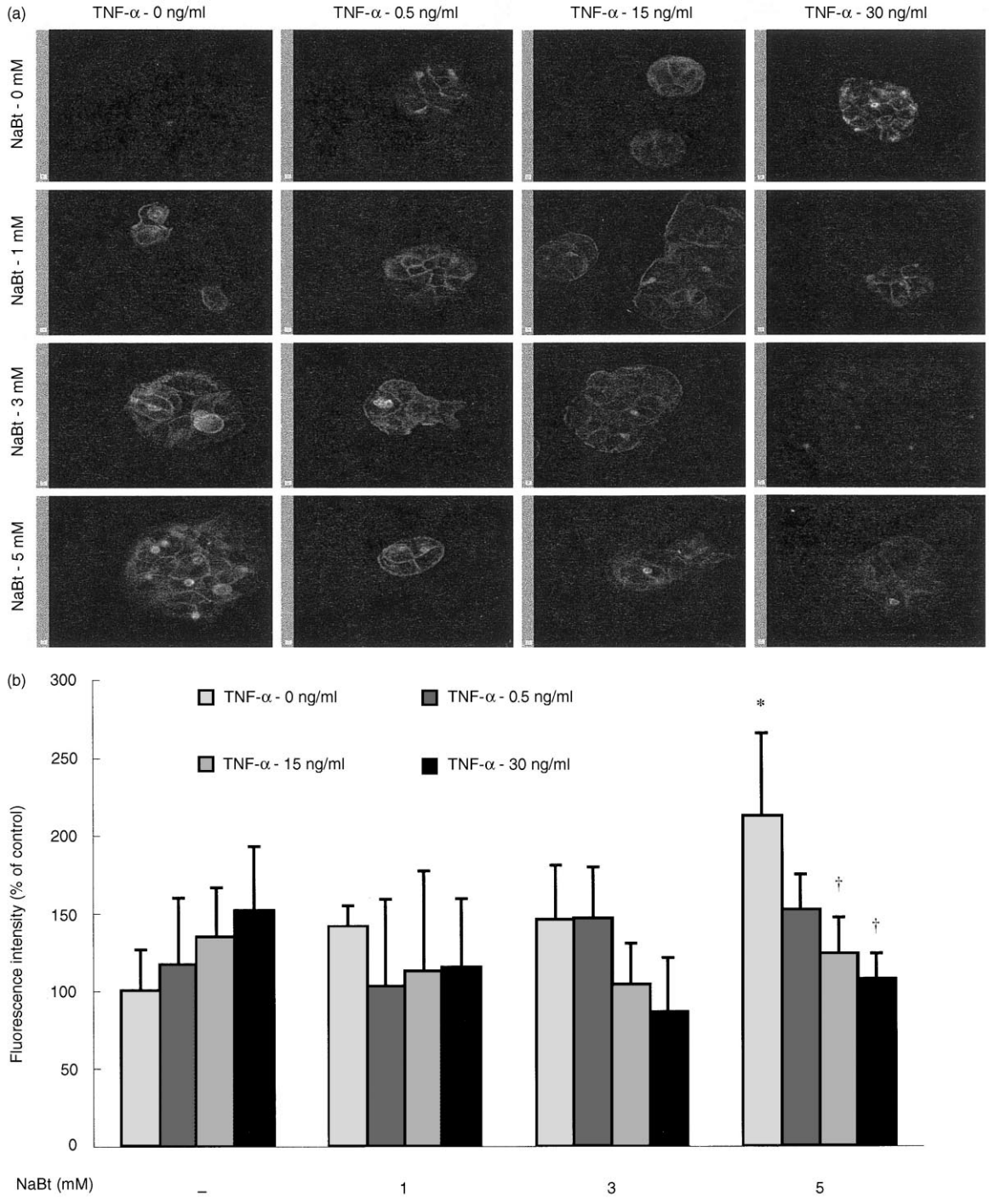


Fig. 4. F-actin content in HT-29 cells treated for 72 h with either 1, 3 or 5 mM of NaBt, 0.5, 15 or 30 ng/ml of TNF-α, or with their combinations. F-actin was stained with FITC-labelled phalloidin. (a) Sections scanned on confocal laser microscope (the results from a representative experiment). (b) Relative green fluorescence intensity. The values are means ±SEM of three independent experiments. *$P < 0.05$ versus untreated control (considered as 100%); †$P < 0.05$ versus relevant doses of NaBt alone.

relative green fluorescence was 2.1-fold higher than in the control). The treatment of cells with TNF-α alone also induced polymerisation of F-actin; however, these effects were not statistically significant. After combined treatment of the cells with TNF-α and 5 mM of NaBt the accumulation of F-actin was prevented (Fig. 4a). This effect was statistically significant at concentrations of 15 and 30 ng/ml of TNF-α (1.7- and 2.0-fold decrease versus control; Fig. 4b).

