Butyric Acid-Induced T-Cell Apoptosis Is Mediated by Caspase-8 and -9 Activation in a Fas-Independent Manner

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Received 20 July 2000/Returned for modification 28 August 2000/Accepted 22 November 2000

Our previous study demonstrated that butyric acid, an extracellular metabolite of periodontopathic bacteria, induced apoptosis in murine thymocytes, splenic T cells, and human Jurkat cells. In this study, we examined whether CD95 ligand-receptor interaction is involved in butyric acid-induced T-cell apoptosis. Flow cytometry analysis indicated that expression of Fas in Jurkat and T cells from peripheral blood mononuclear cells was not affected by butyric acid treatment. Furthermore, the expression of Fas and FasL protein in Western blotting was not affected by butyric acid treatment. Coincubation with blocking anti-Fas antibodies prevented Fas-induced apoptosis but not butyric acid-induced apoptosis. Anti-FasL antibodies also did not prevent butyric acid-induced apoptosis at any dose examined. Although cytotoxic anti-Fas antibody affected butyric acid-induced apoptosis, a synergistic effect was not seen. Time-dependent activation of caspase-8 and -9 was recognized in butyric acid- as well as Fas-mediated apoptosis. IETD-CHO and LEHD-CHO, specific inhibitors of caspase-8 and -9, respectively, completely blocked Fas-mediated apoptosis and partially prevented butyric acid-induced apoptosis. These results suggest that the Fas-FasL interaction is not involved in butyric acid-induced apoptosis and that caspase-8 and -9-dependent apoptosis plays an important role in butyric acid-induced apoptosis, as well as Fas-induced apoptosis.

Butyric acid, one of the short chain fatty acids, suppresses the proliferation of a variety of cancer cell lines in vitro (14, 20). Our previous study (16) demonstrated that short-chain fatty acids, especially volatile fatty acids present in the culture filtrates of Porphyromonas gingivalis, Prevotella loescheii, and Fusobacterium nucleatum, markedly inhibited murine T- and B-cell proliferation and cytokine production. Furthermore, we found that a representative volatile fatty acid, butyric acid, induced cytotoxicity and apoptosis in murine tissue and human T and B cells through a mechanism dependent on caspase-3 (18, 19). Butyric acid induces deacetylation of histones, which leads to alteration of chromosomal structure and gene expression (14). However, the precise mechanism of butyric acid-induced apoptosis has not been elucidated.

One of the most-studied apoptosis pathways is mediated by the ligation of the plasma membrane molecule Fas (APO-1, CD95). Fas is a type I transmembrane glycoprotein belonging to the nerve growth factor/tumor necrosis factor receptor superfamily (8). Fas is expressed on activated T and B cells (25) and thymocytes (33). The interaction of Fas and its ligand plays an important role in the regulation of programmed cell death of T and B lymphocytes (7). When Fas is trimerized by its natural ligand, FasL, either in soluble form (21) or expressed in the membrane of effector cells (1), several intracellular adapter proteins are recruited to the clustered receptors. These molecules, known as FADD/MORT 1, bind to intracellular Fas domains, known as death domains, and recruit one or several cysteine proteases with Asp specificity (caspases), such as caspase-8 (FLICE/MACH/Mch-5) or caspase-10 (FLICE/Mch-4) (4, 11). The recruitment of these proteases induces their autocatalytic processing and activation, which finally leads to the cleavage and activation of caspase-3, the apoptotic executioner. Recently, it was proposed that butyrate can induce apoptosis in human cancer cells (5) and mouse colonic cells (10) via the Fas-FasL system. Activation of caspases is also a key event during the intermediate and terminal phases of apoptosis (30). Caspases implicated in apoptosis are currently divided into activator caspases and effector caspases. The activator caspases currently include caspase-8, -9, and -10, whereas caspase-3, -6, and -7 execute the final cell death. The involvement of individual activator or effector caspases in butyric acid-induced cell death and their exact order within the apoptotic cascade are not known in detail.

