Antagonism of CD95 signaling blocks butyrate induction of apoptosis in young adult mouse colonic cells

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Fan, Yang-Yi, J Ianhu Zhang, Rola Barhoumi, Robert C. Burghardt, Nancy D. Turner, Joanne R. Lupton, and Robert S. Chapkin. Antagonism of CD95 signaling blocks butyrate induction of apoptosis in young adult mouse colonic cells. Am. J. Physiol. 277 (Cell Physiol. 46): C310–C319, 1999.—There is great interest in utilizing butyrate as a chemopreventive agent for colon tumorigenesis because of its ability to promote apoptosis in colon tumor cell lines. Because CD95 (APO-1/Fas) transduces signals resulting in apoptosis, we tested the hypothesis that butyrate-dependent colonicocyte apoptosis is mediated by this death receptor. Butyrate (1 mM) exposure for 24 h upregulated expression of Fas and its ligand in young adult mouse colon (YAMC) cells. To delineate the proapoptotic effect of butyrate and to avoid the confounding effects of detachment from the extracellular matrix, adherent cell apoptosis was monitored as loss of plasma membrane asymmetry and dissipation of mitochondrial membrane potential (ΔΨmt) by laser cytometry. Soluble Fas receptor protein (Fas:Fc chimera) and caspase inhibitors (z-VAD-fmk and z-EDT-fmk) blocked butyrate induction of apoptosis. Treatment with Fas agonistic antibody (clone J o-2) significantly induced cell death, indicating that Fas in colonicocytes is functional. In addition, butyrate promoted apoptosis by inducing loss of ΔΨmt and phospholipid asymmetry of the plasma membrane after 12 and 24 h of exposure, respectively, before cell detachment. Therefore, Fas receptor-dependent signal transduction is involved in butyrate induction of apoptosis in colonicocytes.

anoikis; plasma membrane asymmetry; mitochondrial membrane potential; colon cancer; Fas; APO-1

MAINTENANCE OF THE COLONIC epithelium requires a dynamic equilibrium between cell proliferation and cell death (32). Until recently, colon cancer development was thought to occur primarily through increased cell proliferation at several stages of the tumorigenic process, and little emphasis was placed on programmed cell death (apoptosis). The emphasis has now shifted, and there is considerable interest in the relationship between colonic apoptosis and malignant transformation (13, 18). Inhibition of apoptosis is now thought to be an integral component of the genesis of colorectal adenomas and carcinomas (4, 16, 31). Recent studies of humans demonstrate that reduced apoptotic ability may predispose individuals to increased risk for colon cancer (16).

In certain biological systems, apoptosis plays a central role in animal development by sculpting anatomical structures and deleting unneeded tissue (21). In these cases, apoptosis is a dominant mechanism, involving a high percentage of cells. In contrast, in the large intestine, apoptosis occurs with very low frequency, i.e., less than one apoptotic cell per crypt (8, 18). Although apoptosis is not a dominant feature in histological material, there is compelling evidence that it is a central feature in the regulation of cell number and the elimination of nonfunctional, harmful, or abnormal cells in the colon (8, 18, 32, 37).

Apoptosis-signaling death receptors are characterized by an intracellular region referred to as the death effector domain (DED), which is required for the transmission of the cytotoxic signal (1, 34). Currently, five distinct death receptors are known, including CD95 (APO-1/Fas), tumor necrosis factor (TNF) receptor (TNFR) 1, TNFR-related apoptosis-mediated protein, and TNF-related apoptosis-inducing ligand receptors 1 and 2 (1, 34). With regard to the colonic mucosa, the Fas receptor is considered an important candidate for regulating apoptosis (28, 29, 38, 42). Whereas Fas is strongly expressed throughout the normal colon (28), it is downregulated and progressively reduced during malignant transformation (6, 28). In addition, colon carcinoma cells can acquire mechanisms to escape Fas-mediated apoptosis. These include antagonism of Fas, inhibition of Fas capping, and activation of antiapoptotic programs (42). With regard to Fas activation, binding either to its natural ligand, CD95L (Fas-L), or with agonistic antibodies induces apoptosis in some cells. However, Fas-L expression is virtually absent in the normal colon (29). Thus the biological role for Fas-mediated apoptosis in the colon is still obscure.

