

Research Article

Acetylcholinesterase in intestinal cell differentiation involves G2/M cell cycle arrest

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Abstract. Here we examine differentiation of the intestinal cell line Caco-2 following exposure to sodium butyrate (NaBT), using alkaline phosphatase (ALP) activity and carcinoembryonic antigen (CEA) levels as markers of differentiation. We show that acetylcholinesterase (AChE) activity and RNA levels increase during differentiation. Treatment with AChE

inhibitors or knockdown of AChE levels by shRNA markedly decrease ALP and CEA levels in a concentration- and time-dependent manner. Finally, our observations suggest that NaBT-induced differentiation of intestinal cells involves AChE-induced cell cycle arrest.

Keywords. Differentiation, acetylcholinesterase, cell cycle, butyrate sodium, G2/M.

Introduction

The epithelium of the gastrointestinal tract is a complex and dynamic tissue composed of numerous cell types with important cellular functions, including digestion, absorption, barrier and immune function, and peptide secretion. The mammalian intestinal mucosa undergoes a process of continual renewal characterized by active proliferation of stem cells localized near the base of intestinal crypts, and progression of these cells along the crypt-villus axis with concomitant cessation of proliferation, and subsequent differentiation into one of four primary cell types. Several experimental models have been used to study the molecular mechanisms involved in the regulation of cell growth and differentiation of the normal and neoplastic human intestinal epithelium

[1–4]. During enterocyte-like differentiation, the human colon carcinoma cell line Caco-2 exhibits a variety of differentiated cell characteristics, including dome formation, presence of microvilli, and a polarized columnar morphology with an apical brush border [5–7]. Caco-2 has thus provided a useful *in vitro* model to delineate mechanisms involved in the terminal differentiation of enterocytes.

Butyrate, a short-chain fatty acid produced in the colonic lumen by microbial fermentation, is taken up by cells of the intestinal epithelium, and serves as a major source of energy for the colonic mucosa [8, 9]. Studies have shown that sodium butyrate (NaBT) improves intestinal epithelial barrier function *in vitro* and promotes sodium absorption *in vivo* [10–13], while its absence is associated with atrophy of the mucosa and apoptosis of colonocytes [14]. NaBT stimulates growth of normal epithelial cells, but inhibits proliferation and induces rapid cell differentiation in colon cancer cell lines. Thus, colonic

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NaBT is thought to maintain mucosal differentiation and plays an important protective role in colorectal carcinogenesis [15–18]. However, the molecular mechanisms underlying the antineoplastic effects of NaBT towards carcinogenic cells have not been elucidated.

Acetylcholinesterase (AChE) is most commonly known for its role in terminating cholinergic signaling by the hydrolysis of acetylcholine to choline and acetate. Interestingly, the expression of AChE is not restricted to cholinergic tissues, AChE may therefore play noncholinergic roles during development and tissue morphogenesis. Numerous studies have identified roles for AChE in mediating neuronal interactions, migration, axon formation, and differentiation [19–21]. AChE also has been shown to promote neurite outgrowth and differentiation, as well as amyloid deposition [22–24]. Importantly, AChE has also been shown to increase during the differentiation of non-neural tissues, such as bone, muscle, and the hematopoietic system [25–27]. AChE may therefore participate in cellular functions that are distinct from its established catalytic role.

In this study, we investigate the expression and function of AChE during differentiation of colonic cancer cells. Our results suggest that AChE activity and RNA levels increase during differentiation. Moreover, downregulation of AChE can partially inhibit NaBT-induced differentiation. Finally, we show that NaBT-induced differentiation involves AChE-mediated cell cycle arrest.

Materials and methods

Cell culture and NaBT treatment. Caco-2 cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Differentiation was induced by treating cells with 1 mM NaBT (Sigma, St. Louis, MO). The AChE inhibitor huperzine A and the cholinesterase inhibitor eserine were added 24 h prior to the addition of NaBT to the culture medium.

Plasmid construction and transfection. The full-length human AChE cDNA (GenBank accession no. M55040) was subcloned into the pcDNA3.1A (Invitrogen, Carlsbad, CA) and pEGFP-N1 (Clontech, Mountain View, CA) vectors. To generate an AChE shRNA expressing plasmid, sequences 966 (5'-GAAAGCGTCTTCCGGTTCT-3') and K (5'-GAGTGTCTGCTACCAATAT-3') were synthesized and subcloned into the pSuperior vector (Oligoen-

gine, Seattle, WA). Cells were seeded in 6-well plates and transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Characterization of cell differentiation. ALP activity was measured spectrophotometrically at 405 nm using *p*-nitrophenol phosphate (Merck, Darmstadt, Germany) as substrate [28]. CEA levels were measured using a commercially available kit according to the manufacturer's instructions (Shanghai Kehua Bio-engineering Co. Ltd). Levels of ALP and CEA were normalized by comparing with the total protein which was determined with a bicinchoninic acid (BCA) assay. Finally, ALP activity and CEA levels were expressed as a multiple relative to untreated controls.

Determination of AChE activity. Relative AChE activity was determined spectrophotometrically at 405 nm using a modified Ellman's assay as described [29, 30]. Cells were suspended in extraction buffer (50 mM potassium phosphate, pH 7.4, 1 M NaCl, 0.5% Tween-20). Lysates were clarified by centrifugation at 12000 rpm for 10 min at 4 °C, and supernatant protein concentration was determined by BCA assay. Tetraisopropyl pyrophosphoramidate (iso-OMPA, 75 µM) was pre-incubated with cell supernatant for 30 min to inhibit BuChE activity. One unit (U) of AChE activity represents the hydrolysis of 1 µmol of substrate per minute at 37 °C. The level of AChE activity in undifferentiated Caco-2 cells is 6.87 ± 0.62 mU/mg. Specific AChE activity was defined as the number of units of AChE activity versus corresponding relative protein concentration. Control values were averaged and then normalized to one.

