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Cellular Events Involved in Butyric Acid-Induced T Cell Apoptosis

Tomoko Kurita-Ochiai, Shigeru Amano, Kazuo Fukushima, and Kuniyasu Ochiai

We have previously demonstrated that butyric acid induces cytotoxicity and apoptosis of murine thymocytes, splenic T cells, and human Jurkat T cells. Therefore, to determine the apoptotic signaling pathway induced by butyric acid, we investigated the contribution of reactive oxygen species (ROS), mitochondria, ceramide, and mitogen-activated protein kinases in butyric acid-induced human Jurkat cell apoptosis. After exposure of cells to butyric acid, a pronounced accumulation of ROS was seen. Pretreatment of cells with the antioxidant N-acetyl-cysteine or 3-aminobenzamide attenuated butyric acid-induced apoptosis through a reduction of ROS generation. Cytochrome c, apoptosis-inducing factor, and second mitochondria-derived activator of caspases protein release from mitochondria into the cytosol were detected shortly after butyric acid treatment. Exposure of cells to butyric acid resulted in an increase in cellular ceramide in a time-dependent fashion. In addition, butyric acid-induced apoptosis was inhibited by DL-threo-dihydrosphingosine, a potent inhibitor of sphingosine kinase. Using anti-extracellular signal-regulated kinase (ERK), anti-c-Jun N-terminal kinase (JNK), and anti-p38 phosphospecific Abs, we showed a decrease in ERK, but not in JNK and p38 phosphorylation after treatment of cells with butyric acid. Pretreatment of cells with the JNK inhibitor SP600125 attenuated the effect of butyric acid on apoptosis, whereas no effect was seen with the p38 inhibitor SB202190 or the ERK inhibitor PD98059. Taken together, our results indicate that butyric acid-induced T cell apoptosis is mediated by ceramide production, ROS synthesis in mitochondria, and JNK activation in the mitogen-activated protein kinase cascade. Finally, these results were further substantiated by the expression profile of butyric acid-treated Jurkat cells obtained by means of cDNA array. The Journal of Immunology, 2003, 171: 3576–3584.

Adult periodontitis is a chronic destructive disease involving host inflammatory responses to Gram-negative bacteria. Recent studies in periodontal medicine suggest an association between human periodontal disease and certain systemic disorders such as diabetes mellitus, pneumonia, heart disease, and preterm birth (1). Severe destructive adult periodontitis is caused by a mixed infection and the combination of certain periodontopathogens, for example, Porphyromonas, Prevotella, and Fusobacterium spp. are important in pathogenesis (2). These bacteria produce a variety of virulence factors, such as proteases, LPS, fimbriae, and butyric acid.

Butyric acid has also been shown to be produced by bacterial fermentation of dietary fibers in the human colon. It is used by colorectal cells as the most important source in the production of energy (3). Moreover, butyric acid inhibits in vitro cell growth by favoring cell cycle arrest and it promotes differentiation in normal as well as transformed cells (4). Besides these effects, butyric acid induces apoptosis in a number of cancer cells (5, 6). Emerging evidence indicates that bacterial modulation of apoptosis is an important part of pathogenesis (7). Specific pathogens or their extracellular products may directly induce the apoptosis of host cells (8).

We have previously shown that butyric acid-induced cytotoxicity and apoptosis in murine and human T and B cells involves mechanisms that are dependent on caspases 3, 8, and 9 and that are Fas independent (9–12). Butyric acid inhibits deacetylation of histones, which leads to alteration of chromosome structure and gene expression (13). However, the precise mechanism of butyric acid-induced apoptosis has not been elucidated.

Apoptosis is frequently accompanied by the generation of reactive oxygen species (ROS), resulting in part from cytochrome c departure from mitochondria and attendant disruption of electron transport with enhanced generation of one electron-reduced species of molecular oxygen within the cell (14). ROS represent attractive candidates for final common mediators of apoptosis, yet a specific role for ROS in the execution or resolution of the apoptotic program has not been established.

