Treatment of spinal muscular atrophy by sodium butyrate

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Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of the anterior horn cells of the spinal cord, leading to muscular paralysis with muscular atrophy. No effective treatment of this disorder is presently available. Studies of the correlation between disease severity and the amount of survival motor neuron (SMN) protein have shown an inverse relationship. We report that sodium butyrate effectively increases the amount of exon 7-containing SMN protein in SMA lymphoid cell lines by changing the alternative splicing pattern of exon 7 in the *SMN*2 gene. *In vivo*, sodium butyrate treatment of SMA-like mice resulted in increased expression of SMN protein in motor neurons of the spinal cord and resulted in significant improvement of SMA clinical symptoms. Oral administration of sodium butyrate to intercrosses of heterozygous pregnant knockout-transgenic SMA-like mice decreased the birth rate of severe types of SMA-like mice, and SMA symptoms were ameliorated for all three types of SMA-like mice. These results suggest that sodium butyrate may be an effective drug for the treatment of human SMA patients.

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of anterior horn cells of the spinal cord, leading to muscular paralysis with muscular atrophy. Clinical diagnosis of SMA is based on findings of progressive symmetric weakness and atrophy of the proximal muscles. Affected individuals usually are classified into three groups according to the age of onset and progression of the disease. Children with type I SMA are most severely affected and usually have SMA symptoms before the age of 6 months and rarely live beyond 2 years. Type II and type III SMA are milder forms and the age of onset of symptoms varies between 6 months and 17 years. SMA is one of the most common fatal autosomal recessive diseases in children with a carrier rate of 1–2% in the general population and an incidence of 1 in 10,000 newborns (1). No specific treatment is currently available for SMA patients.

Two survival motor neuron (SMN) genes (*SMN*) are typically present on 5q13: *SMN1* (also known as SMN^T, *SMNtel*) and *SMN2* (also known as SMN^C, *SMNcen*). Loss-of-function mutations of both copies of the telomeric gene, *SMN1,* are correlated with the development of SMA (2–5). The nearly identical centromeric gene, *SMN2*, appears to modify disease severity in a dose-dependent manner, as SMN protein levels from this gene are correlated with disease severity (6, 7). However, the expressed amount of intact SMN protein from *SMN2* does not provide adequate protection from SMA (8).

Although *SMN1* and *SMN2* encode identical proteins, all three forms of proximal SMA are caused by mutation in the *SMN1* gene, but not in the *SMN2* gene (2–5). The differences between these highly homologous genes are in their RNA expression patterns (9–12). Most *SMN2* transcripts lack exons 3, 5, or most frequently, 7, with only a small amount of full-length mRNA generated. On the other hand, the *SMN1* gene expresses mostly a full-length mRNA, and only a small fraction of its transcripts are spliced to remove exons 3, 5, or 7 (11, 12). Recent studies also have shown that an AG-rich exonic splice enhancer in the center of *SMN* exon 7 is required for constitutive inclusion of exon 7 (13). These findings also imply that the low levels of full-length SMN protein produced by *SMN2* are insufficient to protect against disease development (6, 7). Clearly, the total amount of full-length oligomerization-competent SMN protein is a critical SMA determinant, and the amount of SMN protein correlates with the severity of pathologies (14). In addition, there is a strong correlation between the *SMN2* copy number and phenotype in human SMA and SMA-like mice (5–7, 15, 16).

We recently developed a SMA mouse model that genotypically and phenotypically mimics human SMA (15). The severity of pathology in the knockout-transgenic mice is correlated with the amount of intact SMN protein. The difference between *SMN1* and *SMN2* gene expression is the number of full-length transcripts and the amount of SMN protein, and all 5q-linked SMA patients have at least a single intact copy of *SMN2*. Drugs that modify the pattern of *SMN2* transcript in SMA patients to increase full-length *SMN* mRNA expression and the amount of SMN protein may have a therapeutic effect on SMA patients. As a step toward designing a therapeutic protocol for SMA patients, we used Epstein–Barr virus-transformed lymphoid cell lines from SMA patients to screen a series of drugs for their possible effect on the expression of the *SMN2* gene. One drug that was found to be effective was then used to treat our SMA-like mice to determine its potential for the treatment of human SMA.

