Butyrate Induces Necrotic Cell Death in Murine Colonic Epithelial Cell MCE301

Tsukasa MATSUMOTO,a,b,1 Tomoyuki HAYASAKI,b,1 Yoshibko NISHIMURA,c Masahiko NAKAMURA,b
Tadahiro TAKEDA,c Yoshiaki TABUCHI,a Masuo OBINATA,a Toshihiko HANAWA,b and Haruki YAMADA*,a,b

* Kitasato Institute for Life Sciences & Graduate School of Infection Control Sciences, Kitasato University; Tokyo 108–8641, Japan; b Oriental Medicine Research Center, The Kitasato Institute; Tokyo 108–8642, Japan; c Kyoritsu University of Pharmacy; Tokyo 105–8512, Japan; d Graduate School of Pharmaceutical Sciences, Kitasato University; Tokyo 108–8641, Japan; e Life Science Research Center, University of Toyama; Toyama 930–0194, Japan; and f Institute of Development, Aging and Cancer, Tohoku University; Miyagi 980–0872, Japan.

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Recent findings have suggested that organic acids produced by anaerobic intestinal bacteria might contribute to the pathogenesis of colonic ulcers. In this study, it was shown that butyrate caused potent cytotoxicity in the murine normal colonic epithelial cells MCE301 at physiological concentrations. Several markers of apoptosis, such as phosphatidyl serine externalization, cytochrome c release, DNA fragmentation, and chromatin condensation were negative after butyrate exposure. Inhibitor of caspases failed to protect against butyrate cytotoxicity. By transmission electron microscopy, marked swollen mitochondria and vacuolization within the cytoplasm was observed by treatment of butyrate. Collective, these data indicated that butyrate-induced cell death caused through a necrosis-like process. Butyrate induced cell death was reduced partially by treatment with prednisolone or 5-aminosalicylates in a concentration dependent manner. These results suggest that (1) butyrate induces necrotic cell death but not apoptotic cell death, and (2) the necrotic cell death induced by butyrate may be useful as a novel in vitro model of ulcerative colitis to screen useful drugs for the treatment of the disease.

Key words butyrate; ulcerative colitis; necrosis; 5-aminosalicylic acid; colonic epithelial cell; MCE301 cell

Ulcerative colitis (UC) is a disease of unknown etiology characterized by frequent relapses or remissions of inflammation in the colonic mucosa. It has been reported that spontaneous colitis, which consistently develops in knockout and transgenic murine models, does not occur when these mice are maintained in germ free conditions,1,2 and suggested the involvement of bacteria in disease development.3,4 Recently, it has been shown that Fusobacterium varium were identified in the mucosa of a significant number of patients with UC, and specific antibody against F. varium was detected in the sera from patients with UC.5 It has also been reported that F. varium culture supernatants contained high concentrations of butyrate, and the enema containing either butyric acid or F. varium culture supernatants caused UC-like lesions in mice.6 However, the role of butyrate on the pathogenesis of UC has not been fully elucidated.

Butyrate produced in the colonic lumen by anaerobic microbial fermentation of undigested carbohydrates, has been shown to induce proliferation, differentiation, cell cycle arrest, and apoptosis in colonic epithelial cells.7–12 In spite of many studies, the effect of butyrate on colonic epithelial cells is not fully understood. A major limitation to studying the effect of butyrate using primary cultured colonic epithelial cells is the difficulty in maintaining viability of the cells after isolation. Therefore, most studies have been employed using colonic cancer cells. These cell lines have proven useful in analyzing function of colonic epithelial cells. However, butyrate has often been observed to exert paradoxical effects. The results have varied according to the cell line used, and because of their malignant phenotype, their properties and functions may not be representative of colonic epithelial cells in vivo.

The mouse colonic epithelial cell line, MCE301, has been established from a primary culture of gastric mucosal cells of transgenic mice harboring a temperature-sensitive simian virus 40 (tsSV40) large T-antigen gene.13 The cells were not transformed, as judged by the absence of anchorage-independent growth in soft agar and lack of tumor formation in nude mice. Because MCE301 cells have been shown to retain many of the characteristics of normal colonic epithelial cells,13 the studies using MCE301 cells provide useful information to understand various aspect of colonic epithelial cell function and cellular response to various stimuli.

Recent findings have suggested that butyrate produced by anaerobic intestinal bacteria might contribute to the formation of UC-like lesions. In the present study, the effect of butyrate on cell viability of colonic epithelial cells was examined using MCE301 cells in vitro, and it was found that butyrate induced necrotic cell death in MCE301 cells. Results of this study support that the necrotic cell death of MCE301 cells induced by butyrate may be useful as a novel in vitro model of UC to screen useful drugs for the treatment of the disease.