The aim of this study was to determine whether butyric acid requires the Fas-FasL system or caspase-8 and -9 activation to induce T cell death in vitro. Our findings indicate that caspase-8 and -9-dependent apoptosis plays an important role in butyric acid-induced apoptosis but is independent of the Fas-FasL interaction.

MATERIALS AND METHODS

Reagents. Highly purified butyric acid was purchased from Sigma Chemical Co. (St. Louis, Mo.). Solutions of butyric acid ranging in concentration from 0.62 to 5 mM were diluted in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) medium and adjusted to pH 7.2 with sodium hydroxide. Cytotoxic anti-human Fas immunoglobulin (IgM) monoclonal antibody (MAb) (clone CH-11), nontoxic blocking mouse anti-human Fas IgG MAb (clone ZB4), and nontoxic blocking hamster anti-human Fas, IgG MAb (clone 4H9) were from MBL Co. (Nagoya, Japan).

T-cell preparation. Peripheral blood mononuclear cells (PBMC) were separated from the heparinized venous blood of healthy adults by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation. T cells were then separated from PBMC by immunomagnetic cell sorting. PBMC were incubated for 45 min at 4°C with a mixture of MABs to CD14, CD16, CD19, and CD56 (Stemcell Technologies, Vancouver, Canada). The cells were then washed, and
antibody-loaded cells were depleted by negative magnetic selection using anti-
mouse IgG-coated magnetic beads (Dynal, Oslo, Norway). T cells isolated in this
fashion were typically >98% CD3+ cells as analyzed by flow cytometry (Becton
Dickinson, San Jose, Calif.). The human T lymphoma cell line Jurkat was kindly
provided by Dr. Y. Sueishi (Center-Hayama Cancer Institute, Japan). Jurkat cells
were cultured at 37°C in a moist 5% CO2 atmosphere in complete medium
consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf
serum, 2 mM l-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per
ml, and 0.05 mM 2-mercaptoethanol.

Cell proliferation assay. Jurkat T cells (2 × 10^6 cells/ml) were cultured in
together in complete medium with either 1.25 or 2.5 mM butyric acid or 2.5 mM
together in flat-bottomed, 96-well plates (100 μl/well). For apoptosis inhibition
assays, cells were preincubated for 1 h with blocking anti-Fas MAb (ZB4) (1 to 100
μg/ml), with blocking anti-Fas, MAb (4H9) (1 to 100 μg/ml), or with their isotype controls (mouse IgG1 and hamster IgG; Southern
Biotechnology Associates Inc., Birmingham, Ala.) before adding butyric acid or
cytotoxic anti-Fas MAb (CH-11). After incubation for 42 h, 20 μl of 3-(4,5-
dimethyl-2-thiazoly)-2,5-diphenyl) tetrazolium bromide (MTT) (5 mg/ml in
phosphate-buffered saline [pH 7.2, Sigma]) was added to each well. After 6 h
of incubation, the supernatants were decanted, and the formazan precipitates were
solubilized by the addition of 150 μl of 100% dimethyl sulfoxide (Sigma) and
placed on a plate shaker for 10 min. Absorbance at 550 nm was determined
in a Bio-Rad (Hercules, Calif.) protein assay. Equal amounts (25
μg/ml) of protein from each sample were separated into 106 cells/ml) in 24-well tissue culture plates (Falcon; Becton
Dickinson Labware, Lincoln Park, N.J.) with various concentrations of butyric acid, in the
presence or absence of anti-Fas MAB (CH-11 [10 ng/ml]). After incubation
for 21 h, the cells were harvested, centrifuged at 4°C for 5 min, and washed twice
with ice-cold PBS. The cells were resuspended in 400 μl of hypotonic lysis buffer
(0.2% Triton X-100, 10 mM Tris, 1 mM EDTA [pH 8.0]) and centrifuged for
15 min at 13,800 × g (26). Half the supernatants, which contained small DNA
fragments, as well as the pellet containing large pieces of DNA and cell debris,
were used for the diphenylamine (DPA) assay (see below).