Butyrate, a short-chain fatty acid derived in the colon from microbial fermentation of diet-derived complex carbohydrates, promotes differentiation and apoptosis in a variety of colon tumor cell lines (2, 17, 44). As a result, there is great interest in utilizing butyrate as a chemotherapeutic and chemopreventive agent (7, 9). However, the specific mechanisms by which butyrate induces apoptosis have not been fully elucidated. In addition, an early feature of colonic cells undergoing apoptosis is detachment from the extracellular matrix (18, 44). This subset of apoptosis is known as anoikis (18). The phenomenon is similar to what occurs in the colonic epithelium in vivo, and there is good evidence that it occurs in both immortalized and colonic carcinoma cell lines in vitro (2, 20, 44). However, it is unknown whether butyrate induction of apoptosis is
mediated by disrupting interactions between colono-
cytes and the extracellular matrix.

In the present study, we utilized the young adult mouse colon (YAMC) cell line, which was isolated from transgenic mice bearing a temperature-sensitive muta-
tion of the simian virus 40 (SV40) large T antigen gene (43), to determine whether butyrate stimulation of colonic apoptosis is mediated by CD95 (APO-1/Fas). Conditionally immortalized YAMC cells are a relevant model to examine the molecular mechanisms by which diet-derived factors affect relative cancer risk (2, 43). Our results demonstrate that butyrate induces an apoptotic phenotype in adherent colono-
cytes as defined by loss of plasma membrane phospholipid asymmetry in the absence of cell leakage, dissipation of mitochon-
drial membrane potential, and upregulation of Fas and Fas-L expression. In addition, we demonstrate that there is antagonism of Fas signaling by caspase inhibi-
tors (z-VAD-fmk and z-IETD-fmk) and that soluble Fas protein blocks butyrate induction of apoptosis in both adherent and nonadherent (floating) populations of cells. Therefore Fas functions as a transducer of buty-
rate-induced cell death in colonic cells.

MATERIALS AND METHODS

Materials. RPMI 1640 and Hanks’ balanced salt solution (HBSS) were from Mediatech (Herndon, VA). Fetal bovine serum was from Hyclone (Logan, UT). Insulin, transferrin, selenium, and linoleic acid were obtained from Collaborative Biomedical Products (Bedford, MA). Glutamax and recombi-
nant mouse interferon-γ (IFN-γ) were from Gibco BRL (Grand Island, NY). Cellular DNA fragmentation ELISA and the cell death detection ELISA were from Boehringer Mannheim (Indianapolis, IN). Hamster anti-mouse Fas monoclonal antibody (clone O-2), annexin V-FITC, 10× binding buffer, and propidium iodide (PI) were obtained from Pharmingen (San Diego, CA). Hamster IgG was from Jackson Immuno-
Research Labs (West Grove, PA). The general caspase inhibitor (z-VAD-fmk) and caspase 8 inhibitor (z-IETD-fmk) were from Enzyme Systems Products (Livermore, CA). Prepared polyacrylamide gradient gels were from Novex (San Diego, CA). Electroblotting polyvinylidene difluoride membranes were obtained from Millipore (Burlington, MA). Bicinchoninic acid (BCA) protein assay and SuperSignal chemiluminescent detection reagents were from Pierce (Rockford, IL). Antibodies were obtained from the following suppliers: rabbit anti-
Fas M-20 and rabbit anti-Fas-L C-178, Santa Cruz Biotechnol-
ogy (Santa Cruz, CA); mouse anti-glycerdehyde-3-phosphate dehydrogenase (GAPDH) MAB374, Chemicon (Temecula, CA); mouse anti-SV40 DP01, Oncogene (Cambridge, MA); and peroxidase-conjugated secondary antibodies, Kirkegaard & Perry (Gaithersburg, MD). Rhodamine 123 was obtained from Molecular Probes (Eugene, OR). Soluble recombinant human Fas (Fas:Fc) at 2.8 µg/ml or Fas-related TNFR (TNFR:Fc) at 2.8 µg/ml (5, 10) was co-in-
cubated with butyrate for 24 h. At the end of the incubation period, adherent-cell viability was assessed by simultaneous staining with fluorescein diacetate and PI (22), and floating cells were harvested to measure levels of DNA fragmentation. Floating cells were lysed and centrifuged at 2,000 × g to sediment intact nuclei. Supernatants containing low-molecu-
lar weight DNA were subsequently analyzed by ELISA. Values of absorbance at 450 nm (A450) were normalized by the number of adherent cells per dish. For the cell death detection ELISA, cells were seeded as described above. The following day, instead of being prelabeled overnight with 5-bromo-2’-
deoxyuridine, cells were provided fresh medium with or withou