Ceramide is a membrane sphingolipid that has recently emerged as a second messenger involved in the induction of apoptosis (15). In mammalian cells, ceramide can be generated by two mechanisms: 1) from sphingomyelin by activation of acidic or neutral sphingomyelinases and 2) from N-acetylation of dihydrosphingosine by ceramide synthase (16). Ceramide formation can be induced by serum starvation, UV irradiation, γ-irradiation, oxidative stress, and TNF receptor engagement (17).

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Dentistry at Matsudo.

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3 Abbreviations used in this paper: ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; AIF, apoptosis-inducing factor; Smac, second mitochondria-derived activator of caspases protein; NAC, N-acetylcysteine; 3AB, 3-aminobenzamide; DHS, DL-threo-dihydrosphingosine; DPA, diphenylamine; PL, propidium iodide; DCFH-SPP, sphingosine 1 phosphate; ASK1, apoptosis signal-regulating kinase 1; DCFH-DA, 2′,7′-dichlorofluorescin-diacetate.
Mitogen-activated protein kinase (MAPK) signal transduction pathways in mammalian cells include the extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK. ERK is generally associated with proliferation and growth factors. In contrast, JNK and p38 MAPK are induced by stress responses and cytokines and can mediate differentiation and cell death (18–20). Although the emerging evidence suggests that JNK/stress-activated protein kinase and/or p38 MAPK pathways function primarily to promote apoptotic cell death, this issue is controversial based on findings in various systems (21–23). Thus, much remains to be clarified about the biochemical regulation and cellular role of the apoptotic kinase cascades.

In the present study, we explored the intracellular molecular mechanism of butyric acid-induced T cell apoptosis. Specifically, we investigated the possible roles and relationships of ROS, mitochondria, ceramide, and MAPK in butyric acid-induced apoptosis in Jurkat cells. Our results suggest that butyric acid-induced apoptosis is mediated by ROS synthesis in mitochondria and ceramide production, followed by JNK activation in MAPK cascade in Jurkat cells.

Materials and Methods

Cell lines, Abs, and reagents

The human T lymphoma cell line Jurkat was kindly provided by Fujisaki Cell Center-Hayashibara (Okayama, Japan). The cells were cultured at 37°C in a moist 5% CO2 atmosphere in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 mM 2-ME.

Absorbance at 550 nm was determined on an MT32 spectrophotometric microplate reader (Corona Electric, Ibaraki, Japan). Peroxidase-labeled anti-rabbit antisera against apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspases protein (Smac) were purchased from MBL (Nagoya, Japan). Anti-phospho-JNK, anti-phospho-p38 MAPK, anti-phospho ERK, anti-JNK, anti-p38, and anti-ERK were obtained from New England Biolabs (Beverly, MA).

Highly purified butyric acid was purchased from Sigma-Aldrich (St. Louis, MO). Solutions of butyric acid were diluted to 5 mM in DMEM (Life Technologies, Grand Island, NY) and adjusted to pH 7.2 with sodium hydroxide.

Cell proliferation assay

Jurkat cells (2 × 10^5 cells/ml) were treated in complete medium with different concentrations of butyric acid in flat-bottom, 96-well plates (100 μl/ml). After incubation for 20 or 44 h, 20 μl of MTT (5 mg/ml in PBS, pH 7.2) was added to each well. After 4 h of incubation, the supernatants were decanted and the formazan precipitates were solubilized by the addition of 150 μl of 100% DMSO and placed on a plate shaker for 10 min.

DNA fragmentation assay

The DPA reaction was performed by the method of Paradones et al. (25). The colorimetric reaction was quantified spectrophotometrically at 575 nm with a model UV-160A UV spectrophotometer (Shimazu, Tokyo, Japan). The absorbance at 550 nm was determined on an MT32 spectrophotometric microplate reader (Corona Electric, Ibaraki, Japan).

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Absorbance at 550 nm was determined on an MT32 spectrophotometric microplate reader (Corona Electric, Ibaraki, Japan).

