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The Important Role of Caspase-10 in Sodium Butyrate-Induced Apoptosis

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Butyrate, a short chain fatty acid, exhibits a wide variety of biological effects including the inhibition of cell growth, change of cellular morphology and the induction of apoptosis. Sodium butyrate-induced apoptosis has been reported to associate with the up-regulation of pro-apoptotic Bax expression, and the down-regulation of anti-apoptotic Bcl-2 and Bcl-XL expressions. However, in some cases, butyrate has also been shown to cause apoptosis without change in Bcl-2, Bcl-XL and/or Bax. This study investigates the detailed mechanisms of sodium butyrate-induced apoptosis. The effect of sodium butyrate was analyzed in the induction of caspase activities, formation of caspase active forms and mRNA levels in human breast cancer cell line MRK-nu-1. Induction of activities of caspase-3, -10 and, to some extent, -8 and formation of DNA fragmentation were observed with sodium butyrate in a dose- and/or time-dependent manner. The levels of caspase-10 mRNA expression markedly increased in a time-dependent manner by the treatment of sodium butyrate, whereas caspase-8 mRNA expression was not changed. Inhibitors of caspase-8 and caspase-10 reduced caspase-3 activity and subsequent DNA fragmentation induced by sodium butyrate. These caspase inhibitors also inhibited the cleavage of pro-caspase-3 to the active forms indicated by Western blotting analysis. Pyrrolidine dithiocarbamate also inhibited the induction of caspase-10 mRNA expression and caspase-3 activation. Contrary to other reports, levels of Bcl-2, Bcl-XL and Bax mRNA expressions were not distinctly changed by even 5 mM sodium butyrate treatment. Our results suggest that sodium butyrate may trigger apoptosis via the induction of the caspase-10 expression.

Butyrate, a short chain fatty acid, produced by bacterial fermentation from dietary carbohydrate in the colon, exhibits a wide variety of biological effects including the inhibition of cell growth, change of cellular morphology and the induction of apoptosis [32, 35].

Choi reported that exposure of sodium butyrate (SB) resulted in apoptotic cell death accompanied up-regulation of pro-apoptotic Bax expression and down-regulation of anti-apoptotic Bcl-2 and Bcl-XL in human monocytic leukemia U937 cells [7]. Apoptotic signals caused by SB were transmitted to caspase-3, which subsequently activate nuclease and produce DNA fragmentation, a typical marker of apoptosis [2, 3]. Mandel and Kumar reported that butyrate-induced apoptosis in human breast cancer cell line MCF-7 was closely linked to the down-regulation of Bcl-2 mRNA and protein [23]. They also showed that stable Bcl-2 over-expressing MCF-7 cells exhibited a suppression of butyrate-induced apoptosis. Natoni et al. demonstrated that SB treatment resulted in a marked down-regulation of Bcl-XL expression, mitochondrial membrane depolarization, and cytochrome c release to cytoplasm followed by activation of caspase-9 and caspase-3 in human pancreatic cancer cells [25]. Wang et al. also demonstrated that down-regulation of FLIP and up-regulation of caspase-8 allowed apoptosis [37]. Certain findings may indicate that butyrate attacks mitochondria and the released cytochrome c induces a later part of apoptosis, e.g., nuclear and DNA fragmentation [5, 11, 22].

However, Ma *et al.* reported that butyrate-induced apoptosis was blocked by the addition of pan-caspase inhibitor, ZVAD [31]. They indicated that a caspase cascade plays a major role in butyrate-induced apoptosis. Furthermore, Daehn *et al.* reported that butyrate-induced apoptosis is associated with up-regulation of cell surface expression of death receptor Fas and activation of extrinsic caspase-3 activity [9]. The intrinsic caspase pathway did not play an important role in apoptotic cascade, as caspase-9 was not detected. They concluded butyrate is a potent inducer of Fas associated apoptosis in a keratinocyte cells. Rahmani *et al.* showed that ectopic expression of a mutant dominant-negative caspase-8 resulted in a significant decrease in SB-induced apoptosis, and over-expression of Bcl-2 did not decrease the apoptosis [29]. Up to that point, the role of caspase cascade in butyrate-induced apoptosis still remained to be discovered.