SR proteins (Ser-Arg proteins) constitute a family of pre-mRNA splicing factors that are highly conserved throughout the metazoa (17, 18). These proteins have multiple functions in splicing. Biochemical experiments have provided strong evidence that SR proteins play essential roles in general, or constitutive, splicing. They seem to be equally important in splicing regulation, through their ability to modulate selection of alternative splicing sites in a concentration-dependent manner, which contributes to activation (and repression) of splicing through interaction with elements in the pre-mRNA known as splicing enhancers (or silencers) (19–21). Recently, Lorson and Androphy (13) demonstrated that an AG-rich exonic splice enhancer in the center of *SMN* exon 7 is required for inclusion of exon 7, and Hofmann *et al.* (22) further demonstrated that Htra2-β1, an SR-like splicing factor, promoted the inclusion of *SMN* exon 7, stimulating full-length *SMN2* expression. Htra2-β1 specifically functioned through and bound to an AG-rich exonic splicing enhancer in *SMN* exon 7 (22). In the present study, we have explored the relationship between the drug's effect and SR protein.

Materials and Methods

Cell Culture. We established Epstein–Barr virus-transformed lymphoid cell lines from different SMA-type patients (five cases

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Abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neuron; RT-PCR, reverse transcriptase–PCR.

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each for types I, II, and III) with deleted *SMN1* genes by using the following procedures (4). Lymphocytes were collected from whole blood of patients by Ficoll hypaque separation. The buffy coat was collected and washed twice with 5 ml PBS. The pellet was resuspended in 5 ml RPMI medium containing 0.5 ml Epstein–Barr virus, 50 μl phytohemagglutinin (0.5 mg/ml), and 50 μl cyclosporine (0.2 mg/ml). Cells were incubated at 37°C with 5% CO_2 until they became viable.

Mice. Five independent human *SMN2* gene transgenic mice were generated and crossed with mice heterozygous for the *Smn* locus knockout. Transgenic mice that were also homozygous for the knockout alleles ($Smn^{-/-}$ *SMN2*) were then generated by crossing with the above mice. These knockout-transgenic mice developed progressive motor-neuron disease similar to that in human SMA patients. The SMA-like mice were classified into three groups based on their phenotypes, which were judged by three authors (J.-G.C., H.-M.H.-L., and H.L.). Mice with the most severe pathological form (type 1) did not develop furry hair and died before postnatal day 10; mice with intermediate severity (type 2) showed poor activity and variable symptoms and died at ≈2–4 weeks; the type 3 mice survived and bred normally, but had short and enlarged tails (15). SMA-like mice (nonpregnant and pregnant) were supplied with sterile water ad libitum and rodent pellets. The sodium butyrate-treated group received sodium butyrate at a concentration of 0.8 mg/ml or 8 mg/ml in distilled water (with no other substances added), beginning immediately after diagnosis or after 15 days of gestation in SMA-like pregnant mice. Both groups consumed ≈5–10 ml per day per mouse. After 1–12 weeks of treatment, the mice were killed, and their organs or tissues were quickly removed and frozen in liquid nitrogen.

Reverse Transcriptase–PCR (RT-PCR) Analysis. RT from total RNA was performed by using a random primer 5′-TN_{10}-3′ and Moloney murine leukemia virus RT. PCR was then used to amplify the single-stranded cDNA by using one or three pairs of primers covering the entire *SMN* coding region. The first primer pair used to amplify the fragment from the 5′ untranslated region to exon 4 was: forward primer, P1, 5′-CGCTGCGCATCCGCGGGTTTGCTATGGC-3′ and reverse primer, P2, 5′-TCCCAGTCTTGGCCCTGGCAT-3′. The second primer pair used to amplify exons 4–6 was: forward primer, P3, 5′-AACATCAAGCCCAAATCTGC-3′ and reverse primer, P4, 5′-GCCAGTATGATAGCCACTCATGTACCATG-3′. The third primer pair amplified from exon 6 to exon 8 was: forward primer, P5, 5′-CTCCCATATGTCCAGATTC-TCTTGATGATGC-3′ and reverse primer, P6, 5′-ACTGCCTCACCACCGTGCTGG-3′. P1 and P6 were used to amplify the full-length *SMN* cDNA. The PCRs were performed as described (15).