T cell culture for apoptosis

Jurkat cells (1 × 10^6/well) were cultured in 1 ml of complete medium in 24-well tissue culture plates (Falcon; BD Labware, Lincoln Park, NJ) with 5 mM butyric acid. For apoptosis inhibition assays, cells were preincubated for 1 h with the antioxidant NAC or 3AB or with the ceramide inhibitor erinacine-A (New England Biolabs, Beverly, MA). Briefly, Jurkat cells (1 × 10^6) in 1 ml of medium were cultured as indicated for 21 h, washed, and then stained with PI and annexin V-FITC in annexin binding buffer and analyzed with CellQuest software (BD Biosciences, San Jose, CA) by FACScan Calibur within 1 h. Data from 10^6 cells were analyzed for each sample.

Flow cytometric measurement of ROS production using 2′,7′-dichlorofluorescin-diacetate (DCFH-DA)

To measure the production of ROS, Jurkat cells (1 × 10^6 cells/ml of culture medium) were incubated for 30 min at 37°C in the dark with 10 μM DCFH-DA (Calbiochem), as described previously (26). The 2′,7′-dichlorofluorescin fluorescence resulting from the oxidation of DCFH-DA was measured in 10,000 cells on a logarithmic scale of fluorescence of four decades of log by using a FACScan flow cytometer (BD Biosciences) at excitation and emission wavelengths of 488 nm and 524/44 nm, respectively.

Measurement of caspase protease activity

After incubation of cells (1 × 10^5/well) in 24-well tissue culture plates for the indicated times with 5 mM butyric acid, the cells were harvested and washed as described above, and the caspase-3, -8, and -9 activities were measured using a caspase fluorometric protease assay kit (MBL). Levels of released 7-amino-4-trifluoromethylcoumarin were measured with a BioLumin 960 spectrophotofluorometer (Molecular Dynamics Japan, Tokyo, Japan) with excitation at 400 nm and emission at 505 nm. Antioxidant NAC was administered 1 h before the addition of butyric acid.

Preparation of mitochondria-free cytosolic extracts and whole cell extracts

Cytosolic extracts were prepared using an ApoAlert Cell Fractionation Kit (Clontech). Briefly, cells were harvested by gently scraping and then were incubated in a fractionation buffer mix on ice for 10 min. The cells were then homogenized in an ice-cold Dounce homogenizer by 50 gentle strokes. The homogenates were centrifuged at 700 g for 10 min at 4°C, and the supernatants were transferred to fresh tubes and centrifuged at 10,000 × g for 25 min at 4°C. The mitochondria-free supernatants (cysotolic fraction) were frozen at −70°C until further analysis. Whole cell extracts were obtained by dissolving the cells in lysis buffer (10 mMTris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, and 2 μg/ml leupeptin), followed by centrifugation at 14,000 × g for 10 min at 4°C. The supernatant was collected and stored at −70°C.

Western blotting

The protein content of cytosolic or whole cell extracts was measured using the Bio-Rad (Hercules, CA) protein assay. Equal amounts (25 μg) of protein from each sample were separated by sodium dodecyl sulfate-12.5% PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking with PBS containing 5% nonfat dry milk and 0.1% Tween 20, the membranes were exposed to the primary Abs overnight at 4°C on a shaker. Before and after incubation with the HRP-conjugated secondary Abs (Amersham Pharmacia Biotech, Piscataway, NJ), the membranes were washed extensively using PBS/Tween. Ab binding was detected by ECL (Amersham Pharmacia Biotech) and quantitatively analyzed by use of NIH Image 1.61 software.
Lipid extraction and ceramide assay

After harvesting the cells at the indicated times, the lipids were extracted by the method of Bligh and Dyer (27), and ceramide measurement using 1,2-diacylglycerol kinase were performed as described (28). The solvent system used to separate ceramide phosphate was chloroform/acetone/methanol/acetic acid/H2O (10:4:3:2:1). The level of ceramide was determined by comparison with a concomitantly run standard curve comprised of known amounts of ceramide (Sigma-Aldrich).