In the present study, we showed caspase-10 mRNA level was increased by the treatment with SB. Inhibitor of caspase-10 completely inhibited the executor caspase-3 activity determined by both enzyme activity and Western blotting assay. Increase of mRNA level of caspase-10 induced by SB was entirely abolished by pyrrolidine dithiocarbamate (PDTC). PDTC also blocked caspase-3 activation. These results indicate that induction of caspase-10 is critical for SB-induced apoptosis.

MATERIALS AND METHODS

Materials

Sodium butyrate, Z-IETD-fmk, Z-AEVD-fmk and MG-132 were obtained from Sigma-Aldrich (ST. Louis, MO, USA). Ac-DEVD-MCA and Ac-IETD-MCA were obtained from Peptide Institute (Osaka, Japan). Ac-AEVD-AFC was from BioVision (Mountain View, CA, USA). SN 50 was obtained from EMD Biosciences (San Diego, CA, USA). Anti-caspase-3 antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated mouse anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antibody was obtained from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LightCycler-FastStart DNA Master SYBR Green I Kit was obtained from Roche Diagnostics GmbH (Basel, Switzerland).

Cell culture and induction of apoptosis

Human breast cancer cell line MRK-nu-1 was provided from the Institute of Development, Aging and Cancer, Tohoku University. The cells were maintained in suspension in 250 ml Nunc flasks (non-treated SI, Nunc) in RPMI 1640 supplemented with 10% fetal calf serum, at 37°C in a 5% CO₂ humidified atmosphere. At the onset of experiments, cells were transferred into culture dishes for suspension cells (Sumilon Celltight X, Sumitomo Bakelite, Tokyo, Japan) at the indicated density for various periods (Figure legends). Cell death was induced by treatment with SB dissolved in distilled water. For treatments with inhibitors of caspases or nuclear factor kappa-B (NF-kappaB), cells were incubated with each inhibitor for 1 h, and then, treated with SB for the indicated times.

Assay of caspase activity

MRK-nu-1 cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5) and 0.2% Triton X-100 on ice and clarified by centrifugation. The enzyme activity was assayed as described previously [38] with 50 μ M fluorescent substrates, Ac-DEVD-MCA (for caspase-3), Ac-IETD-MCA (for caspase-8) and Ac-AEVD-AFC (for caspase-10). Fluorescent AMC and AFC product formation was measured with excitation at 380 nm and emission at 460 nm using MTP-32 plate reader (Corona Electric, Hitachinaka, Japan), and with excitation at 400 nm and emission at 505 nm using ARVO MX-fla (PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA), respectively. Caspase activity was expressed as the amount of AMC or AFC cleaved per mg protein per min. Protein concentration of the lysate was determined with a Bio-Rad DC protein assay system.

Detection of DNA fragmentation

DNA was isolated by the method reported by Hayashi *et al.* [14] with some modifications. Briefly, cells were lysed in cold buffer containing 20 mM Tris-HCl (pH 7.8), 2 mM EDTA and 0.5% Triton X-100. After centrifugation, supernatant samples were treated with RNase A and proteinase K. Subsequently, fragmented DNA was precipitated with 5 M NaCl and isopropanol. To assay the DNA

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fragmentation pattern, samples were subject to 2% NuSieve/agarose (3:1) gel electrophoresis and bands were stained with SYBR Green 1 nucleic acid gel stain.

Real-time polymerase chain reaction

Total RNA was extracted from MRK-nu-1 cells using Sepasol RNA I-super (Nakarai Tesque, Kyoto, Japan) following the protocol provided by the manufacturer. The cDNA was synthesized from 1 µg RNA with Oligo-dT primer using a First strand cDNA synthesis kit for RT-PCR (Roche Diagnostics GmbH) as instructed by the manufacturer. Polymerase chain reaction (PCR) for caspase-8, caspase-10 and internal control, GAPDH, was performed using a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics GmbH). Primer sequences were as follows:

