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Inhibition of histone deacetylase activity enhances Fas receptor-mediated apoptosis in leukemic lymphoblasts

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Abstract

We recently reported that butyrate, an inhibitor of histone deacetylases, is capable of inducing Fas-independent apoptosis in the acute lymphoblastic leukemia cell line CCRF–CEM. Here we demonstrate that butyrate enhances Fas-induced apoptosis in this cell line. The application of different histone deacetylase inhibitors revealed that tetra-acetylated histone H4 is associated with the amplifying effect of butyrate on Fas-induced cell death. FasL, Fas, FADD, RIP, caspase-8, caspase-3, Bid, $FLIP_{S+L}$, FLASH and FAP-1, proteins known to act within the Fas-apoptosis cascade, showed no changes in their expression levels in cells treated with butyrate compared with untreated cells. Analyses of Fas-oligomerization and Western blotting as well as enzyme activity assays of caspase-2, caspase-3 and caspase-8 suggest that butyrate enhances Fas-induced apoptosis downstream of Fas but upstream of caspase-8 activation. In immunoprecipitation experiments a 37 kD butyrate-regulated protein was detected which specifically interacts with caspase-8. *Cell Death and Differentiation* (2001) **8**, 1014–1021.

Keywords: Fas (CD95/APO-1); death-inducing signaling-complex (DISC); butyrate; histone deacetylase; apoptosis

Abbreviations: Fas, Fas receptor; FasL, Fas ligand; TNF, tumor necrosis factor α; SCFA, short-chain fatty acid; ROS, reactive oxygen species; PBS, phosphate buffered saline; TBS, TRIS buffered saline; SDS, sodium dodecyl sulfate; DISC, death-inducing signaling-complex; FACS, fluorescence-activated cell sorter

Introduction

In the past few years histone acetylation has been increasingly recognized as being involved in the regulation of gene transcription (reviewed in[1,2]). Furthermore, numerous studies have reported that inhibitors of histone deacetylases, such as the short-chain fatty acid (SCFA) butyrate, cause arrest of cell division and induction of differentiation markers in animal cells[3–5] but induce programmed cell death in a cell-type specific manner, and even in the butyrate-sensitive cell types follow different mechanisms of apoptosis. Butyrate-induced apoptosis in certain colonocytes occurs via a Fas-dependent pathway[6] whereas butyrate-induced apoptosis of leukemic lymphoblasts does not.[7] Moreover, butyrate resensitizes Fas-resistant colonocytes to this form of cell death.[8,9]

Although the underlying mechanisms have not yet been elucidated, these findings suggest that histone acetylation has an impact on sensitivity to Fas-induced apoptosis. Fas-induced apoptosis is probably the best understood form of a cellular apoptosis program. FasL-induced oligomerization of Fas enables the homotypical binding of adaptor proteins like FADD (Mort1) to the receptors death domain and the recruitment of caspase-8 (FLICE), via homotypical interaction of death effector domains.[10,11] The protein complex thus formed at the cytosolic side of the plasma membrane consisting of Fas, FADD and caspase-8 is called the death-inducing signaling-complex (DISC). Most likely by bringing several procaspase-8 molecules into physical proximity, a proteolytic activation cascade is initiated.[12,13] Autocatalytically activated, caspase-8 cleaves and activates effector caspases such as caspase-3 (YAMA, CPP32),[14] which execute the proteolytic cleaving of cellular substrates. Another target protein of caspase-8 is Bid, that, when cleaved, translocates from the cytosol to the mitochondria, and initiates mitochondrial changes characteristic for apoptosis, such as permeability transition and leakage of cytochrome *c*[15] (reviewed in[10]).

Additionally, an increasing number of regulatory proteins have been identified that either enhance or inhibit Fas-induced apoptosis (reviewed in[16]), such as FLIP (CASPER, I-FLICE, Flame, CASH, CLARP, MRIT, Usurpin), RIP, Sentrin, FAP-1 and FLASH. In human cells, two forms of FLIP, $FLIP_S$ and $FLIP_L$ have been identified. Both $FLIP_L$ (containing two death effector domains and a caspase-like domain in which the active center cystein residue is substituted by tyrosin) and $FLIP_S$ (containing only two death effector domains) interact with the adaptor protein FADD and caspase-8, thereby blocking downstream signaling.[17,18] RIP, Sentrin, Fas-associated phosphatase-1 (FAP-1) and FLASH are Fas-binding proteins. Sentrin and FAP-1 inhibit apoptosis[19–21] while RIP and FLASH have been reported to promote apoptosis.[22,23] The expression of the genes encoding these proteins might be modulated by histone acetylation and have therefore to be taken into

consideration when the actions of histone deacetylase inhibitors on Fas-induced cell death are studied.

In this study we demonstrate that inhibitors of core histone deacetylases enhance Fas- as well as TNF-induced apoptosis in an acute lymphoblastic leukemia cell line. In order to elucidate the mechanism of action we analyzed their influence on the Fas signal pathway at different stages and show that butyrate-induced stimulation of Fas-apoptosis is associated with a threshold degree of histone hyperacetylation. We provide evidence that Fas-induced apoptosis is enhanced downstream of Fas but upstream of caspase-8 activation.

