ABSTRACT

Acute myeloid leukemia (AML) is a disease characterized by a block of maturation. Genes coding for core binding factors are rearranged in a considerable subset of AML cases and result in an altered interaction of core binding factor (CBF) subunits with transcriptional coregulators (NCoR/SMRT). Recruitment of histone deacetylase is also altered in AML, and a subsequent transcriptional repression of target genes involved in myeloid maturation is determined. We determined here the effects of two histone deacetylase inhibitors, sodium butyrate and the stable prodrug xylitol butyrate derivative (D1), on a t(8;21)-positive cell line (Kasumi-1) as well as primary AML blasts. Exposure (24–96 h) to butyrate (1 mM) of Kasumi-1 cells induced histone H4 acetylation, whereas H3 acetylation was unchanged. Induction of morphological and immunophenotypic granulocytic maturation (96 h), also confirmed by an increased expression of CAAT/enhancer binding protein α, was observed. Inhibition of proliferation and apoptosis via activation of caspase-9 was also observed. In primary AML blasts, butyrate (0.5 mM) increased histone H4 acetylation of 18 of 19 cases tested. Terminal granulocytic maturation was observed in all cases (5 of 5) characterized by chromosomal translocations involving CBF, whereas in non-CBF cases, maturation was incomplete (4 of 8) or absent (4 of 8). Our data indicate the possibility to effectively remove, in CBF AML cases, the maturation block generated by histone deacetylase stable recruitment, contributing to a possible development of molecularly targeted therapies of AML.

INTRODUCTION

Acute myeloid leukemia (AML) is biologically a highly heterogeneous disease. Multiple recurrent chromosome and gene rearrangements have been identified and contribute to delineation of prognostically distinct categories of AML (1). One such category has been defined recently as core binding factor (CBF) AML. Patients with CBF AML (whose cells most commonly exhibit either t(8;21)q22; q22) AML1/ETO or inv(16)(p13q22) CBFB/MYH11) constitute approximately 15–20% of adults younger than 60 years with de novo AML (2, 3). The function of the genes involved in these translocations (4–7) is modified as result of the fusion with inappropriate partners and results in disruption of their role in promoting transcriptional activity, by recruiting an active histone deacetylase (HDAC; Ref. 8) through interactions with the nuclear corepressors NCoR and Sin3A (9–11). Quite recently, this molecular phenomenon has been recognized in acute promyelocytic leukemia (APL; PML/RARα and PLZF/ RARα positive) as well as CBF AML. This molecular alteration thus represents a shared pathogenetic mechanism (12). On this basis, efforts are directed to tailor derepressive, maturative therapy for specific AML subtypes by the use of histone deacetylase inhibitor (HDACi) such as suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), valproic acid (13, 14), and several others under investigation.

Butyrate has long been known to be a HDACi able to induce maturation in normal and tumor cells and to have a partial activity in vivo in the therapy of myelodysplastic syndromes, refractory AML and APL (15–17). Recently, monosaccharide ester derivatives of butyric acid characterized by high molecular stability have been developed and studied (18). We demonstrated previously that sodium butyrate and monosaccharide esters inhibit cell growth and induce incomplete maturation and apoptosis in AML cells (19). We provide here evidence for the acetylating activity of sodium butyrate and a xylitol butyrate derivative (D1) in two AML cell lines and in primary AML blasts. Histone acetylation was paralleled by terminal granulocytic maturation, apoptosis, and inhibition of proliferation in the CBF AML cell line Kasumi-1 and in the CBF AML primary blasts, whereas in other non-CBF AML cases, maturation was incomplete or absent.

