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Short-chain fatty acid derivatives stimulate cell proliferation and induce STAT-5 activation

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Current chemotherapeutic and butyrate therapeutics that induce fetal hemoglobin expression generally also suppress erythropoiesis, limiting the production of cells containing fetal hemoglobin (F cells). Recently, selected short-chain fatty acid derivatives (SCFADs) were identified that induce endogenous γ-globin expression in K562 cells and human burst-forming units–erythroid and that increase proliferation of human erythroid progenitors and a multilineage interleukin-3–dependent hematopoietic cell line. In this report, γ-globin inducibility by these SCFADs was further demonstrated in mice transgenic for the locus control region and the entire β-globin gene locus in a yeast artificial chromosome and in 2 globin promoter-reporter assays. Conditioned media experiments strongly suggest that their proliferative activity is a direct effect of the test compounds. Investigation of potential mechanisms of action of these SCFADs demonstrates that these compounds induce prolonged expression of the growth-promoting genes *c-myb* and *c-myc*. Both butyrate and specific growth-stimulatory SCFADs induced prolonged signal transducer and activator of transcription (STAT)-5 phosphorylation and activation, and *c-cis* expression, persisting for more than 120 minutes, whereas with IL-3 alone phosphorylation disappeared within minutes. In contrast to butyrate treatment, the growth-stimulating SCFADs did not result in bulk histone H4 hyperacetylation or induction of $p21^{Waf/Cip}$, which mediates the suppression of cellular growth by butyrate. These findings suggest that the absence of bulk histone hyperacetylation and p21 induction, but prolonged induction of *cis, myb, myc,* and STAT-5 activation, contribute to the cellular proliferation induced by selected SCFADs. (Blood. 2001;97:3259-3267)

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Introduction

Butyrate analogs have been of interest as potential therapeutics for the β-globin disorders following the demonstration that butyrate could transcriptionally activate developmentally silenced fetal and embryonic globin genes in many experimental conditions.[1-11] In clinical trials, arginine butyrate stimulated hemoglobin F (HbF) synthesis to levels above 20% and induced a nearly 2-fold increase in the amount of HbF/cell and in proportions of red blood cells expressing HbF (F reticulocytes) in patients with sickle cell anemia.[10-12] With constant use, however, the activity of the drug in maintaining substantial increases in HbF-containing erythrocytes gradually decreased.[13] We hypothesized that this tachyphylaxis was due to the coincident cellular growth-inhibitory properties of butyrate and developed intermittent or "pulse" regimens (4 days of use per month) to surmount this problem.[12] Although this regimen proved effective, it also imposed severe limitations on the amount of drug that can be delivered to the patient. An orally bioavailable transcriptional inducer of γ-globin that does not have cellular growth-inhibitory properties and could be administered more frequently to stimulate a further increase in the proportion of HbF-containing cells (F cells) would be therapeutically advantageous for β-globin disorders.[14]

Butyrate (at 1-6 mM) inhibits cellular proliferation and entry into S phase, in a wide variety of cell types,[15-22] consistent with a reversible cell cycle arrest at the G_1 phase of the cell cycle.[23-27] The precise mechanisms of butyrate-induced G_1 arrest have not been completely elucidated. Mitogen-stimulated progression through the replicative cell cycle is mediated by intracellular signal transduction events involving generation of second messengers, activation of protein kinase cascades, and gene transcription.[28-30] It is probable that butyrate-induced growth arrest results from perturbation of these crucial mitogenic signaling events. Indeed, several investigators have demonstrated effects of butyrate on mitogenic signaling events that are considered important for normal cell cycle progression. Butyrate blocks cyclin D1 induction/activation in response to mitogens or $p21^{Ras}$, blocks downstream signals generated by cyclin D1, and induces the cyclin-dependent kinase inhibitor $p21^{Waf/Cip}$.[31,32] Levels of $p21^{Waf/Cip}$ are generally low in quiescent cells but rise in response to mitogenic stimulation or treatment with butyrate.[31]

To improve upon arginine butyrate as a therapeutic for induction of the fetal globin gene (γ-globin), we designed several short-chain fatty acid derivatives (SCFADs) and tested them to determine (1) the types of functional groups that could be substituted for a carbon yet retain γ-globin gene–inducing actions and (2) what substitutions of these types of structures prolong the half-life of the compound in vivo. To confer resistance to β-oxidation and to β-glucuronidation, substitutions with methyl groups,

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Submitted June 29, 2000; accepted January 16, 2001.

Supported by National Institutes of Health grants DK-52962, HL-61208, and CA-084193.

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addition of halogens or methoxy and phenoxy groups, and branched chains, which result in steric hindrance to cleavage by the enzymes of oxidative metabolism, have been effective. Three prototype compounds, α-methylhydrocinnamic acid, phenoxyacetic acid, and 2,2 dimethylbutyric acid induce both γ-globin expression and growth of erythroid progenitors from normal subjects, hemoglobinopathy patients, and fetal samples.[11,33-35] These third-generation fatty acid derivatives share some, but not all, of the biochemical, molecular, and functional properties of the parent compound, butyrate.[11,33-35] For example, these compounds increased fetal globin production but did not inhibit erythroid colony (burst-forming unit–erythroid, or BFU-E) growth, and certain compounds even stimulated human BFU-E proliferation beyond that induced by high concentrations of a panel of hematopoietic growth factors.[33] Used in vivo, one compound increases total red blood cell counts in baboons by 2 g/dL in 2 weeks, despite ongoing phlebotomy.[34-35]

To investigate potential mechanisms of action of these compounds, we analyzed their effects on the 32D cell line, which is dependent upon interleukin (IL)-3 for growth, undergoes apoptotic cell death in the absence of IL-3, and survives but does not proliferate when IL-3 concentrations are reduced by 50-fold below the levels required for maximal proliferation.[36-38] No experimental condition or growth factor has previously been found to abrogate the IL-3 dependency of this cell line. Under conditions of IL-3 depletion, 32D cells can also proliferate in response to erythropoietin (EPO) or terminally differentiate into mature granulocytes in the presence of granulocyte colony-stimulating factor (G-CSF).[36-39] Strikingly, some of the SCFADs that stimulate γ-globin expression also supported proliferation of this multilineage cell line and prevented apoptotic cell death when IL-3 was withdrawn.

The molecular basis for the mitogenic or IL-3–sparing activity of these prototype SCFADs has not yet been established. The growth and differentiation processes of hematopoietic cells are regulated through the interaction of several hematopoietic growth factors, including EPO and IL-3, with their specific cell surface receptors. These cytokine receptors activate the Janus kinase (JAK) family of tyrosine kinases.[40] The activation of JAKs leads to the recruitment of a class of latent cytoplasmic transcriptional factors known as signal transducers and activators of transcription (STATs). After phosphorylation of the STATs, they dissociate from the receptor, form heterodimers or homodimers through reciprocal SH2 domain–phosphotyrosine interactions, undergo nuclear translocation, and activate specific genes through binding to specific DNA promoter sequences.[40] Both the IL-3 receptor and the EPO receptor signal through activation of JAK-2 with subsequent activation of STAT-5.[41] STAT-5 activity is normally tightly controlled, peaking 5 to 30 minutes after stimulation and returning to baseline within 1 to 2 hours.