Results

Butyrate enhances Fas- and TNF-induced apoptosis

To determine whether butyrate interacts with the cell death pathway triggered by the cell-death receptors Fas and TNF, we examined the effects of butyrate on Fas- as well as TNF-induced apoptosis in the acute lymphoblastic leukemia cell line CEM–C7H2 within incubation periods at which butyrate itself does not induce apoptosis. Figure 1A shows that incubation with the agonistic anti-Fas antibody CH-11 for 6 h led to apoptosis in 15% of the cells. In the presence of 1 mM butyrate, added 2 h prior to CH-11, the number of apoptotic cells increased to 30% and with 10 mM to 45%, whereas butyrate by itself had no apoptosis-inducing effect. Analysis of time-dependence (Figure 1B) shows that the amplifying effect of butyrate was already weakly present 4 h after butyrate addition to CH-11-treated cells and increased within the following 5 h. Figure 1C summarizes the data from an experiment where the effects of butyrate on TNFα-induced cell death were investigated. Similar to butyrate-mediated enhancement of Fas-induced apoptosis, 15% of the cells treated with TNFα for 6 h showed an apoptotic phenotype and addition of butyrate 2 h prior to TNFα resulted in an increase in apoptosis, e.g. in the presence of 10 mM butyrate the percentage of apoptotic cells increased to 35%. The percentages of apoptosis, as shown in the figures are based on measurements of propidium iodide fluorescence as described in Material and Methods. Similar results were obtained when AnnexinV-binding to cells or forward/sideward light scattering of nuclei was measured for quantification of apoptosis (data not shown).

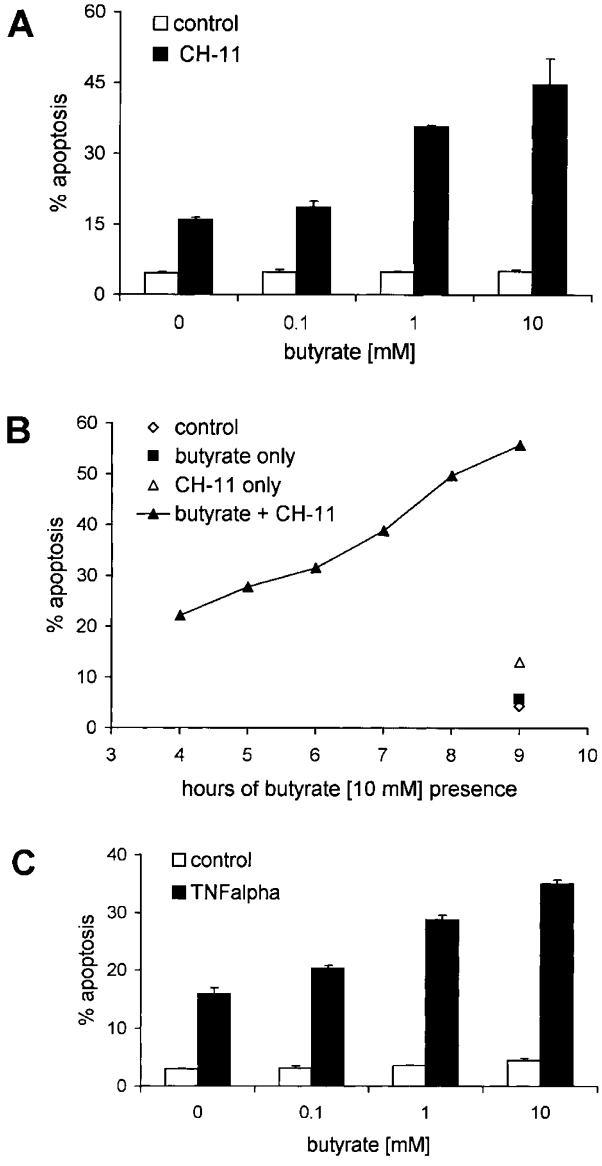

Figure 1 Butyrate enhances Fas- and TNF-induced apoptosis. (**A, C**) CEM-C7H2 cells were cultured with butyrate at the indicated concentrations for 8 h in the presence or absence of 50 ng/ml CH-11 agonistic anti-Fas antibody (**A**) or 100 ng/ml of recombinant TNFα (**C**) applied 2 h after butyrate addition. The percentage of apoptotic cells was determined by nuclear propidium iodide staining and FACS analysis. Shown are means $\pm$ S.D. of representative experiments. All experiments were carried out at least three times. (**B**) Shows the time dependence of the butyrate effect on Fas-induced apoptosis. In the respective cultures, 50 ng/ml CH-11 antibody was present for 6 h, while butyrate was present for the times indicated; i.e., 4–9 h. Apoptosis was determined by nuclear propidium iodide staining and FACS analyses. A representative experiment is shown

Other short-chain fatty acids and trichostatin A mimick the butyrate effect at concentrations inhibiting histone deacetylases

Since sodium butyrate is believed to mediate its major biologic effects through inhibition of histone deacetylases, we tested the ability of other inhibitors of histone deacetylases (the two other SCFAs acetate and propionate, and the structurally unrelated inhibitor trichostatin A) to mimick the butyrate effect on Fas-induced apoptosis. Figure 2A shows that trichostatin A, like butyrate, enhanced Fas-induced apoptosis. Comparison of the data from Figure 2B, showing the influence of butyrate, propionate and acetate on Fas-induced apoptosis, with Figure 2C, showing the degree of histone hyperacetylation caused by these short-chain fatty acids, demonstrates that the effect on Fas-induced apoptosis is correlated with the degree of histone acetylation. The clear appearance of tetra-acetylated histone H4, observed with 1 mM of butyrate and 10 mM of butyrate and propionate, coincided with enhancement of Fas-induced apoptosis. Taken together, the data suggest that histone hyperacetylation might be related to this phenomenon.