starch and centrifuged for an additional 24 h. Floating cells were harvested to measure apoptosis. Cells were rinsed once with sterile PBS, and then 1× binding buffer containing 2.5 mM CaCl2 (diluted from 10× binding buffer) was added to the well. Cells were subsequently incubated with 25 µl of annexin V-FITC and 50 µl of PI for 15 min in the dark at room temperature. After 15 min, the solution was aspirated and the cells were gently rinsed with 1× binding buffer twice. Binding buffer (200 µl) was added to each well, and the dual-labeled cells were visualized by phase-contrast and fluorescence microscopy with a Nikon Diaphot inverted microscope. For each well, cells stained with either annexin V-FITC, PI, or both were counted from three representative fields, then normalized by the total cell number in that same

under an IFN-inducible promoter, mouse H-2Kb class 1 gene (43). Cells were cultured under permissive (33°C) or nonpermissive (39°C) conditions as previously described (2).

Apoptosis in nonadherent cells. Either cellular DNA frag-
mentation ELISAs or cell death detection ELISAs were used to measure apoptosis in floating-cell populations (2). Briefly, for the cellular DNA fragmentation ELISA, 30,000 cells were seeded into 35-mm dishes and incubated for 24 h. Nonadher-
cent cells were rinsed off with HBSS, and adherent cells were provided fresh medium containing 10 µM 5-bromo-2’-
deoxyuridine and incubated overnight. The following day, fresh medium containing sodium butyrate (0, 1, or 5 mM), hamster anti-mouse Fas monoclonal antibody (clone O-2; at 5 or 10 µg/ml), or hamster IgG (10 µg/ml) was added to cells, and the dishes were incubated for 24 h at 33 or 39°C. The level of butyrate was selected on the basis of luminal composition data from the rodent colon, where normal buty-
rate levels are in the 1–4 mM range (45). This is also the range within which the large majority of in vitro butyrate studies have been conducted (17, 19, 20). Above these levels, cytotoxic effects have been observed (2, 35).

In selected experiments, cells were preincubated with the general caspase inhibitor z-VAD-fmk (25, 50, and 75 µM) or caspase 8 inhibitor z-IETD-fmk (5 and 10 µM) 1 h before butyrate treatment. In addition, in separate experiments, soluble recombinant human Fas (Fas:Fc) at 2.8 µg/ml or Fas-related TNFR (TNFR:Fc) at 2.8 µg/ml (5, 10) was co-
incubated with butyrate for 24 h. At the end of the incubation period, adherent-cell viability was assessed by simultaneous staining with fluorescein diacetate and PI (22), and floating cells were harvested to measure levels of DNA fragmentation. Floating cells were lysed and centrifuged at 2,000 × g to sediment intact nuclei. Supernatants containing low-molecu-
lar weight DNA were subsequently analyzed by ELISA. Values of absorbance at 450 nm (A450) were normalized by the number of adherent cells per dish. For the cell death detection ELISA, cells were seeded as described above. The following day, instead of being prelabeled overnight with 5-bromo-2’-
deoxyuridine, cells were provided fresh medium with or withou

field under phase-contrast conditions. Typically, the total cell numbers in representative fields varied between 100 and 300 cells.