We show in this report that certain SCFADs, which induce γ-globin expression and stimulate growth of the IL-3–dependent 32D cell line—with kinetics similar to EPO—also induce the expression of the STAT-5–inducible growth-related immediate early genes c-*myc* and c-*myb* and the STAT-5–dependent transcript *c-cis*. The growth-stimulatory SCFADs and the parental compound butyrate augment both the level of STAT-5 activation and its duration. Unlike butyrate, however, these derivatives have no obvious histone deacetylase (HDAC)-inhibitory activity and do not induce $p21^{Waf1/Cip}$, which contributes to the cell cycle arrest imposed by butyrate. Thus, the new compounds share STAT-5 activation and downstream transcript induction with butyrate but lack its specific growth-inhibitory activities.

Materials and methods

Reporter gene assays

To determine if the SCFADs act at the level of transcription, K562 cells stably transfected with a construct containing the 1.4-kilobase (kb) *Kpn*I-*Bgl*II fragment of the human HS2 of the locus control region (LCR) linked to the γ-globin promoter and the enhanced green fluorescent protein (EGFP) reporter gene were used as previously described.[42] Because EGFP messenger RNA (mRNA) has long stability, positive changes average 2- to 4-fold, and weak inducers are not detectable in this system.[43] The cells were cultured in Iscove's modified Dulbecco's medium, antibiotics, and 2 mM glutamine (Life Technologies, Rockville, MD) containing 10% fetal calf serum and 100 μg/mL Hygromycin-B (Sigma, St Louis, MO) as selection for the transfected construct, and they were treated with 0.5 to 2 mM concentrations of neutral solutions of the SCFADs for 18 hours. Compounds studied included butyric acid, α-methylhydrocinnamic acid, 3-(3,4-dimethoxyphenyl) propionic acid, 2,2 dimethylbutyrate, and phenoxyacetic acid (Aldrich Chemical, Milwaukee, WI). GFP fluorescence was assayed using a Cytofluor 2300 plate reader (Millipore, Bedford, MA). Each condition was assayed in quadruplicate, and each plate was analyzed immediately after the addition of the SCFAD to obtain a baseline (background) measurement. The increase of EGFP fluorescence is represented in arbitrary fluorescence units.

Selected SCFADs were also tested in a second reporter assay, using GM979 cells that were stably transfected with a construct containing the human μLCR linked to the β- and γ-globin gene promoters, each linked in turn to the Renilla luciferase and the firefly luciferase, respectively, as previously described.[44] GM979 cells were cultured in RPMI 1640 media (Life Technologies) with 10% fetal calf serum and G418 (900 μg/mL) (Sigma) alone or with arginine butyrate or 1 of 3 SCFADs described above, at 0.2 to 1 mM concentrations. The 2 luciferases, as reporters for the β- and γ-globin promoters, were assayed in cell lysates with the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's directions, using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA). Proliferation of the untreated and the SCFAD-treated GM979 cells was assessed by enumeration and CellTiter96 (Promega).

Analysis of γ-globin mRNA in transgenic mice

Transgenic mice containing the LCR and the entire β-globin gene locus in a 240-kb yeast artificial chromosome were used to evaluate γ-globin inducibility by the SCFADs in vivo.[45] The animals were treated either with sodium butyrate (1000 mg/kg/d) continuously administered by a subcutaneous osmotic pump (Alza, Palo Alto, CA) or with 1 of 3 prototype test compounds, administered as aqueous solutions in a volume of less than 500 μL, by a single daily intraperitoneal injection for 5 or 7 days. A derivative of acetic acid, butyric acid, and propionic acid were each tested in 2 to 3 transgenic mice. Fifty microliters of blood was collected by retro-orbital puncture daily for 10 to 13 days, and globin mRNA was assayed by ribonuclease protection assay, as previously described.[45] Control mice received the same volume of normal saline for the same duration of time. All procedures were performed with approval of the Institutional Animal Care and Use Committee of the University of Southern Alabama. Statistical analyses were performed using repeated measures analysis of variance with the GB STAT software program to compare the proportions of γ-globin mRNA obtained in each animal at baseline to γ-globin mRNA obtained 3 times sequentially during treatment with test compounds.

Cell culture conditions

The IL-3–dependent 32D cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2mM glutamine (Life Technologies) in 5% CO_2/95% air at 37°C. These IL-3–dependent cells required 25 U/mL

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recombinant murine IL-3 (Biosource International, Camarillo, CA) for optimal proliferation; a 50-fold lower concentration (0.5 U/mL) was required to maintain minimal viability.[34,36] Test compounds were dissolved in medium, neutralized to pH 7.4, and added to the cells at a final concentration of 1 mM. Cells were withdrawn from 25 U/mL recombinant murine IL-3, washed, and resuspended in 0.5 U/mL recombinant murine IL-3 (minimal survival conditions). Recombinant human EPO (3 U/mL) and G-CSF (100 U/mL) (both from Amgen, Thousand Oaks, CA) were also added, and proliferation was assessed. A cell density of 2.5×10^5/mL to 10×10^5/mL was maintained by passing the cells into fresh media at 3-day intervals, with or without the test compounds described above or 2 additional compounds, 2,2 dimethylmethoxyacetic acid (T. E. Neesby, Fresno, CA) and DL-α-amino-n-butyric acid (Sigma), or by concentrating the cells if apoptosis occurred. Viability was assessed with trypan blue dye exclusion on days 1, 3, 6, and 9 of culture. The proportion of viable or apoptotic cells and the fraction of cells in each phase of the cell cycle were assessed by incubation with propidium iodide and fluorescence-activated cell sorter analysis.[34]

Conditioned media experiments

To evaluate whether effects on cell growth might be mediated indirectly through autocrine factors produced by the treated cells, 32D cells were cultured as described above in IL-3 (0.5 U/mL), which supports minimal survival but not proliferation, and the following prototype compounds: arginine butyrate, α-methylhydrocinnamic acid, 2,2-dimethylbutyrate, 3-(3,4-dimethoxyphenyl) propionic acid, and phenoxyacetic acid for 48 hours. The cells were pelleted at 500*g* and resuspended in fresh media with 20 μg/mL aprotinin without any SCFADs for 24 hours. Media from this cellular growth were collected, 0.2 μM filtered, and new 32D cells were cultured in the conditioned media, which were replaced at 3-day intervals. Proliferation was assessed on days 1, 5, and 8 of culture using CellTiter96 (Promega). To determine if autocrine growth-inhibitory proteins were produced by the 32D cells treated with butyrate, the conditioned media were assayed for tumor necrosis factor-α and interferon-γ using enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN) according to the manufacturer's directions.