MATERIALS AND METHODS

Cells and Culture Conditions. Heparinized peripheral blood and bone marrow samples were obtained after informed consent from 19 patients with AML. French-American-British diagnosis included 5 M1, 6 M2, and 8 M4 (20). T lymphocytes were removed after Ficol-Isoaque centrifugation by E-rosetting with sheep RBCs and AML cells, which were then cryopreserved in DMSO, as described previously (21). After being thawed and washed, AML blasts were depleted of adherent cells after incubation in serum-free medium in plastic culture flasks (250 ml; Greiner) and then cultured. After this procedure, blast content reached 98–100% in each sample, as revealed by morphological analysis. Cytogenetic analysis and reverse transcription-PCR to establish the presence of AML-associated fusion genes were performed according to standard methods (22). Primary AML blasts, Kasumi-1 cells (a human AML1/ETO-positive cell line derived from a myeloblastic leukemia; Ref. 23), and the human pre-osteoclastic leukemia cell line FLG 29.1, derived from a monoblastic leukemia (kindly provided by Dr. Bernabei, AOUC Careggi, Firenze, Italy; Ref. 24), were cultured in RPMI 1640 supplemented with 50 units/ml penicillin, 50 µg of streptomycin, and 10% FCS at 37°C in a humidified atmosphere containing 5% CO2. Kasumi-1 cells (0.4 × 106/ml) and AML blasts (1 × 106/ml) were incubated in the presence of different doses of sodium butyrate (Sigma) or D1 (O-n-butanoyl-2,3-O-isopropylidene-a-t-manno-furanoside; kindly provided by Chiesi Pharmaceuticals, Varese, Italy).

Cell Proliferation and Maturation. Cell proliferation was evaluated and quantified by cell counting. Differentiation was evaluated by morphological, cytochemical, and immunological examinations. Before and after treatment of culture, cytosin preparations of AML cells were stained with May-Grünwald-Giemsa. Morphology of cells was then examined by light microscopy using a ×100 lens and immersion oil. At least 200 cells/slide were counted in duplicate, and the percentages of blasts, promyelocytes, myelocytes, metamyelocytes, band forms, granulocytes, monoblasts, monocytes, and macrophages were scored. Maturation morphological signs were considered to be appearance of cytoplasmic granules, loss of cytoplasmic basophilia, chromatid condensation, and nuclei segmentation. Nucleated cell number and viability were determined by counting with Türk solution and trypan blue dye exclusion. Picnosis of nuclei and cytoplasmic condensation were also scored as specific
signs of apoptosis accompanying maturation (25). For nitroblue tetrazolium (NBT)-reduction assay, a solution containing 12-O-tetradecanoylphorbol 13-acetate (Sigma) and NBT (Sigma) was added to cell suspensions as described previously (26), and the percentage of positivity was determined by scoring 500 cells. CD34, CD15, CD11b, and CD14 cell surface markers were analyzed by flow cytometry. Cells were subjected to centrifugation and washed in PBS. Phycocerythrin (PE)-labeled CD34 or PE-labeled CD11b (Becton Dickinson, San Jose, CA) and FITC-labeled CD15 or FITC-labeled CD14 antibodies (Becton Dickinson) were added, and cells were incubated for 30 min in the dark at room temperature. After centrifugation in PBS for 5 min, cells were resuspended in 500 μl of 1× antibody binding buffer (PBS with 0.1% sodium azide). Cytofluorimetric analysis was performed in a FACScan (Becton Dickinson) by the use of CellQuest software. Results are expressed as mean fluorescence intensity (MFI), which is the ratio of the mean fluorescence channel of the labeled specific antibody to the control antibody channel.

**Annexin-V Binding Assay.** To quantify apoptosis after treatment with butyrates, cells were subjected to centrifugation and resuspended in 100 ml of 1× binding buffer (HEPES buffered saline solution with 2.5 mm CaCl₂). PE-labeled Annexin-V (Roche Diagnostics) and propidium iodide (PI) were added, and cells were incubated for 15 min in the dark at room temperature. After the addition of 400 ml of 1× binding buffer and agitation, flow cytometer analysis was performed using a FACSscan (Becton Dickinson). Cells positively stained with Annexin-V and PI-negative were considered apoptotic.