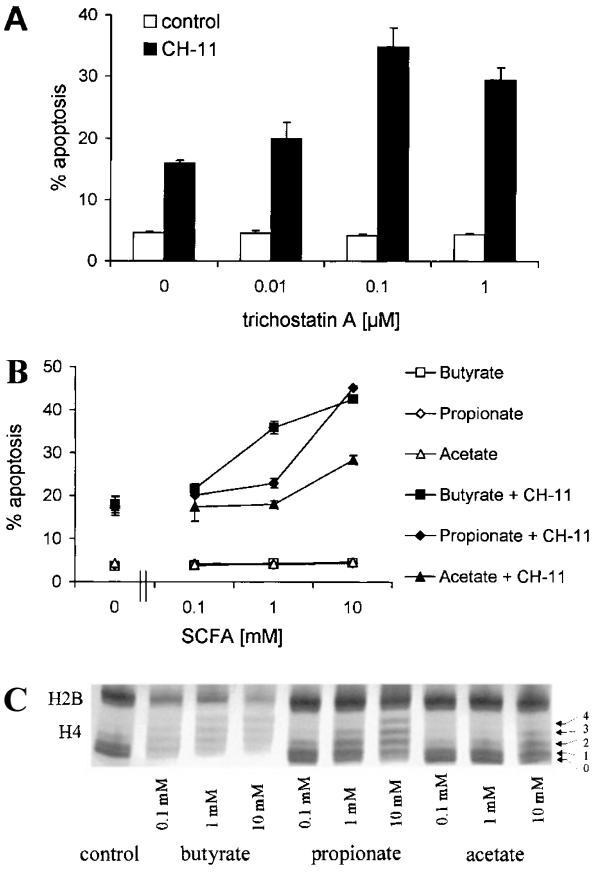

Figure 2 Enhancement of Fas-induced apoptosis can be achieved by different histone deacetylase inhibitors and correlates with the degree of histone hyperacetylation. CEM-C7H2 cells were treated with trichostatin A (**A**); or acetate, propionate and butyrate, respectively, (**B**) at the indicated concentrations for 8 h; 50 ng/ml of CH-11 antibody was added 2 h after application of butyrate. Subsequently, the cells were subjected to apoptosis determination by nuclear propidium iodide staining and FACS analysis. Shown are means ± S.D. of representative experiments performed in triplicates. (**C**) Shows histone extracts prepared from untreated CEM-C7H2 (control) and cells treated for 8 h with 0.1 mM, 1 mM or 10 mM butyrate, propionate or acetate resolved on 12% acidic urea – Triton X-100 polyacrylamide gel and stained with Coomassie Blue R-250. The numbers on the right side of the figure (0, 1, 2, 3 and 4) refer to non-, mono-, di-, tri- and tetra-acetylated histone H4

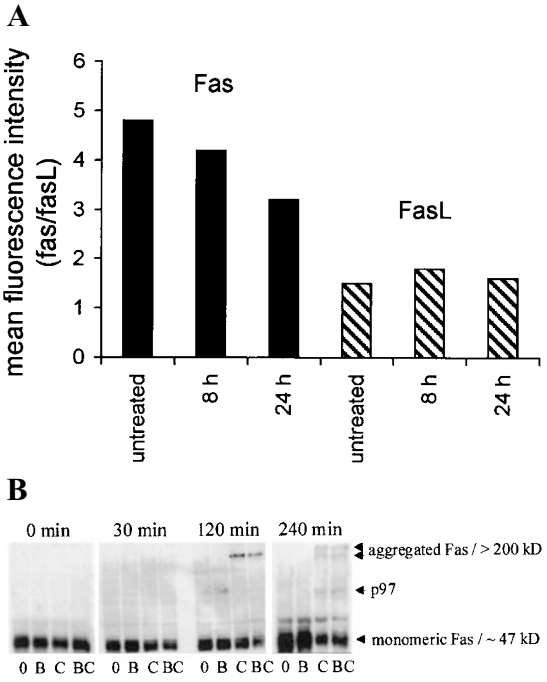

Figure 3 Butyrate downregulates Fas-receptor and does not modulate Fas-oligomerization kinetics. (**A**) Shows FACS analysis of CEM-C7H2 cells incubated with 10 mM butyrate for the indicated times, stained with an FITC-labeled anti-human Fas or anti-human Fas-ligand antibody compared with isotype controls. On the y-axis the ratio specific-antibody/isotype-control is plotted. (**B**) Shows the kinetics of Fas-oligomerization and the appearance of the aggregation-dependent band p97 in the presence or absence of butyrate or Fas-agonistic antibody CH-11 for the indicated timepoints, analyzed by Western blotting as described in Material and Methods. Butyrate (10 mM) was added 2 h prior to the addition of CH-11 (0 min). 0, untreated; B, butyrate only; C, CH-11 only; BC, butyrate and CH-11

Butyrate does not increase Fas protein levels and does not affect Fas-oligomerization during Fas-induced apoptosis

As the acetylation status of nucleosomal histones has been shown to be an important regulator of transcriptional activity, we examined whether essential players of the Fas-apoptosis pathway were regulated by induction of histone hyperacetylation. The first obvious candidate-protein which might be regulated by butyrate and thereby lead to enhanced Fas-apoptosis was Fas itself. However, rather than an upregulation of the Fas-receptor, which one would expect if Fas were the mediator of butyrate-mediated amplification of Fas apoptosis, we observed a slight decrease of the Fas-specific staining intensity from 4.8 to 4.2 within 8 h, and to 3.2 after 24 h of butyrate (10 mM) treatment (Figure 3A). FasL expression was not significantly altered by butyrate (Figure 3A). In order to investigate differences in Fas-receptor oligomerization during Fas-induced apoptosis in the presence or absence of butyrate, we performed Western blot analyses for Fas mono- and oligomers (and p97) as described by Kamitani *et al*.[24] These analyses revealed that induction of Fas-oligomerization induced by CH-11 was not enhanced by butyrate (Figure 3B).

