Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway

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**Background:** During the process of tumorigenesis most colon cancer cells acquire resistance to apoptosis. The short chain fatty acid butyrate is well established as an antitumour agent which selectively induces apoptosis in colon cancer cells but not in normal intestinal epithelial cells.

**Aims:** To analyse the signalling pathway of butyrate induced apoptosis.

**Methods:** Using Caco-2 cells we focused on the bcl family of proteins, mitochondrial pathway, and caspase signalling cascade involved in butyrate induced apoptosis. Techniques employed included western blots, immunofluorescence, as well as experiments with peptide inhibitors of specific caspases.

**Results:** Butyrate induced a clear shift of the mitochondrial bcl rheostat towards a proapoptotic constellation, as demonstrated by upregulation of proapoptotic bax accompanied by reduced antiapoptotic bcl-x, levels. This was associated with translocation of cytochrome-c from the mitochondria to the cytosol, resulting in activation of the caspase cascade via caspase-9. Key executioner enzymes were caspases-3 and -9. No effect of butyrate on regulatory proteins of the inhibitor of apoptosis family was observed.

**Conclusions:** Butyrate induced Caco-2 cell apoptosis via the mitochondrial pathway. Upregulation of bax and translocation of cytochrome-c were upstream of the caspase cascade. Subsequently, this cascade was activated via the formation of an apoptosome.

**MATERIAL AND METHODS**

**Cell culture and reagents**

Caco-2 cells (passages 16–26, ATCC) were cultured at 37°C in a humidified atmosphere of 5% CO2 in minimal Eagle medium supplemented with 5% heat inactivated fetal calf serum (Gibco, Grand Island, New York, USA) and 1% penicillin/streptomycin, as previously described.

**Abbreviations:** SCFA, short chain fatty acid; AIF, apoptosis inducing factor; PS, phosphatidyl serine; ECL, enhanced chemiluminescence; VDAC, voltage dependent anion channel; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; IAP, inhibitor of apoptosis; XIAP, X-linked inhibitor of apoptosis.
**Apoptosis assays**

Caco-2 cells were cultured in 24 multiwell plates to subconfluent and stimulated with butyrate (0.01–100 mM) for up to 48 hours. After stimulation with butyrate with or without caspase inhibitors for periods of up to 48 hours, apoptosis was monitored using the annexinV/propidium iodide and TUNEL assay, as previously described. Butyrate induced apoptosis was quantified by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany). In parallel, morphological characteristics of apoptosis were monitored in butyrate treated Caco-2 cells by confocal and immunofluorescence microscopy, after staining with the DNA dye Hoechst 33342. Expression of phosphatidyl serine (PS) on the outer leaflet of the plasma membrane was monitored by immunofluorescence after binding of FITC labelled annexin-V to PS in the presence of calcium. These studies were performed using a Leica immunofluorescence microscope (Leica, Bernstein, Germany) equipped with a video documentation system. To analyse the role of specific caspases in butyrate induced apoptosis, cells were treated with the following caspase inhibitors: the broad range inhibitor zVAD-fmk (200 µM), the caspase-9 specific inhibitor LEHD-FMK (1–300 µM), the caspase-8 specific inhibitor IETD-FMK (1–300 µM), as well as the caspase-3 specific inhibitor DEVD-FMK (1–200 µM).

**Western blots**

The effect of butyrate on expression of bcl-2, bcl-xl, bid, bak, and bax, the inhibitors of apoptosis (IAP), cIAP1 and cIAP2, and X-linked inhibitor of apoptosis (XIAP), and activation of different caspases were determined by western blots. Butyrate stimulated and unstimulated (control) cell lysates were prepared using an ice cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors containing antipain, bestatin, chymostatin, leupeptin, pepstatin, phosphoramidon, pepstatin, and aprotinin (Boehringer, Mannheim, Germany). Equivalent protein samples were resolved on 8–14% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Germany). All antibodies were diluted in Tris buffered saline/Tween 20-1% milk powder. This step was followed by incubation with the corresponding horseradish peroxidase conjugated antibody (antimouse-IgG 1:5000, anti-rabbit-IgG 1:6000; Biosource, Germany). Bands were read by enhanced chemiluminescence (ECL-kit, Amersham, Germany). In addition, mitochondrial expression of bak was analysed after separation of this compartment, as described below.