cDNA expression array

Total RNA was prepared using the Atlas pure total RNA isolation kit (Clontech) following the manufacturer’s instructions. For analyzing gene expression in Jurkat cells, the Atlas cDNA expression array (Clontech) was used as described in the user’s manual. Briefly, 5 μg of total RNA was converted into 32P-labeled first strand cDNA by means of Moloney murine leukemia virus reverse transcriptase. Unincorporated 32P-labeled nucleotides were removed by chromatography on a Chroma Spin-200 (Clontech). cDNA fractions of highest activity were pooled for hybridization to each Atlas membrane. Equal amounts of cpm (∼4–5 × 10^6 cpm) were used in each pair of experiments (control and treated cells). After prehybridization, hybridization with probes, and washing, the membranes were sealed in sample bags and exposed to x-ray film for 1–21 days. Several exposures were taken to select conditions of the highest signal-to-background ratio. Primary array data were acquired by a laser scanner, and the relative intensity of signals was then determined by the NIH Image 1.61 software. Only signals that differed from the control by at least twofold in all three independent experiments were taken into consideration. The data were expressed as the mean of three experiments.

Statistics

Multiple-group comparisons were made using a one-way ANOVA followed by a post hoc intergroup comparison by the Bonferroni-Dunn test. where appropriate, Student’s t test was used to compare two groups.

Results

The effect of butyric acid on viability and apoptosis in human Jurkat T cells

Butyric acid inhibited Jurkat cell proliferation in a dose-dependent manner, as shown in Fig. 1A. This effect was observed at concentrations as low as 0.62 mM (79 ± 1% vs control, p < 0.01). Maximal inhibition by butyric acid was noted at a dose of 5 mM (51 ± 3% (24 h) and 9 ± 0.01% (48 h) vs control, p < 0.01). Under control conditions, near-confluent Jurkat cell monolayers showed a spontaneous apoptosis rate of 7.0 ± 0.2% after 21 h (Fig. 1B). Incubation with butyric acid strongly induced Jurkat cell apoptosis in a dose-dependent manner. A maximal apoptotic response of 38 ± 2% was observed with 5 mM butyric acid after 21 h of incubation (Fig. 1B). Fig. 1C shows the results of bivariate FITC-annexin V-PI flow cytometry of Jurkat cells after incubation with butyric acid for 17 and 21 h. The lower left quadrant represents the early apoptotic cells, which are PI negative and annexin V positive, indicating the translocation of phosphatidyl serine to the external cell surface but retaining the integrity of the cytoplasmic membrane (29). The upper right quadrant represents the non-viable necrotic and late-stage apoptotic cells, which are positive for annexin V binding and PI uptake. After 17 h of incubation in the presence of butyric acid, there were 21% early-stage apoptotic cells and 7.9% late-stage apoptotic and necrotic cells. Furthermore, by 21 h of treatment, the number of early- and late-stage apoptotic

FIGURE 1. A. Dose-dependent effects of butyric acid on cell proliferation. Jurkat cells were cultured with the indicated concentration of butyric acid for 24 (○) or 48 h (●). Cellular proliferation was determined by an MTT assay and is expressed as the percentage of the absorbance obtained without butyric acid. The results are expressed as the mean ± SEM of three different experiments with triplicate cultures. *p < 0.05; **p < 0.01 (related to the activity of culture without butyric acid). B. Dose-response curve of butyric acid-induced apoptosis. Jurkat cells were cultured with the indicated concentration of butyric acid for 21 h. Harvested cells were assayed by the DPA assay. The data are expressed as the mean ± SEM of three different experiments with triplicate cultures. *p < 0.05; **p < 0.01 (related to the activity of culture without butyric acid). C. Annexin V-FITC staining of apoptotic cells in butyric acid-treated Jurkat cells. Jurkat cells were double stained with annexin V-FITC and PI after treatment with 5 mM butyric acid (BA) for the indicated times, and then they were analyzed by flow cytometry. Annexin V− PI− cells are the early apoptotic cells and annexin V− PI+ cells are the late apoptotic cells. The figure is representative of five experiments with similar results.
and necrotic cells was markedly increased, by up to 35% and 14.8%, respectively.