caspase-8 forward primer,	5'-ATGCAAACTGGATGATGACA-3';
caspase-8 reverse primer,	5'-GATTATCTTCAGCAGGCTCTT-3';
caspase-10 forward primer,	5'-AATCTGACATGCCTGGAG-3';
caspase-10 reverse primer,	5'-ACTCGGCTTCCTTGTCTAC-3';
Bcl-2 forward primer,	5'-GCTCTAAAATCCATCCAG-3';
Bcl-2 reverse primer,	5'-CCTCTCCATCATCAACTT-3';
Bax forward primer,	5'-CCCGAGAGGTCTTTTTC-3';
Bax reverse primer,	5'-GCCTTGAGCACCAGTTTG-3';
Bcl-XL forward primer,	5'-TTACCTGAATGACCACCTA-3';
Bcl-XL reverse primer,	5'-ATTTCCGACTGAAGAGTGA-3';
GAPDH forward primer,	5'-CCCATCACCATCTCCAGGAGC-3';
GAPDH reverse primer,	5'-CCAGTGAGCTTCCCGTTCAGC-3'.

All sequences of the primer pairs were designed by Nihon Gene Research Lab (Sendai, Japan). The 10 µl PCR reaction mixture contained 1 µl of LightCycler-FastStart DNA Master SYBR Green I, 4 mM (caspase-8 and GAPDH) or 5 mM (caspase-10) MgCl₂, 0.5 µM of each primer, and 5 ng of sample cDNA. Real-time PCR reactions were run on LightCycler (Roche Diagnostics GmbH) with the following amplification protocol. PCR amplification began with a 10 min pre-incubation step at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 64°C (caspase-8), 63 °C (caspase-10), 62°C (Bax), 50°C (Bcl-2), 60°C (Bcl-XL) or 55°C (GAPDH) for 10 s, and elongation at 72°C for 6 s (caspase-8), 6 s (caspase-10), 12 s (Bax), 10 s (Bcl-2), 10 s (Bcl-XL) or 19 s (GAPDH). The relative concentration of PCR product derived from the target gene was calculated using software of the LightCycler System. Results were expressed relative to the number of GAPDH transcripts used as an internal control. The quality of the PCR products was checked by 2% agarose gel electrophoresis. In all cases, single bands of the expected size were observed. The specificity of each PCR product was further assessed by melting curve analysis.

Western blotting

Cells were lysed in a boiling lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM sodium orthovanadate and 1% SDS and clarified by centrifugation. Cell lysates (40 µg protein/lane) were separated on 12% SDS-polyacrylamide gels under reducing conditions, transferred to Sequi-Blot PVDF Membrane (Roche Diagnostics GmbH), and probed with anti-caspase-3 followed by goat anti-rabbit antibody coupled to horseradish peroxidase, and with horseradish peroxidase-conjugated anti-GAPDH antibody. Products were visualized using the ECL system (GE Healthcare, Buckinghamshire, England).

Statistical analysis

The results shown derive from experiments repeated three or four times, unless otherwise indicated. Results are expressed as mean ± SD. Statistical significance analysis was performed using the two-tailed Student's *t*-test. Values of *p* < 0.05 were considered statistically significant.

RESULTS

SB-induced apoptosis in MRK-nu-1 cells

Induction of apoptosis by SB in human breast cancer cell line MRK-nu-1 was confirmed with activities of caspase-3, -8 and -10 and DNA fragmentation. MRK-nu-1 cells were incubated with various concentration of SB for 24 h. SB-induced activities of caspase-3 and -10 in a dose-dependent manner. The activities of caspase-3 and -10 remarkably increased with 5 mM SB. Caspase-8 activity

also increased, but only to a small extent compared with caspase-10 (Fig. 1a). We also examined the activation of caspase-3, -8 and -10 by 5 mM SB treatment for 6 to 48 h. SB induced activities of caspase-3, -8 and -10 in a time-dependent manner (Fig. 1b). The increase in activities of caspase-3 and -10 was detected at 12 h and continued up to 24 h. The SB-induced caspase activities gradually decreased after 24 h. This result reflected the decrease in the number of cells due to the progress of apoptotic cell death. In the apoptotic cascade, activation of caspase-3 causes DNA fragmentation, which is one of the characteristics of apoptosis. In gel electrophoresis, a typical ladder pattern of inter-nucleosomal DNA fragmentation was clearly observed in MRK-nu-1 cells treated with SB at the concentration of >2 mM (Fig. 1c). The extent of the formation of DNA fragment was slightly reduced by the treatment with 4 mM and 5 mM of SB. This result was also due to the reduction of cell number.