**Cytochrome-c translocation**

To monitor the shift in cytochrome-c from the mitochondria to the cytosol, the compartments were separated by ultracentrifugation prior to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blots. Cell pellets were lysed by three cycles of freeze-thawing in a buffer containing 20 mM HEPES/KOH (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 1 mM NaEDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSE, and 250 mM sucrose, as recently described by Hiura and colleagues. Thereafter, the cytosolic fraction (S-100 fraction) was separated from the mitochondria by ultracentrifugation at 100 000 g (45 000 rpm) at 4°C for 30 minutes. After SDS-PAGE (15% gel) and transfer onto nitrocellulose mem-

**Immunofluorescence studies**

Translocation of cytochrome-c was visualised and monitored by immunofluorescence. Caco-2 cells were grown on glass slides, fixed in 4% ice cold paraformaldehyde after stimulation with butyrate (as above). After intensive rinsing, cells were stained with anticytochrome-c antibody (1:1000; BD-Pharmingen) for two hours at 21°C, followed by a secondary FITC labelled antimouse antibody (1:1000) for 45 minutes at
21°C and photodocumented. Control samples included staining with non-specific isotype mouse IgG, followed by the same secondary antibody and procedure as above. No mitochondrial or cytosol staining was observed in these isotype controls, confirming the high specificity of the primary antibody.

Statistical analysis
Results are reported as mean (SEM) of triplicate samples. Significance was established at 95%, and determined by the Student’s t test for non-paired values and the Mann-Whitney U test for non-parametric values.

RESULTS
Kinetics of butyrate induced Caco-2 cell apoptosis
Incubation of near confluent immature Caco-2 cells with butyrate induced a high rate of apoptosis in a dose and time dependent manner (fig 1A). The proapoptotic effect only occurred above a threshold concentration of 5 mM butyrate. Treatment with butyrate concentrations between 0.1 and 2 mM did not induce Caco-2 cell apoptosis, even after prolonged incubation periods of 48 hours or longer (results not shown). In contrast, butyrate concentrations above 5 mM induced almost complete apoptosis (87 (9)% at this time interval. Morphological analysis (fig 1B,C) confirmed the typical features of apoptotic cell death in butyrate stimulated Caco-2 cells. Onset of butyrate induced apoptosis was about 16 hours (fig 1A) with—as an early sign—loss of membrane asymmetry, as seen by expression of PS at the outer leaflet of the membrane (fig 1C). Confocal microscopy revealed membrane blebbing in these PS positive cells. The characteristic nuclear changes of apoptotic cell death became more apparent after 20–24 hours of butyrate treatment. Butyrate stimulated Caco-2 cells displayed clear nuclear chromatin condensation and fragmentation resulting in the formation of apoptotic bodies.

Signalling cascade in butyrate induced Caco-2 cell apoptosis
Inhibition of the intracellular apoptosis caspase cascade with the broad range caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) completely suppressed the proapoptotic effect of butyrate (fig 2). In order to delineate the molecular events involved, we first examined the “classical caspase cascade” which is activated via a complex of a death receptor, adapter molecules, and caspase-8 (“DISC”). In response to butyrate, no activation of caspase-8 occurred under all experimental conditions tested, even after prolonged
periods of incubation of up to 72 hours (fig 2B). Consistent with this finding was the observation that specific inhibition of caspase-8 with the peptide inhibitor IETD-fmk was also incapable of altering the apoptotic effect of butyrate (48 (9)% versus 46 (11)%). In contrast, we detected clear activation of downstream caspases-3 and -1 in response to butyrate, as demonstrated by western blot (fig 2B). Inhibition of caspase-3 blocked butyrate induced apoptosis (48 (9)% v 12 (6)%; \( p<0.05 \)), indicating that this enzymes was critical to butyrate induced apoptosis. In contrast, we did not observe any effect of butyrate on expression of any caspases, in contrast with a recent report in a rat colon cancer model.