Subcellular Fractionation. Fresh frozen spinal cord, brain, and skeletal muscle samples (500 mg) from different types of SMA mice were fractionated as described (15). Tissues were homogenized with a tight-fitting glass pestle in ice-cold buffer A (10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/0.5 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin) with 0.5% Nonidet P-40 and kept on ice for 15 min. The nuclei were pelleted by centrifugation at 800 *g* for 3 min. The nuclear pellet was resuspended by trituration in 100 μl of buffer B (20 mM Hepes, pH 7.9/0.4 M NaCl/1 mM EDTA/1 mM EGTA/1 mM DTT/1 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin) and kept on ice for 15 min followed by centrifugation at 15,000 *g* for 10 min at 4°C. The supernatant (soluble nuclear extract) was removed, and the insoluble nuclear pellet was further sonicated in sonication buffer (100 mM Tris·HCl, pH 7.4/1% SDS/5 mM EDTA/1 mM DTT/1 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin).

Western Blot Analysis and Histopathological Analysis. Synthetic peptides containing part of human SMN exon 7 (amino acids 279–288) and exon 2 (amino acids 72–84) were used to immunize rabbits and to purify specific antibodies (H2 and H7) from rabbit crude sera with an EAH-Sepharose 4B column (Amersham Pharmacia) according to the manufacturer's instructions. Two mouse anti-SR protein antibodies (anti-SRp20 and 16H3), purchased from Zymed, were used to detect the human SR proteins. Protein samples were loaded on a 5% polyacrylamide stacking gel above a 12% separating gel, and the gel was run with a discontinuous buffer using Laemmli's method. After electrophoresis, proteins were transferred electrophoretically to poly(vinylidene difluoride) membranes (Millipore). After the transfer, the membranes were blocked in TBST (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Tween 20) containing 4% BSA for 2 h at room temperature. Blots were incubated with adequate dilution of anti-SMN exon 2 (H2), anti-SMN exon 7 (H7), or anti-SR protein antibodies in TBST for 2 h at room temperature. The blots were washed for three 20-min periods in TBST and then incubated with a 1:32,000 dilution of an anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in TBST for 1 h at room temperature. The reaction was detected by adding 1.5% 5-bromo-4-chloro-3-indoyl phosphate and 3% nitro blue tetrazolium in a developing buffer (100 mM NaCl/5 mM $MgCl_2$/100 mM Tris·HCl, pH 9.5). Histopathological analysis and immunohistochemical staining were performed as described (15).

Statistics. The intensity of the RT-PCR products containing exon 7, lacking exon 7, or SMN and tubulin in Western blot were analyzed by the Collage Image Analysis System to calculate the ratio of these products (12). Results from multiple experiments are expressed as mean ± standard error. Survival data of treated and untreated mice are presented as a Kaplan–Meier plot using the log rank test. A standard χ^2 test was used to assess differences in the frequency of mild or severe phenotype in the SMA-like mice born from treated and untreated mothers, which analyzed the percentage of type 1 (or 2 + 3) newborn mice as a fraction of the total number of pups. Differences with a P value of <0.05 were considered statistically significant.