FADD, RIP, Sentrin, FAP-1, FLASH, caspase-8, $FLIP_{S+L}$, caspase-3 and bid protein levels are not affected by butyrate-treatment

Since we did not observe upregulation or enhanced clustering of Fas following addition of butyrate, we investigated the expression levels of proteins known to critically act within this pathway such as the death receptor adapter proteins FADD and RIP, the inducer caspase-8, the effector caspase-3, Bid, the FLICE (caspase-8)-inhibitory proteins $FLIP_{S+L}$, Sentrin, Fas-associated phosphatase-1 (FAP-1) and FLASH. However, as demonstrated by Western blot analysis (Figure 4) and Northern blot analysis for FAP-1 and FLASH (data not shown), the expression levels of FADD, RIP, FAP-1, FLASH, caspase-8, $FLIP_{S+L}$, caspase-3 and Bid were not affected by butyrate treatment. Although Sentrin was slightly downregulated within 2 h of butyrate treatment, downregulation did not correlate with the Fas-apoptosis enhancing effect of butyrate.

Butyrate promotes enhanced caspase-3 and caspase-8 but not caspase-2 activation during Fas-induced apoptosis

Next, we analyzed the activation of caspases-2, -3 and -8 during the initiation phase of Fas-mediated caspase-activation in the presence or absence of butyrate. Figure 5 shows that caspase-3 (Figure 5A) and -8 (Figure 5B) activation mediated by Fas stimulation was enhanced in the additional presence of butyrate. Four hours after CH-11 addition, a clear difference of caspase-3 cleavage products (p20; p17)-intensity and caspase-8 cleavage product (p20)-intensity between butyrate-treated and non-treated could be observed. Corresponding results were observed when caspase-3 and -8-activity was analyzed by *in vitro* enzyme-activity-assays (Figure 6). Direct addition of butyrate to the *in vitro* enzyme-activity assays had no influence on caspase activity (data not shown). Figure 5C shows that a proteolytic fragment of active caspase-2 becomes detectable 2 h after CH-11 addition but neither the timepoint nor the activation-intensity is affected in the additional presence of butyrate.

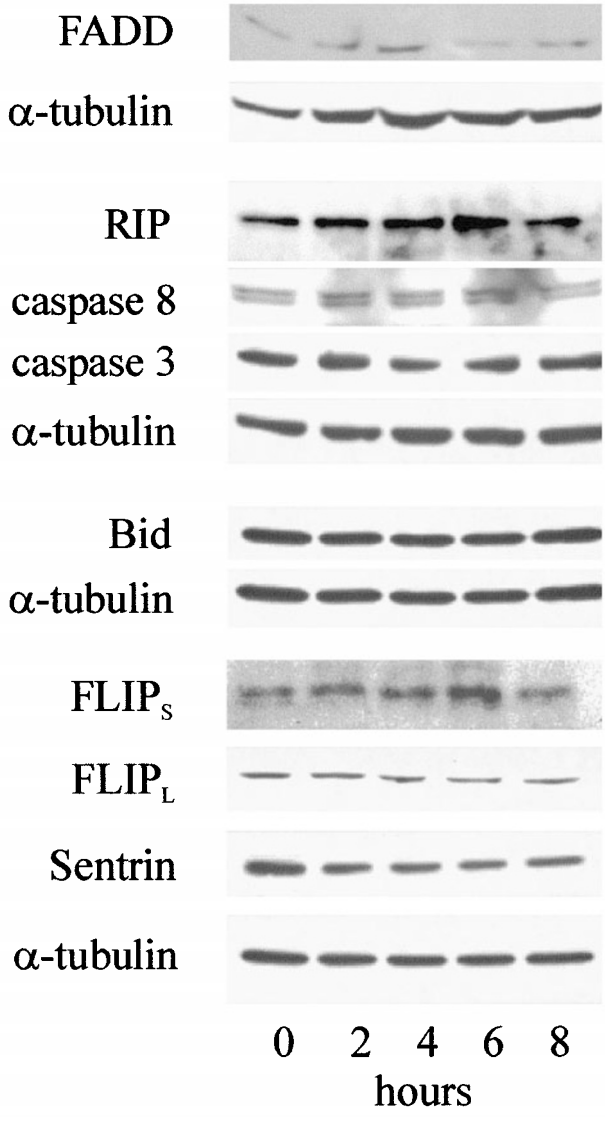

Figure 4 FADD, RIP, FLIP$_{S+L}$, Sentrin, caspase-8, caspase-3 and Bid expression are not affected by butyrate treatment. Western blot analysis of CEM-C7H2 leukemic lymphoblasts treated with 10 mM butyrate for the times indicated. Whole cell extracts were prepared, separated on SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and hybridized with antibodies to human FADD, RIP, caspase-8, caspase-3, Bid, FLIP$_{S+L}$ or Sentrin

A 37 kD protein, upregulated by butyrate specifically interacts with caspase-8

After we were able to narrow down the point of action of the protein regulated by inhibition of core histone deacetylases, we performed immunoprecipitations of FADD and caspase-8 of cells metabolically labeled with ^{35}S-methionine/cysteine. As shown in Figure 7 three protein bands upregulated by butyrate were detected (~20 kD, ~37 kD and ~56 kD), one specifically co-precipitating with caspase-8 (~37 kD).