Semiquantitative RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and was reverse-transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI). Resulting cDNAs were for PCR amplification of a 479 bp fragment of acetylcholinesterase-tailed (AChE-T) variant (primers: 5'-CGGGTCTACGCCTACGTCTTTG-3' and 5'-CACAGGTCTGAGCAGCGATCC-3') and a 588-bp fragment of GAPDH (5'-CCACCCATGGCAAATTCATGGCA-3' and 5'-TCTAGACGGCAGGTCAGGTCCACC-3'). Amplification conditions were as follows: 94 °C for 20 s (2 min for the first cycle), 60 °C for 30 s, and 72 °C for 40 s (10 min for the last cycle) for 28 cycles.

Flow cytometry. Cells were harvested at various time points, washed twice with PBS, fixed in 70% ethanol at 4 °C overnight or 4% paraformaldehyde for 10 min

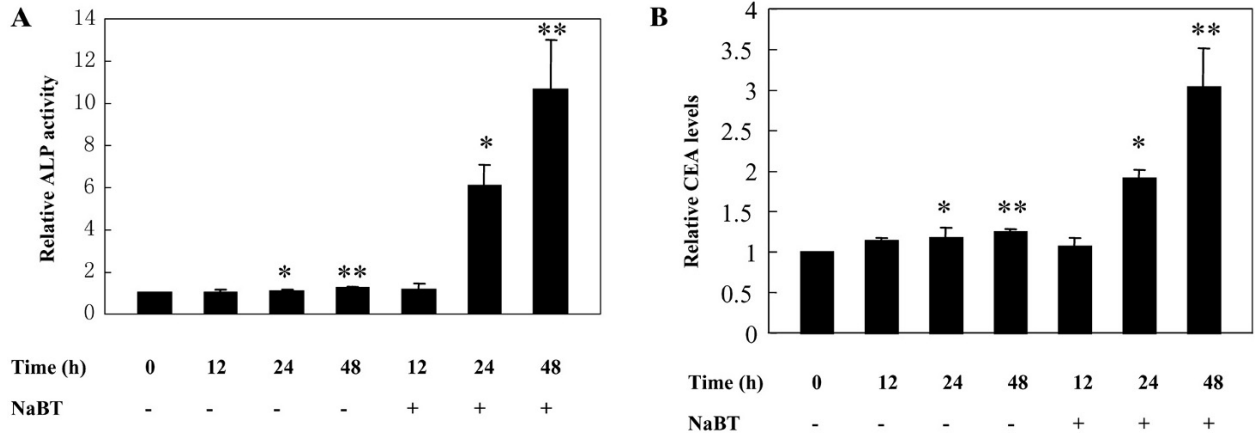


Figure 1. The effect of NaBT on changes in the levels of ALP and CEA. ALP activity (A) and CEA levels (B) was measured in homogenates from Caco-2 cells treated with 1 mM NaBT after the indicated treatment times. Values are expressed as means \pm SD of three independent experiments (* $P < 0.01$ for NaBT-treated 24 h group vs. untreated 24 h group; ** $P < 0.01$ for NaBT-treated 48 h group vs. untreated 48 h group).