**Cell Lysis and Western Blotting.** Cells were cultured (5 × 10⁶/ml) in RPMI 1640 supplemented with 10% FCS and then treated or not with butyrates. Cells were then washed once with ice-cold PBS and solubilized by incubating for 10 min at 95°C in Laemmli buffer (62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 0.005% blue bromphenol, and SDS 2%). Lysates were clarified by centrifugation (20,000 × g; 10 min at room temperature). Protein concentration in the supernatants was determined, and 30-μg aliquots of each sample were boiled for 10 min, in the presence of 100 mM 2-mercaptoethanol, before being separated by SDS-PAGE in a 15% polyacrylamide gel and then transferred onto nitrocellulose membranes (Hybond-ECL; Amersham) by electroblotting. Histone H4 (H4) and histone H3 (H3) acetylation were determined by incubating membranes in PBS containing 0.1% Tween 20 and 5% BSA (T-PBS/5%BSA; 3–5 h at room temperature), and then in a 1:1000 dilution of a polyclonal anti-acetylated H4 antibody (Upstate Biotechnology) or polyclonal anti-acetylated H3 antibody (Santa Cruz Biotechnology) in T-PBS/5%BSA (16–18 h at 4°C). To verify equal loading of samples per lane, H4 expression was determined by stripping and incubating the same membranes in T-PBS/5%BSA (3–5 h at room temperature) and then in a 1:1000 dilution of a polyclonal anti-H4 antibody (Upstate Biotechnology) in T-PBS/5%BSA (16–18 h at 4°C). A pan-acetylated protein polyclonal antibody (Santa Cruz Biotechnology) was used in the same blotting conditions. A non-poisoning binding protein (C/EBPα) in a 1:1000 dilution of a polyclonal anti-C/EBPα (Santa Cruz Biotechnology) or polyclonal anti-caspase-9 (Santa Cruz Biotechnology) or monoclonal anti-caspase-3 (Santa Cruz Biotechnology). Secondary antibodies, horseradish peroxidase conjugated, were anti-rabbit or anti-mouse IgG (Sigma). Antibody-coated protein bands were visualized by ECL chemiluminescence detection (Amersham, Buckinghamshire, United Kingdom).

**Real-Time Quantitative PCR (RQ-PCR).** After RNA extraction by Trizol as specified by the manufacturer, samples were submitted to reverse transcription (Expanded reverse transcriptase; Roche). Expression levels of C/EBPα were measured relative to an exogenously applied quality-control nucleic acid template. Real-time PCR product accumulation was monitored using the intercalating dye SYBR Green I, which exhibits a higher fluorescence upon binding to the minor groove of double-stranded DNA. The following primers were used (27): C/EBPα forward 5'-CTTCAACACGAGCTTGCTGGCCGA-3' and reverse 5'-AGCTGGCTGGGTCATCCTACC-3'. To compensate variations of RNA integrity and differences in the reverse transcription step, the C/EBPα transcript was normalized to β2-microglobulin as an internal control gene. For β2-microglobulin, the following primers were used: forward, 5'-GGAATTTGATTTGAGAGCATC-3'; and reverse, 5'-CAGTGGCCTGGCTGCTAAATATGACTA-3'. Granulocyte/macrophage colony stimulating factor (GM-CSF) gene transcription was also evaluated, and the primers used were as follows (28): forward, 5'-ATGGGTGCAGAGGTGCCTGC-3'; and reverse, 5'-CTGGTTCCCAGAGCTCACAAGGGG-3'. Reaction mixtures contained QuantiTec SYBR Green PCR Master Mix (Qiagen) and 0.4 pmol/μl of primers. The following protocol was applied, using an i-Cycler (Bio-Rad): 2 min at 50°C to allow destruction of contaminants by UNG, 15 min at 95°C to activate Hot Startaq DNA polymerase (Sigma), and amplification was carried out for 40 cycles: denaturation 30 s at 95°C, annealing for 30 s at 60°C, and elongation 30 s at 72°C. Because Sybr Green is not a sequence-specific method, to discriminate between specific and non-specific PCR products, a melting curve analysis was performed. Relative C/EBPα and GM-CSF gene expression was calculated by comparing ΔCt as described previously (29).