**Butyric acid-induced apoptosis depends on generation of ROS**

To determine whether ROS serve as effectors of butyric acid-mediated apoptosis, intracellular ROS levels were measured by FACS using 2',7'-dichlorofluorescein fluorescence. Butyric acid-treated cells showed higher basal levels of ROS compared with nontreated cells (Fig. 2A). To establish whether the up-regulation of ROS was necessary for butyric acid-induced apoptosis, the antioxidant NAC was used to pretreat butyric acid-sensitized cells. Treatment of cells with 5 mM NAC for 1 h followed by butyric acid sensitization resulted in ROS levels similar to those in untreated cells. These data provide evidence that ROS production occurred during butyric acid-induced apoptosis.

To test whether augmented ROS levels play a role in mediating the death signal of butyric acid, the two antioxidants NAC and 3AB were used to treat the cells before the addition of butyric acid. Both NAC and 3AB showed a protective effect against butyric acid-induced apoptosis in a dose-dependent manner (Fig. 2B).

These data suggest that the generation of ROS is essential for the induction of apoptosis by butyric acid.

Our previous study demonstrated that caspase-8 and -9-dependent, as well as caspase-3-dependent, apoptosis plays an important role in butyric acid-induced T cell death (12). To investigate the intracellular mechanism of caspase activation in butyric acid-induced cytotoxicity, the effect of pretreatment with NAC on butyric acid-induced caspase activation was examined. As shown in Fig. 2C, caspase-3, -9, and especially -8 activities were dose dependently reduced by pretreatment with NAC. The pretreatment with NAC decreased butyric acid-induced caspase-8 activity to near the control levels. These results indicate that ROS production plays a role as a regulator of caspase activation in butyric acid-induced apoptosis.

**Mitochondria participation in butyric acid-induced T cell apoptosis**

Recent evidence indicates that mitochondria play a pivotal role in apoptosis in multicellular organisms by releasing apoptotic factors...
such as cytochrome c, which activates the caspase effector pathway, Smac/Diablo (direct IAP binding protein with low pi), which neutralizes a set of caspase inhibitors known as the inhibitor of apoptosis proteins, and AIF, which is involved in a caspase-independent cell death pathway. Because mitochondria play an important role in many forms of apoptosis and are also a major source for ROS production (30), we investigated the involvement of mitochondria as the source of ROS. To detect whether cytochrome c, AIF, and Smac release are involved in butyric acid-induced apoptosis, we next examined the distributions of these apoptotic factors (Fig. 3). Cytochrome c was not present in the cytosolic fraction of untreated cell lysates but was evident in cytosolic extract prepared immediately after butyric acid treatment, increasing steadily up to 5 h after treatment. Cytosolic levels of AIF and Smac were also detected immediately and increased throughout the 3-h period after butyric acid treatment. These data suggest that butyric acid induces the release of several mitochondrial apoptotic proteins, indicating that butyric acid induces apoptosis via the mitochondrial apoptotic pathway.

Ceramide production in butyric acid-induced T cell apoptosis

Because ROS induces ceramide-mediated apoptosis in human lung epithelial cells (31, 32), we examined the involvement of ceramide generation in butyric acid-induced T cell apoptosis. Exposure of cells to 5 mM butyric acid increased cellular ceramide in a time-dependent manner (Fig. 4A). By 24 h, a 210% increase in ceramide production over the basal level was seen. Breakdown of sphingomyelin through sphingomyelinases results in the formation of ceramide, which is subsequently metabolized to sphingosine and sphingosine 1 phosphate (SPP) (33, 34). With respect to the sphingomyelinase pathway, several studies (33, 34) have shown that ceramide displaces several of its biological activities via an SPP-dependent pathway. Therefore, we also tested whether the increase in ceramide production in butyric acid-treated Jurkat cells was SPP dependent. We used DHS, which is a potent inhibitor of sphingosine kinase, to examine the effect of kinase inhibition on ceramide-mediated T cell apoptosis by butyric acid. Butyric acid-induced Jurkat cell apoptosis was eliminated by DHS in a dose-dependent manner (Fig. 4B). These results suggested that the involvement of SPP in ceramide-mediated T cell apoptosis by butyric acid.