Immunoblot analysis. For time course Western blot analysis, 1 × 10⁶ YAMC cells were cultured in T-75 flasks in the presence of 1 mM sodium butyrate for 0, 8, or 24 h. After culture, the medium was removed and adherent cells were washed with HBSS three times. Cells were scraped into 3 ml of homogenization buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 2 mM EDTA, 1 mM EGTA, 50 µM NaF, 100 µM sodium orthovanadate, 25 µg/ml aprotinin, 25 µg/ml leupeptin, 25 µg/ml pepstatin) (30, 40). Cell lysates were passed through a 25-gauge needle three times and incubated on ice for 30 min. Cellular debris was removed by centrifugation (13,600 g, 15 min) at 4°C. The cell extracts were aliquoted and frozen at −80°C. The protein concentration of each extract was determined against a standard by the BCA protein assay. Cell extracts were treated with pyronin sample buffer and subjected to Tris-glycine gel electrophoresis by using 10–20% gradient polyacrylamide gels as described by Laemmli (24). After electrophoresis, gels were electrobblotted onto a Millipore Immobilon-P transfer membrane with a Hoefer Mighty Small Transphor unit (Pharmacia, Piscataway, NJ) at 400 mA for 75 min. After transfer, the membrane was processed by the method of Davidson et al. (11). Membranes were incubated with primary antibody (rabbit anti-Fas, rabbit anti-Fas-L, mouse anti-GAPDH, or mouse anti-SV40) diluted in blocking buffer at 4°C overnight. Dilution of the primary antibody was titrated for each protein. Peroxidase-conjugated secondary antibody (1:10,000) incubation was for 1.5 h at room temperature and was followed by luminescence development with the SuperSignal reagent mixture. Blots were exposed to Kodak Biomax MR film, scanned, and quantitated with IQ software (BioImage, Ann Arbor, MI) for steady-state levels of homogenates for each target protein was loaded onto the gel to ensure that the response was quantitative.

Assessment of mitochondrial membrane potential in adherent cells. The potentiometric probe rhodamine 123 was used to provide a relative measure of mitochondrial membrane potential (ΔΨm) (12) as previously described (3). YAMC cells in two-well cover glass chamber slides were incubated with butyrate for 0, 6, 12, or 24 h and subsequently loaded with 2.5 µM rhodamine 123 for 15 min in an incubator at 33°C. Cells were washed three times with culture medium without serum, the analysis of rhodamine 123 fluorescence intensity was then performed at an excitation wavelength of 488 nm, and the emitted fluorescence of adherent cells was monitored at 530 nm with an Ultima confocal microscope (Meridian Instruments, Okemos, MI). Data from at least eight areas per well and from four wells per treatment group were collected in each experiment.

Data analysis. Statistical significance was calculated by one-way ANOVA. When P values were <0.05 for the treatment effects, means were separated by Duncan’s multiple range test.

RESULTS

Butyrate induces loss of plasma membrane asymmetry in adherent colonocytes. To characterize YAMC cell apoptosis after butyrate treatment, we examined phosphatidylserine (PS) externalization in adherent colonocytes. Elevated levels of apoptotic cells were detected 24 h after exposure to the permissive temperature, 33°C (Fig. 1 A and B). Because PS exposure occurs early after the onset of apoptosis (1, 34), these data demonstrate for the first time that a significant number of apoptotic cells are seen in the adherent cell populations (Fig. 1A). Because detachment from the matrix occurs relatively early in the process of apoptosis (15, 44), the level of apoptosis in nonadherent (floating) cells was also quantitated by DNA fragmentation ELISA. Butyrate treatment (24 h) increased apoptosis in the nonadherent population as well (Fig. 1B). These
Fig. 2. Classification of fluorescent staining patterns in adherent cells after 24 h of butyrate treatment (magnification, ×200). A: early-stage apoptotic (annexin V⁻/PI⁻) cells (green). B: late-stage apoptotic (annexin V⁺/PI⁻) cells (orange). C: necrotic (annexin V⁻/PI⁺) cells (red).
data are consistent with previous reports that colonocytes undergo apoptosis after butyrate exposure (2, 20, 44). As a positive control, in cells grown at the nonpermissive temperature (39°C), the numbers of adherent early- and late-stage apoptotic cells [cells positive for annexin V and negative for PI (annexin V+/PI−) and annexin V+/PI+, respectively] and necrotic (annexin V−/PI+) cells were significantly (P < 0.05) increased (Figs. 1A and 2). Late-stage apoptosis or secondary necrosis (annexin V+/PI+) is likely the consequence of an apoptotic process in culture where no phagocytes are present to remove dying cells (41, 44). The effect of butyrate treatment on the total percentage of apoptotic cells per culture (adherent + floating populations) is described in Table 1. On average, there were 10-fold more apoptotic cells in the floating-cell population than in the adherent-cell population. However, with butyrate treatment there were >20-fold more apoptotic cells in the floating-cell population than in the adherent-cell population.