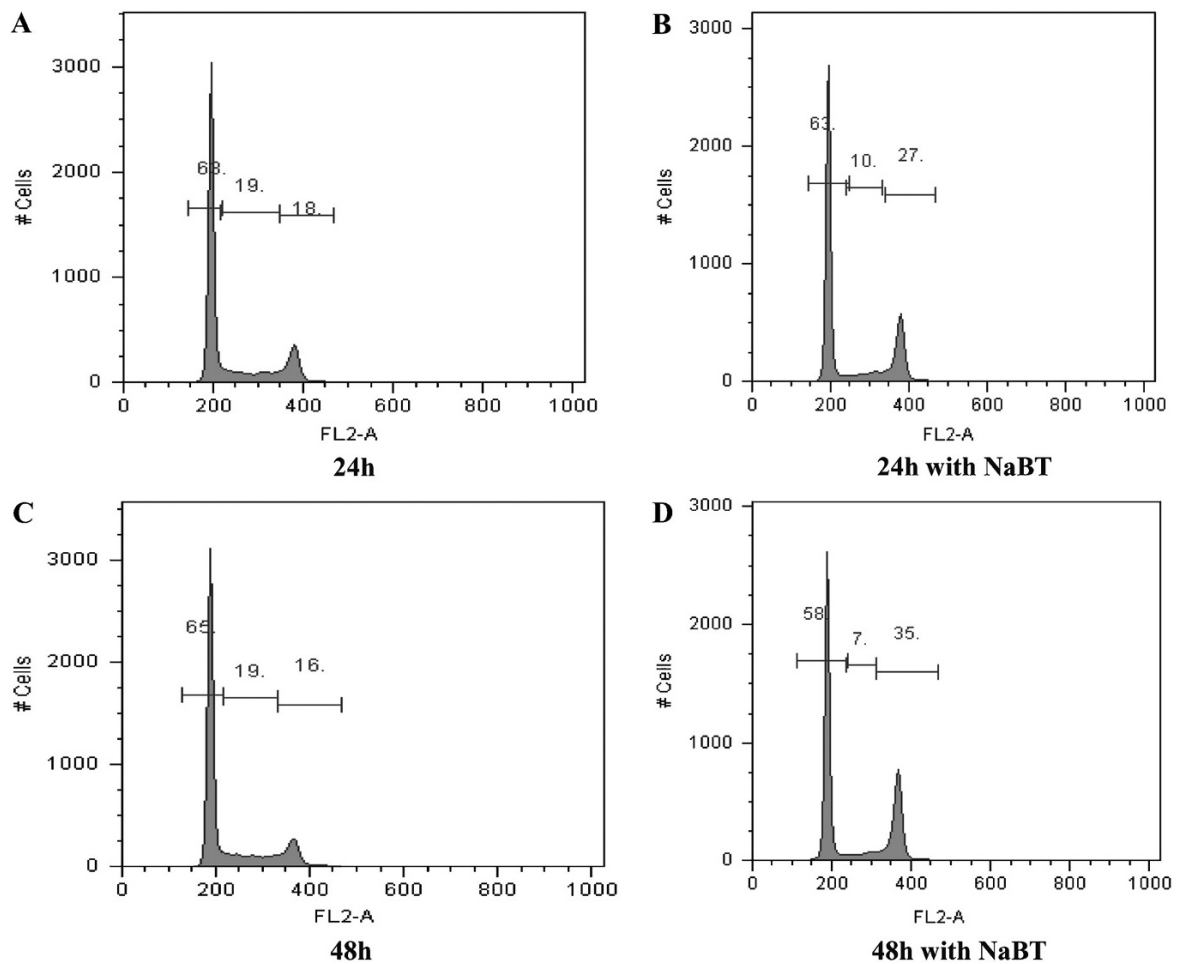


Figure 2. Analysis of the cell cycle distribution of Caco-2 cells treated with NaBT. (A) Untreated cells at 24 h. (B) Cells treated with NaBT for 24 h. (C) Untreated cells at 48 h. (D) Cells treated with NaBT for 48 h.

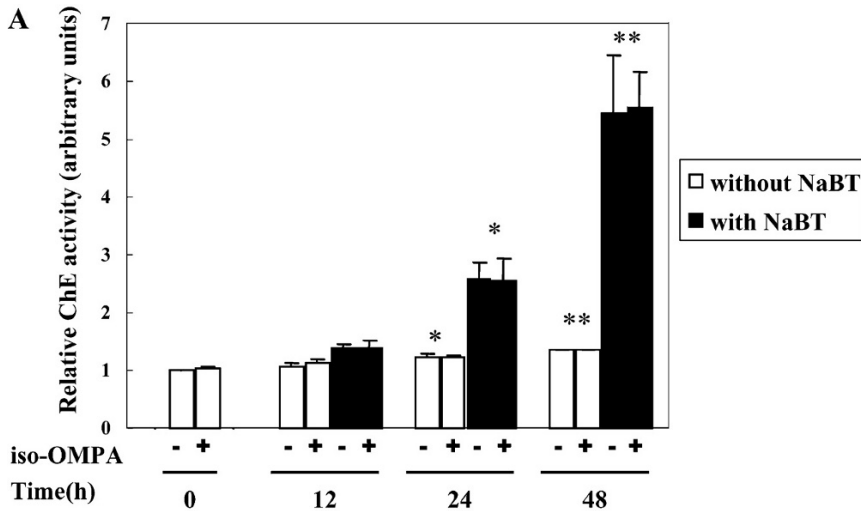
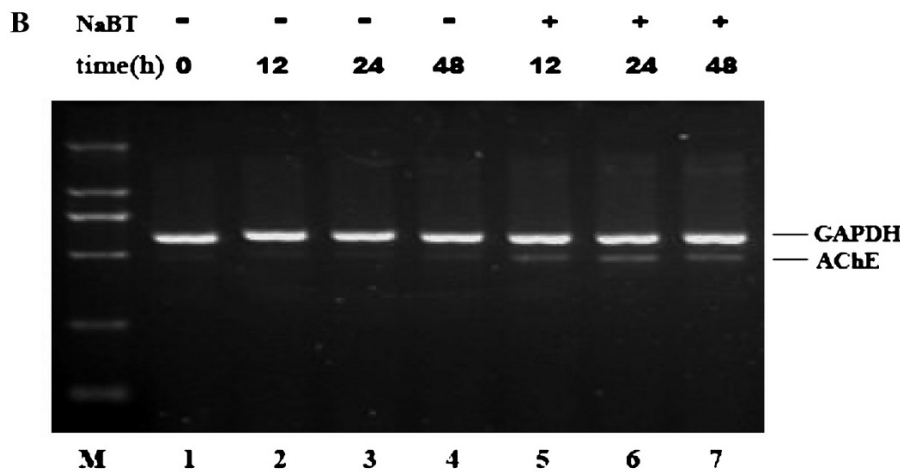


Figure 3. NaBT induces upregulation of AChE. (A) ChE activity in Caco-2 cells exposed to 1 mM NaBT after the indicated times. (B) Semiquantitative RT-PCR analysis of AChE expression following NaBT treatment for the indicated time periods, with GAPDH expression as an internal control. Shown are means \pm SD for three independent experiments (* $P < 0.01$ for NaBT-treated 24 h group vs. untreated 24 h group; ** $P < 0.01$ for NaBT-treated 48 h group vs. untreated 48 h group)



at room temperature (for analysis of GFP fluorescence), and then permeabilized with 0.1% Triton in 0.1% sodium citrate. Cells were then collected by centrifugation and stained by addition of propidium iodide (50 $\mu\text{g/ml}$). RNase A (200 $\mu\text{g/ml}$) was then added and samples were incubated for 30 min at 37°C. Cell cycle analysis was performed using the BD FACSCalibur (Becton Dickinson, San Jose, CA), and cell cycle phase was analyzed using Flowjo and FCS3.0 software.