Northern blot analysis

Expression of several growth-related genes in the presence or absence of SCFADs was evaluated. Northern blot analysis was performed on mRNA extracted from 32D cells cultured in the presence or absence of SCFADs, G-CSF, or EPO under concentrations of low IL-3 (0.5 U/mL), high IL-3 (25 U/mL), or no IL-3. The methods used for RNA blot analysis have been described elsewhere.[31] Briefly, after exposure to test compounds for 1 to 11 days, 1×10^7 to 5×10^7 cells were harvested, washed in phosphate-buffered saline, lysed with RNA denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.1 M β-mercaptoethanol, and 0.5% N-lauroylsarcosine), and frozen at −80°C until further processing. RNA was isolated and Northern blots prepared as previously described.[31] DNA probes for *c-myb*, *c-myc*, and β-actin were obtained from American Type Culture Collection (Manassas, VA).[31] The *c-cis* probe was the generous gift of Dr Olivier Hermine, and the *p21* probe was the generous gift of Drs Steven Farmer and Bert Vogelstein.[46] The *Eco*RI fragment of *c-myb*, *Pst-1* fragment of *c-myc* exon-2, *Pst*I insert of β-actin complementary DNA, *Hin*dIII-*Eco*RI fragment of *c-cis*, and *Eco*RI fragment of *p21* clone were radiolabeled using a random-primer labeling kit (Promega). Globin mRNA levels were quantitated by densitometric scanning of the autoradiograms using an LKB-Ultrascan laser densitometer (Pharmacia LKB, Piscataway, NJ).

Western blot analysis of STAT-5 phosphorylation

Cultured in RPMI medium, 32D cells were washed twice and resuspended at a density of 7.5×10^5/mL with test compounds at 1 mM overnight. To induce STAT-5, the cells were then incubated briefly with IL-3 (25 U/mL), and cellular extracts were prepared at 0, 5, 30, 60, and 120 minutes after IL-3 addition; 1×10^7 cells were lysed with 1 mL ice-cold RIPA buffer (1% Nonidet P-40 [NP-40], 10% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS] in phosphate-buffered saline), 100 mM NaF, 2 mM Na_3VO_4, 10 μg/mL leupeptin, and 1 mM AEBSF (ICN Biochemicals, Irvine, CA). Cellular extracts were immunoprecipitated with 2 μg of a STAT-5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4° C for 18 hours. Immune complexes were recovered by adding 20 μL protein A–agarose beads (Santa Cruz Biotechnology), and the beads were washed 3 times with 1 × RIPA buffer and once with 1 × TNE (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM Na_3VO_4, 2 mM $ZnCl_2$, and 1 mM ethylenediaminetetraacetic acid). Immune complexes were eluted in 40 μL SDS sample buffer by heating at 95°C for 5 minutes, separated on 12% SDS polyacrylamide gels, and transferred to nitrocellulose membranes (Life Technologies). The membranes were blocked with TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween) containing 5% nonfat dry milk and incubated with an antiphosphotyrosine antibody PY99 (Santa Cruz Biotechnology). Bound antibodies were visualized using the Luminol reagent (Santa Cruz Biotechnology) according to the manufacturer's directions. Membranes were incubated at 65°C for 30 minutes with reprobing solution (62.5 mM Tris-HCl [pH 6.8], 0.1 M β-mercaptoethanol, 2% SDS) to strip the antibody and reprobed with STAT-5 antibody to assess the amount of total STAT-5 protein.[31]

Analysis of histone H4 acetylation

Acetylation of histone proteins was evaluated in human K562 cells cultured with the test compounds at 2 mM for 18 hours. The nuclei were isolated in RSB buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 2 mM $CaCl_2$, 5 mM sodium butyrate) with 0.5% NP-40 on ice for 10 to 15 minutes and washed with RSB without NP-40. For antibody detection of acetylated histone proteins, isolated nuclei were lysed in Laemmli's protein loading buffer, and total nuclear proteins were electrophoresed on an SDS-containing 20% polyacrylamide gel. Resolved proteins were transferred onto nitrocellulose membrane and subjected to immunoblotting. Acetylated histone H4 protein was detected as previously described.[31,47,48] An antiacetylated histone H4 with a weak cross-reactivity to acetylated histone H2B rabbit polyclonal immunoglobulin G (Upstate Biotechnology, Lake Placid, NY) was used as the primary antibody at 0.75 μg/mL; goat antirabbit immunoglobulin G (1:1000 dilution) was the secondary antibody.

Results

We previously demonstrated that certain SCFADs stimulated γ-globin synthesis in human BFU-E above the levels found in untreated cultures from the same subject.[33] Furthermore, the same derivatives increased endogenous γ-globin mRNA in K562 cells up to 3-fold.[34] Although butyrate stimulates γ-globin expression at the transcriptional level,[49] a transcriptional mechanism had not previously been studied for the new SCFADs. A γ-globin promoter-driven EGFP reporter system was used here to test for potential transcriptional activation by the SCFADs.[42] EGFP is highly stable, and transcriptional changes in this system average 2- to 4-fold.[43] Four prototype compounds, α-methylhydrocinnamic acid, 2,2 dimethylbutyrate, 3-(3,4-dimethoxyphenyl) propionic acid, and DL-α-amino-n-butyric acid, all induced EGFP by 2- to 3-fold (Figure 1). Exposure to butyrate for longer than one day consistently decreased EGFP expression, which may result from its growth-inhibitory and cytostatic effects.

Both cell proliferation and human γ-globin expression resulting from exposure to prototype derivatives were also examined in GM979 cells, which have extremely low (< 1%) γ-globin promoter activity relative to β-globin promoter activity.[44] Treatment with butyrate increased γ-globin by 230% over baseline levels, and treatment with dimethylbutyrate, α-methylhydrocinnamic acid, and 3-(3,4-dimethoxyphenyl) propionate increased γ-globin promoter activity by 60%, 30%, and 30%, respectively, above control

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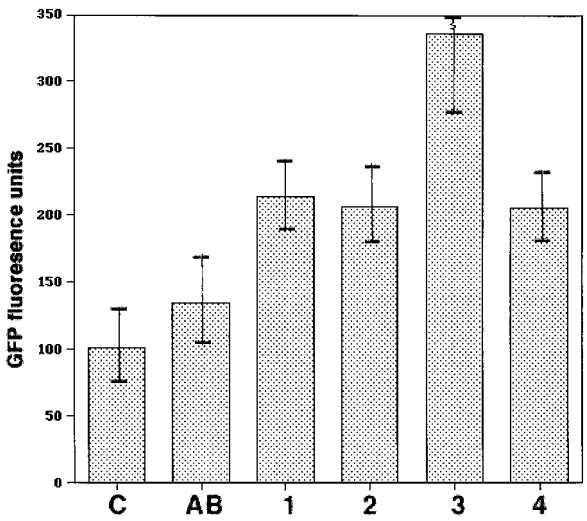