MATERIALS AND METHODS

Materials Murine normal colonic epithelial cells MCE301 were cultured in Dulbecco’s modified Eagle medium (DMEM)/Ham F-12 (1 : 1) medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 5% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, U.S.A.), 10 μg/ml insulin, 5.5 μg/ml transferrin, 2 μg/ml ethanolamine, 5 μg/ml sodium selenite, and 10 ng/ml epidermal growth factor at 37°C in a humidified atmosphere of 5% CO2 in air. After reached to confluent, the cells were cultured at 39°C in DMEM/Ham F-12 alone for 3 d, and then subjected to experiment. All experiments using MCE301 cells were carried out at 39°C. Human colorectal carcinoma Caco-2 cells were...
kindly provided Prof. T. Itoh (Kitasato University, Tokyo, Japan) and were cultured at 37 °C in DMEM (Sigma) supplemented with 5% FBS. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Dojindo (Kumamoto, Japan). DeadEnd Fluorometric TUNEL System was obtained from Promega (Madison, WI, U.S.A.). Sodium butyrate, propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were from Sigma. Alexa Fluor594 phallloidin was from Molecular Probes (Eugene, OR, U.S.A.).

**Cell Viability Measurement** The viability of cultured cells was evaluated using MTT assay, and lactate dehydrogenase (LDH) release assay.

(a) MTT Assay: The cells were grown on 96-well culture plates with 200 μl of medium. After treatment with tested samples for a period of time, 20 μl of MTT solution (5 mg/ml) was added and incubated for 4 h. The reaction was stopped by the aspiration of the MTT-containing medium. The formed formazan crystals were dissolved in 100 μl of isopropanol containing 40 mM HCl and then diluted with equal volume of water. Absorbance was measured in a microplate reader (Bio-Rad M-450) at 570 nm with a reference filter at 405 nm.

(b) Measurement of Lactate Dehydrogenase: Lactate dehydrogenase (LDH) released to cell culture media by dead cells was measured using the CytoTox-ONE® TM Homogeneous Membrane Integrity Assay Kit (Promega) according to the manufacturer’s instructions. The results are expressed as percent of LDH released in samples relative to samples in which cells were lysed with 1% Triton X-100.

**Apoptosis Assays** MCE301 cells were cultured in Collagen Type I-Coated Chamber slide (IWAKI, Tokyo, Japan) and stimulated with butyrate (8 mM). Apoptosis was monitored using the annexin V and TUNEL assay. In parallel, morphological characteristics of apoptosis were monitored in butyrate treated MCE301 cells by immunofluorescence microscopy, after staining with the DNA dye DAPI. Expression of phosphatidyl serine on the outer leaflet of the plasma membrane was monitored by immunofluorescence after binding of FITC labeled annexin V to PS. These studies were performed using an immunofluorescence microscope (type BX41, Olympus, Tokyo, Japan). To analyze the role of specific caspases in butyrate induced cell death, cells were treated with the following caspase inhibitors: the broad range caspase inhibitor zVAD-FMK, the caspase-9 specific inhibitor zLEHD-FMK, the caspase-8 specific inhibitor zIETD-FMK, the caspase-3 specific inhibitor zDEVD-FMK, as well as the caspase-1 specific inhibitor zYVAD-FMK.

**Cytchrome c Release Assay** To monitor the translocation of cytochrome c from the mitochondria to the cytosol, the compartments were separated prior to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. In brief, cells were harvested and washed once with ice-cold PBS and then the mitochondria and cytosol fractions were prepared using Mitochondria/Cytosol Fractionation Kit (BioVision, Mountain View, CA, U.S.A.) according to the manufacturer’s instructions. Each fraction was subjected to SDS-PAGE, transferred onto nitrocellulose membrane, and analyzed by Western blotting with anti-cytochrome c Ab (clone 7H8.2C12, LAB VISION, Fremont, CA, U.S.A.).

**Measurement of Mitochondrial Membrane** Potential Mitochondrial membrane potential (ΔΨm) was measured by the use of the green fluorescent probe 5,5',6,6'-tetrachloro-1',3',3'-tetraethylbenzimidazolylcarbocyanine iodide, JC-115) (B-Bridge International, San Jose, CA, U.S.A.) according to the manufacturer’s instructions, and green and red fluorescence were observed by fluorescence microscopy.