DNA fragmentation assay. The DPA reaction was performed by the method of
Paradinas et al. (29). Perchloric acid (0.5 M) was added to the other half of
the DNA (resuspended with 200 μl of hypotonic lysis buffer) and to the pellets
containing uncult the supernatants containing DNA fragments, and then 2
volumes of a solution containing 0.088 M DPA, 98% (vol/vol) glacial acetic acid,
1.5% (vol/vol) sulfuric acid, and a 0.5% (vol/vol) concentration of 1.6% acetal-
dehyde solution were added. The samples were stored at 4°C for 48 h. The
colorimetric reaction was quantified spectrophotometrically at 575 nm in
a model UV-160A UV spectrophotometer (Shimazu Co. Ltd., Tokyo, Japan).
The percentage of fragmentation was calculated as the ratio of DNA in the super-
natants to the total DNA.

Flow cytometry analysis. PBMC (4 × 10^5) and Jurkat cells (1 × 10^6) in
1 ml of medium were cultured for the indicated times with or without 5 mM
butyric acid. To measure Fas expression, cells (10^6) were then harvested and stained
with fluorescein isothiocyanate isothiocyanate-labeled anti-human Fas MAb (clone DX2) or
with an isotype control (mouse IgG1) (Becton Dickinson) for 30 min at 4°C. After
washing in PBS, the samples were analyzed with a FACSscan apparatus within
1 h. Data from 10^4 cells were analyzed for each sample.

Western blotting. Cells were lysed in lysis buffer (10 mM Tris-HCl [pH 7.4],
150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenyl-
methylsulfonyl fluoride, 8 μg of aprotinin per ml, 2 μg of leupeptin per ml)
and centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was collected and
the amount of protein was measured using the Bio-Rad (Hercules, Calif.) pro-
tein assay. Equal amounts (25 μg) of protein from each sample were separated by
sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis and trans-
ferred to a polyvinylidene fluoride membrane (Millipore, Bedford, Mass.). Western
blots were probed with mouse anti-human Fas or FasL MABs, or with their
isotype controls (mouse IgG1) obtained from Transduction Laboratories (Lex-
ington, Ky.). Primary antibodies were detected using a goat-anti mouse horse-
radish peroxidase-conjugated secondary antibody (Amersham, Little Chalfont,
United Kingdom). Detection of chemiluminescence was performed with an ECL
Western blot detection kit (Amersham), according to the supplier’s recommenda-
tions.

Measurement of caspase protease activity. After incubation of cells (10^6 per
well) in 24-well tissue culture plates for the indicated times with 5 mM butyric
acid or 10 ng of cytotoxic anti-Fas MAB (CH-11) per ml, all the cells were
collected, washed as described above, and the caspase-8 and -9 activities were
measured using a caspase fluorometric protease assay kit (MBL Co.). Levels of
released 7-amin-4-trifluoromethylcoumarin (AFC) were measured with a Bio-
Lumin 960 spectrophotofluorometer (Molecular Dynamics Japan, Tokyo, Japan) with
excitation at 400 nm and emission at 505 nm. The results are expressed as the
mean ± SEM of three different experiments with triplicate cultures. Values
significantly different from the corresponding negative control without stimu-
lants, or the corresponding inhibitor-free anti-Fas antibody or butyric acid values
at < 0.05 are indicated. Inhibition of caspase-9 with IETD-cleaving activity and
of caspase-9 with LEHD-cleaving activity was achieved using caspase-8 inhibitor
Ac-IETD-CHO and caspase-9 inhibitor Ac-LEHD-CHO (Peptide Institute, Inc.,
Osaka, Japan), respectively, administered 1 h before the addition of butyric acid or
anti-Fas antibody.