Results

Sodium Butyrate Changes the Processing of *SMN2* Gene Transcripts. Epstein–Barr virus-transformed lymphoid cell lines from all three types of SMA patients were established and used for drug screening. Several drugs were tested to investigate their potential effect on the expression of the *SMN2* gene by using RT-PCR. Among them, sodium butyrate was able to change the expression pattern of the *SMN2* gene. The amount of exon 7-containing *SMN* mRNAs increased in lymphoid cells cultured with 5 ng/ml to 500 μg/ml of sodium butyrate (Fig. 1*a*). The maximal effect was found after 4 h of stimulation (Fig. 1*b*). Sodium butyrate-treated lymphoid cells from all types of SMA patients showed an increased number of full-length *SMN* transcripts (Fig. 1*c*). To understand the mechanism involved in this change in full-length *SMN* transcript levels, separate RT-PCRs were used to examine the patterns of alternative splicing in exons 3, 5, and 7. We found that the alternative splicing pattern of exons 3 and 5 was unchanged after sodium butyrate stimulation (Fig. 1 *d* and *e*), but that the alternative splicing pattern of exon 7 of the *SMN2* gene changed to the *SMN1* pattern (Fig. 1*f*). Therefore, addition of sodium butyrate in the culture resulted in an increased number of full-length *SMN* mRNA transcripts.

Sodium Butyrate Increases Exon 7-Containing SMN Protein in SMA Lymphoid Cells. To determine whether sodium butyrate-induced expression pattern changes of *SMN2* resulted in an increased amount of exon 7-containing SMN protein, we used different concentrations of sodium butyrate to treat the lymphoid cell