**Transmission Electron Microscopy** Ruthenium red en bloc staining was performed by a prescribed method. In brief, the cultured cells were treated by immersion in a fixative composed of 4% formaldehyde and 1% glutaraldehyde in 60 mM phosphate buffer (pH 7.4) for 12 h. After exchanging the fixative for 0.1 M cacodylate buffer (pH 7.2), the sample was treated with 2% osmium tetroxide solution containing ruthenium red (TAAB Laboratories Equipment, Aldermaston, England) in the dark for 3 h. The samples were dehydrated in ethanol solutions, then embedded in Epon mixture. Semi- and ultra-thin sections were made using an LKB ultramicrotome. The specimens were stained with lead citrate and uranyl acetate and examined under a JEOL 1200EXII electron microscope at an accelerating voltage of 80 kV.

**Statistical Analysis** Data were expressed as mean±S.D., and differences between groups were analyzed by ANOVA followed by post hock analysis using Scheffe’s test using a personal computer with the StatView-J program for Macintosh (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

When the effects of several organic acids on cells viability were examined using MCE301 cells at a concentration 8 mM, only butyrate induced the reduction of cell viability in a time-dependent manner, as determined by the MTT assay (Fig. 1). Incubation of MCE301 cells with butyrate induced a high rate of cell death in a concentration-dependent manner (Fig. 2). This butyrate induced cell death was observed by not only MTT assay but also LDH release assay (Fig. 2). However, butyrate did not induce cell death in human colorectal carcinoma Caco-2 cells under the same concentration of butyrate (8 mM) as MCE301 cells. The cell death in Caco-2 cells by butyrate was observed at concentrations above...
20 mM (data not shown). The basis of this resistance to butyrate of Caco-2 cells is unclear. In this study, four days post-confluence Caco-2 cells were used. It has been reported that according to the spontaneous differentiation culture of Caco-2 cells, the ability of butyrate to induce apoptosis becomes markedly attenuated.17) The differentiation status of Caco-2 cells may involve in this resistance to butyrate.

The butyrate-induced reduction of cell viability of MCE301 cells was assumed to be apoptosis, because the induction of apoptosis in colon carcinoma cells by butyrate has been well known.18—21) The pro-apoptotic effects of butyrate are mediated via up-regulation of pro-apoptotic proteins, such as Bax and Bak, down-regulation of Bcl-Xl and the activation of caspases such as caspase-3.22) One of the major apoptosis pathways is triggered by the release of mitochondrial apoptogenic protein, cytochrome c. Cytosolic cytochrome c binds to the CED-4 homolog Apaf-1 and induces caspase-9-dependent activation of caspase-3.23) To elucidate whether cytochrome c release pathway contributes to butyrate-induced cell death, butyrate-treated MCE301 cells were lyzed, and then cytosolic and mitochondrial fractions were subjected to immunoblotting with anti-cytochrome c antibody. Although the cells were stimulated with butyrate, the cytochrome c was detected in only mitochondrial fraction and not detected in cytosolic fraction (Fig. 3A). Because the translocation of cytochrome c from the mitochondria to the cytosol was not observed by stimulation of butyrate, it was suggested that the cytochrome c release pathway is not necessary for butyrate-induced cell death. In order to investigate whether the butyrate-induced cell death occurs via the caspase pathway, the effect of zV AD-FMK, a potent cell-permeable pan-caspase inhibitor was examined. The caspase inhibitor zV AD-FMK did not block the cell death induced by butyrate (Fig. 4). In addition to zV AD-FMK, the caspase-9 specific inhibitor zLEHD-FMK, the caspase-8 specific inhibitor zIETD-FMK, the caspase-6 specific inhibitor zVEID-FMK, and the caspase-3 specific inhibitor zDEVD-FMK, and the caspase-1 specific inhibitor zYVAD-FMK also did not block the cell death (Fig. 4). Thus, caspase activation may not be required in the cell death induced by butyrate. To confirm if butyrate induces the apoptotic cell death against MCE301 cells, we analyzed several apoptotic markers after treatment with butyrate.

Since nuclear disintegration and degradation of chromosomal DNA are cardinal features of apoptosis, the DNA fragmentation and the nuclear morphology of butyrate-treated cells were analyzed. Whether butyrate induced nuclear condensation, the butyrate-treated cells were examined by fluorescent microscopy after DAPI staining, and an increase in the number of cells with nuclear condensation was not observed (Fig. 3B). To explore whether butyrate treatment resulted in DNA fragmentation, the TUNEL staining was performed as a hallmark of apoptosis, and an increase in the

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**Fig. 2. Effect of Butyrate on Survival of MCE301 Cells**

MCE301 cells were cultured with various concentrations of butyrate at 39 °C for 72 h. Cell survival was measured by MTT assay and LDH assay. Results are expressed as the mean±S.D. of triplicate determinations. *p<0.05, **p<0.01, ***p<0.001.