**RESULTS**

Butyrate Treatment Induces Histone H4 Acetylation in Kasumi-1 and FLG 29.1 Cells. Butyrate treatment of the Kasumi-1 cell line markedly increased histone H4 acetylation (Fig. 1), which was undetectable in untreated cells. A marked histone H4 acetylation (Fig. 1A) was observed after 24 h of treatment with 1 mM sodium butyrate, whereas a higher dose (2 mM) of D1 was necessary to obtain the same effect (Fig. 1B). H4 acetylation was induced maximally after 3 h by sodium butyrate and maintained throughout the time frame considered. Longer incubation times were needed by 1 mM D1 to reach histone H4 acetylation levels comparable with those induced by sodium butyrate (Fig. 1E). Similar levels and kinetics of histone H4 acetylation were obtained in the FLG 29.1 leukemic cell line not expressing CBF rearrangement (Fig. 1, I and J). Histone H3 acetylation was evaluated in parallel, and it was not significantly modified by butyrate treatment at any of the time points analyzed (Fig. 1F). We then determined whether the acetylation of proteins different from histones was affected by butyrate treatment by blotting with an
anti-pan-acetylated antibody (Fig. 1H). We found that the only protein whose acetylation was apparently altered after treatment with butyrates comigrated with H4, whereas the acetylation of the protein band corresponding to H3 was unchanged. Thus, the marked difference in the pattern of acetylation between H4 and H3 histones was confirmed. Total histone H4 expression was not altered by butyrate treatment (Fig. 1G).

**Butyrate Treatment Inhibits Cell Proliferation and Induces Apoptosis in Kasumi-1 Cells.** Butyrates are known to inhibit cell proliferation. We thus determined whether Kasumi-1 cell proliferation was affected by butyrate treatment (Fig. 2). The number of viable cells in culture was determined after a 3-day incubation (Fig. 2A). Untreated cells underwent a 2.5-fold increase (8 × 10⁶ cells/culture) with respect to time zero (3 × 10⁶ cells/culture). Treatment with 1 mM sodium butyrate or D1 not only abrogated this amplification but reduced cell number below the time-zero value (1.5 and 2 × 10⁶ cells/culture, respectively). We then verified whether such a marked inhibition of cell growth was paralleled by the induction of programmed cell death (Fig. 2B). In the absence of butyrate treatment, the percentage of apoptotic cells, revealed as Annexin V-positive cells (light gray), was 7.5 ± 1.9% after 24 h of culture. The addition of 1 mM sodium butyrate or D1 increased the level of apoptotic cells to 48.6 ± 5.4% and 29.9 ± 6.7%, respectively. Similar results were obtained by the cytotoxicometric measuring of the pre-G₁ peak after propidium iodide incorporation into DNA (not shown). In the same set of experiments, the counts of cells negative for both propidium iodide and Annexin V provided the percentages of viable cells (white), which were in keeping with the data obtained by trypan blue staining (Fig. 2A). Cell positivity for both propidium iodide and Annexin V provided the percentage of necrotic cells (dark gray).

The effect of butyrate treatment on the activation of the apoptotic caspase cascade is shown in Fig. 3. The cleavage of p35 procaspase-9 (Fig. 3A), which follows its activation, took place after 12 h and was complete after 24 h of treatment with both butyrate and D1 derivative. Procaspase-9 cleavage was indicated by the disappearance of the p35 inactive enzyme. Procaspase-3 was activated as well, following a 24- or 48-h treatment with sodium butyrate or D1 (Fig. 3B), and the cleavage of the p34 proenzyme was paralleled by the appearance of the p11 active enzyme. It is worth noting that sodium butyrate was more effective than D1 after a 24-h treatment, in keeping with the results of Fig. 2B. Butyrate treatment markedly affected cell morphology (Fig. 3D). The treatment of Kasumi-1 cells (left) with sodium butyrate (middle) or D1 (right) for 24 h determined massive apoptosis.