Real-time PCR analysis of mRNA expression in SB-treated cells

We demonstrated that caspase-8 and -10 were activated in SB-treated MRK-nu-1 cells as mentioned above. It is important to analyze the step of the activation of these caspases. We tried to examine the expression of caspase-8 and -10 mRNA in SB-treated MRK-nu-1 cells by real-time PCR. In the time course experiments with 5 mM SB, levels of caspase-10 mRNA tended to increase at 3 h and then increased with the time dependent manner (Fig. 2). Treatment with SB resulted in a dramatic increase in levels of caspase-10 mRNA by approximately 5-, 8.5- and 12-fold compared with controls after 6, 9 and 12 h, respectively, whereas the levels of caspase-8 mRNA was not changed (data not shown). These results could suggest that caspase-10 gene expression plays an important role in SB-induced apoptosis.

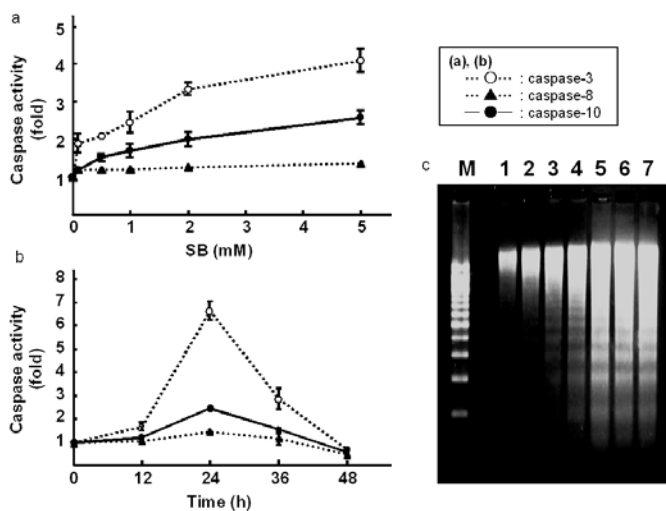


Figure 1. SB-induced caspases activation and DNA fragmentation. (a) Dose-dependent analysis of caspase-3, -8 and -10 activities. MRK-nu-1 cells (1×10^6) were treated with various concentrations of SB for 24 h. Caspase activity was subsequently measured as described in Materials and Methods. Each caspase activity was expressed as a relative to the control. Specific activities in control (vehicle only-treated) were: caspase-3, 123.0 ± 2.1 ; caspase-8, 632.5 ± 25.5 ; caspase-10, 85.9 ± 5.8 pmol/mg protein/min. Values are mean $\pm$ SD ($n = 4-5$). (b) Time-dependent analysis of caspase-3, -8 and -10 activities. Cells (1×10^6) were treated with 5 mM for the indicated periods. Caspase activity was assayed and expressed as (a). Specific activity in control (0 time) was: caspase-3, 73.6 ± 2.8 ; caspase-8,

224.9 ± 5.9 ; caspase-10, 87.3 ± 4.8 pmol/mg protein/min. (c) Dose-dependent formation of DNA fragments. Cells (1×10^6) were treated with the various concentrations of SB for 24 h. DNA was electrophoresed on a 2% NuSieve/agarose (3:1) gel, and stained with SYBR green I. Lane 1, 0 mM; 2, 0.5 mM; 3, 1.0 mM; 4, 2.0 mM; 5, 3.0 mM; 6, 4.0 mM; 7, 5.0 mM. M; 200 bp ladder marker.