Discussion

The aim of the present study was to determine the effect of the histone deacetylase inhibitor butyrate on Fas-induced apoptosis in acute lymphoblastic leukemia cells. In previous studies it was reported that butyrate sensitizes colon cancer and renal carcinoma cell lines to Fas-induced apoptosis.[8,9,25] We show here that butyrate enhances Fas-induced cell death also in leukemic lymphoblasts, suggesting that this is not an entirely cell type-specific, but rather a general effect of butyrate. Good correlation of Fas-apoptosis enhancement with the histone deacetylase inhibiting ability of structurally related (acetate and propionate) and structurally unrelated (trichostatin A) drugs suggests that a threshold degree of chromatin hyperacetylation with subsequent modulation of gene transcription may be responsible for this effect. The induction or repression of complex transcription-regulatory cascades appears to be unlikely because amplification of Fas-induced apoptosis was already visible 4 h after butyrate addition to the cells. It appears to be more likely that butyrate-mediated histone hyperacetylation acts directly on transcription of the gene(s) encoding the protein(s) responsible for the observed effect. The question whether butyrate downregulates a Fas-apoptosis inhibitory protein or upregulates a positive regulator of apoptosis could not be answered because both, inhibitors of transcription (actinomycin D) as well as of translation (cycloheximide), enhanced Fas-induced apoptosis in the absence of butyrate (data not shown). Based on these considerations we investigated whether butyrate modulates the expression of proteins known to be important players in Fas-induced cell death. Protein levels of FasL, Fas, FADD, RIP, caspase-8, FLIP$_{S+L}$, Bid, caspase-3 and mRNA levels of FLASH and FAP-1 were not affected, suggesting that these proteins are not directly linked to histone hyperacetylation-mediated amplification of Fas-induced apoptosis. Sentrin seemed to be downregulated 2 h after butyrate-addition to the cells, but poor correlation with the time dependence of fas-apoptosis enhancement by butyrate suggests no important role in this phenomenon.

To functionally define the point of action of the butyrate-regulated protein within this apoptosis pathway, we first

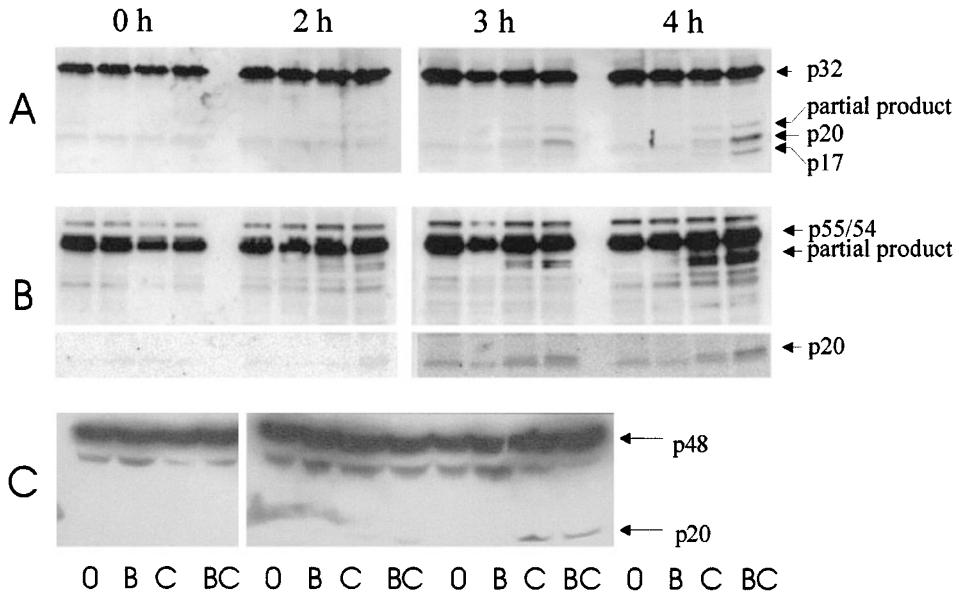

Figure 5 Butyrate increases caspase-3 and caspase-8 but not caspase-2 activation during Fas-induced apoptosis. CEM-C7H2 cells were treated with 10 mM butyrate 2 h prior to addition of 50 ng/ml CH-11 antibody (timepoint 0 h) and compared with untreated, butyrate, or CH-11 only treated cells. Cells were harvested at the indicated timepoints and samples for Western blotting were prepared. Western blots for caspases-3 (**A**), -8 (**B**) and -2 (**C**) were performed as described in Material and Methods. 0, untreated; B, butyrate only; C, CH-11 only, BC, butyrate and CH-11. Representative experiments are shown

analyzed Fas-oligomerization. No differences in the kinetic of Fas-oligomerization in the presence or absence of butyrate during induction of Fas apoptosis was observed, which points to a more downstream site of action. Analyses of caspase-3-activation (Western blotting) and caspase-3-activity (caspase-activity assays) in the presence or absence of butyrate during Fas-induced apoptosis showed that butyrate enhanced both. This, together with the observation that caspase-3-protein levels remained unchanged, pointed to a more upstream site.