**Butyrate Treatment Induces Myeloid Maturation in Kasumi-1 Cells.** To establish whether butyrate-induced acetylation was paralleled by cell maturation in Kasumi-1 cells, we determined cell morphology (May-Grünewald/Giemsa staining), NBT reduction, immunophenotypic modifications (Fig. 4), and expression of C/EBPα (Fig. 5). In the absence of butyrate (Fig. 4A, left), cells appeared as undifferentiated blasts, with negligible spontaneous maturation (see “Materials and Methods”) of viable cells (9.6 × 10⁶ cells/8 × 10⁶ total cells)
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Fig. 4. Butyrate treatment induces myeloid maturation in Kasumi-1 cells. A, cells were incubated in the absence (left) or the presence of 1 mM sodium butyrate (but, middle) or D1 (right) for 4 days. Cytospin preparations were stained with May-Grünwald/Giemsa and examined by light microscopy. The corresponding absolute number are reported in the table. The relative percentages of NBT-positive cells are also reported. Data represent the averages of four independent experiments (bars, SE), in which four randomly chosen fields for slide were examined. B, CD34, CD15, and CD11b cell surface expression was evaluated by flow cytometry using PE (for CD34 and CD11b) or FITC (for CD15) conjugated monoclonal antibodies. Cells were cultured for 72 h in the absence or the presence of sodium butyrate or D1 and MFI was calculated. Data represent the averages of four independent experiments; bars, SE.

and table; Fig. 4A). Treatment of cells with sodium butyrate (Fig. 4A, middle) or D1 (right) for 72 h determined a marked granulocytic maturation (6.15 x 10^4 cells/1.5 x 10^8 total cells and 84 x 10^4 cells/2 x 10^8 total cells of viable cells, respectively; see also the table in Fig. 4A). In particular, butyrates caused a decrease of cell size and nucleus:cytoplasm ratio, nuclear changes, and appearance of cytoplasmic granules. It is worth noting that, after treatment with sodium butyrate, 28 ± 1.8% of cells reached the level of terminally mature neutrophils. NBT positivity increased from 0 to 52.5 ± 1.2% of viable cells after sodium butyrate treatment and from 0 to 51.5 ± 2.1% with D1.

Maturation was also evaluated by flow cytometric analysis of the expression of typical markers of myeloid maturation of Kasumi-1 cells (Fig. 4B). After addition of sodium butyrate or D1 to cultures, the percentage of CD34-expressing cells was marginally decreased (from 96.2 ± 9.7% in untreated cultures to 82.3 ± 5.1% and 83.8 ± 3.7%, respectively). MFI of immature CD34 cells was 2.4 ± 0.1 for butyrate and 2.6 ± 0.1 for D1 (control MFI, 3.6 ± 0.1; Fig. 4B, top panels). Little effect on CD11b and CD15 could be measured. The percentage of CD15-expressing cells was 27.1 ± 3.4% in untreated cultures but increased up to 37.3 ± 5.0% and 41.3 ± 5.5%, respectively, after sodium butyrate and D1 addition. MFI was 1.2 ± 0.1 for sodium butyrate and 1.2 ± 0.1 for D1 (untreated control, 1.0 ± 0.0; Fig. 4B, middle panels). CD11b expression was enhanced by sodium butyrate or D1 treatment (3.6 ± 2.6% and 4.8 ± 3.5% of cultured cells, respectively), with respect to control cultures (1.7 ± 1.3% of cells). MFI for CD11b expression in treated cells was 1.3 ± 0.1 for sodium butyrate and 1.4 ± 0.1 for D1 incubation (MFI untreated control, 1.2 ± 0.1). These results are consistent with what was shown previously (30) for other maturation inducers [all-trans retinoic acid (ATRA) and TSA]. Consistently, CD14 expression was undetectable in Kasumi-1 cells, either constitutively or after butyrate treatment, confirming the absence of any sign of monocyctic maturation in these cells. FLG 29.1 cells, notwithstanding the induction of histone acetylation (Fig. 1A), were absolutely irresponsive to butyrates in terms of maturation, as we have already demonstrated (19).