Figure 1. Induction of γ-globin gene transcription by the SCFADs. K562 cells stably transfected with a construct containing HS2 linked to the γ-globin gene promoter and the reporter gene EGFP were cultured with certain SCFADs for 24 hours. The total fluorescence of 2.5×10^5 cells per well was measured in a cytofluorometric plate reader and is expressed in arbitrary units including the standard deviation of each condition performed in quadruplicates. Test compounds were added at 1 mM. C, untreated control cells; AB, arginine butyrate; 1, α-methylhydrocinnamic acid; 2, 2,2 dimethyl butyric acid; 3, 3-(3,4-dimethoxyphenyl) propionic acid; 4, DLα–amino-n-butyric acid.

levels. After 2 days of culture, an increase in cell proliferation was demonstrated in GM979 cells treated with α-methylhydrocinnamic acid or dimethylbutyrate of 66% and 50%, respectively, over control growth; cell growth decreased in cells treated with arginine butyrate and 3-(3,4 dimethoxyphenyl) propionic acid by 50% and 20% of control cell number, respectively. These studies were consistent with our previous findings that 2 of these SCFADs are mitogenic and 1 was growth-inhibitory, and all induced γ-globin promoter expression in this cell line.

To test the activity of the SCFADs in inducing γ-globin expression in vivo, human γ- and β-globin mRNA was assayed in transgenic mice treated with the test compounds. An increase in relative expression of γ-globin mRNA from 1.5- to 3.3-fold above baseline was observed in all 8 animals treated with the SCFADs. The protocol consisted of one daily treatment of 500 mg/kg/d for 5 to 7 days, and induction was observed on days 4 to 7 of treatment, with γ-globin induction continuing through 10 to 13 days of study, when blood sampling was discontinued. These responses were similar to responses induced by treatment with sodium butyrate at 1000 mg/kg/d, which resulted in a mean 1.9-fold increase in γ-globin mRNA over baseline, and these findings were statistically significant (Table 1). These data demonstrate that certain SCFADs induce expression of the human γ-globin genes to a similar or greater degree than does butyrate in vivo. Taken together, these results from 3 different experimental systems strongly suggest the γ-globin induction by the SCFADs is through transcriptional activation.

After establishing induction of the human γ-globin gene promoter by the SCFADs, we next investigated potential effects of the SCFAD on expression of several growth-related genes, including c-*myb* and c-*myc*. EPO induced these genes in 32D cells in culture (observed only on day 11), and mitogenic concentrations of IL-3 induced *c-myb* and *c-myc* throughout the culture period (observed on days 1 and 11, Figure 2A). G-CSF did not support the production of either transcript, with levels less than in control conditions on day 1. During culture for 11 days under conditions (0.5 U/mL IL-3) that support only cell survival and not proliferation, expression of *c-myb* was reduced to 40% of control conditions at day 1, and the steady-state transcript levels of c-*myc* fell to 25% to 40% of control over the treatment period (Figure 2A). Culture in the presence of the stimulatory SCFADs (eg, α-methylhydrocinnamic acid, 2,2 dimethylbutyrate, 2,2 dimethylmethoxyacetic acid, phenoxyacetic acid, or DL-α-amino-n-butyric acid) induced *c-myb* levels by 2.5- to 4-fold above control levels within one day and persisted for the treatment period. *C-myc* increased 2- to 3-fold after treatment with the SCFADs to equal that induced by the high mitogenic concentrations of IL-3 (25 U/mL) and reached relative levels of up to 3.5-fold in response to phenoxyacetic acid and α-methylhydrocinnamic acid (Figure 2B). Early effects on *c-myc* expression (day 1) were observed with 2,2 dimethylbutyrate, phenoxyacetic acid, and α-methylhydrocinnamic acid. In contrast, arginine butyrate had no effect on *c-myc* or *c-myb* expression and caused cell death, even in the presence of 0.5 U IL-3/mL. No viable cells were recovered on day 11 of culture with arginine butyrate.

To determine whether the proliferative effects of the SCFADs on 32D cells were associated with activation of any of the second messengers involved in signaling pathways of IL-3, we examined the activation of STAT-5 in 32D cells treated with arginine butyrate, 3 stimulatory derivatives, or the nonmitogenic SCFAD 3-(3,4 dimethoxyphenyl) propionic acid. IL-3 induces activation of STAT-5 through tyrosine phosphorylation. Pretreatment of 32D cells with arginine butyrate or the mitogenic SCFADs resulted in increased and sustained levels of phosphorylation of the STAT-5 proteins after IL-3 stimulation, as assessed by immunoblotting using an anti-pTyr antibody. Sixty minutes after IL-3 stimulation, 32D cells pretreated with the SCFADs showed significantly higher levels of phosphorylation of STAT-5 than did the control cells, and this phosphorylation was sustained for 2 hours, compared with the more transient phosphorylation induced by IL-3 alone (Figure 3A,B). Treatment with the fourth derivative, 3-(3,4 dimethoxyphenyl) propionic acid, did not result in cellular proliferation, as occurred with the other SCFADs, and phosphorylation of STAT-5 was not observed in cells treated with this compound for the same duration. The lack of both a mitogenic effect and STAT-5 activation

Table 1. Induction of γ-globin mRNA by short-chain fatty acid derivatives in transgenic mice

Animal	Compound tested	Baseline (γ/γ + β)	Treatment (γ/γ + β)	Fold increase	*P* value
W1P	AMHCA	0.33	0.81	2.5	.04
W2P	AMHCA	0.14	0.21	1.5	.02
8618	AMHCA	0.03	0.10	3.3	.05
W2P	DMB	0.17	0.36	2.1	.03
W1P	DMB	0.05	0.08	1.6	.04
1-1P	PAA	0.23	0.42	1.8	.02
5-3P	PAA	0.40	0.80	2.0	.01
7-1P	PAA	0.30	0.71	2.4	.03
3638	SB	0.07	0.12	1.7	.05
8633	SB	0.06	0.13	2.2	.03
8636	SB	0.10	0.19	1.9	.03

The γ-globin mRNA in mice transgenic for the LCR and the entire human β-globin gene locus in a yeast artificial chromosome. Mice were treated with SB 1000 mg/kg/d continuously for 7 days or with 1 of 3 prototype SCFADs (AMHCA, DMB, PAA) given in aqueous solutions at neutral pH as intraperitoneal injections once daily for 5 or 7 days. Doses of the SCFADs were 500/mg/kg, except for animal 1-1P, which received 1000/mg/kg for 1 day and 500/mg/kg for 4 days, and animal 7-1P, which received daily doses of 250/mg/kg for 5 days. Baseline and 3 peak on-treatment γ-globin mRNA levels were compared using repeated measures analysis of variance.