Effects of inhibitors of caspase-8 and -10 on SB-induced apoptosis

To confirm the role of caspase-10 in SB-induced apoptosis, MRK-nu-1 cells were treated with caspase-8 (Z-IETD-fmk) and -10 (Z-AEVD-fmk) specific inhibitors and analyzed to determine the activation of executor caspase-3. Both Z-IETD-fmk and Z-AEVD-fmk completely inhibited the induction of caspase-3 activity (Fig. 3a). It is possible that the caspase inhibitors show the inhibitory effect on the other caspase activity *in vitro*. We further examined the effect of the inhibitors on the activation of caspase-3 by Western blotting analysis. Z-IETD-fmk and Z-AEVD-fmk clearly blocked the formation of caspase-3 active forms (Fig. 3a). The effect of Z-IETD-fmk or Z-AEVD-fmk was assessed by the formation of DNA fragments in SB-treated MRK-nu-1 cells. As shown in Fig. 3b, both

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Z-IETD-fmk and Z-AEVD-fmk inhibited the formation of DNA fragments induced by SB treatment, indicating that caspase-8 and -10 are acting upstream of caspase-3.

Effect of PDTC on expression of caspase-10 mRNA and activation of caspase-3 in SB-treated cells

PDTC, which is well known as an antioxidant and an inhibitor of transcription factor NF-kappa B, has been reported to reduce apoptosis [26]. To investigate the effect of PDTC on SB-induced apoptosis, PDTC was added 1 h before the treatment of SB and the expression of caspase-10 mRNA and caspase-3 activation were examined. PDTC significantly inhibited the induction of caspase-10 mRNA expression (Fig. 4a). The extent of the inhibitory effect of PDTC increased in a dose-dependent manner, although SB and/or PDTC did not cause significant differences in the levels of caspase-8 mRNA in the same cells (Fig. 4a). Furthermore, PDTC inhibited the induction of caspase-3 activation and the cleavage of pro-caspase-3 to 17 kDa and 19 kDa active forms induced by SB in a dose-dependent manner (Fig. 4b). When the expression of caspase-10 mRNA was considerably suppressed by the treatment with 200 μ M PDTC, the activation of caspase-3 was almost completely blocked. These facts also indicate the participation of caspase-10 in SB-induced apoptosis.

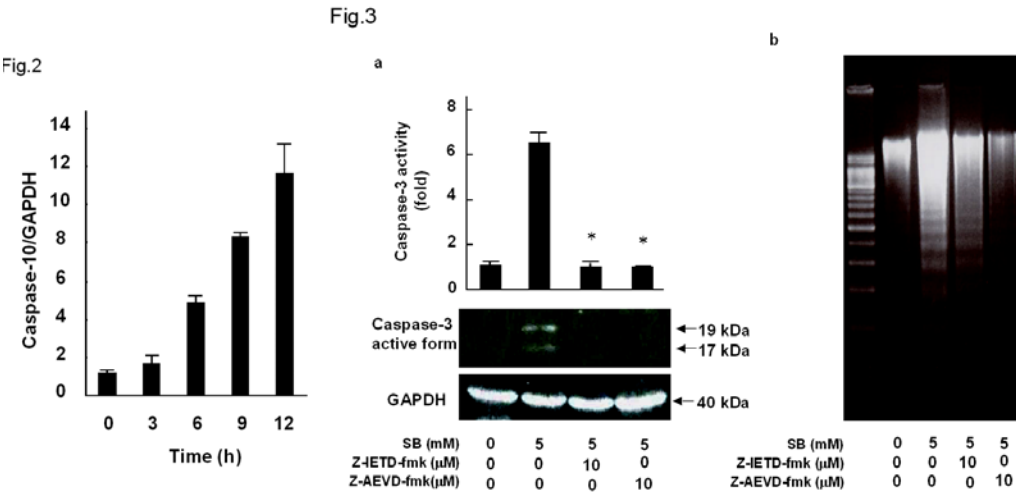


Figure 2. Time-dependent caspase-10 mRNA expression analyzed by real-time PCR. Cells (5×10^5) were treated with 5 mM SB for the indicated periods. The copy number of caspase-10 mRNA was adjusted by that of GAPDH. Results were indicated as a relative to the control. Values are mean $\pm$ SD (n = 5).