The expression of the C/EBPα transcription factor is strictly related to initial granulocytic maturation (31). We thus analyzed its pattern of expression during butyrate-induced maturation of Kasumi-1 cells (Fig. 5). C/EBPα gene expression was analyzed by RQ-PCR. C/EBPα transcript was found strongly increased after 3 and 6 h of incubation. After 48 h, the level of C/EBPα transcript was markedly lower, to increase again thereafter (Fig. 5A). Accordingly, C/EBPα protein expression reached maximal levels after 12 h of butyrate treatment (1.4- and 2.8-fold higher than at time zero, respectively, as revealed by band densitometry), to decrease thereafter (Fig. 5B). We then determined whether butyrates determined specific derepression of increased expression of retinoic acid receptor (RARE) and p53, by lysis of cells before or after incubation for the indicated times, in the absence or the presence of sodium butyrate or D1. Proteins were then subjected to SDS-PAGE and immunoblotting with anti-C/EBPα antibodies (upper panel). Protein loading was verified by stripping and reprobing the same membrane with anti-H4 antibodies (lower panel). C/EBPα densitometry values reported in the table were normalized with respect to the H4 content and are expressed in function of the value obtained for unstimulated cells.

Fig. 5. Butyrate treatment induces expression of C/EBPα in AML1/ETO-positive Kasumi-1 cells. A, expression of C/EBPα gene induced by butyrate was quantified by RQ-PCR. Expression at the indicated times was calculated after normalization with respect to an internal control (β2-microglobulin). [ ], untreated cells; □, cells treated with sodium butyrate; ■, cells treated with D1. B, expression of C/EBPα protein was quantified by lysing cells before or after incubation for the indicated times, in the absence or the presence of 1 mM sodium butyrate (but) or D1. Proteins were then subjected to SDS-PAGE and immunoblotting with anti-C/EBPα antibodies (upper panel). Protein loading was verified by stripping and reprobing the same membrane with anti-H4 antibodies (lower panel). The C/EBPα densitometry values reported in the table were normalized with respect to the H4 content and are expressed in function of the value obtained for unstimulated cells.

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AML1-controlled genes. GM-CSF gene expression, as revealed by RQ-PCR, was modestly increased (with a 2-ΔΔCt of 1.25 and 1.99 for sodium butyrate and D1, respectively) after 6-h butyrate treatment.

**Butyrate Treatment Induces H4 Acetylation and Myeloid Maturation in Primary AML Blasts.** We then analyzed the effects of butyrate treatment on primary AML blasts (Table 1). H4 acetylation was absent or faint in untreated AML blasts in 18 of 19 cases (and marked in case 18). Treatment with 0.5 mM sodium butyrate or D1 for 72 h induced H4 acetylation in all 18 AML cases. Fig. 5 shows several patterns of H4 acetylation after treatment of primary AML blasts with butyrates. In all cases, treatment with both sodium butyrate and D1 markedly induced H4 acetylation, after either 24 or 72 h. In one case (no. 10), D1 was more effective than sodium butyrate, even after only 24 h of treatment. Butyrate effects on maturation were determined in only 13 cases, by morphology and immunophenotype. AML blasts were 4 of 13 cases (31%) did not modify their phenotype into a more mature one, despite the presence of butyrate-induced H4 acetylation (Table 1, nos. 10–13; Fig. 6, no. 10). In only 5 of the 9 maturing cases, a significant number of AML blasts differentiated to terminally mature neutrophils (Fig. 6, nos. 1–5). All of these 5 cases were molecularly characterized as CBF AML. The remaining AML cases had a normal karyotype and absence of AML/ETO or CBF/MYH11 molecular rearrangements. Within the group that underwent significant maturation, we mention: case 3, where of 12 × 10⁶ cells, no mature cells were scored in the untreated culture, whereas after 72 h of D1 treatment, mature granulocytic cells were 4.5 × 10⁶ of 9 × 10⁶ cells; case 4, where of 3.3 × 10⁶ untreated cells 0.16 × 10⁶ cells were mature, whereas after D1 treatment, mature granulocytic cells were 0.27 × 10⁶ of 0.4 × 10⁶ cells; case 5, where of 15 × 10⁶ cells, untreated culture yielded 0.29 × 10⁶ mature cells, whereas after D1 exposure, mature granulocytic cells were 5 × 10⁶ of 10 × 10⁶ cells. On the other hand, the primary cultures whose slides did not score a significant percentage of morphologically maturing cells, as indicated in Table 1, contained finally only negligible absolute numbers of mature cells.