Because significant populations of adherent cells express the initial features of apoptosis before detachment (Fig. 1A), a kinetic analysis of butyrate-induced apoptosis was made over a 0- to 36-h incubation period (Fig. 3). The data indicate that early apoptotic cells (annexin V+/PI−) are not detected in the adherent population at 12 h.

Butyrate exposure increases Fas and Fas-L protein levels. We determined whether butyrate upregulates functionally important death signaling molecules in YAMC cells. As shown in Fig. 4, A and B, after 8 h of butyrate treatment, Fas and Fas-L protein levels were increased by 52 and 47% of the control, respectively. At 24 h, butyrate increased Fas and Fas-L protein levels by 55 and 96% of the control, respectively. In contrast, butyrate did not affect the expression of SV40 large T antigen or the housekeeping gene, the GAPDH gene, at either time point.

Mitochondrial function-dependent apoptosis. Because alterations in mitochondrial membrane potential have been implicated in apoptosis (20), we determined whether butyrate exposure initiates a process whereby the ΔΨm is dissipated (Fig. 5). At both 1 and 5 mM

Table 1. Effect of butyrate treatment on relative levels of apoptosis in adherent and floating cell populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Control</th>
<th>Butyrate, 33°C</th>
<th>Vehicle, 39°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherent</td>
<td>0.33 ± 0.06</td>
<td>0.72 ± 0.08</td>
<td>2.90 ± 0.39</td>
</tr>
<tr>
<td>Floating</td>
<td>2.92 ± 0.24</td>
<td>17.49 ± 0.66</td>
<td>13.57 ± 0.33</td>
</tr>
<tr>
<td>Total*</td>
<td>3.24 ± 0.44</td>
<td>18.21 ± 1.35</td>
<td>16.47 ± 1.30</td>
</tr>
</tbody>
</table>

Data are mean percentages of apoptotic cells per culture (n = 4–21) ± SE from 5 separate experiments. Cells were treated with butyrate (1 mM) or vehicle and incubated at 33°C or were treated with vehicle and incubated at 39°C (nonpermissive temperature) for 24 h. *Percent apoptotic cells = [(no. of adherent apoptotic cells + no. of floating apoptotic cells)/no. of adherent cells] × 100.

Fig. 3. Kinetic analysis of butyrate-induced apoptosis. YAMC cells were treated with 1 mM butyrate and incubated up to 36 h. Adherent-cell apoptosis was determined at 0, 4, 8, 12, 24, and 36 h after butyrate treatment by annexin V-FITC and PI dual labeling (see Fig. 1 legend). Values are normalized relative to total no. of adherent cells per culture field (n = 4 wells). *Significantly different (P < 0.05) from control (no butyrate).

Fig. 4. Butyrate increases Fas and Fas-L protein levels. YAMC cells were treated with butyrate (1 mM) and incubated at 33°C for 0, 8, or 24 h. Adherent-cell lysates were harvested, and protein expression was analyzed by immunoblotting. A: representative immunoblot analysis of Fas, Fas-L, simian virus 40 (SV40), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B: densitometric analysis of butyrate effects on Fas, Fas-L, SV40, and GAPDH expression. Data were pooled from 3 separate experiments and are expressed as means ± SE. Protein levels were standardized against the 0-h control; n = 7–9 for each assay. Different letters over bars indicate significant differences (P < 0.05).
butyrate, \( \Delta \Psi_{\text{mt}} \) in adherent cells was dissipated but not collapsed after 12 h of exposure. Therefore butyrate is capable of inducing mitochondrial damage after an initial lag period, before exposure of PS on the outer cell membrane leaflet.

**Fas mediates apoptosis in YAMC cells.** Because Fas is a receptor that transduces signals resulting in cell death, we asked how YAMC cells would respond when cultured with an agonistic Fas antibody (Jo-2). The agonistic anti-Fas (5 or 10 µg/ml) or control IgG was added to colonocytes. Cultures were maintained for 24 h at the permissive temperature (33°C), after which nonadherent cells were harvested and apoptosis was measured by DNA fragmentation ELISA (Fig. 6). Overall, Fas-mediated killing was elevated by 150–280%, in a dose-dependent manner, relative to the control. These data indicate that an apoptosis-inducing receptor, Fas, is functional in YAMC cells.