Statistical analysis. All experiments were repeated at least three times. Data were presented as the mean \pm standard deviation (SD). Significant differences between treatment groups were determined using an unpaired Student's *t* test. Differences were considered statistically significant at $P < 0.05$ or $P < 0.01$.

Results

NaBT-induced Caco-2 cell differentiation and cell cycle arrest. Caco-2 cells were incubated with or without NaBT and harvested at 12, 24, and 48 h after treatment. ALP and CEA, two brush border enzymes that have been used as markers of epithelial cell differentiation [31, 32], were measured to determine the extent of differentiation. As shown in Figure 1A, at 24 h, ALP activity had increased by approximately 6-fold in NaBT-treated cells, compared with untreated cells, and by ~10-fold by 48 h of exposure to NaBT. NaBT treatment also led to increased CEA levels by 24 h, which was approximately 3 times higher than untreated cells by 48 h (Fig. 1B). These observations suggest that NaBT induces differentiation in a time-dependent manner.

To further characterize this differentiation, we examined whether NaBT can induce cell cycle arrest by examining DNA content by flow cytometry. As shown in Figure 2, the fraction of cells in the G2/M phase

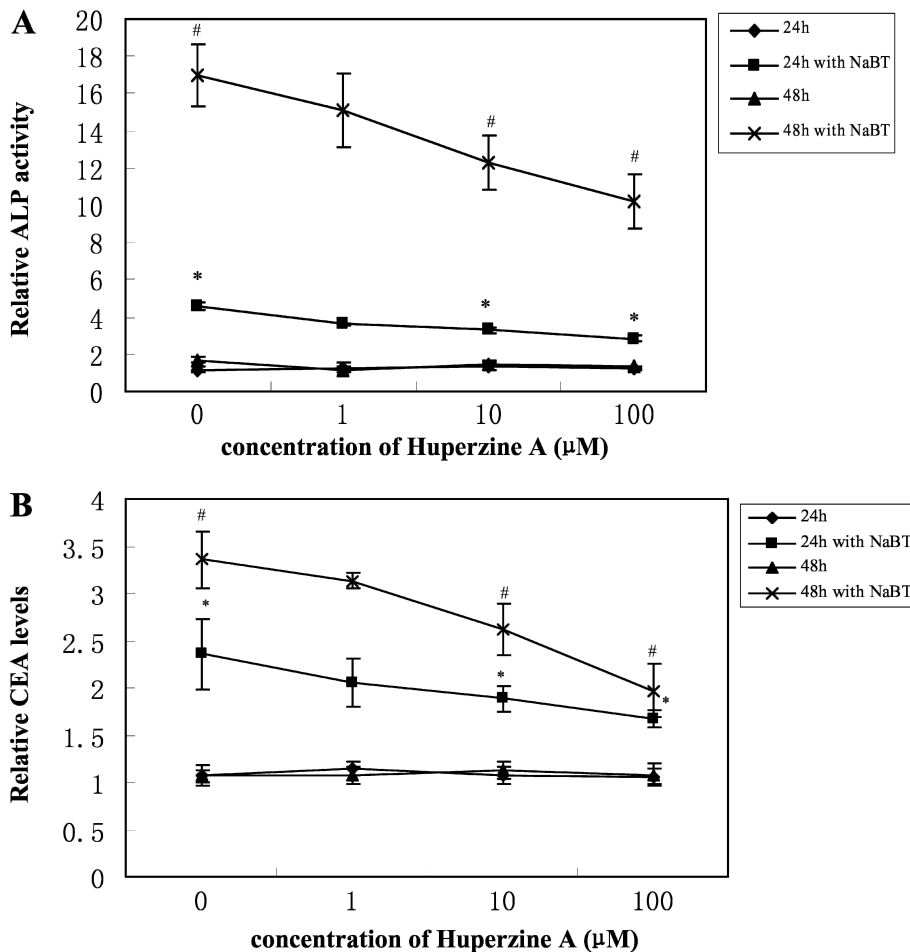


Figure 4. Effects of Huperzine A on ALP and CEA levels during NaBT-induced differentiation. Caco-2 cells were incubated with Huperzine A for 24 h before exposure to NaBT. After indicated times, (A) ALP activity and (B) CEA levels were determined. Shown are means \pm SD for three independent experiments (* P < 0.05 for NaBT-treated 24 h group vs. inhibitor-treated 24 h groups; # P < 0.05 for NaBT-treated 48 h group vs. inhibitor-treated 48 h groups).

increased (~27% in G2/M), while the fraction in the S phase decreased, following NaBT treatment for 24 h compared with untreated cells (~18% in G2/M) (Fig. 2A, B). By 48 h of treatment, no obvious change in cell cycle distribution was observed in untreated cells (Fig. 2C), while in treated cells, the G2/M population was further increased (~35% in G2/M) and both G0/G1 and S-phase populations decreased (Fig. 2D).

NaBT-induced differentiation is associated with changes in esterase activity and expression of AChE.