**DISCUSSION**

This study demonstrated that butyrates as single agents are able to determine terminal granulocytic maturation in AML blasts. We showed here, for the first time to our knowledge, that AML blasts from stable cell lines and primary cultures lack constitutive H4 histone acetylation and that the in vitro treatment with sodium butyrate or the xylitol butyrate ester D1 always induces H4 acetylation. Acetylation was already evident after a 3-h stimulation with 1 mM butyrate, became marked after 6 h, and was maintained after 96 h, consistent with previous reports concerning apicidin, SAHA, and TSA (33–35). In primary cultures of AML blasts (non-APL), butyrates induced H4 acetylation in 18 of 19 cases. The only case in which acetylation could not be induced was characterized by an abnormally high constitutive H4 acetylation. All of the other cases tested showed a massive increase of H4 acetylation after treatment with sodium butyrate or D1, whose effects were usually delayed in accordance to its being a produg (18). Cell maturation was evaluated after a 72-h butyrate treatment of cell lines and primary blasts from 13 AML cases. In particular, roughly 50% of Kasumi-1 cells revealed morphological maturation together with enhanced NBT reduction. Moreover, these data were supported by changes in the expression pattern of surface maturation antigens. Effective induction of maturation was confirmed by the demonstration of transient expression of both transcript and protein of C/EBPα, a transcription factor determinant for granulocytic maturation (36). The derepression of C/EBPα induced by butyrate treatment is particularly notable, not only as a specific sign of the restoration of a parasynthetic maturative program but also because of the fact that silencing of this gene is typical of AML1-ETO-positive AMLs (36, 37) and that its altered expression has a strong prognostic significance (38). The decline in CEBPα gene and protein expression after 48-h butyrate exposure may be attributable to its pivotal role in the very early stages of granulocytic maturation, a role played later in the maturative pathway by other transcription factors such as C/EBPδ (39). In further support of an effective

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**Table 1. Morphological and genetic features of primary AML blasts from AML patients**

<table>
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*FAB, French-American British classification. All cases were tested by conventional karyotyping, all M2 and M4 cases were also tested by RT-PCR for the fusion genes AML1-ETO or CBF/MYH11, respectively. Maturation (as evaluated by morphological criteria): −, 0–25%; +, 25–50%; ++, 50–70%; ++++, 70–100% of mature metamyelocytes and granulocytes over the value obtained for untreated control cultures.
induction of granulocytic maturation, the expression of GM-CSF gene, physiologically under the control of AML1 and silenced by AML1/ETO, was induced, although modestly, by butyrate exposure.

Among primary AML cases, cells derived from the cases of CBF AML underwent a marked morphological maturation, yielding >50% of terminally mature granulocytic cells. In primary AML cultures, absolute cells number was lower after butyrate treatment than in untreated cultures, confirming the butyrate inhibition of cell growth observed previously (19). Notwithstanding this decrease in the number of viable cells, the total recovery of mature cells was significantly higher than that of untreated controls. Parameters of spontaneous growth (in the absence of exogenous growth factors) are highly variable in primary AML cultures (32), thus rendering a homogeneous representation of absolute cell numbers as cumbersome. AML with different French-American-British subtypes not presenting molecular rearrangements of CBF showed scanty signs of maturation after butyrate treatment.