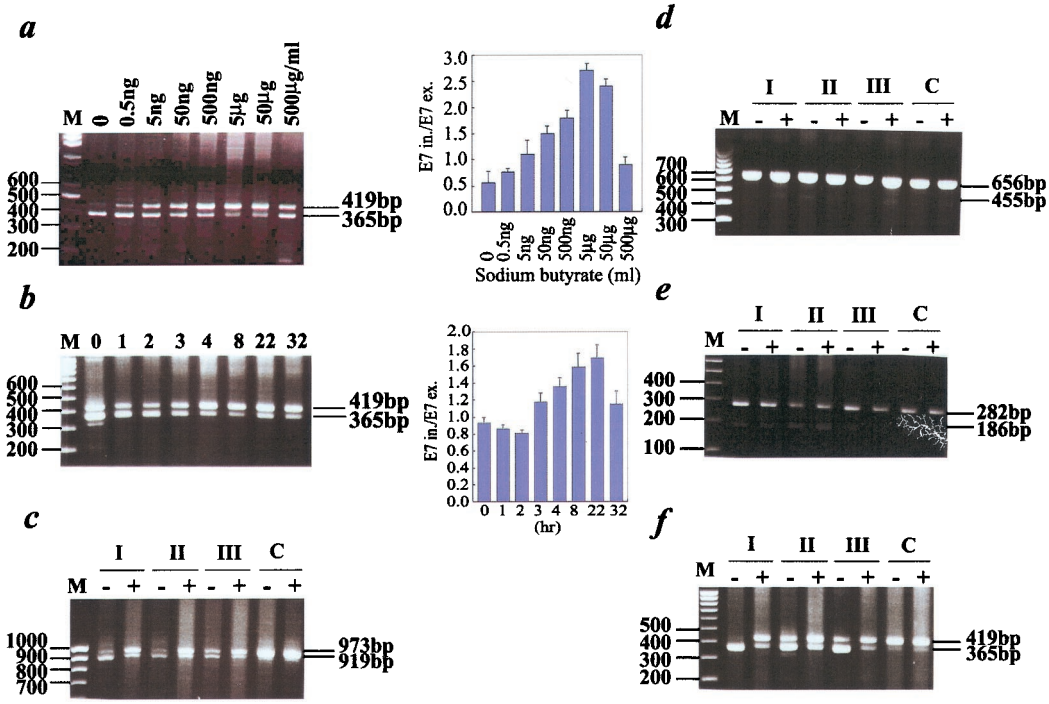

Fig. 1. Effects of sodium butyrate on the expression of the human *SMN2* gene in lymphoid cell lines of SMA patients with *SMN1* deletion. (*a*) RT-PCR analyses of exons 6–8 of the *SMN2* gene showed that the exon 7-containing transcript was increased with 5 ng/ml to 500 μg/ml sodium butyrate treatment. Quantitative analysis of the exon 7-containing transcript is shown on the right, in which the ratio of exon 7 inclusion to exon 7 exclusion is indicated (mean ± SD; $n = 3$). M: 100 bp-ladder marker. E7 in.: exon 7 inclusion; E7 ex.: exon 7 exclusion. (*b*) The SMA lymphoid cell lines were treated with sodium butyrate for 1, 2, 3, 4, 8, 22, and 32 h. RT-PCR analyses of exons 6–8 of the *SMN2* gene showed that the exon 7-containing transcript was increased after 4-h treatment with 500 ng/ml sodium butyrate. A quantitative analysis of the exon 7-containing transcript is shown on the right (mean ± SD; $n = 3$). (*c*) RT-PCR amplification of whole cDNAs (exons 1–8) of *SMN2* genes from different types of SMA lymphoid cell lines showed that the full-length transcript of the *SMN2* gene is very similar to the transcript of the *SMN1* gene after sodium butyrate treatment. (I, II, and III represent different types of SMA lymphoid cell lines; −, untreated; +, treated; C, normal). (*d–f*) RT-PCR analyses of exons 1–4 (*d*), 4–6 (*e*), and 6–8 (*f*) for *SMN2* gene expression showed that only the transcript pattern of exon 7 was changed due to alternative splicing. There was no change for exons 3 and 5.

lines (three cases each for types I, II, and III) established from different types of SMA patients. In both cytosolic and nuclear fractions, Western blot analysis indicated that sodium butyrate also increased the intact SMN protein after 4-h stimulation with 0.5 ng/ml to 500 μg/ml of sodium butyrate (Fig. 2). However, a decreasing effect was found in the cytosolic fraction when more than 5 μg/ml sodium butyrate was used.

Sodium Butyrate Increases Specific SR Proteins in SMA Lymphoid Cell Lines. SR proteins are known to play an important role in the processes of alternative splicing of genes (17, 18), and previous studies have identified a splicing enhancer element in exon 7 of the *SMN* gene (10, 13). To investigate sodium butyrate-induced expression pattern changes of *SMN2* involving the SR protein, we used different antibodies for SR proteins to detect SR protein expression patterns after sodium butyrate treatment. The results showed that two SR proteins of about 27 kDa were induced after treatment, which were detected by using mouse anti-SR protein 16H3 antibody. However, no difference was found by using the mouse anti-SRp20 antibody (Fig. 3*a*). These induced SR protein reactions were blocked by either a specific mitogen-activated protein kinase inhibitor (PD98059) or an inhibitor of protein phosphatases (okadaic acid) (Fig. 3*b*). All lymphoid cell lines (three cases each for types I, II, and III), which were established from different types of SMA patients, showed similar results.

Treatment of Types 2 and 3 SMA-Like Mice with Sodium Butyrate. To investigate whether the *in vitro* effects of sodium butyrate on lymphoid cell lines also occur in SMA-like mice *in vivo*, we used sodium butyrate to treat types 2 and 3 SMA-like mice (15 mice each). Sodium butyrate was administered to SMA-like mice via a 0.8 mg/ml or 8 mg/ml solution available ad libitum in their drinking water for 1–12 weeks. The amount of sodium butyrate consumed by SMA-like mice was estimated to be ≈4–80 mg/day. The sodium butyrate-treated type 2 SMA-like mice survived 4–5 days longer than the untreated ones (Fig. 4). Some of the treated type 2 mice ultimately died from infection caused by traumatic injury of the paralytic hindlimbs.

Our previous study showed that tails of untreated types 2 and 3 SMA-like mice had decreased diameters of muscle fibers, atrophy of muscle bundles, group atrophy, and subcutaneous edema (15). In the present study, after sodium butyrate treatment, the tails of types 2 and 3 SMA-like mice showed nearly normal muscle patterns. Grossly, the tails of treated mice were slightly shorter than normal, and treated mice rarely developed chronic necrosis from the tip of the tail toward the root (2% for the treated group vs. 50% for the untreated group). Histopathologically, the tails of treated mice had few atrophied muscle bundles, and group atrophy and subcutaneous edema were rarely present (Fig. 5). Western blot analysis showed that the exon 7-containing SMN protein level was elevated in different tissues, including motor neurons of the spinal cord (Fig. 6 *a* and *b*). Immunohistochemical studies showed that both exon 2-containing (Fig. 6 *c* and *d*) and exon 7-containing (Fig. 6 *e* and *f*) proteins were increased, which may have resulted from an increase in the total amount of intact SMN protein. Because the severity of the pathological changes in SMA patients and SMA-like mice is correlated with the amount of intact SMN protein present in the spinal cord (6, 7, 15, 16), the effect of oral sodium butyrate,