AMHCA indicates α-methylhydrocinnamic acid; DMB, dimethylbutyric acid; PAA, phenoxyacetic acid; SB, sodium butyrate; SCFAD, short-chain fatty acid derivative; LCR, locus control region.

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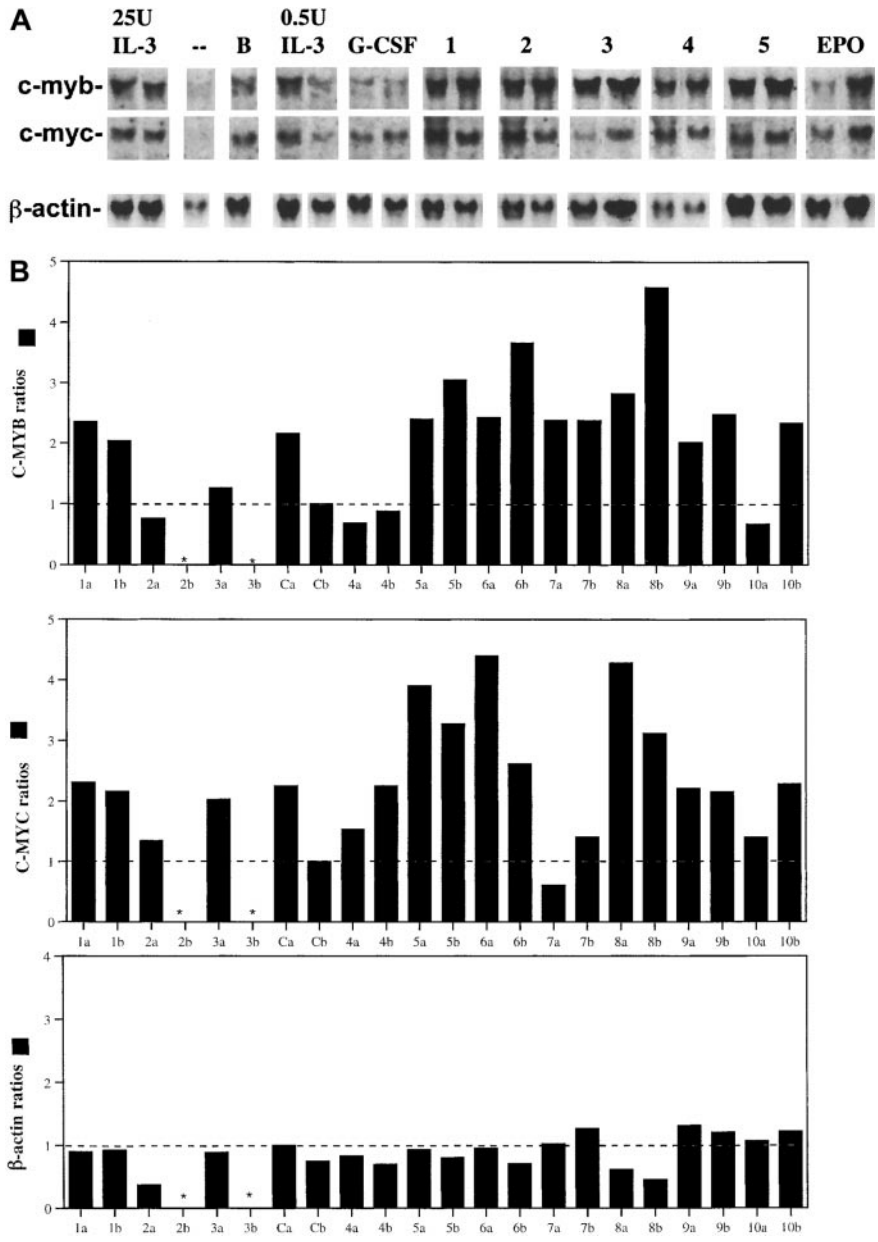

Figure 2. Relative steady-state levels of *c-myb, c-myc*, and β-actin transcripts in 32D cells with hematopoietic growth factors or SCFADs. (A) Cells were cultured for 11 days in high IL-3 (25 U/mL, proliferating conditions), low IL-3 (0.5 U/mL, growth-arresting conditions) or no IL-3 (—), death-inducing conditions. SCFADs were tested at 1mM concentrations with 0.5 U/mL IL-3, growth-arresting conditions. Cells were harvested for RNA analysis at days 1 and 11. The first lane of each pair represents RNA from day 1 cultures, the second lane, RNA derived at day 11 (except with arginine butyrate and no IL-3, where RNA was available only at day 1, with no cells surviving to day 11). Total cellular RNA was separated by agarose gel electrophoresis, transferred to nitrocellulose and hybridized with [^{32}P]-labeled probes specific for *c-myc*, *c-myb,* or β-actin, as a control for loading. The autoradiograms are shown here. Experimental conditions: 25U IL-3, cells cultured in 25 U/mL IL-3; —, cultured without any IL-3; in all the other conditions, cells were cultured in 0.5 U/mL IL-3, which is sufficient for survival, but not proliferation. The control was 0.5 U IL-3, with 0.5 U/mL IL-3; B, 0.5 U/mL IL-3 plus 1mM arginine butyrate; G-CSF, 0.5 U/mL IL-3 plus 100 U/mL G-CSF; 1, 0.5 U/mL IL-3 plus 1mM α methyl hydrocinnamic acid; 2, 0.5 U/mL IL-3 plus 1mM 2,2 dimethyl butyric acid; 3, 0.5 U/mL IL-3 plus 1mM 2-2 dimethylmethoxyacetic acid; 4, 0.5 U/mL IL-3 plus 1mM phenoxyacetic acid; 5, 0.5 U/mL IL-3 plus 1mM DL-α-amino-n-butyric acid; EPO, 0.5 U/mL IL-3 plus 3 U/mL erythropoietin. (B) Quantitation of *c-myc, c-myb,* and β-actin transcript expression levels by densitometric analysis. The treatment conditions identified by number correspond to those described in panel A, and a represents mRNA levels from day 1 cultures; b, day 11 cultures; *, no sample available due to death of the cells by day 11. The data for each transcript are expressed as relative to the levels of that specific transcript found in RNA from day 11 cultures under control conditions (low IL-3, 0.5 U/mL), which were arbitrarily given a value of one. The dashed line in each graph represents the levels of *c-myc, c-myb,* or *β-actin* transcripts in the cells at day 11 under control conditions. The conditions were the same as in part A, and conditions 3-10 had 0.5U/mL IL-3 in addition to 1mM of the SCFA derivatives or cytokines added. 1, 25 U/mL IL-3; 2, no IL-3; 3, arginine butyrate; C, control had 0.5 U/mL IL-3 alone; 4, 100U/mL G-CSF; 5, α methylhydrocinnamic acid; 6, 2,2 dimethylbutyric acid; 7, 2,2 dimethylmethoxyacetic acid; 8, phenoxyacetic acid; 9, α-amino-n-butyric acid; 10, 3U/mL erythropoietin.

by this growth-inhibitory compound is consistent with STAT-5 activation being directly related to cell proliferation induced by the mitogenic SCFADs.