AChE activity is known to increase as Caco-2 cells reach confluence during differentiation [33]. We examined the activity of cholinesterase (ChE) during NaBT-induced differentiation in Caco-2 cells. ChE activity increased significantly during the course of NaBT-induced differentiation, and it was not affected by BuChE activity inhibitor iso-OMPA (Fig. 3A). This result indicated that ChE activity during the course of NaBT-induced differentiation was attributed to AChE. Compared with untreated cells, AChE activity increased around 2.5-fold within the first 24 h and

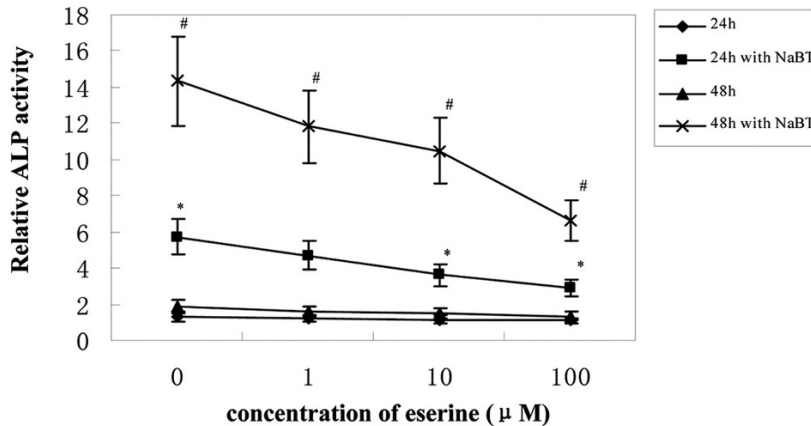
reached levels as high as 5-fold induction after 48 h of treatment.

Then we examined the effects of NaBT on relative levels AChE expression by semiquantitative RT-PCR. Although AChE expression could hardly be detected in undifferentiated cells, treatment with NaBT led to a pronounced increase in the levels of AChE-T variant mRNA (Fig. 3B). In contrast, levels of GAPDH, which was used as an internal control, did not change during differentiation. Interestingly, AChE mRNA levels were significantly increased by 12 h (Fig. 3B), while levels in AChE activity were significantly increased by 24 h (Fig. 3A), indicating gradual upregulation of AChE mRNA and activity.

AChE inhibitors block NaBT-induced Caco-2 differentiation.

To assess the requirement of AChE for NaBT-induced Caco-2 cell differentiation, we used the AChE inhibitor huperzine A and the ChE inhibitor eserine. In the absence of NaBT, pretreatment of cells with huperzine A for 24 h did not affect ALP or CEA levels, while incubation with 10 μ M huperzine A during NaBT treatment decreased ALP

A



B

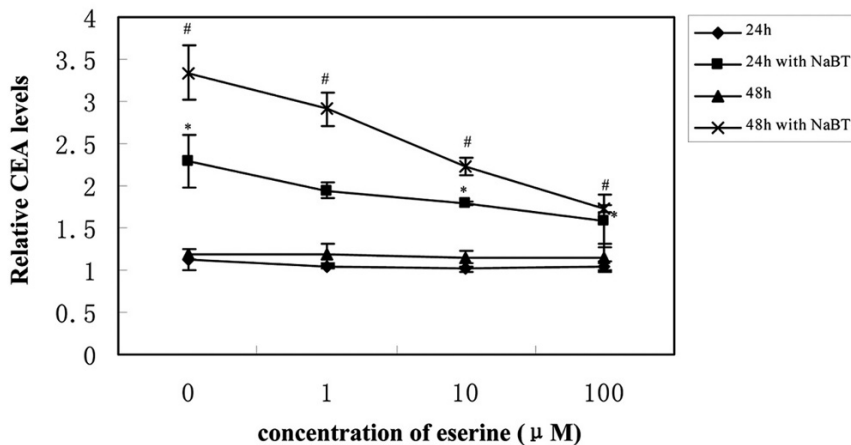


Figure 5. Effects of eserine on ALP and CEA levels during NaBT-induced differentiation. ALP activity (A) and CEA levels (B) in Caco-2 cells incubated with eserine for 24 h prior to exposure to NaBT. Shown are means \pm SD for three independent experiments (* $P < 0.05$ for NaBT-treated 24 h group vs. inhibitor-treated 24 h groups; # $P < 0.05$ for NaBT-treated 48 h group vs. inhibitor-treated 48 h groups).

and CEA levels, and treatment with 100 μ M huperzine A led to more decreased ALP and CEA levels (Fig. 4). As observed with huperzine A, treatment with 10 μ M eserine decreased ALP and CEA levels during NaBT-induced differentiation, ALP and CEA levels decreased more following treatment with 100 μ M eserine, but pretreatment for 24 h with eserine did not affect ALP or CEA levels (Fig. 5).

The effects of AChE knockdown on NaBT-induced Caco-2 cell differentiation and cell cycle phase. To further examine the role of AChE in NaBT-induced colonocyte differentiation, we used shRNA to knock down expression of AChE in Caco-2 cells. We examined the consequences of two individual AChE shRNA candidates, termed K [34] and 966. Their efficacy in silencing AChE expression was evaluated by examining AChE activity. Caco-2 cells were transfected with shRNA or control vectors for 24 h, and

were then treated with NaBT for a further 24 h. Interestingly, NaBT-induced increases in AChE activity were reduced about 30% by transient transfection with shRNA 966, compared with control vector, while transfection with shRNA K was more effective (Fig. 6A). Furthermore, NaBT-treated cells transiently transfected with either 966 or K exhibited reduced ALP activity (Fig. 6B) and CEA levels (Fig. 6C). These observations suggest that AChE is required for NaBT-induced differentiation.