Figure 3. Effects of caspase-8 inhibitor (Z-IETD-fmk) and caspase-10 inhibitor (Z-AEVD-fmk) for the caspase-3 activation. Cells (1×10^6) were pretreated with 10 μ M of each specific inhibitor for 1 h, followed by 5 mM SB for 15 h. (a) Effects of caspase-8 and -10 inhibitors on caspase-3 activation. Upper panel: Caspase-3 activity was expressed as a relative to the control. Caspase-3 activity in control (vehicle only-treated) was 37.5 ± 3.8 pmol/mg protein/min. Values are mean $\pm$ SD (n = 4-5). *Indicates $p < 0.01$ compared with controls. Lower panel: Western blotting of caspase-3 active form. (b) DNA fragmentation in the presence of caspase inhibitors. DNA fragmentation was detected as mentioned in Fig. 1c.

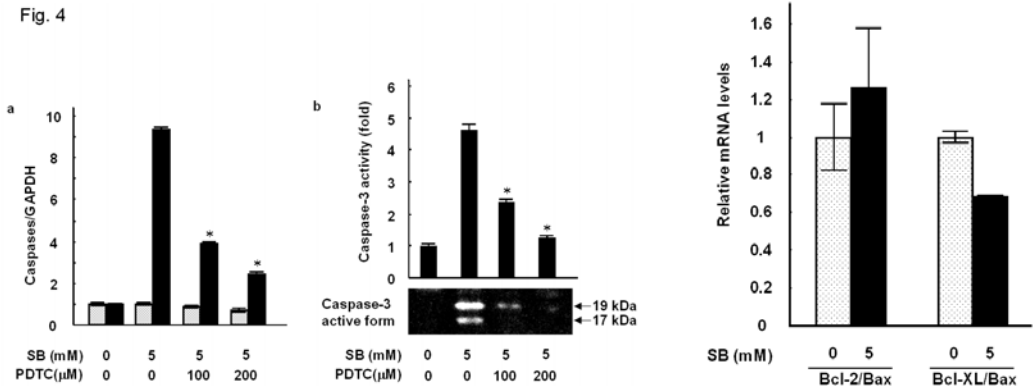


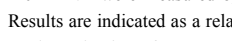
Figure 4. Effects of PDTC on the induction of caspase-10 mRNA expression. (a) Effect of PDTC on levels of caspase-8 and -10 mRNA. Cells (5×10^5) were pretreated with 100 or 200 μ M PDTC for 1 h, followed by 5 mM SB for 9 h. Levels of caspase-8 and -10 mRNA were measured by real-time PCR. The copy number of caspase-8 and -10 mRNA were adjusted by that of GAPDH. Results are indicated as a relative to the control. Values are mean $\pm$ SD (n = 4-6).  (▨, caspase-8; ▤, caspase-10). (b) Effect of PDTC on the activation of caspase-3. Cells (1×10^6) were pretreated with 100 or 200 μ M PDTC for 1 h, followed by 5 mM SB for 15 h. Upper panel: Caspase-3 activity was measured and expressed as a relative to the control. Caspase-3 activity in control (vehicle only-treated) was 47.1 ± 1.4 pmol/mg protein/min. Values are mean $\pm$ SD (n = 4-6). Lower panel: Western blotting of caspase-3 active forms. *Indicates $p < 0.01$ compared with controls (a,b).

Figure 5. Effect of SB on the expression of Bcl-2 family genes analyzed by real-time PCR. Levels of Bcl-2, Bcl-XL and Bax mRNA were analyzed by real-time PCR. Cells (5×10^5) were treated by 5 mM SB for 9 h. Results are indicated by the ratio of Bcl-2/Bax or Bcl-XL/Bax and shown as a relative to the control. Values are mean $\pm$ SD (n = 5-6).

Real-time PCR analysis of effect of SB on Bcl-2 family expression

There are many reports claiming the important roles of anti-apoptotic protein Bcl-2 or Bcl-XL and pro-apoptotic protein Bax in SB-induced apoptosis [5, 11, 21]. We investigated the expression of mRNA of Bcl-2 family by real-time PCR. Bcl-2 mRNA level was not changed by the treatment with 5 mM SB. But Bcl-XL mRNA level was decreased to about 60% of the control. However, pro-apoptotic Bax mRNA also decreased to about 80% of the control. Thus, Bcl-XL/Bax ratio was not drastically changed. Bcl-2/Bax ratio was not changed in the SB-treated cells (Fig. 5).