Downstream of Fas-oligomerization and upstream of caspase-3-activation mitochondria occupy a central role in this signal transduction pathway. Measurements of mitochondrial trans-membrane potential and inhibition of fas-induced apoptosis by regulatable transgenic expression of Bcl2 clearly demonstrated that it is not an amplifying mechanism at the level of mitochondria that leads to this phenomenon but most likely that the butyrate-regulated protein acts upstream of the branch to the mitochondria, as butyrate enhanced mitochondrial transmembrane potential breakdown during Fas-induced apoptosis as well as the non-bcl2-inhibitable death signal bypassing the mitochondria (data not shown).

Similarly to the data from caspase-3 activation, caspase-8 Western blots, caspase-8-enzyme activity assays and the observation that caspase-8 protein status remained unchanged suggested that the butyrate-regulated factor acts upstream or at the level of caspase-8 activation, but downstream of Fas-oligomerization. Proteins known to interfere with the signaling pathway at this site are RIP, FLIP, FLASH and FAP-1. Analyses of RIP and $FLIP_{S+L}$ protein levels however, showed no changes during butyrate-treatment of cells and FAP-1 and FLASH mRNA Northern blot analyses also showed unchanged mRNA levels. In a previous work cycloheximide-mediated enhancement of Fas-induced apoptosis was investigated,[26] showing that FLIP, RIP and XIAP were downregulated. The mechanisms of butyrate-mediated fas-apoptosis enhancement are however, according to the data presented, different, as RIP and FLIP were not regulated and XIAP-(inhibiting caspases-3, -7 and -9 which are activated downstream of caspase-8 activation during Fas-induced cell death) – downregulation could not be responsible for enhanced caspase-8 activation. Additionally, the recently cloned SADS protein, which has been shown to enhance Fas- but not TNF-induced apoptosis by stabilizing the caspase-8-FADD interaction,[27] is also unlikely to be the causative protein for the butyrate effect, as butyrate as well as TSA enhanced TNF-induced cell death also.

Interestingly, caspase-2 activation during Fas-induced apoptosis was unaltered in the presence or absence of butyrate. The mechanisms by which caspase-2 is activated via Fas remain unclear. The present data suggest however, that caspase-2 is activated upstream from, or in parallel to, caspase-8, but not downstream via a caspase-8-dependent mechanism, as butyrate affected caspase-8 but not caspase-2 activation. Furthermore, it may be assumed that caspase-2 binds to the Fas-receptor-oligomer via adaptor proteins. If the adaptor protein were not FADD, which cannot be excluded, the possible frame of action for the butyrate-regulated protein would be downstream from Fas and upstream from caspase-8 activation. If FADD were the adaptor for caspase-2 binding to Fas, the frame would be downstream of FADD and upstream of caspase-8 activation.

Based on these considerations and in order to detect possible causative proteins we performed immunoprecipi-

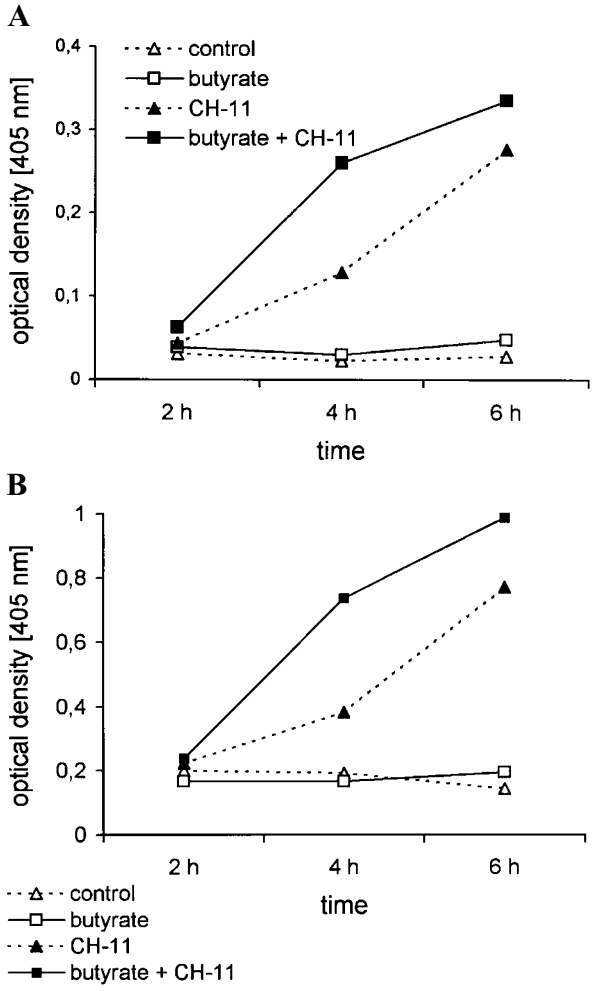

Figure 6 Butyrate increases caspase-3 and caspase-8 activity during Fas-induced apoptosis. This figure shows the analysis of caspase-3 and caspase-8 activity during Fas-induced apoptosis in the presence or absence of butyrate by photometrical evaluation of the cleavage of the chromogenic caspase-3 substrate DEVD-pNA or the chromogenic caspase-8 substrate IETD-pNA. (**A**) Shows that caspase-3 activation mediated by Fas stimulation was enhanced in the additional presence of butyrate. Four hours after CH-11 addition, optical density increased from 0.023 in untreated and 0.03 in butyrate-treated cells to 0.129 in cells treated with CH-11 and to 0.26 in cells treated with butyrate and CH-11. The enhancement of caspase-3 activity could also be observed 6 h after CH-11 addition. For caspase-8 optical density increased from 0.193 in untreated and 0.167 in butyrate-treated cells to 0.384 in cells treated with CH-11 and to 0.736 in cells treated with butyrate and CH-11 4 h after CH-11 addition. Direct addition of butyrate to the *in vitro* assays did not affect caspase-8 activity (data not shown)

tations of FADD and caspase-8. As demonstrated in Figure 7, a protein upregulated by butyrate, within a time period required for amplification of Fas-induced apoptosis, with an approximate molecular mass of 37 kD specifically co-precipitated with caspase-8. Concerning a hypothetical function of this protein we suggest that it binds to the non-DISC-bound part of caspase-8, because the 37 kD protein could not be immunoprecipitated with anti-FADD-antibodies, which should be possible if the protein was associated with the DISC. Thereby it might enhance