Cis (cytokine-inducible SH2-containing protein) is one of the STAT-5 target genes in hematopoietic cells. Northern blot analysis demonstrated that treatment with mitogenic (25 U/mL, data not shown) or 0.5 U/mL levels of IL-3 resulted in induction of *c-cis* mRNA in 32D cells within 1 hour of exposure; a profound decline in expression was observed by 3 hours after exposure to the growth factor. After exposure to either butyrate or 2 growth-stimulatory SCFADs, *c-cis* was induced by 2-fold compared with the cells under control culture conditions, and expression persisted beyond 3 hours in cells treated with butyrate and the stimulatory SCFADs (Figure 4).

Conditioned media experiments demonstrated no inhibitory effects on proliferation from cells treated with butyrate or stimulatory effects from conditioned media obtained from cells treated with the SCFADs. Further investigation of potential autocrine production of growth-inhibitory proteins in media conditioned from butyrate-treated cells did not detect the presence of 2 common cellular growth inhibitors, tumor necrosis factor-α, and interferon-γ. These findings strongly suggest that the observed effects on cell growth are directly mediated by the test compounds and butyrate.

Butyrate and other SCFADs are known inhibitors of HDACs, and their effects on both gene transcription and cell growth are often attributed to this biochemical activity. To determine if the growth-stimulatory SCFADs also possess global HDAC inhibitory activity, cells treated with 4 different mitogenic SCFADs were examined for bulk histone H4 acetylation by immunoblot analysis using a specific antiacetylated histone H4 antibody. Acetylated histone H4 was only detected after butyrate treatment and was not observed in untreated cells or in cells treated with the SCFADs (Figure 5). This represents a major difference from the activity of butyrate, although it cannot be entirely ruled out whether these compounds have a very weak inhibitory activity or inhibit a subset of cellular histone acetylases that do not contribute to bulk histone acetylation. A second mechanism whereby butyrate has been

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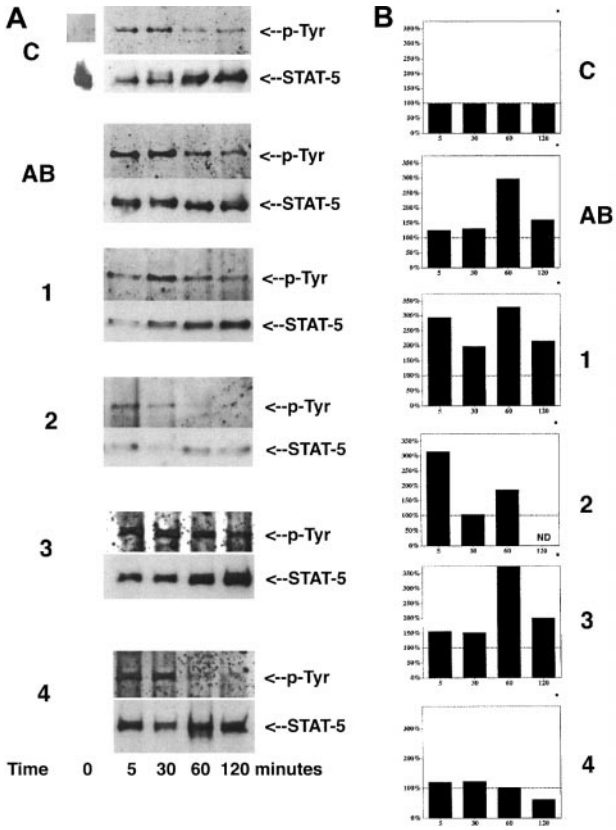

Figure 3. Activation of STAT-5 by treatment with SCFADs. (A) Immunoblot analysis of total STAT-5 protein and phosphorylated STAT-5 protein in 32D cells. 32D cells were deprived of IL-3 for 18 hours, in the presence of various SCFADs, and then stimulated with 25 U/mL IL-3. Total cellular protein extracts harvested at 0, 5, 30, 60, and 120 minutes after IL-3 stimulation were treated with anti-Stat5 antibody, and the immunoprecipitated complexes were separated by SDS-PAGE and transferred to nitrocellulose. Filters were probed first with an antiphosphotyrosine, developed, stripped, and subsequently probed with an anti–STAT-5 antibody and developed. The figure is an autoradiogram of the ECL exposures. Treatments included Control (C); arginine butyrate at 1mM (AB); phenoxyacetic acid at 1mM (1); α methylhydrocinnamic acid at 1mM (2); 2,2 dimethyl butyric acid at 1mM (3); and 3-(3,4-dimethoxyphenyl) propionic acid at 1mM (4). (B) Densitometric quantitation of the films of the ECL immunoblots, expressed as arbitrary units, after subtraction of background and normalization for total Stat5 protein present in the sample. The dashed lines in all bar graphs indicate the level of phosphorylated STAT-5 protein remaining in the IL-3–only control at each specific time point. C, control; AB, arginine butyrate at 1mM; 1, phenoxyacetic acid at 1mM; 2, α-methyl hydrocinnamic acid at 1mM; 3, 2,2 dimethyl butyric acid at 1mM; and 4, 3-(3,4-dimethoxyphenyl) propionic acid at 1mM.

demonstrated to inhibit cell growth involves induction of p21$^{Waf/Cip}$ expression. We therefore compared the activity of butyrate and the mitogenic SCFADs on p21$^{Waf/Cip}$ expression. Butyrate treatment strongly induced p21$^{Waf/Cip}$ mRNA in 32D cells within one day of exposure; the SCFADs had no effect on p21 transcript levels (Figure 6). A schema of these findings, in relation to established cell cycle regulators, is illustrated in Figure 7.