We then analyzed the cell cycle progression of GFP-positive Caco-2 cells transfected with K, 966, or control pSuperior vectors. After NaBT treatment, the percentage of cells in G2/M increased (Fig. 7B) compared with untreated cells (Fig. 7A); transient transfection of K or 966 decreased the percentage of G2/M phase in the NaBT-treated cells (Fig. 7D, F). Transient transfection of K or 966 did not affect cell cycle progression in untreated cells, compared with cells transfected with

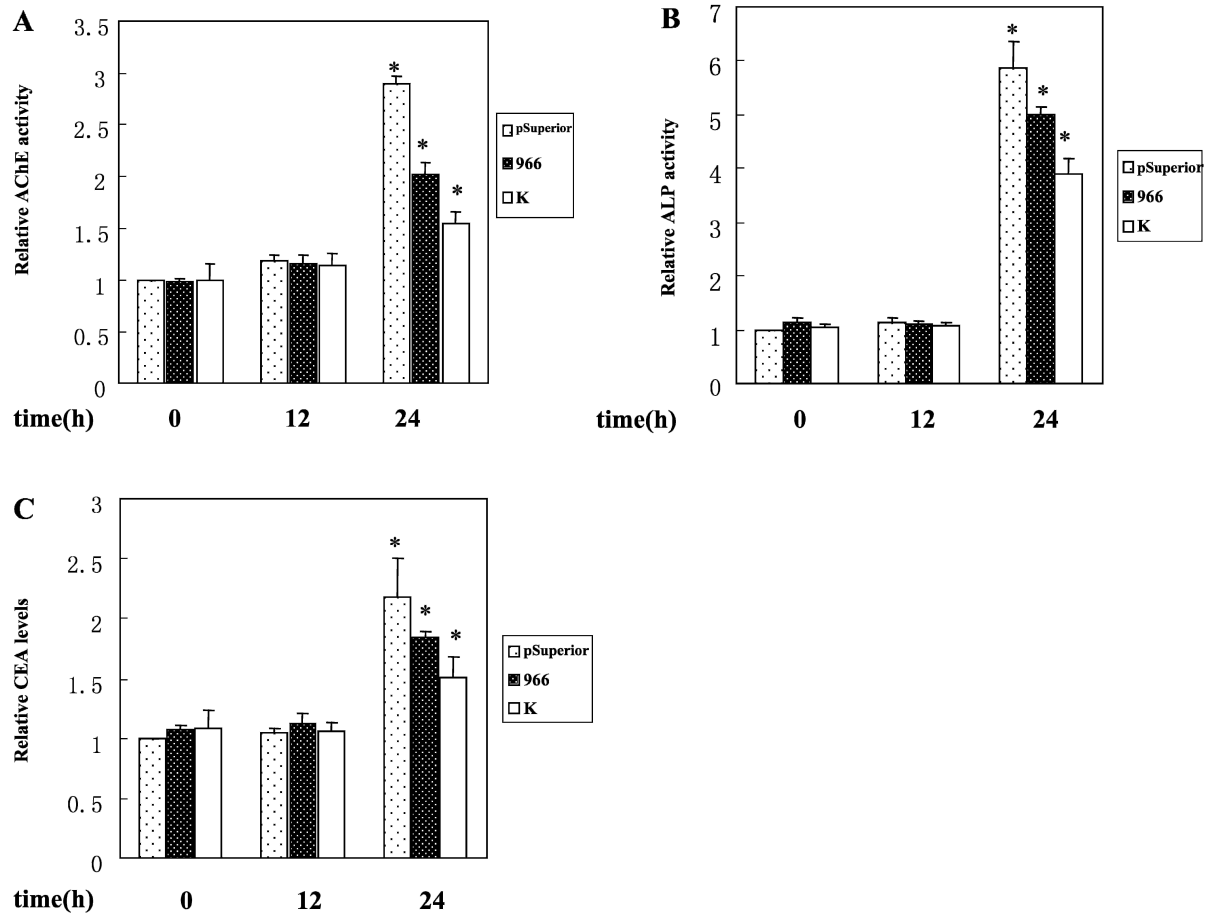


Figure 6. shRNA-mediated knockdown of AChE decreases ALP and CEA levels during NaBT-induced differentiation. AChE activity (A), ALP activity (B), and CEA levels (C) following transient transfection of AChE shRNA 966, K, and control vectors for 12 h, and treatment with NaBT at indicated times. Shown are means \pm SD for three independent experiments (* $P < 0.05$ for NaBT-treated 24 h pSuperior group vs. NaBT-treated 24 h K group or NaBT-treated 24 h 966 group).

control vector (Fig. 7C, E). In contrast, the percentage of cells in both G_0/G_1 and S phases increased during NaBT-induced differentiation following transient transfection with K and 966, compared to cells transfected with control vector (Fig. 7G).

The effects of AChE overexpression on cell cycle progression.

To examine whether AChE-T variant is sufficient to alter cell cycle progression, we constructed an AChE expression plasmid by cloning AChE-T variant cDNA into the vector pEGFPN1, then examined cell cycle progression in Caco-2 cells transfected with either pEGFP-N1-AChE or the control vector pEGFP-N1 by flow cytometry. As expected, the percentage of cells in the G2/M phase increased following AChE overexpression (Fig. 8). While 53% of cells overexpressing AChE were in the G2/M phase (Fig. 8B), only 34% of control cells had reached the G2/M phase (Fig. 8A). Accordingly, the fraction of cells in both G_0/G_1 and S phases decreased with AChE overexpression (Fig. 8C). The level of AChE activity

in pEGFP-N1-AChE transfected cells was about nine times higher than that in pEGFP-N1 transfected cells as well as untransfected cells (Fig. 8D).

Discussion

Butyrate is a metabolic by-product formed in the gastrointestinal tracts of mammals. Although butyrate serves as a source of oxidative fuel for normal colonocytes, addition of it to neoplastic cells inhibits hyperproliferation and drives terminal differentiation and/or apoptosis [16–18]. Consistent with its anti-carcinogenic role *in vitro*, increased butyrate production is closely correlated with the reduction of tumor mass in animal models [35, 36]. Although several studies have revealed that NaBT can induce apoptosis [37–39], we suppose that NaBT-induced cell differentiation or apoptosis depends on its dosage. When we used 1 mM NaBT to treat Caco-2 cells, we did not detect distinct apoptotic cell fractions in flow cytometry.