Effect of NF-kappaB inhibitors on SB induced apoptosis

PDTC was usually used as a convenient tool for investigating the participation of NF-kappaB. Thus, it is conceivable that NF-kappaB somehow plays a role in the process of SB-induced apoptosis. We investigated the effect of SN50, an inhibitor of NF-kappaB p50 subunit nuclear translocation [37], and MG-132, an inhibitor of IL-1-induced NF-kappaB activation [1], on the SB-induced apoptosis. Cells were pretreated with MG-132 and SN50 for 1 h, followed by 5 mM SB for 9 h for real-time PCR analysis and 15 h for caspase assay. SN50 and MG-132 had no effect on the induction of caspase-3 activity and the expression of caspase-10 mRNA. Amounts of mRNA and enzyme activities were corrected by subtracting the values from those of the controls. The amount of SB-induced caspase-10 mRNA and caspase-3 activity was taken as 100%. Caspase-3 activity in controls (vehicle only-treated) was 122.9 ± 4.4 (pmol/mg protein/min) (Table 1).

Table. 1

Treatment	Caspase-10 mRNA levels(%)	Caspase-3 activity(%)
Control	0.0 ± 1.4	0.0 ± 2.7
SB	100.0 ± 11.5	100.0 ± 3.6
SB+MG-132	113.9 ± 7.5	90.5 ± 9.7
SB+SN50	104.8 ± 11.1	88.3 ± 0.7

DISCUSSION

The naturally occurring short chain fatty acid sodium butyrate (SB) produced from dietary carbohydrate exhibits a wide variety of biological effects towards cell growth, cell morphology and gene expression. Exposure of cells to SB resulted in the growth inhibition and induction of apoptosis in a dose-dependent manner as detected by caspase-3 activity and the formation of DNA fragmentation. The incidence in SB-induced apoptosis was associated with the up-regulation of pro-apoptotic Bax expression, and down-regulation of anti-apoptotic Bcl-2 and Bcl-XL expressions. In fact, in the last few years, many authors have indicated that SB is potentially effective for the down-regulation of Bcl-2 and Bcl-XL and the up-regulation of Bax [28-30]. However, SB does not modify cell levels of Bcl-2,

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Bcl-XL, Bcl-xS and Bax in human colonic carcinoma derived cells [15]. SB also induces tumor cell sensitization to the apoptotic effect of the combination of TNF-alpha [16] and IFN-gamma [18], but it does not modify the level of the FADD/Mort1 adaptator molecule, at the connection between Fas- and TNF-dependent apoptosis pathways [4, 13]. These results indicate the presence of a process other than modifying Bcl-2 family genes in SB-induced apoptosis.

The aim of the present study was to determine the precise mechanism of SB-induced apoptosis. We performed experiments in which the effect of SB was evaluated for the induction of caspases with the treatment of SB. Our results, showing that SB inhibits cell growth and induces apoptosis, not only confirm but add new information to previous observations on the effects of SB. SB induced caspase-3, -8 and -10 as shown in Fig. 1a, b in a time- and dose-dependent manner. DNA fragmentation was observed by the treatment of SB in a dose-dependent manner (Fig. 1c). Recently, studies have identified caspase-10 as an important initiator caspase, in addition to caspase-8, in death receptor signaling pathways [19, 24, 36]. Analysis of transcriptional level of caspase-8 and -10 inductions was performed using real-time PCR. Nearly 5 times of caspase-10 mRNA was induced in 6 h of incubation with SB. Twelve h treatment with SB induced more than 10 times of caspase-10 mRNA shown in Fig. 2. Whereas, the level of caspase-8 mRNA was not changed the same concentration of SB (data not shown).