### 3.4.3. E-cadherin protein expression

The expression of E-cadherin protein was detected in the cells treated with TNF-α, NaBt or their combinations for 24 or 48 h (Fig. 5). The opposite effects of TNF-α (slightly decreasing expression) and NaBt (slightly increasing expression) and the tendency to higher E-cadherin expression after combined treatment of HT-29 cells with TNF-α and NaBt were observed, but no significant difference to the untreated control was shown in any case.

## 4. Discussion

The balance between cell growth, differentiation and cell death maintains tissue homeostasis in the colonic crypt. Deregulation of these processes plays an important role in colonic carcinogenesis. There is evidence that, in addition to endogenous growth regulators like TNF-α, dietary factors like butyrate may also take part in the regulation of cytokinetics in this tissue. In accord with other authors [10,12,13], we have demonstrated in this paper that NaBt: (1) mediated $G_0/G_1$ arrest of the cell cycle of HT-29 colon adenocarcinoma cells and thus inhibited their growth; and (2) induced differentiation of these cells, which was characterised by an elevated ALP activity and E-cadherin expression, and by morphological changes accompanied by polymerisation of F-actin. While it has been shown that NaBt-mediated arrest of the cell cycle is connected with the induction of the inhibitor of cyclin-dependent kinases $p21^{WAF/Cip1}$ [18], the mechanisms of NaBt-induced differentiation are less clear and mostly speculative [19]. Moreover, NaBt is able to slightly stimulate apoptosis in several colon carcinoma cell lines, including HT-29 cells [20,21].

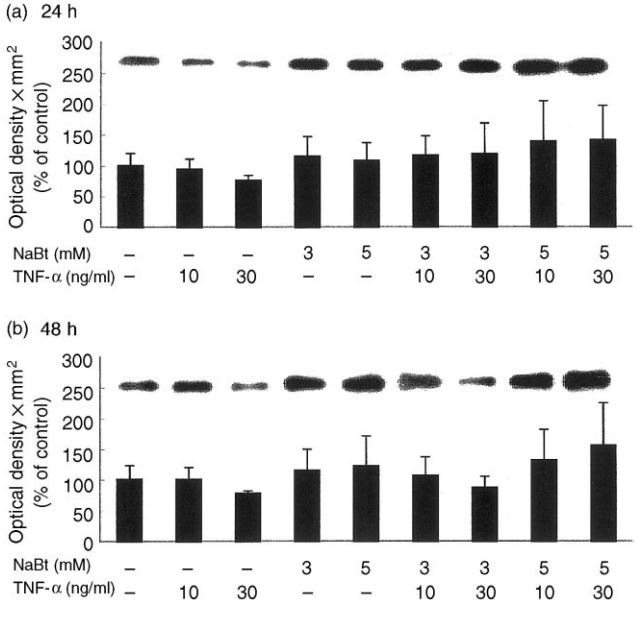


Fig. 5. E-cadherin protein expression of HT-29 cells treated for (a) 24 or (b) 48 h with either NaBt (3 or 5 mM), TNF-α (10 or 30 ng/ml) or their combinations. Densitometric quantifications of Western blots are demonstrated. The values are means ±SEM of three independent experiments. The bands show the results from a representative experiment.

In our experiments, cell growth was also suppressed after higher concentrations of the TNF-α used, but without any effect on cell cycle parameters. This discrepancy could be explained by a slower rate of cell progression through the cell cycle. It was shown in other human colon carcinoma cells that there is minimal or no sensitivity to TNF-α even at a concentration of 100 ng/ml [22]. Since the effects of a combined treatment of the HT-29 cells with NaBt and TNF-α on the cell cycle and cell growth were none or only additive, it was concluded that there is no significant interaction between these factors affecting cell proliferation.