The moderate effect of butyrates on the expression of maturation antigens CD11b, CD15, and CD34 is consistent with previous observations (40). Some authors reported recently that the treatment of AML-derived cell lines with HDACi has only a partial effect in relieving the differentiation block (41) but is especially effective in AML1/ETO-positive cell lines (42) and that fresh blasts from AML patients do not undergo significant differentiation upon in vitro treatment with various HDACi (30), whereas combined treatment with valproic acid, ATRA, or decitabine was successful in restoring granulocytic maturation (43, 44). These data indicate that more than one molecular event may be necessary to fully restore myeloid maturation. On the other hand, our results demonstrated for the first time that treatment with HDACi as a single agent is sufficient to induce significant, although incomplete, maturation confined to only a subset of primary (non-APL) AML cells.

To validate the efficacy of butyrates in terms of specific inducers of granulocytic maturation for Kasumi-1 cells, we also evaluated the maturation response of Kasumi-1 cells to ATRA. Mature granulocytic cells after 72 h culture in the presence of 1 μM ATRA were 25%, with 40% NBT positivity. Moreover, in preliminary experiments performed in our laboratory with primary AML blasts, granulocyte-colony stimulating factor (G-CSF) strongly synergized with butyrates to induce maturation. The molecular mechanisms underlying this successful combination of differentiating agents with respect to less effective ones, i.e., cAMP plus G-CSF, vitamin D3 plus ATRA, remain still to be dissected. The main goal of the present research in this field should be to identify, by gene profiling, the series of transcription factors and the group of target genes whose expressions are elicited specifically and with precise timing by the different molecules known as maturation inducers in vitro.

The ability of butyrates alone to remove maturation block appears to preferentially occur in the subset of CBF AML, although the number of cases studied is limited, and conclusions have to be cautious. This seems to indicate that there are AML subtypes in which the permanent recruitment of HDAC is the main leukemogenic alteration, whereas other mechanisms (i.e., hypermethylation of CpG islands) may be present in other cases and hamper translation of genes responsible for maturation (45, 46). The combination of HDACi and agents such as decitabine, 5-azacytidine (30, 44, 47), ATRA (30), or G-CSF (47) is required to remove the maturation blockage of AML cells, restore gene transcription, and complete the maturation program (48, 49).

To complete the complex picture of the activity and interactions of HDACi, one has to consider that HDAC may regulate gene expression independently of their direct effects on chromatin, acting on selective targets (14). The best studied and efficient HDACi entered into Phase I and II clinical trials is SAHA (50). SAHA induces p21waf1 protein expression by selectively acetylating the promoter of the gene (34) and not simply by hyperacetylating histones. Therefore, the selectivity of SAHA, and HDACi in general, would not be attributable to the cell type and to specific molecular alterations but to the reinduction of selective gene expression (51). In this sense, our results are intriguing, because we demonstrate a sharp difference in histone H3 and H4 acetylation induced by butyrates. This observation seems to indicate a selectivity and specificity of action of butyrates as HDACi, which may be fundamental for future clinically tailored applications. The precise role of the different histones in target gene transcription must, therefore, be further investigated and clarified.

We also demonstrated that butyrate treatment induced massive apoptosis in Kasumi-1 cells. Using different approaches, we observed the early appearance of butyrate-induced apoptosis, similar to what was observed for other HDACi (33, 52, 53). In our study, the evidence for procaspase-9 cleavage seems to clearly indicate the mitochondrial pathway as the one responsible for butyrate-induced apoptosis (54).

A decade ago, it was shown that combined exposure to ATRA and G-CSF in vitro could restore maturation, although incomplete, in a high percentage of non-APL AML cases (55, 56). These findings heralded a new therapeutic strategy based on the possibility of reinduction of non-APL AML cells susceptible to overcoming the maturation block when efficiently stimulated. Unfortunately, at that stage, and even later (19), the molecular background of butyrate sensitivity had not been elucidated yet, and those AML cases had not been exhaustively studied from the molecular point of view. Given their good tolerance in clinical trials and established antitumor activity, more HDACi are being synthesized (57). Their characterization with respect to specific cellular targets and the pattern of molecular sensitivity will contribute to transforming the clinical use of HDACi from an empirical treatment to a molecularly tailored therapy.

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GRANULOCYTIC MATURATION AFTER BUTYRATE TREATMENT OF LEUKEMIC BLASTS


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