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**Fig. 3. Effect of Butyrate on Apoptotic Makers**

(A) Analysis of cytochrome c release. MCE301 cells were cultured in the presence of 8 mM butyrate for 24 h, and then mitochondrial fraction and cytosolic fraction were prepared. The presence of cytochrome c in each fraction was analyzed by Western blotting with anti-cytochrome c Ab. (B) Analysis of chromatin condensation. MCE301 cells were cultured in the presence of 8 mM butyrate for 24 and 48 h, and then the cells were stained with DAPI (blue) and Alexa Fluor594 phalloidin (red). Apoptotic cells exhibiting morphological features of chromatin condensation were not observed. (C) DNA fragmentation was evaluated by TUNEL assay. MCE301 cells were cultured in the presence of 8 mM butyrate for 24 and 48 h, and then the cells were stained with TUNEL (green) and PI (red). DNA fragmentation was negligible.

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20 mM (data not shown). The basis of this resistance to butyrate of Caco-2 cells is unclear. In this study, four days post-confluence Caco-2 cells were used. It has been reported that according to the spontaneous differentiation culture of Caco-2 cells, the ability of butyrate to induce apoptosis becomes markedly attenuated.17) The differentiation status of Caco-2 cells may involve in this resistance to butyrate.

The butyrate-induced reduction of cell viability of MCE301 cells was assumed to be apoptosis, because the induction of apoptosis in colon carcinoma cells by butyrate has
TUNEL positive cells was also not observed (Fig. 3C). The expression of phosphatidyl serine at the outer leaflet of the membrane is an early cellular event of apoptotic cell death. The expression of phosphatidyl serine could not be detected by using annexin V staining (data not shown). Taken together, the data demonstrated that butyrate induced cell death may not be apoptosis, because several apoptotic markers were negative after butyrate exposure, and chemical inhibitors of caspase failed to protect against butyrate-induced cell death.

One of the most convincing and standard methods to detect non-apoptotic cell death is to examine the ultrastructure of cells by transmission electron microscopy. Therefore, the morphology of butyrate treated-cells was examined. Both the nuclei and organelles (e.g. mitochondria) of untreated control cells appeared normal (Fig. 5). MCE301 cells treated with 8 mM butyrate for 24 h showed the phenotypic changes associated with necrosis. Thus, some swollen mitochondria were frequently recognized at this duration (Fig. 5). At 48 h after exposure to butyrate, marked vacuolization within the cytoplasm was also frequently observed (Fig. 5). However, any remarkable morphological alteration due to apoptosis such as chromatin condensation and nuclear membrane rupture of nucleus was not observed by treatment with 8 mM butyrate for 24 and 48 h incubation (Fig. 5). Altogether, these data indicated that butyrate-induced cell death is a necrosis. To our knowledge, this is the first observation that butyrate induces necrotic cell death in colonic epithelial cells.

The mechanism of butyrate-induced necrotic cell death is unknown now. However, the butyrate-induced necrotic cell death in MCE301 cells is not a non-specific accidental disruption of cellular function, because present study was examined by using the physiological concentration of butyrate. In this study, we examined the level of mitochondrial membrane potential ($\Delta \Psi_m$) as a mitochondrial function in MCE301 cells using a membrane potential-sensitive dye JC-1. The green fluorescent probe, JC-1 exists as a monomer at low membrane potential and at higher potentials, JC-1 forms red fluorescent aggregates. In MCE301 cells, butyrate markedly lowered mitochondrial $\Delta \Psi_m$ (Fig. 6). Because it has been reported that a catastrophic decrease in bioenergy production due to mitochondrial dysfunction induced necrosis,

Predonisolone and 5-aminosalicylic acid (5-ASA) have widely been used for the treatment of UC patients. It is interesting to determine whether butyrate-induced cell death could be prevented by treatment of therapeutic drug, therefore the effect of predonisolone and 5-ASA on butyrate-induced necrotic cell death in MCE301 cells was examined. In the presence of prednisolone or 5-ASA, butyrate induced cell death was reduced in a dose dependent manner (Fig. 7). The data presented here may have important clinical relevance. However, much remains to be determined about the mechanisms of action of predonisolone and 5-ASA on butyrate-induced cell death.

Recently, it has been reported that the enema containing butyrate caused UC-like lesions in mice. This study demonstrated that butyrate caused potent cytotoxicity in the murine normal colonic epithelial cells MCE301 cells at physiolog-
cal concentrations. Butyrate-induced cell death in MCE301 cells was partially but significantly reduced by clinically used therapeutic drug, such as prednisolone or 5-ASA. These findings and the results support that the necrotic cell death in MCE301 cells induced by butyrate may be useful as a novel in vitro model of UC to screen useful drugs for the treatment of the disease. In order to develop a new murine model that mimics clinical features of UC induced by a butyrate, experiments are currently underway, and it must await further study.

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