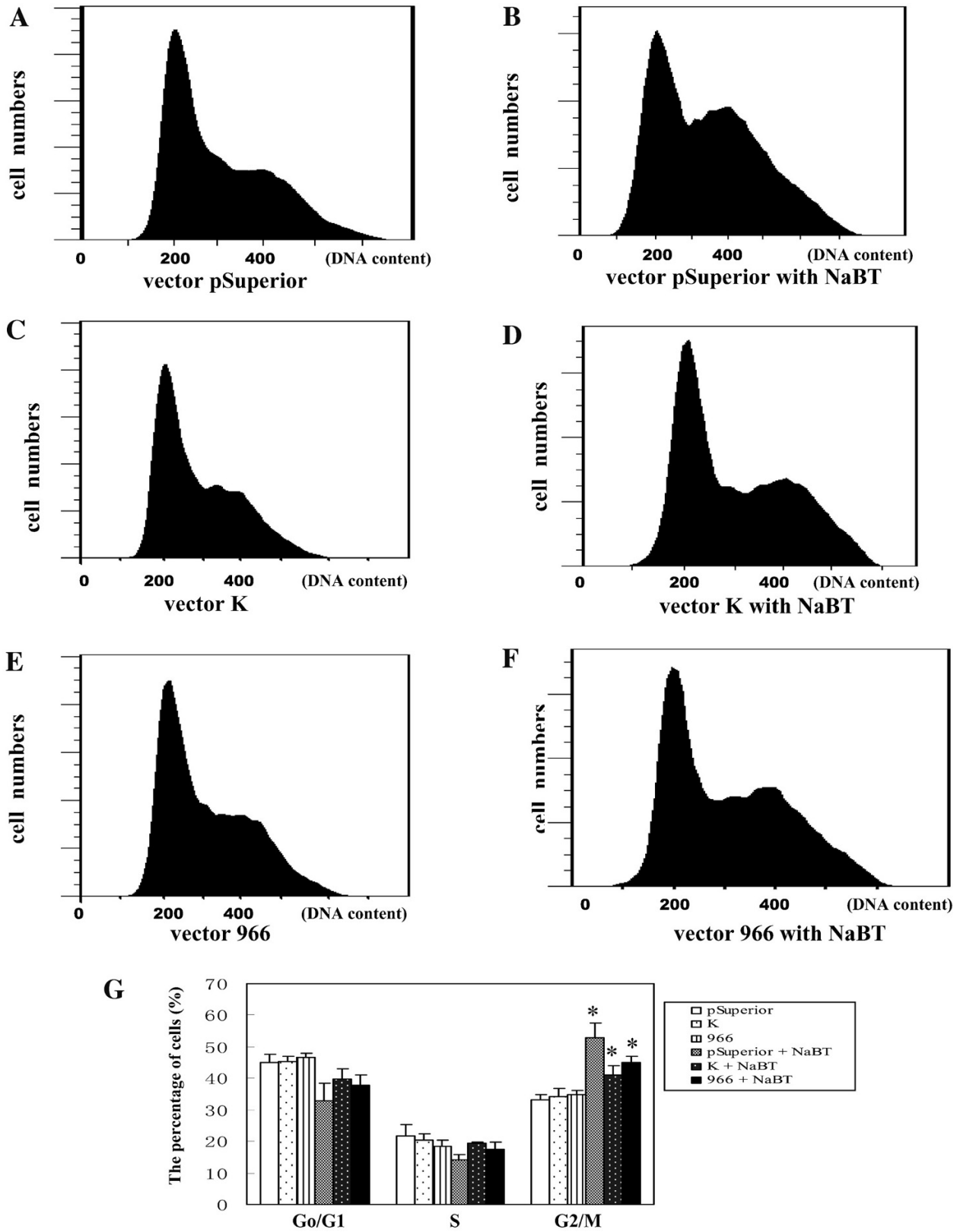


Figure 7. shRNA-mediated knockdown of AChE leads to altered cell cycle distribution following NaBT treatment. Cells transfected with control vector were treated without (A) or (B) with NaBT. (C) Cells transfected with AChE shRNA K without or (D) with NaBT. (E) AChE shRNA 966 were treated without or (F) with NaBT. (G) Bars showing the proportion of cells in G0/G1, S, and G2/M phases of the cell cycle after transient transfection of AChE shRNA 966, K, or control vectors. Shown are means \pm SD for three independent experiments (* $P < 0.01$ for NaBT-treated 24 h pSuperior group vs. NaBT-treated 24 h K group or NaBT-treated 24 h 966 group).