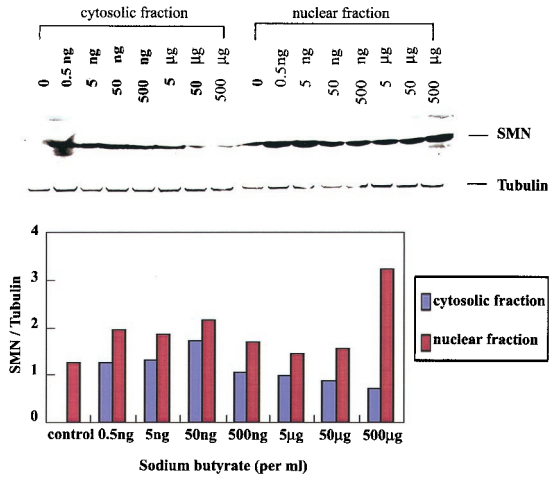

Fig. 2. Effects of sodium butyrate on the expression of the human SMN protein in lymphoid cell line of a representative SMA type I patient. (*Upper*) Western blot analysis showed a gradual increase in exon 7-containing SMN protein expression in nuclear fraction after 0.5 ng/ml to 500 μg/ml sodium butyrate treatment. The amount in the cytosolic fraction decreased with increasing doses (5, 50, and 500 μg) of sodium butyrate. (*Lower*) Quantitative analysis of SMN and tubulin ratios are shown. The control represents an untreated SMA control.

particularly in the spinal cord, may be of therapeutic value for SMA patients.

We also used 16 mg/day and 40 mg/day sodium butyrate solution for the treatment and found no definite toxicity at 16 mg/day sodium butyrate treatment, whereas mice that received 40 mg/day sodium butyrate treatment died because of dehydration.

Treatment of Intercross Heterozygous Knockout-Transgenic Mice after Pregnancy. Because the survival time of type 1 and some type 2 SMA mice is short, evaluation of the therapeutic effect of sodium butyrate is difficult. To overcome these problems, sodium butyrate (4–80 mg/day) was administered ad libitum in

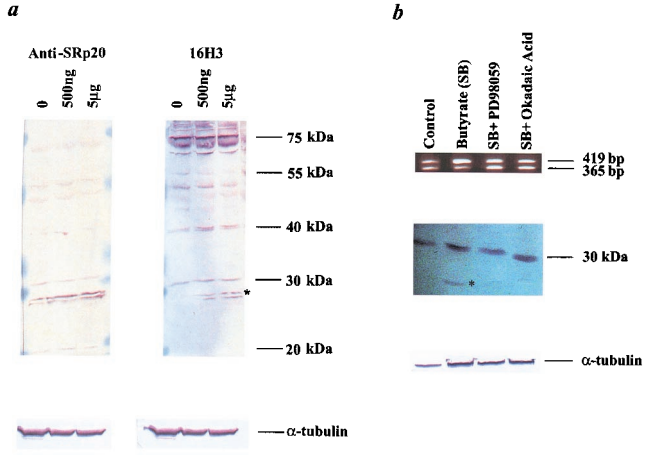

Fig. 3. Effects of sodium butyrate on the expression of human SR proteins in the lymphoid cell line of a representative SMA type I patient. (*a*) Western blot analysis showed two SR proteins (*) were induced after 500 ng and 5 μg sodium butyrate stimulation, which were detected by mouse anti-SR protein mAb (6H3), but no difference was found by using mouse anti-SRp20 mAb. (*b*) The induced SR proteins (*) disappeared after adding mitogen-activated protein kinase inhibitor PD98059 or phosphate inhibitor (okadaic acid), and the expression pattern of *SMN2* also changed to untreated status. The control represents an untreated SMA control.