Statistics. Multiple-group comparisons were made using a one-way analysis
of variance followed by post hoc integroup comparison by the Bonferroni-Dunn
test. Where appropriate, Student’s t test was used to compare two groups.

RESULTS

Expression of Fas in Jurkat and PBMC-T cells after butyric acid treatment. To test whether the Fas-FasL system might
mediate butyric acid-induced apoptosis, Jurkat and T cells from PBMC (PBMC-T cells) were cultured for the indicated
times with 5 mM butyric acid, and Fas expression was assessed by
flow cytometry (Fig. 1). Jurkat cells endogenously express
Fas on their surface, and Fas expression was not affected by 6
or 16 h butyric acid treatment (Fig. 1A). Although normal
peripheral blood T cells express substantial amounts of Fas
(Fig. 1B) and upregulate its expression within 16 h of treat-
ment with anti-CD3 MAB (data not shown), the expression was
not affected by butyric acid treatment (Fig. 1B). These results
indicate that Fas expression is not associated with butyric acid-
induced apoptosis. Furthermore, we analyzed the effect of
butyric acid on Fas and FasL protein expressions in Jurkat cells
by Western blotting (Fig. 2). Jurkat cells constitutively ex-
pressed Fas and FasL proteins, and this expression was not
affected by butyric acid treatment.

Effect of anti-Fas or anti-FasL MABs on butyric acid-
induced apoptosis. It was recently proposed that the Fas-FasL
system is involved in the cytotoxicity exerted by several drugs,
including butyric acid (10, 23). To test this hypothesis, apopto-
sis was induced in Jurkat cells by treatment with butyric acid in
the presence or absence of different concentrations of blocking
anti-Fas MAB (ZB4 [1 to 100 μg/ml]). To assess the blocking
activity of this antibody, cells were also treated with a cytotoxic
anti-Fas IgM MAB (CH-11 [10 ng/ml]). After 24 h of incuba-
tion, cell viability was determined by the MTT assay. As shown
in Fig. 3, while all the cytotoxicity induced by CH-11 antibody
was prevented by the blocking antibody in a dose-dependent
manner, there was no inhibition of butyric acid-induced cell
death.

We next tested whether the FasL system, which is the main
mediator of activation-induced cell death in Jurkat T cells (22),
could be involved in the butyric acid-induced apoptosis. Jurkat
cells were cultured with butyric acid in the presence or absence
of different concentrations of anti-FasL MAB (4H9 [1 to 100
μg/ml]) and examined for cell viability (Fig. 4). Anti-FasL
MAB did not prevent butyric acid-induced apoptosis at any
dose examined.

Cytotoxic anti-Fas MAB also affects butyric acid-induced
apoptosis. To examine the effect of cytotoxic anti-Fas antibody
on butyric acid-induced apoptosis, Jurkat cells were treated
with anti-Fas MAB (CH-11) in the presence of butyric acid.
When Jurkat cells were cultured in the presence of 0.625 to 5.0
mM butyric acid for 21 h and quantified by the DNA fragmen-
FIG. 1. Effect of butyric acid on Fas expression in Jurkat cells and PBMC. Jurkat cells (A) and PBMC-T cells (B), which express Fas constitutively, were treated with 5 mM butyric acid (BA) for the indicated times, and Fas expression was determined using flow cytometric analysis (logarithmic scale) after incubation with fluoresceinated monoclonal anti-Fas and an isotype control. The figure is representative of five experiments with similar results. FITC, fluorescein isothiocyanate.

A. Jurkat

Control (6 h)

Control (16 h)

BA (6 h)

BA (16 h)

B. PBMC

Control (21 h)

BA (21 h)

FITC, fluorescein isothiocyanate.
tation assay, a dose-dependent increase (21.5 to 35.8%) in DNA fragmentation was seen (Fig. 5). With 5 mM butyric acid, a maximal increase (35.8 ± 2.0%) in DNA fragmentation was induced in Jurkat cells. The addition of 10 ng of anti-Fas MAb (CH-11) per ml potentiated butyric acid-induced DNA fragmentation (58.5 to 72.4%) in Jurkat cells, and increased DNA fragmentation (36.6 to 38.9%) was observed in all the cultures treated with various concentrations of butyric acid (P < 0.05).