Effect of MAPK on butyric acid-induced T cell apoptosis

Recent evidence indicates that ROS is a key mediator in activating the members of the MAPK pathways, such as p38 and JNK, which contributes to progression of cell apoptosis (35–37). Therefore, we examined the influence of butyric acid on JNK, p38, and ERK phosphorylation. Jurkat cells were treated with 5 mM butyric acid for the indicated times (Fig. 5A) and cellular extracts were subjected to immunoblot analysis using phosphospecific Abs against the respective MAPKs. These Abs specifically recognize the activated, diphosphorylated form of JNK, p38, and ERK. ERK phosphorylation increased 15 min after exposure to butyric acid, then decreased to baseline levels, and finally a down-regulation was observed at 6 h. The levels of JNK phosphorylation increased markedly by 0.5 h after the start of treatment with butyric acid, then decreased to baseline levels, and finally a down-regulator was observed at 6 h. The levels of JNK phosphorylation increased markedly by 0.5 h after the start of treatment with butyric acid, although the levels varied with time after treatment. Furthermore, p38 phosphorylation increased slightly with time. All blots were stripped and reprobed with the respective Abs recognizing JNK, p38, and
assayed by the DPA assay. The results are expressed as the mean then were treated with 5 mM butyric acid for 21 h. Harvested cells were PD98059 did not modulate the effect of butyric acid (Fig. 5).

Involvement of p38 and JNK in butyric acid-induced cell death

To examine whether the activation of p38 and JNK and the inhibition of ERK were essential for butyric acid-induced cell death, Jurkat cells were pretreated with serial concentrations of the p38 inhibitor SB202190, the JNK inhibitor SP600125, or the ERK inhibitor PD98059 for 1 h and then were treated with 5 mM butyric acid for 21 h. Harvested cells were assayed by the DPA assay. The results are expressed as the mean ± SEM of three different experiments with triplicate cultures. **, p < 0.01 (related to the activity of the culture without MAPK inhibitors).

Discussion

ROS production frequently occurs in cells exposed to UV light, γ-rays, H$_2$O$_2$, or cytokines (39, 40). In many experimental situations, the induction of apoptosis is accompanied by a rise in intracellular ROS (14). Furthermore, the observed inhibition of apoptosis by different antioxidants such as NAC (41, 42), ascorbate (43), and α-tocopherol (43) suggests that ROS production plays a role in apoptosis in diverse cell lines (42, 44). Our results also show that ROS is involved in butyric acid-induced apoptosis in Jurkat cells. Butyric acid-induced apoptosis is partially inhibited by antioxidants such as NAC or 3AB, and this inhibition correlated with a decrease in butyric acid-induced ROS formation. However, because butyric acid-induced apoptosis was not completely inhibited by NAC or 3AB, it is possible that ROS and undefined signals other than ROS play an important role in butyric acid-induced cell death. Because butyric acid-induced caspase-3, -8, and -9 activation was reduced by NAC, it is possible that the caspases act downstream of ROS. Furthermore, the finding that caspase-8 activity was reduced near the control levels by NAC suggests that the production of ROS is completely related to the activity of caspase 8. However, the partial depression of caspase-3 and caspase-9 suggests that a factor other than ROS may be involved in the observed caspase activity. It is possible that ROS formation involves a caspase-independent pathway.

There are two major pathways through which apoptosis is induced: one involves death receptors and is exemplified by Fas-mediated caspase-8 activation, and another is the stress- or mitochondria-mediated caspase-9 activation pathway. Our previous study (12) indicates that the Fas-Fas ligand interaction is not involved in butyric acid-induced apoptosis and that caspase-8 and caspase-9-dependent apoptosis plays an important role in butyric
acid-induced as well as Fas-induced apoptosis. Therefore, in butyric acid-induced apoptosis, death receptor- and stress-mediated signals seem to lead to the production of ROS followed by caspase activation.