In agreement with the results of Giardina and associates [14] we have shown that dual treatment with TNF-α and NaBt significantly increased the number of dead and apoptotic HT-29 cells. In our experiments, this effect was achieved with lower doses of TNF-α (0.5–30 ng/ml) and after a longer duration of treatment (24–72 h). Potentiation of apoptosis induced by Fas ligand after the treatment of HT-29 cells with IFNγ, TNF-α and NaBt has also been reported [23].

Interestingly, we have shown for the first time that TNF-α attenuated NaBt-induced differentiation parameters, namely ALP activity and F-actin polymerisation — which are considered as the main differentiation markers for HT-29 cells. While NaBt alone enhanced dramatically the cytoplasmic activity of ALP, TNF-α alone had no effect; however, it significantly decreased the effect induced by NaBt. In addition to increased ALP activity, our results demonstrated that NaBt also increased the relative F-actin content in HT-29 cells, which demonstrated changes of their intracellular organisation similar to that of normal enterocytic cells [24]. A similar effect of NaBt has already been observed by Ryan and Higgins [25] in transformed rat fibroblasts. Although TNF-α alone did not influence ALP activity in our experiments, it tended to increase F-actin content, as was also shown by Chazaud and associates [26] in human endothelial cells. However, similar to the ALP activity, after a combined treatment of cells with TNF-α and NaBt the relative F-actin content was suppressed in our experiments.

E-cadherin is a membrane protein which facilitates cell–cell connections. A downregulation of both E-cadherin mRNA and protein expression has been observed in colorectal carcinomas, and it correlates with poor outcome and increased invasiveness [27]. In spite of the results described above, our data did not indicate any significant differences in the expression of E-cadherin within the framework of our experimental conditions. Butt and colleagues [28] found that NaBt enhanced E-cadherin protein expression in HT-29 cells, but our results demonstrated only a slight insignificant increase in comparison with the control. In contrast, TNF-α alone slightly and insignificantly decreased the expression of this adhesive molecule.

The mechanisms of the interaction between NaBt and TNF-α which caused a decrease in HT-29 cell differentiation remain unclear and can only be derived from other cell systems. From the reports of Velazquez and associates [19] and Larric and Wright [29] it could be supposed that NaBt and TNF-α might share similar signalling pathways including phospholipase C and protein kinase C. It means that the interaction resulting in decreasing differentiation could occur at this level. However, additional experiments showed that the supplementary reduction of ALP activity after combined treatment with NaBt and TNF-α was similar, even if TNF-α was added 1, 6 or 24 h before or after NaBt (data not shown). This suggests that the mechanism of NaBt and TNF-α interaction is more complex and provides space for further investigation. Concerning E-cadherin expression it can be derived from other reports that the expression of this molecule depends on many factors (e.g. confluence of the cells) [30] and that the effects of TNF-α may vary according to the cell system used.

In summary, our results demonstrated that TNF-α does not interfere with the effects of NaBt on cell cycle distribution and cell growth. In contrast, TNF-α potentiated the apoptosis and suppressed the differentiation of colon carcinoma HT-29 cells that was induced by NaBt. In spite of the fact that all these cell processes are connected, the individual steps could be (at least in part) regulated independently [31]. Our previous results with human myeloid HL-60 cells demonstrated synergistic potentiation of differentiation by combined treatment with all-*trans* retinoic acid (ATRA), TGF-β1 and inhibitors of 5-lipoxygenase without changes in cell cycle, cell growth and apoptosis [32]. We have also shown that potentiation of antiproliferative and differentiation effects of ATRA by inhibitor of cytochrome P-450 is accompanied by decreased apoptosis and an increased level of the anti-apoptotic bcl-2 protein in HL-60 cells [33].

Our findings call attention to the role of TNF-α in the development of adenoma or carcinoma during prolonged chronic inflammation of the colon or during the treatment of cancer patients with TNF-α via suppression of the beneficial effects of butyrate. In this context, the beneficial effect of apoptosis potentiation also remains debatable because of a possible impairment of the balance between cell proliferation, differentiation and apoptosis whose rigorous regulation is very important in the colon crypt. Therefore, a further clarification of the exact mechanisms regulating colon cell kinetics is necessary.

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