Since the addition of anti-Fas MAb (CH-11) alone also induced DNA fragmentation (38.9 ± 2.2%) in Jurkat cells, these results indicate that the increased DNA fragmentation with the addition of anti-Fas MAb is due to the apoptosis activity of anti-Fas MAb itself.

**Activation of caspase-8 and -9 in butyric acid-induced and cytotoxic anti-Fas-induced apoptosis.** There is growing evidence suggesting that activator caspases, especially caspase-8 and -9, play essential roles in the apoptotic process. The requirement for caspase-8 and -9 in Fas- and butyric acid-induced apoptosis was determined by their capacity to cleave the caspase-8 substrate IETD-AFC and the caspase-9 substrate LEHD-AFC. Analysis of protease activation during the cell death induced by treatment of Jurkat cells with cytotoxic anti-Fas (CH-11) MAb or with butyric acid resulted in a time-dependent increase in caspase-8 and -9 protease activities (Fig. 6A and B). The increase in caspase-8 protease activity began about 6 h after the addition of anti-Fas (CH-11) MAb and peaked after 16 to 21 h, reaching levels more than three times those of control populations. Treatment with butyric acid also increased caspase-8 activity time-dependently, although the increase was slight (1.8-fold) compared with anti-Fas treatment. The increase in caspase-9 protease activity also began about 6 h after the addition of anti-Fas (CH-11) MAb and peaked after 21 h, reaching levels more than four times those of the control. Treatment with butyric acid also increased caspase-9 activity about 16 h after its addition. The enhanced caspase-8 proteolytic activity induced by treatment of Jurkat cells with anti-Fas MAb or butyric acid was inhibited to below the control levels in a dose-dependent manner by treatment with the caspase-8 inhibitor IETD-FMK, indicating that

\[\text{FIG. 2. Western blot analysis of human Fas and FasL in Jurkat cells.} \]

\[\text{FIG. 3. Effect of antagonistic (ZB4) anti-Fas antibody on butyric acid- and agonistic (CH-11) anti-Fas antibody-induced cytotoxicity.} \]

Jurkat cells were preincubated in either control medium or medium supplemented with the indicated concentration of antagonistic anti-human Fas MAb (ZB4) and an isotype control for 1 h and then were incubated for 48 h with 1.25 or 2.5 mM butyric acid (BA), or 10 ng of cytotoxic anti-Fas MAb (Fas, CH-11) per ml in the presence or absence of ZB4 antibody, as indicated. Cell viability 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 means ± SEMs (error bars) of three different experiments with triplicate cultures. Values significantly different (P < 0.05) from the corresponding ZB4-free butyric acid values or Fas values are indicated by asterisks.
IETD-FMK inhibits the activation of caspase-8 protease induced by anti-Fas MAb or butyric acid (Fig. 6C). Pretreatment with the caspase-9 inhibitor LEHD-FMC also decreased butyric acid-induced and Fas-induced caspase-9 activity to near the control levels (Fig. 6D). However, IETD-FMK and LEHD-FMC, which completely block Fas-mediated apoptosis, partially prevented butyric acid-induced apoptosis (Fig. 6E and F).

DISCUSSION

Since the Fas-FasL system plays a major role in the homeostasis of the immune system (7) and because lymphocytes are exquisitely sensitive to butyric acid (17, 18), we tested whether the lymphocytic response to butyric acid is involved in the Fas-FasL system. Fas expression can be induced in response to various cytokines, including gamma interferon and tumor necrosis factor alpha (21, 32), to wild-type p53 activity (28) and to cytotoxic drugs (23, 24). In contrast, alkyl-isophospholipids (8), doxorubicin (12), and hydrogen peroxide (9) induce apoptosis independently of Fas signaling.