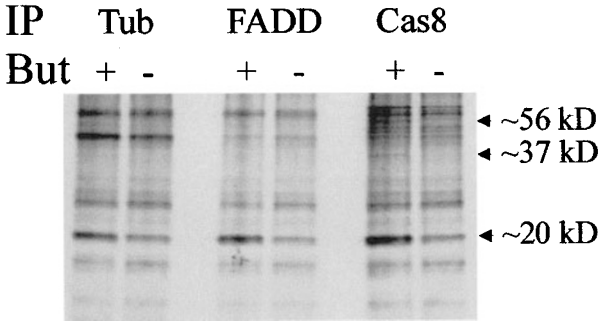

Figure 7 Immunoprecipitation of FADD and caspase-8. For the detection of proteins, regulated by histone hyperacetylation, at that site where, according to the functional analyses they are expected to act, CEM-C7H2 cells were incubated with 75 μCi/ml ^{35}S-methionine/cysteine-label in the presence or absence of butyrate (10 mM) for 8 h and thereafter immunoprecipitations of FADD, caspase-8 or α-tubulin as a control were performed as detailed in Materials and Methods. Precipitates were resolved on SDS polyacrylamide gels; gels were dried and analyzed by exposure to X-ray films (IP, antibody used for immunoprecipitation; But, butyrate; Tub, α-tubulin; cas8, caspase-8)

caspase-8 activation by transfering non-DISC-bound caspase-8 to the DISC. This opportunity would represent a novel mechanism in controlling caspase-activation and controlling a cell-death pathway. According to this model, inhibition of core histone deacetylases should also enhance TNFα-induced cell death. In fact butyrate (Figure 1C) as well as trichostatin A (data not shown) enhanced TNFα-induced cell death with a similar time- and dose-dependence as in the case of Fas-induced apoptosis. Isolation and characterization of this protein will be needed to test the mentioned hypothesis. Since two inhibitors of histone deacetylases, namely suberoylanilide hydroxamic acid and the butyrate pro-drug tributyrin have entered phase I clinical trials,[28,29] interactions with physiological death receptor signals are of clinical relevance. Furthermore, death receptor signals have recently been shown to constitute a prominent mechanism in the immune defense against tumors;[30–32] thus agents resensitizing cells to or enhancing death receptor signaling may become useful therapeutic tools. In this context, we investigated the interaction of histone deacetylase inhibition with Fas-induced apoptosis. Our analysis showed that butyrate and other histone deacetylase inhibitors enhance Fas-induced apoptosis in lymphoblastic leukemia cells and that this phenomenon is clearly associated with histone hyperacetylation. Furthermore, this event can be pinpointed downstream of Fas but upstream of caspase-8 activation, which is strengthened by the discovery of a butyrate-regulated caspase-8-binding protein.

Material and Methods

Reagents

Fas apoptosis-inducing antibody, clone CH-11 was purchased from Biomol, Hamburg, Germany. Sodium butyrate was prepared by titrating butyric acid (Fluka Chemie AG, Buchs, Switzerland) with

sodium hydroxide to pH 7.3. All other reagents, including sodium acetate, sodium propionate, trichostatin A[33], and propidium iodide were from Sigma (Vienna, Austria), unless indicated otherwise.

Cell lines and cell culture

CEM–C7H2 is a glucocorticoid-sensitive subline of CCRF–CEM–C7[34]. For culture and experiments the cells were grown in 5% CO_2, saturated humidity, at 37°C in RPMI 1640 supplemented with 10% fetal or bovine calf serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Quantification of apoptosis

For detection and/or quantification of apoptosis, reduction of nuclear propidium iodide fluorescence together with forward/sideward light scattering analysis[35] and the Annexin V method[36] were used. FACS analysis of nuclear propidium iodide fluorescence and forward/sideward light scattering analysis have been described previously.[37] Briefly, 5×10^5 cells were permeabilized and stained with 750 μl propidium iodide (50 μg/ml in 0.1% Triton X-100/0.1% sodium citrate) and subjected to analysis in an argon laser-equipped FACScan (Becton Dickinson, San Jose, CA, USA) using either propidium iodide fluorescence intensity or forward/sideward light scattering as parameters. Cell debris and small particles were excluded from analysis. Based on propidium iodide staining, cells in the sub-G1 marker window were considered to be apoptotic. Using forward/sideward light scattering as parameters, apoptotic cells appear smaller (lower forward scatter values) and more granulated (higher sideward scatter values) than living cells.