Several inhibitory proteins have recently been described which block the caspase cascade at specific levels. The molecules XIAP as well as cIAP1 and 2 selectively block caspases-3 and -9. Therefore, we examined expression of XIAP and cIAP1/2 in Caco-2 cells in order to test the hypothesis that butyrate resulted in their downregulation. As shown in fig 3, Caco-2 cells expressed all three IAP proteins. However, even high concentrations of butyrate failed to alter levels of these regulatory proteins.

**Butyrate induced the translocation of mitochondrial cytochrome-c to the cytosol**

An important mechanism to activate and stimulate the caspase cascade is via the mitochondrial pathway. A crucial step in this signalling cascade is the translocation of mitochondrial cytochrome-c into the cytosol. This step allows the formation of a so-called apoptosome, a complex of cytochrome-c, APAF-1, in association with the zymogen caspase-9. This complex then results in the activation of the caspase cascade via cleavage of caspase-9. We observed that butyrate induced a clear shift of cytochrome-c from the mitochondria to the cytosol, as shown by western blot (fig 4A). This efflux was detectable after incubation with butyrate at concentrations of 10–100 mM in a dose dependent manner. Immunofluorescence techniques confirmed the translocation of cytochrome-c into the cytosol in response to butyrate (fig 4B).

To test the hypothesis that translocation of cytochrome-c was an initial event and upstream of the caspase cascade, we blocked this signalling cascade using the broad range inhibitor zVAD-fmk (200 \( \mu \)M). At this zVAD-fmk concentration, butyrate induced Caco-2 cell apoptosis was totally blocked (fig 2B). Despite complete inhibition of the caspase signalling cascade, translocation of cytochrome-c occurred in an unaltered way (fig 4C). However, no induction of downstream caspase-3 and Caco-2 cell apoptosis was observed, confirming the necessity of specific caspases to propagate the apoptotic signal.

AIF is another intramitochondrial molecule that can induce the apoptotic signalling machinery by translocation to the cytosol.
cytochrome-c. In contrast with cytochrome-c, we observed no shift in AIF from the mitochondrial fraction to the cytosol in response to butyrate (fig 4B). All AIF remained in the mitochondrial compartment.

We then set out to confirm that translocation of cytochrome-c is involved in activation of the caspase cascade via caspase-9. Specific inhibition of caspase-9 with the peptide inhibitor LEHD-fmk resulted in a dose dependent block of induction of apoptosis, as shown in fig 2A. Western blot analysis confirmed that caspase-9 was activated after stimulation with butyrate (fig 2B).

Effect of butyrate on the bcl-2 gene family

There is increasing evidence that the bcl gene family plays a key role in regulating the release of mitochondrial cytochrome-c. Various antiapoptotic members, such as bcl-2 or bcl-xL, are thought to protect a cell against the translocation of cytochrome-c whereas proapoptotic molecules, such as bax, bak, and bid, promote the efflux of cytochrome-c. In the present study, we therefore examined whether butyrate altered the balance of these pro- and antiapoptotic molecules in Caco-2 cells. Western blot analysis showed marked downregulation of bcl-xL, even at butyrate concentrations as low as 1 mM, whereas no effect on bcl-2 protein occurred even despite maximal concentrations (fig 5). No effect on proapoptotic bax or bid molecules was observed in response to butyrate. On the other hand, bak was strongly induced in a dose dependent manner above a threshold concentration of 5–10 mM butyrate. After separation of the cytosolic from the mitochondrial compartment, clear upregulation of bak in the mitochondrial could be observed in response to butyrate stimulation (fig 5). Further fractional analyses revealed however that there was no shift of bax or bid from the cytosolic to the mitochondrial compartment (data not shown).

DISCUSSION

It is well established that butyrate is a potent inducer of apoptosis in colon carcinoma cells. In the present study, we analysed the signalling sequence in Caco-2 cells undergoing apoptosis after stimulation with butyrate. We observed activation of the caspase cascade after an interval of 12–16 hours, in concert with previous reports. The signalling cascade emerged from the mitochondria resulting in initial activation of caspase-9 and subsequently stimulation of downstream caspases-3 and -1. In contrast, no formation of a DISC occurred in response to butyrate, and caspase-8 was not activated. Specific inhibition of this particular caspase with peptide inhibitors did not alter the apoptotic response of Caco-2 cells to butyrate whereas inhibition of caspases-9 or -3 significantly suppressed Caco-2 cell apoptosis.