In the present study we have demonstrated that Fas and FasL expression does not involve butyric acid-induced apoptosis (Fig. 1 and 2). Coincubation with blocking anti-Fas or anti-FasL MAbs could not prevent butyric acid-induced apoptosis (Fig. 3 and 4). In addition, PBMC-T cells, which express membrane Fas (Fig. 1) but are resistant to Fas-mediated apoptosis, were efficiently killed by butyric acid (17). Therefore, in spite of the presence or absence of functional Fas, it can be concluded that butyric acid-induced apoptosis occurs independently of activation of the Fas-FasL system since no correlation between sensitivity to Fas and butyric acid was found. There-
**FIG. 6.** Butyric acid induces activation of caspase-8 and -9 in Jurkat cells. (A and B) Jurkat cells were cultured with or without 10 ng of cytotoxic anti-Fas MAb (Fas) per ml or 5 mM butyric acid (BA) for the indicated times. Cell extracts were prepared and caspase-8 (A) and -9 (B) activities were measured as described in Materials and Methods. (C and D) Jurkat cells were treated with the indicated concentrations of Ac-IETD-CHO (C) or Ac-LEHD-CHO (D) for 1 h and then treated with 10 ng of cytotoxic anti-Fas MAb (Fas) per ml or 5 mM butyric acid (BA) for 21 h. Cell extracts were prepared and caspase activities were measured as described in Materials and Methods. (E and F) Jurkat cells were treated with the indicated concentrations of Ac-IETD-CHO (E) or Ac-LEHD-CHO (F) for 1 h and then treated with 10 ng of cytotoxic anti-Fas MAb (Fas) per ml or 5 mM butyric acid (BA) for 21 h. Harvested cells were assayed by the DPA assay. Error bars, SEMs.
fore, cytotoxic anti-Fas MAb also affected butyric acid-induced apoptosis, but no synergistic effect was seen (Fig. 5).

Caspase activation plays a central role in the execution of apoptosis. The two best-studied pathways of caspase activation are the cell surface death receptor pathway, such as Fas-mediated apoptosis, and the mitochondrion-initiated pathway (6). In this study, time-dependent activation of caspase-8 and -9 was recognized in butyric acid- as well as Fas-mediated apoptosis. These data show for the first time that butyric acid activates a key element of the Fas signaling pathway independently of Fas/FADD activation. Hence, the expression of FasL or activation of Fas is not essential for the initial triggering of the apoptotic cascade by butyric acid. Although the precise mechanism of butyric acid-induced apoptosis remains unclear, our previous study indicated that butyric acid treatment decreased Bcl-2 and Bcl-2 XL expression in murine splenic T cells (unpublished data). The Bcl-2 family regulates apoptosis via the release of cytochrome c from mitochondria (16). The up-regulation of anti-apoptotic Bcl-2 or its close homologue Bcl-XL is known to inhibit apoptosis (3), whereas the down-regulation of Bcl-2 or its antagonization by dimerization with Bax promotes programmed cell death (27). This indicates that the butyric acid-induced pathway is closely involved with the mitochondrial Bcl-pathway, known to be critical to apoptosis in other models (15, 25). Sodium butyrate is reported to inhibit the mitochondrial Bcl-pathway, known to be critical to apoptosis, but no synergistic effect was seen (Fig. 5).

In conclusion, our data indicate that Fas-FasL interaction is not involved in butyric acid-induced apoptosis and that caspase-8 and -9-dependent apoptosis plays an important role in butyric acid- as well as Fas-induced apoptosis. The specific mechanisms by which butyric acid initiates the apoptosis signaling pathway is currently being investigated.

ACKNOWLEDGMENTS

This work was supported in part by a grant to promote multidisciplinary research projects; grants-in-aid (11671818) of scientific research from the Ministry of Education, Science, and Culture of Japan; and a Suzuki Memorial Grant (00-1004) from the Nihon University School of Dentistry at Matsudo.

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