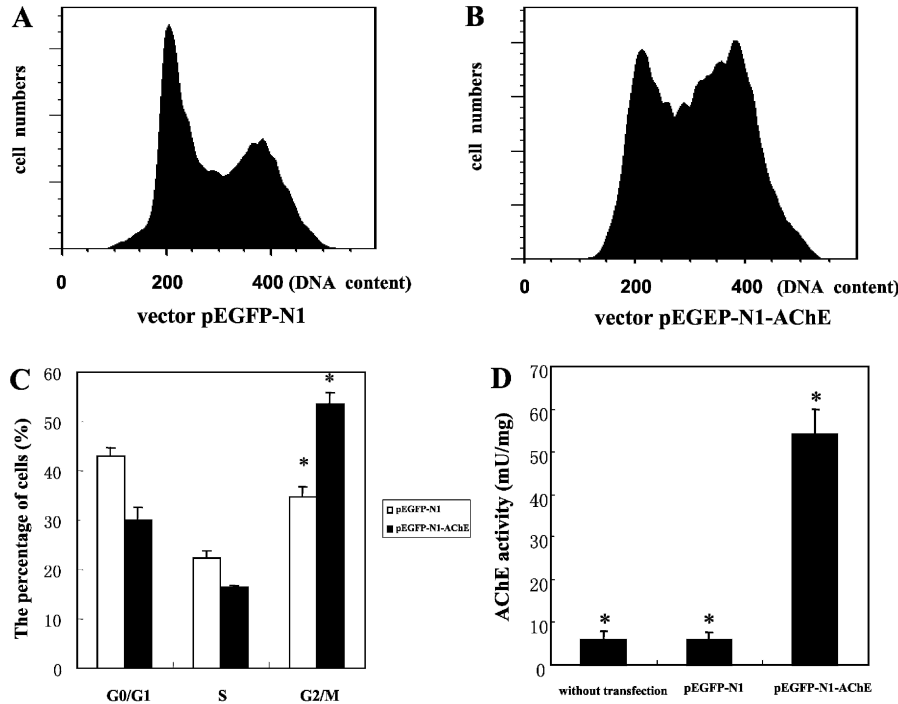


Figure 8. Overexpression of AChE induces G2/M arrest in Caco-2 cells. Cells were transiently transfected with pEGFP-N1 (A) or pEGFP-N1-AChE (B) vectors and cell cycle phase was determined by flow cytometry. (C) Bars showing the proportion of pEGFP-N1 or pEGFP-N1-AChE transfected cells in G0/G1, S, and G2/M phases of the cell cycle. (D) The levels of AChE activity in transfected and untransfected cells. Shown are means \pm SD for three independent experiments (* $P < 0.05$ for pEGFP-N1 group vs. pEGFP-N1-AChE group or no transfection group).

etry assay (Fig. 1). Cleaved caspase-3 was, however, detectable following treatment with high concentrations of NaBT (data not shown). NaBT can block cell cycle progress at G1/S or the G2/M checkpoint depending on colon cancer cell lines from different origins [40–42]. Here, we observed that NaBT primarily affected progression during the G2/M phase in Caco-2 cells, consistent with previous reports [43, 44].

The primary function of AChE is to hydrolyze acetylcholine (ACh) and thus terminate cholinergic neurotransmission. Recently, increasing evidence has suggested that AChE has a number of biological roles in addition to its function during cholinergic neurotransmission [45, 46]. Moreover, AChE has been shown to be expressed by some non-neural cell types, including myocytes and hematopoietic cells. It was taken as a differentiation marker in megakaryocytes [47]. AChE upregulation was also observed correlated with the differentiation state of osteoblasts and staurosporine-treated human prostate cancer cells [48, 49]. Expression of AChE in cancers of the colon, sigmoid colon, and in rectal cancers was found to be reduced compared to normal tissues [50, 51]. However, to date no studies have addressed the role of AChE in the transformation of colon epithelial cells. Using NaBT-induced differentiation of Caco-2 cells as a model, we observed increased ALP and CEA levels, and an inhibition of G2/M cell cycle progression. Notably, AChE activity was enhanced, as previously reported [33]. We also found that the AChE-T variant

was upregulated during this process. These results provide supporting evidence that AChE expression is upregulated during differentiation. The interruptive effects of AChE shRNA and AChE inhibitors on Caco-2 differentiation validated the role of AChE in modulating colon cell differentiation.

Both the AChE inhibitor huperzine A and the ChE inhibitor eserine efficiently inhibited AChE activity and reduced ALP and CEA levels. These findings strongly suggest that catalytic activity of AChE is required for differentiation. Furthermore, the AChE substrate ACh was suggested to enhance cell proliferation by overstimulating cholinergic receptors (AChRs) in colon cancers [52–54]. Muscarinic acetylcholine receptor (mAChR) expression was markedly reduced, and the agonist carbachol was able to inhibit Friend murine erythroleukemia cell (MELC) differentiation [55]. Thus the emergence of AChE might provoke cell differentiation by influencing AChRs via ACh. The cholinergic receptors and cholinergic responses may be involved in this process. On the other hand, during myogenic differentiation of C2C12 cells, expression of AChE-T protein and enzymatic activity were dramatically increased, but the level of the tetrameric globular form of AChE (G_4 AChE) was decreased. The production of G_4 AChE is controlled by the level of proline-rich membrane anchor (PRiMA) expression [56]. There is currently no evidence of PRiMA expression in differentiated Caco-2 cells. Whether PRiMA is expressed and contributes to changes in AChE molecular forms

during Caco-2 differentiation may be examined in future studies.

In our experiments, treatment with AChE shRNA abrogated the NaBT-induced block in G2/M, suggesting that AChE regulates differentiation by regulating cell cycle progression. Consistent with this observation, AChE overexpression blocked cell cycle progression in the G2/M phase. These results suggest that AChE is both necessary and sufficient to alter cell cycle progression, yet it is not clear whether this effect is direct or indirect. AChE has been reported to bind basement membrane proteins such as laminin, collagen IV, and the transcriptional co-repressor CtBP [34, 57–60]. Upregulation of AChE is likely to affect the expression of basement membrane proteins that interact with AChE, leading to changes in cascade signals. Integrin, a collagen/laminin receptor, has been shown to activate Ras/MAP kinase cascades, cell cycle progression, and to prevent apoptosis [61]. CtBP/BARS and CtBP1 have been shown to regulate Golgi fragmentation and mitosis, since anti-sense and siRNA directed against CtBP caused a reduction of mitotic index and an accumulation of cells in G2 of the cell cycle [62]. These observations suggest that AChE acts indirectly on cell cycle progression, but this hypothesis must be tested in future studies.

In conclusion, AChE is likely to play an important role in colon cancer progression, and may thus serve as a promising diagnostic marker or target for therapeutic intervention.

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