Discussion

Butyric acid and the analog phenylbutyrate inhibit cell growth, and administration of butyrate as a therapeutic must accordingly be limited to 4 days per month in sickle cell disease.[12] We have demonstrated that selected SCFADs can stimulate hematopoietic cell growth and abrogate requirements for the multipotential growth factor IL-3. In this report, we have extended the study of the

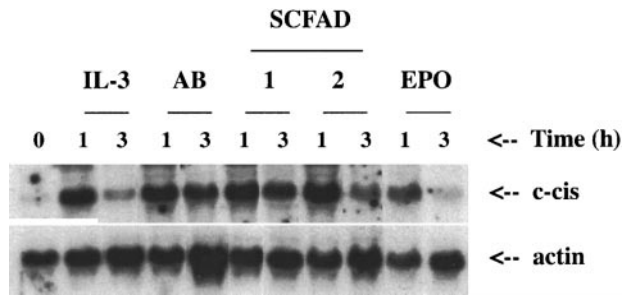

Figure 4. Northern blot analysis of *c-cis* expression in 32D cells with and without treatment with the SCFA derivatives. After the addition of 0.5 U/mL IL-3 at time zero, mRNA was prepared from the 32D cells at 0, 1, and 3 hours, as labeled above each lane. In the control lane (IL-3) 0.5 U/mL IL-3 was added at time 0, and in the EPO lane 3 U/mL was added at time 0 after starvation overnight. AB lanes had a concentration of 1 mM arginine butyrate; 1, 1 mM α methylhydrocinnamic acid; 2, 1 mM 2,2 dimethylbutyric acid; the cells were starved overnight in these concentrations and at time zero 0.5 U/mL IL-3 was added.

growth-stimulatory effects of specific SCFADs. We demonstrate here that these derivatives further stimulate the activity of a chromosomal γ-globin promoter in 2 reporter assays and in vivo in an animal model to a degree similar to that observed with treatment with butyrate. The compounds also induce the growth-related genes *c-myb* and *c-myc* and a STAT-5 target gene, *c-cis*. Although these 2 activities are molecularly distinct, the combination of a likely transcriptional induction of γ-globin expression by these compounds and their stimulatory effects on erythropoiesis should result in an increase in F cells that is severalfold greater than that achievable by arginine butyrate, as similar dosing restrictions should not be required if these compounds were administered as therapeutics.

The SCFADs do not appear to stimulate mitogenesis though autocrine mechanisms: Hematopoietic progenitor cells that do not respond to IL-3 or EPO still respond to the SCFADs, and conditioned media experiments did not yield any evidence of indirect growth stimulation by SCFAD-conditioned media or growth inhibition by arginine butyrate-conditioned media. Our finding that the magnitude and duration of STAT-5 phosphorylation is enhanced by both the growth-stimulatory SCFADs and by butyrate, but not by a nonmitogenic SCFAD, strongly suggests that STAT-5 may be a mediator of the IL-3–sparing effects of these agents. There appears to be some specificity to the action of the growth-stimulatory SCFADs for STAT-5. There are 6 STAT family members with redundancy in the ability of these members to activate similar profiles of genes when ectopically expressed.[44,50,51] We have previously found that STAT-2 is *not* phosphorylated in response to the SCFADs (or in response to EPO or IL-3). Although knockout studies indicate that STAT-5 activation may not be

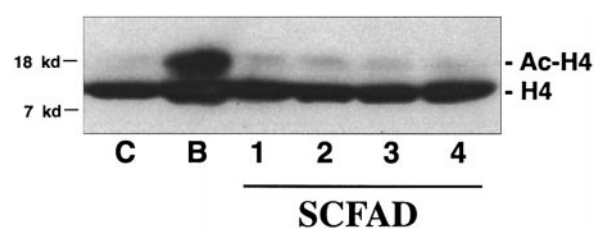

Figure 5. Histone deacetylase inhibitory activity of butyrate compared to SCFA derivatives. Immunoblot analysis of total nuclear proteins from K562 cells separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody to acetylated histone H4. Cells were left untreated (C) or treated for 18 hours with the following test compounds at 1 mM: arginine butyrate (B); α methyl hydrocinnamic acid (1); 2,2 dimethylbutyric acid (2); phenoxyacetic acid (3); or 3-(3,4-dimethoxyphenyl) propionic acid (4).

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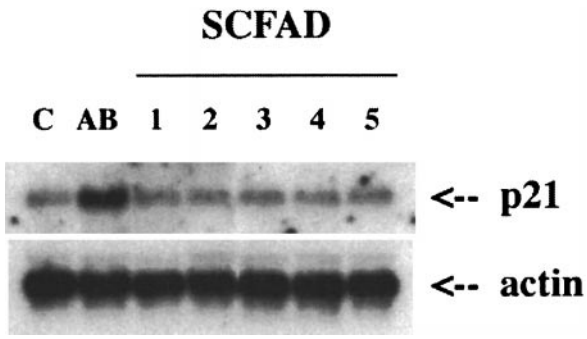

Figure 6. Northern blot analysis of p21 expression in 32D cells with and without treatment with SCFADs. The mRNA was prepared from cells cultured for 1 day. p21 expression was induced 3-fold with arginine butyrate (AB) compared to control (C) 0.5 U/mL IL-3 alone. In contrast, the short-chain fatty acid derivatives, 2,2 dimethylbutyric acid (1); α-methylhydrocinnamic acid (2); DL-α amino-n-butyric acid (3); phenoxyacetic acid (4); and 2,2 dimethylmethoxyacetic acid (5) were not significantly different from the control cells treated with 0.5 U/mL IL-3 alone.

required for IL-3 signaling in mice, use of a dominant-negative STAT-5 (Tyr694Phe, deficient in DNA binding) has established the need for STAT-5 activation in IL-3 and EPO signaling in the 32D cells studied here.[50-53] Regardless of whether it is required in IL-3 signaling, STAT-5 activation clearly suffices to induce the proliferation of IL-3 or GM-CSF–dependent primary cells and cell lines.[50,54-57]

Butyrate or the SCFADs do not increase levels of STAT-5 protein. The phosphorylation status of the STATs is the only known regulator of their DNA binding and transcriptional activity.[55] Thus, the augmentation of STAT-5 activity by stimulatory SCFADs could be due to (1) increases in the activity of STAT-5 kinases, (2) alteration of phosphorylated STAT-5 half-life,[58] or (3) changes in the activity of the phosphatase that inactivates STAT-5. Regarding the first potential mechanism, the best-characterized STAT kinases are the JAKs,[40] and no changes were found in JAK activation with these compounds (unpublished data, 1998). With respect to the second possible mechanism, butyrate has been reported to inhibit the 26S proteosome and induce accumulation of ubiquitinated proteins, promoting their degradation.[59] However, in preliminary studies no effect of SCFADs was found on general proteosome