Mitochondria are involved in the decision of cells to survive or not at several levels. First, mitochondria can activate the cell death machinery by releasing in the cytosol proapoptotic factors such as procaspases, caspase activators (i.e., cytochrome c and Smac/Diaiblo), or caspase-independent factors such as AIF. Furthermore, the importance of mitochondria in apoptosis has been reinforced by studies showing the contribution of ROS in cell death signaling. Because mitochondria are a major source of ROS production, it was of value to investigate the involvement of mitochondria in butyric acid-induced T cell apoptosis. In the current study, butyric acid was found to elicit the immediate release of cytochrome c from mitochondria. Before cytochrome c release, AIF and Smac were released into the cytosol. Our previous study indicated that butyric acid treatment decreased Bcl-2 and Bcl-x
expression in murine splenic T cells (45). The Bcl-2 family regulates apoptosis via the release of cytochrome c from mitochondria (46). The up-regulation of antiapoptotic Bcl-2 or its close homologue Bcl-x
is known to inhibit apoptosis (47), whereas the down-regulation of Bcl-2 or its antagonization by dimerization with Bax-α promotes programmed cell death (48). This indicates that the butyric acid-induced pathway is closely involved with the mitochondrial Bcl pathway, which is known to be critical to apoptosis in other models (49, 50). Our results also suggest that butyric acid induces T cell apoptosis in a caspase-dependent and -independent manner by releasing several mitochondrial apoptotic proteins. Also, in our previous study (12), caspase-8 and -9 inhibitors did not inhibit the cell apoptosis induced by butyric acid as completely as they did the apoptosis induced by anti-Fas Ab. Therefore, some factors other than caspase (such as AIF) may play an important role together with these caspases in butyric acid-induced T cell apoptosis.

In this study, butyric acid increased cellular ceramide in a time-dependent manner. The ceramide increase was bimodal, i.e., in early and late phases of apoptosis. It has been suggested that the bimodal increase is dependent on the cell line and dose used and that the early increase may be the initiator of the apoptotic cascade, whereas the late increase would function as an amplifier (51). Therefore, our results showing a sustained elevation in this signaling molecule by butyric acid suggest that ceramide would function as an amplifier in butyric acid-induced T cell apoptosis. Furthermore, the finding that the sphingosine kinase inhibitor DHS inhibited butyric acid-induced apoptosis demonstrates that ceramide and butyric acid share a common mandatory step toward the executioner phase of apoptosis. These results suggest that in Jurkat cells the induction of ROS by butyric acid triggers the apoptotic pathway by inducing ceramide generation and that elevation of ceramide is sufficient and necessary for the induction of apoptosis.

In several systems, the generation of ROS has been shown to play an important and early role in ceramide-mediated apoptosis (52, 53), whereas supplementation of antioxidants such as reduced glutathione and NAC inhibits the induction of both ceramide levels and apoptosis (53, 54). Therefore, it appears that the generation of ROS and increase in ceramide may be the common effect of several diverse apoptotic stimuli. Furthermore, recent studies suggest that ceramide may affect the permeability of mitochondrial outer membrane and the release of cytochrome c via the lipid raft formation (55).

In this study, we have demonstrated that treatment of Jurkat cells with butyric acid results in a rapid phosphorylation of JNK and p38 MAPK and the suppression of ERK. Furthermore, butyric acid-induced apoptosis was functionally inhibited by the JNK inhibitor SP600125, whereas the inhibition of p38 and ERK did not protect Jurkat cell lines from butyric acid-induced apoptosis. This indicates that JNK, but not p38 and ERK, is involved in butyric acid-induced apoptosis. A number of studies have indicated that activation of JNK plays a crucial role in apoptosis induced by various stimuli. For example, the JNK signaling pathway is essential for neuronal apoptosis in response to excitotoxic stress (56). Others have shown that UV-induced apoptosis in fibroblasts requires JNK for cytochrome c release from the mitochondria (57). In our study, pretreatment of cells with SP600125 prevented butyric acid-induced apoptosis, suggesting the involvement of JNK activation in this process. Recent evidence has shown that JNK is able to phosphorylate and inactivate Bcl-x
and Bcl-2 (58, 59). Therefore, given that the major antiapoptotic function of the Bcl-2 family members resides at the level of the mitochondria, abrogation of Bcl-2/Bcl-x
function may explain the requirement