Butyrate-induced death of YAMC cells is blocked by soluble Fas protein and caspase inhibitors. The ability of butyrate and Fas antibody to induce apoptosis in YAMC cells raised the possibility that butyrate induction of cell death is mediated by Fas. To determine if Fas is involved in butyrate-induced apoptosis, the neutralizing effect of a soluble form of Fas that binds to the Fas-L on the cell surface was utilized. Cells were cultured in the presence of a chimeric molecule consisting of the extracellular region of human Fas and the Fc portion of human IgG (Fas:Fc), as previously described (5). Butyrate induced 9.7- and 3.6-fold increases in early- (annexin V+/PI-) and late-stage (annexin V+/PI+) adherent apoptotic cells, respectively, which were inhibited by soluble Fas:Fc (Fig. 7A). As a control, a chimeric molecule consisting of the extracellular portion of the Fas-related TNFR and the Fc portion of human IgG (TNFR:Fc) was coincubated with butyrate (10). Unlike Fas:Fc, this molecule had no effect on butyrate-induced cell death. In addition, Fas:Fc antagonized butyrate-induced apoptosis in nonadherent (floating) cells (Fig. 7B). Another means of antagonizing Fas-mediated induction of apoptosis is to block the upregulation of key catalytic effectors (caspases) that are activated after Fas ligation (36). We initially determined the effect of z-VAD-fmk, a broad spectrum polypeptide inhibitor of the interleukin-1-converting enzyme (ICE) family of cysteine proteases (caspases) (23). Consistent with the role of caspases in apoptosis, z-VAD-fmk pretreatment blocked the induction of early-
stage apoptosis (annexin V⁺/PI⁻) in adherent cells after 24 h of treatment with 1 mM butyrate (Fig. 8A). In contrast, z-VAD-fmk only partially blocked the induction of early-stage apoptosis (annexin V⁺/PI⁻) after incubation with 5 mM butyrate. This may indicate that butyrate is capable of inducing apoptosis by more than one mechanism. Because FLICE (caspase 8) directly interacts with the DED of Fas and is the first in the cascade of proteases activated by Fas ligation (23, 26), we examined the effect of the peptide inhibitor z-IETD-fmk on butyrate-induced apoptosis. z-IETD-fmk, which inhibits caspase 8 (26), blocked the proapoptotic effect of butyrate in both adherent-cell (Fig. 8B) and nonadherent (floating)-cell populations (Fig. 8C). In addition, z-IETD-fmk blocked butyrate-dependent dissipation of ΔΨ

It has now been clearly established that the transformation of the colonic epithelium to carcinomas is associated with a progressive inhibition of apoptosis (4, 16, 31). Recently, investigators have focused on the mechanisms responsible for the reduction of apoptosis in colon cancer (2, 4, 17, 20, 29). Interestingly, for the rat azoxymethane colon tumorigenesis model, we have demonstrated that apoptosis has greater diagnostic value than cell proliferation in terms of predicting which animals will develop colon tumors (8). These data suggest that the assessment of apoptosis deserves a place in the armamentarium of intermediate biomarkers for colon tumorigenesis. In this context, colon cancer is a disease strongly influenced by environmental factors, with diet being one of the most important modifying agents. Among dietary factors, the protective effect of high-fiber (i.e., complex plant carbohydrate) diets has been attributed, in part, to the production of butyrate by anaerobic fermentation in the colon (9, 17), although this remains a controversial area of research (25). One hypothesis to explain the tumor suppressor activity of butyrate is that this short-chain fatty acid induces apoptosis in a variety of colon carcinogenesis models, providing a protective effect (2, 7, 17, 20, 44). However, the precise mechanism of action of this putative chemotherapeutic agent has not been fully elucidated (2, 7, 9, 17, 20).