Preparation of histones and analysis of histone acetylation

Histones were prepared from 10^7 or 10^8 cells. Acidic extraction of core histones was performed from whole cells with 0.3 M HCl after prior extraction of linker histones and HMG-proteins with 5% perchloric acid. For the extraction procedures with 5% perchloric acid as well as 0.3 M HCl, the pellet was taken up in 20 ml for a first extraction step and the extraction was repeated with 10 ml of the respective acids. In each extraction step the pellets were subjected to homogenization in a Dounce homogenizer by 10 up-and-down strokes. After incubation for 30 min on ice, the homogenate was centrifuged for 15 min at 20 000 $\times$ *g*, 4°C. The supernatants of the two extractions with 0.3 M HCl were united and the core histones precipitated with 25% trichloric acid (final concentration). After incubation on ice for at least 1 h, the precipitate of the core histones was collected by centrifugation at 20 000 $\times$ *g*, 20 min, 4°C. The pellet was washed with HCl-acetone and dried *in vacuo*. Electrophoretic separation of the acetylated forms of histone H4 was performed in acidic urea-Triton X-100 polyacrylamide gels (12% T, 2.6% C, 8 M urea).[38]

Western blotting and immunodetection

Cells were washed in PBS, suspended in sample loading buffer (2% SDS, 10 mM Tris, pH 7.4) boiled for 1 min, and sonified. Equal amounts of cell lysates were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking nonspecific binding sites for 1 h with 5% nonfat milk in TTBS (TBS with 0.1% Tween 20), the membranes were incubated for 2 h at room temperature with antibodies against caspase-2, caspase-3, FADD (Pharmingen, San Diego, CA, USA), caspase-8 (Upstate Biotechnology, Lake Placid, NY, USA) – this antibody detects a nonspecific approximately 19 kD protein as well as the caspase-8 cleavage product p20, which corresponds with the upper band of the double-band marked with p20 in the lower part of Figure 5B (3 and 4 h, treatment C and BC) – Sentrin, $FLIP_{S+L}$ (Alexis Biochemicals, Läufelfingen, Switzerland), RIP (Transduction Laboratories, Lexington, KY, USA) or Bid (Santa Cruz, CA, USA). α-tubulin (Calbiochem-Novabiochem Corporation, San Diego, USA) was used as a standard for the amount of protein applied. All antibodies were dissolved in blocking buffer (TTBS with 5% nonfat milk). The membranes were then washed three times in TTBS and incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse, donkey anti-rabbit (Amersham, Les Ulis, France) or anti-goat antibodies. The membranes were then washed again three times in TTBS and developed, using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Analysis of Fas-oligomerization was performed as described by Kamitani *et al*.[24] (anti-human Fas-death-domain antibody clone 3D5 was obtained from Alexis Biochemicals, Läufelfingen, Switzerland).

Determination of Fas and Fas ligand expression by immunofluorescence and FACS analysis

For the quantification of surface Fas and FasL expression, FITC-labeled murine anti-human Fas (clone UB2, Immunotech, Marseille, France) and FITC-labeled anti-human FasL (clone H11, Alexis, Läeufelfingen, Switzerland) monoclonal antibodies were used. Briefly, 5×10^5 cells were stained with the respective specific reagents (1 μg) or an isotype-matched negative control antibody for 30 min at 4°C, washed and immediately analyzed by flow cytometry.

Analysis of caspase-3 and -8 activity

For measurement of caspase-3 and -8 activity, cells were either not treated or treated with butyrate or CH-11 or both. After incubation for the indicated times (see Result section) cells were washed with PBS, lysed in caspase-buffer (PIPES 20 mM; NaCl 100 mM; DTT 10 mM; EDTA 1 mM; CHAPS 0.1%; Succrose 10%, + protease inhibitors) for 30 min on ice and centrifuged for 20 min, 4°C, 17 000 $\times$ *g* to separate dissolved proteins from nuclei. After determination of protein concentration by the standard Bradford-procedure, 40 μg for caspase-3 or 120 μg for caspase-8 of total protein were suspended in 200 μl of caspase-buffer (with additional 10 μl PMSF [0.1 M] and 20 μl NaF [0.5 M] per 5 ml caspase-buffer); 1 μl of the chromogenic caspase-3 substrate DEVD-pNA or 3 μl of the chromogenic caspase-8 substrate IETD-pNA (Bachem AG, Bubendorf, Switzerland) was added and the reaction mix was incubated for 1 h (caspase-3) or 2.5 h (caspase-8) at 37°C. After incubation the reaction mix was put on ice and subsequently subjected to photometrical analysis (λ=405 nm).

Metabolic labeling of cells and immunoprecipitation of FADD, caspase-8 and α-tubulin

For the immunoprecipitations of FADD, caspase-8 or α-tubulin, 10^7 cells were washed twice with and incubated in labeling medium (nine parts of methionin-free RPMI 1640+one part RPMI 1640+10% FCS); protein labeling was performed with 75 μCi/ml of ^{35}S methionine/cysteine mix (Hartmann Radiochemicals, Germany) for 8 h in the presence or absence of 10 mM butyrate. Thereafter cells were washed twice with cold PBS, lysed (150 mM NaCl, 0.1% Triton X-100, 30 mM Tris, 1 mM PMSF, 10% Glycerol, Peptide Inhibitors) for 15 min on ice. The soluble protein fraction was collected by centrifugation (15 min, 4°C, 10 000 $\times$ *g*) and incubated with 2 μg of the respective antibodies

and 40 μl of protein A/protein G agarose mix (Oncogene Research Products, Darmstadt, Germany) rotating for 2 h at 4°C. Bound proteins were collected by centrifugation. The pellet was washed four times in lysis buffer and subsequently suspended in Western loading buffer and separated in SDS-polyacrylamide gels as described in 'Western blotting and Immunodetection'. Gels were dried for 1 h at 80°C and analyzed by exposure to X-ray films.

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