During the process of tumorigenesis, most cancer cells acquire resistance to apoptosis. One mechanism is by overexpressing antiapoptotic proteins, inhibiting the signalling cascade at various levels. Since caspases-3 and -9 are key enzymes in butyrate induced apoptosis, we looked for molecules which selectively block these enzymes. XIAP in particular, as well as cIAP1 and cIAP2, are known to potently block both caspases, thereby inhibiting apoptosis. However, we observed no change in the expression of these molecules after butyrate treatment and no inhibitory effect on butyrate induced apoptosis. These observations indicate that these molecules are not involved in the regulation of Caco-2 cell apoptosis induced by butyrate. Another important mechanism which may confer resistance to apoptosis are changes in the bcl-2 gene rheostat. Krajewska and colleagues clearly demonstrated in colon cancers that there is significant upregulation of antiapoptotic bcl-xL, concomitant with downregulation of proapoptotic bak resulting in a shift towards an antiapoptotic bcl constellation. Similar to that observation, we found in unstimulated Caco-2 cells antiapoptotic bcl-xL expressed at a high level which was in clear contrast with the non-transformed human intestinal epithelial cells HIEC (our unpublished observations). bcl-2 expression was low in Caco-2 cells as was described in normal intestinal epithelial cells. Butyrate treatment decreased bcl-xL expression in a potent and dose dependent manner. This downregulation of bcl-xL occurred at concentrations as low as 1 mM butyrate. However, no induction of Caco-2 cell apoptosis was detected at these doses. These data indicate that although downregulation of antiapoptotic bcl-xL was insufficient to induce Caco-2 cell apoptosis, it might decrease the threshold to undergo apoptosis. Nita and colleagues reported that resistance to chemotherapeutic agents could be overcome after antisense treatment that suppresses expression of bcl-xL in DLD1 cancer cells. Similarly, Hirose and colleagues found in azoxymethane induced rat colon adenocarcinomas significant upregulation of bcl-xL but moderate downregulation of bcl-2 in tumour cells. These observations confirm that bcl-xL may play a more important antiapoptotic role than bcl-2 in some colon tumour cells as well as in Caco-2 cells.

In contrast, upregulation of proapoptotic bak was a key step in butyrate induced Caco-2 cell apoptosis. We recently showed that suppression of butyrate induced neosynthesis of bak by the protein synthesis inhibitor cycloheximide completely abolished induction of apoptosis in Caco-2 cells. Once bak was upregulated, which takes 12–16 hours, cycloheximide failed to block butyrate induced apoptosis, supporting this observation. In the present study, we were able to add
additional evidence affirming the crucial role of bak and the mitochondrial pathway in butyrate induced Caco-2 cell apoptosis. After butyrate stimulation, clear upregulation of bak protein was observed in the mitochondria. In contrast, no effect of butyrate on two other proapoptotic molecules, bax and bid, was detectable nor did we observe translocation of either molecule from the cytosol to the mitochondria. Increased levels of bak are known to allow the exit of cytochrome-c from the mitochondrial to the cytoplasmic compartment. These observations regulated by the competitive binding of either molecule to bak. Enhanced mitochondrial bak expression allows the efflux of the bcl rheostat, with neosynthesis of mitochondrial following pathway for the induction of apoptosis by butyrate allowing the subsequent activation of the caspase cascade.

Figure 6 Proposed signalling pathway for butyrate induced Caco-2 cell apoptosis. After stimulation with butyrate the mitochondrial bcl rheostat is shifted towards a proapoptotic constellation, with markedly increased mitochondrial bak levels and decreased bcl-xL levels. This shift allows the translocation of cytochrome-c from the mitochondria to the cytosol, allowing the formation of an apoptosome. Thereafter, activation of the caspase cascade via caspase-9 ensues, leading to apoptotic cell death.

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