| Table 1. Differential gene expression by DNA microarray analysis*
<table>
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<th>Gene</th>
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<th>17 h</th>
<th>Function</th>
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<tr>
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<td>-3.6</td>
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<td>2.5</td>
<td>Bcl-2 homologous antagonist/killer</td>
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<td>-3.7</td>
<td>Glutathione-δ-transferase (GST) homolog</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>1.3</td>
<td>-</td>
<td>Apopain precursor; cysteine protease CPP32</td>
</tr>
<tr>
<td>Caspase 6</td>
<td>1.4</td>
<td>-</td>
<td>Cysteine protease MCH1 isoforms alpha + beta</td>
</tr>
<tr>
<td>Caspase 7</td>
<td>1.7</td>
<td>-</td>
<td>ICE-like apoptotic protease 3 (ICE-LAP3); apoptotic protease MCH3</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>2.4</td>
<td>1.6</td>
<td>ICE-like apoptotic protease 5 (ICE-LAP5); MURT1-associated CED3-3 homolog</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>1.6</td>
<td>1.9</td>
<td>ICE-like apoptotic protease 6 (ICE-LAP6); apoptotic protease MCH6</td>
</tr>
<tr>
<td>CRAFT</td>
<td>1.6</td>
<td>-</td>
<td>CD40 receptor-associated factor 1 (CRAFT 1)</td>
</tr>
<tr>
<td>DAPK1</td>
<td>-1.3</td>
<td>-1.5</td>
<td>Death-associated protein kinase 1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>-</td>
<td>-3.0</td>
<td>NF-κB DNA binding subunits</td>
</tr>
</tbody>
</table>

* Jurkat cells were treated for indicated times with or without 5 mM butyric acid. Total RNA was isolated, retrotranscribed, 32P-labeled and hybridized to the cDNA array representing 205 transcripts for known genes. The signals were then analyzed by NIH Image 1.61 software and expressed as fold of increase with respect to the untreated cells.
of JNK for the release of cytochrome c from the mitochondria and the induction of apoptosis (57, 60). p38 MAPK is also activated by various stressful stimuli and is involved in the apoptosis signal (61, 62). However, the p38 MAPK-specific inhibitor SB202190 had no effect on butyric acid-induced T cell apoptosis in our system, although p38 MAPK is preferentially activated in response to butyric acid stimuli.

Oxidative stress induces activation of JNK and p38 MAPK (63). Apoptosis signal-regulating kinase 1 (ASK1) was identified by Ichijo et al. (63) as one of the MAPK kinase kinases that activates JNK and p38 MAPK and induces the stress-mediated apoptosis signal. A constitutive active mutant of ASK1 induces cytochrome c release and activation of caspase-9 and caspase-3, but not caspase-8, indicating that ASK1 executes apoptosis mainly by mitochondria-dependent caspase activation (64). We also found that a high level of ASK1 production could be induced rapidly after treatment of cells with butyric acid (data not shown). Thereby, we concluded that ROS and ceramide production are the major contributors to butyric acid-induced apoptosis. However, there must be other mechanisms independent of ROS and ceramide production that account for butyric acid-induced death of Jurkat cells. Finally, we identified many genes that are up- and down-regulated by butyric acid stimulation of Jurkat cells. The expression profile of butyric acid-treated Jurkat cells by means of cDNA array supported our other experimental findings. The expression profile of the cDNA array was not restricted to Jurkat cells, because similar expression with butyric acid was seen on PBMC T cells (data not shown). The present results provide a basis for understanding the molecular mechanisms for the biological changes that are responsible for butyric acid effects on Jurkat cells.

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References