In order to determine whether caspase-10 plays a role in the caspase cascade in SB-induced apoptosis, we estimated caspase-3 activity in the presence of caspase-8 and -10 inhibitors [17]. We found the caspase-8 and -10 inhibitors to be effective in reducing the SB induced caspase-3 activity almost completely as shown in Fig. 3a. Caspase-3 active forms which were observed in SB-treated cells disappeared in accordance of reduced activity (Fig. 3a). Caspase-8 and -10 inhibitors inhibited the formation of DNA fragments in SB-treated cells (Fig. 3b). These results coincide the data of the study of Chopin et al. [8] showing a range of caspases maybe involved in SB-induced cell death. PDTC, an antioxidant reagent and inhibitor of transcription factor NF-kappaB, has been reported to reduce apoptosis. We tested the effect of PDTC on SB-induced apoptosis. PDTC effectively reduced the expression of caspase-10 mRNA induced by SB shown in Fig. 4a. PDTC also inhibited the SB-induced caspase-3 activity and the conversion to caspase-3 active forms (Fig. 4b).

These data may suggest that caspase-10 plays an important role in the SB-induced apoptosis in human breast cancer cell line MRK-nu-1. Taxol triggered caspase-8 and -10 dependent apoptosis in the CCRF-HSB-2 human lymphoblastic leukemia cell line [10, 27]. As mentioned above, PDTC acts as antioxidant and the antagonist of the NF-kappaB [12]. In fact, several reports showed caspase-8 or caspase-10 is able to activate the transcriptional activity of NF-kappaB [6, 21, 34]. In order to determine whether NF-kappaB involves in the SB-induced apoptosis, we verified the effect of other NF-kappaB inhibitors MG-132 and SN-50. These two compounds did not affect the induction of caspase-10 mRNA expression but neither did they affect caspase-3 activity induced by SB (Table 1). Our results could suggest that the involvement of NF-kappaB in SB-induced apoptosis was unlikely. It is conceivable that PDTC acts as antioxidant against SB-caused oxidation in apoptotic cascades and/or as an inhibitor of conversion of butyrate to a putative active compound [20]. To exclude this possibility, the effect of antioxidants N-acetylcystein, Tiron, mannitol and glutathione were examined. These compounds did not show any inhibiting effect on SB-induced caspase-3 activity (data not shown). The results of the present study indicate that NF-kappaB may not play an important role in SB-induced apoptosis. Furthermore, SB is one of the histone deacetylase (HDAC) inhibitors that modulate several apoptosis related proteins. It is important to clarify whether PDTC affects the HDAC inhibitor activity of SB. PDTC did not release the inhibitory effect of SB on histone deacetylase (data not shown). The fact indicates that induction of caspase-10 mRNA by SB is not due to the HDAC inhibitor activity of SB. Exposure of U937 cells to SB resulted in growth inhibition and induction of apoptosis in a dose-dependent manner [7]. The increase in apoptosis was associated with the up-regulation of pro-apoptotic Bax expression, and down-regulation of anti-apoptotic Bcl-2 and Bcl-XL expressions. SB is also capable of alteration of Bcl-2 family protein expression in several tumor cells [5]. It is important to examine whether SB-induced apoptosis accompanied by the up-regulation of pro-apoptotic Bax expression and down-regulation of anti-apoptotic Bcl-2 or Bcl-XL expressions. We investigated the expression of mRNA of Bcl-2, Bcl-XL and Bax by real-time PCR. Bcl-2 mRNA level

was not changed by the treatment with 5 mM SB. Bcl-2/Bax ratio was not changed in the SB-treated cells. In contrast, the expression of Bcl-XL mRNA was decreased to about 60% of the control. However, pro-apoptotic Bax mRNA also decreased to about 80% of the control. Thus, Bcl-XL/Bax ratio was not drastically changed (Fig. 5). The alteration of the amount of Bcl-2 family may not be indispensable in SB-induced apoptosis. In fact, Sawa *et al.* reported that SB increased the expression of the Bad protein, although the expression of Bcl-2, Bcl-XL, and Bax was not changed by the addition of SB [33].

These results using PDTC and caspase inhibitors seem to indicate that caspase-10 plays an important role in SB-induced apoptosis.

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