The present study was undertaken to determine whether butyrate stimulation of apoptosis is mediated by CD95 (APO-1/Fas). Fas is a 48-kDa transmembrane molecule whose gene belongs to the TNF nerve growth factor receptor gene superfamily (1, 34). The binding of Fas-L to Fas induces trimerization of the Fas receptor, which subsequently recruits caspase 8 through a Fas-associated death domain (MORT 1) adapter (23, 26). Although Fas is strongly expressed throughout the colonic crypt, the limited expression of Fas-L raises questions regarding the biological role of this cell death machinery in the colon (28, 29). Our data demonstrate that butyrate exposure selectively induces expression of Fas-L and Fas. The Fas receptor-ligand pair is functional, because treatment with an agonistic Fas antibody induces apoptosis in YAMC cells.
The direct involvement of Fas and Fas-L in butyrate-induced colonocyte apoptosis is established by the fact that neutralizing soluble Fas protein, when provided immediately before butyrate exposure, blocks butyrate-induced apoptosis in both adherent- and nonadherent (floating)-cell populations. In addition, apoptosis of both adherent and nonadherent cells is inhibited by both a broad-spectrum, ICE-like protease inhibitor (z-VAD-fmk) and a caspase 8 inhibitor (z-IETD-fmk). Caspase 8 directly interacts with the activated trimerized form of the Fas receptor (23, 26). Although the involvement of peptide inhibitors in other pathways cannot be completely ruled out, the antagonism of butyrate-induced apoptosis by z-IETD-fmk and the
neutalizing soluble Fas protein Fas:Fc is consistent with the direct involvement of the Fas receptor.

It has been speculated that butyrate initiates an apoptotic phenotype in colonocytes by disrupting interactions between cells and the extracellular matrix (18, 44). As expected, with butyrate exposure, YAMC cells lose their adherence to the substratum and floating cells undergo DNA fragmentation (Table 1). To delineate the direct proapoptotic effect of butyrate and to avoid the confounding effects of detachment-induced apoptosis (i.e., anoikis), we determined the percentage of adherent cells exhibiting an apoptotic morphology. In the early steps of butyrate-induced apoptosis, YAMC cells are still attached to the culture flask. These cells lose their phospholipid membrane asymmetry and expose PS at the cell surface while maintaining their plasma membrane integrity. This process was monitored by using annexin V-FITC. In addition, because the involvement of mitochondria in apoptotic processes has been demonstrated (1, 19, 20), butyrate-initiated dissipation of the \( \Delta \Psi_{\text{int}} \) in adherent cells was determined by laser cytometry. Although Heerdt et al. (19) have demonstrated that butyrate-initiated dissipation of \( \Delta \Psi_{\text{int}} \) is required for progression through the apoptotic cascade in nonadherent cells, our novel observation that butyrate induces mitochondrial damage before anoikis is consistent with the early involvement of mitochondria in Fas-mediated apoptosis (36). Interestingly, mitochondrial damage occurred after an initial lag period, paralleling the upregulation of Fas-L expression. This is consistent with the idea that protein synthesis or degradation is required for progression through the apoptotic cascade. In keeping with this interpretation, there is evidence that butyrate induction of apoptosis is dependent on the inhibition of histone deacetylase and new protein synthesis (27). In addition, with regard to the selective degradation and posttranslational processing of functionally important proteins, the ubiquitin pathway may play a role in the butyrate-induced apoptotic program (36).

Butyrate is not a classical apoptogenic metabolite, capable of inducing massive apoptosis. It is believed to serve as the primary energy source for colonocytes in vivo (33, 45). It is therefore not altogether surprising that the effect of butyrate on colonocyte apoptosis in adherent cells is numerically small. In comparison, the total number of cells (adherent + floating) undergoing apoptosis after butyrate exposure is comparatively high, \( \sim 18\% \) (Table 1). With regard to the biological relevance of this effect, it is now clear that small changes in apoptosis regulate crypt cell number (18), alter colon cancer risk (4, 8), and modulate tumor behavior (39). In addition, the levels of apoptosis in adherent-cell populations in our model system are similar to reported levels in the human, rat, and mouse colon in vivo (4, 8, 14, 18). Therefore the YAMC cell culture system is a relevant model to examine the mechanisms by which butyrate modulates colon apopto-

In conclusion, butyrate stimulation of colonic apoptosis is mediated by CD95 (APO-1/Fas). Our results also show that although butyrate is capable of disrupting interactions between colonocytes and the extracellular matrix, an anoikis-independent death pathway also exists. Because Fas is downregulated or lost in the majority of colon carcinomas, it remains to be determined whether butyrate treatment or dietary fiber supplementation will prove useful in the fight against colorectal cancer.

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