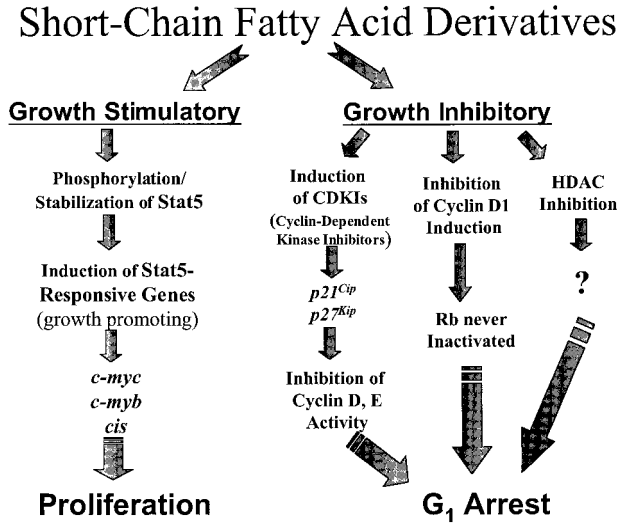

Figure 7. Schema of signaling pathways. Butyrate induces components of both signaling pathways, growth inhibitory and growth stimulatory. Treatment of 32D cells with the selected short-chain fatty acid derivatives results in activation of the growth-stimulatory signaling events shown.

inhibition, making this mechanism less likely. Regarding the third possible mechanism, the nuclear phosphatase responsible for dephosphorylating and inactivating the STATs is not yet known.[60,61] The growth-stimulatory SCFADs do not structurally resemble phosphatase-inhibitors, however, and butyrate itself has been reported to activate, not inhibit, a cellular phosphatase.[62]

The possibility that the SCFADs might act by "augmenting" a subthreshold cytokine response can also be considered. For example, insulin-like growth factor-1 has been shown to augment EPO-induced proliferation of an EPO/IL-3–dependent cell line by enhancing phosphorylation of STAT-5.[52,53] The mechanism appears to be via enhancement of EPO activation of STAT-5 through independent activation of a Ras-mediated pathway by insulin-like growth factor-1. The 32D cells studied here require a very low level of IL-3 (submitogenic) for viability (to prevent apoptosis) and for mitogenic responses to EPO or the SCFADs, and IL-3 in this role may act as a "survival factor" rather than a growth factor. We did not detect any growth stimulation effects from media conditioned by the SCFADs. It is possible, though, that the SCFADs are somehow augmenting this subthreshold IL-3 survival signal into a mitogenic signal.

These studies demonstrated that 2 known target genes of STAT-5, c-*myc* and c-*myb*,[55,63] are activated in cells treated with the stimulatory SCFADs and were not activated by a derivative that did not stimulate cell proliferation. These genes are induced by multiple mitogenic stimuli. The c-*myb* transcription factor has dual functions in hematopoietic cells in that it regulates both genes that prevent apoptosis and genes involved in cellular proliferation.[63,64] The ectopic expression of c-*myb* can prevent growth arrest of differentiating hematopoietic cells. Overexpression of c-*myb* in 32D cells blocks the ability of these cells to terminally differentiate, resulting in indefinite growth in the presence of G-CSF.[64]

Notably, butyrate is as effective at enhancing STAT-5 phosphorylation and c-*myb* and *c-cis* as are the SCFADs, yet butyrate is clearly not growth-stimulatory. In fact, although butyrate arrests normal cells in G_1, it induces apoptosis in transformed cells within 4 hours after G_1-S arrest.[58,65-77] Thus, the activities of butyrate that cause cell cycle arrest may be problematic when the drug is used in patients with hemoglobinopathies unless it is given intermittently, which, in turn, limits the amount of γ-globin and the proportions of F cells that can be induced.

Analysis of the mechanistic differences between butyrate and the growth-stimulatory SCFADs may shed some light on the mechanisms whereby butyrate arrests cells. Transitions between different phases of the cell cycle are regulated by the activities of cyclin-dependent kinases (CDKs). CDK activities are, in turn, dependent upon the expression of appropriate cyclin proteins.[30,78,79] Thus, progression through G_1 and into S phase requires the sequential expression of cyclins D, E, and A and concomitant activation of their CDK partners. The CDK inhibitor, $p21^{Waf1/Cip}$, which negatively regulates cell cycle progression in G_1, is induced to high levels in response to multiple antiproliferative stimuli.[46,80,81] Butyrate-treated cells express high levels of $p21^{Waf/Cip}$ transcripts relative to untreated control cells.[31,82] The marked induction of p21 by butyrate, in contrast to the lack of induction by the growth-promoting SCFADs, may explain why these compounds activate STAT-5, *myb*, and *myc*, yet only butyrate suppresses growth.

Because of the known activity of butyrate as an HDAC inhibitor, this activity has been proposed as the mechanisms of $p21^{Waf/Cip}$ gene induction and the subsequent G_1 cell cycle arrest. Nucleosomal histone acetylation is determined by the opposing actions of acetyltransferase and deacetylase enzymes.[83-86] The

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charge neutralization of core histones is thought to induce a chromatin structure transition that increases accessibility of nucleosomal DNA to sequence-specific DNA-binding proteins. By inhibiting deacetylases with butyrate or with trichostatin A,[47] acetyltransferases act unopposed, leading to global histone hyperacetylation. Yet, despite the "global" changes in acetylation, most cells exhibit only a reversible growth inhibition and trichostatin A is reported to alter the expression of only 2% of cellular genes.[87]

Histone H4 and its acetyl forms are used as indicators of global histone acetylation. In the absence of butyrate, most H4 is a nonacetylated and monoacetylated species. After treatment with 0.5 to 5 mM butyrate or the potent inhibitor of HDAC, trichostatin A, tetra-acetylated H4 is the major isoform among the H4 species. Sodium or arginine butyrate, phenylbutyrate, and phenylacetate are all strong inhibitors of HDACs, inducing bulk H4 acetylation, and all show antiproliferative properties, causing G_1 arrest in normal cells. In contrast to butyrate, none of the growth-promoting SCFADs tested induced bulk H4 hyperacetylation. Although these compounds might still theoretically inhibit a subset of HDACs that have gene-specific effects not detected by measuring bulk histone acetylation, the findings strongly suggest a correlation between bulk HDAC-inhibitory activity and cell cycle arrest. This finding also dissociates global histone hyperacetylation from stimulation of γ-globin gene transcription. Until this time, the small molecules known to stimulate γ-globin expression have been HDAC inhibitors that strongly induce bulk histone hyperacetylation or chemotherapeutic agents that suppress the bone marrow.[88]

In summary, these findings demonstrate that selected SCFADs that induce γ-globin gene expression, growth-related gene expression, and cell proliferation lack 2 activities of butyrate that are associated with cell growth arrest. These candidates for HbF-enhancing therapeutics and related stimulatory SCFADs should not require restricted administration to avoid inhibiting erythropoiesis and thus have potential to produce several-fold more F cells in vivo than do butyrate or phenylbutyrate. These derivatives also have potential as oral therapeutics for stimulating erythropoiesis in anemias of other etiologies.

Acknowledgments

We thank Drs Olivier Hermine, Steven Farmer, and Bert Vogelstein for generously providing probes and Dr George Stamatoyannopoulos for generously providing GM979 cells.

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