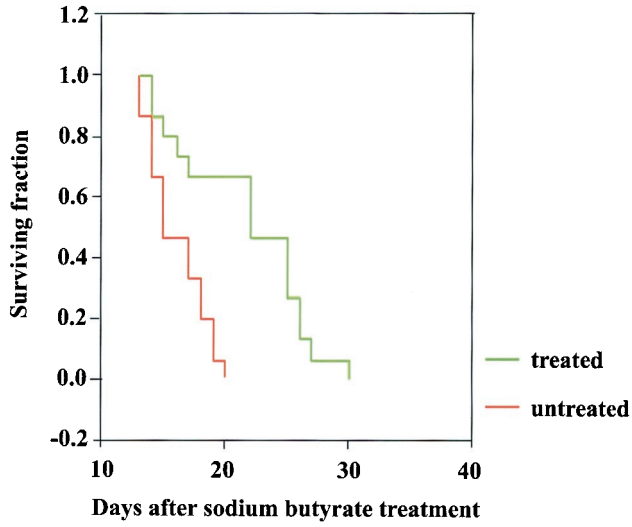

Fig. 4. Survival time of type 2 SMA-like mice after sodium butyrate treatment shown in a Kaplan–Meier survival curve. Sodium butyrate was added to the drinking water after diagnosis of type 2 SMA-like mice that showed poor activity after postnatal day 10; 15 mice were left untreated (red), and 15 mice were treated with sodium butyrate (green) from diagnosis to death. The type 2 SMA-like mice treated with sodium butyrate lived significantly longer than those in the untreated SMA control group (P = 0.0004). Treated group: mean = 21.7 days, range = 14–30 days; untreated group: mean = 15.5 days, range = 13–20 days.

drinking water to pregnant $Smn^{+/-}SMN2$ intercrossed mice, which had previously produced offspring of different types of SMA progeny, especially the severe form (15). Sodium butyrate treatment began on the 15th day postcoitum to avoid a possible teratogenic effect. A total of 21 pups with type 1, 22 with type 2, and 48 with type 3 were born from the treated group; and 35 pups with type 1, 17 with type 2, and 38 with type 3 were born

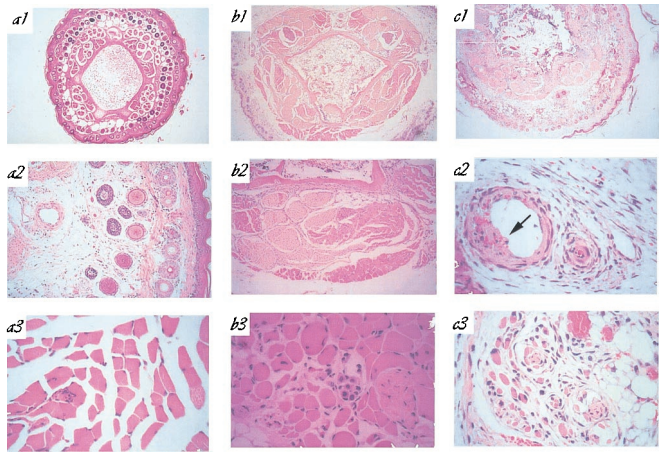

Fig. 5. Histological analysis of a type 3 SMA-like mouse after sodium butyrate treatment. (*a1–a3*) Normal control. (*b1–b3*) Type 3 SMA-like representative mouse after sodium butyrate treatment. (*c1–c3*) Type 3 SMA-like representative mouse with no treatment. Cross sections of tails were stained with hematoxylin and eosin (×40 for *a1*, *b1*, and *c1*; ×100 for *a2* and *b2*; ×400 for *c2*, *a3*, *b3*, and *c3*). The tails of sodium butyrate-treated mice had a few regions of muscular bundle atrophy but no subcutaneous edema (*b1–b3*). The tails of untreated mice had severe muscular bundle atrophy (*c1* and *c3*), thrombus in the vessel walls due to muscular atrophy, resulting in venous blood stasis (*c2*, arrow), and mild subcutaneous edema (*c1*) compared with normal controls (*a1–a3*).

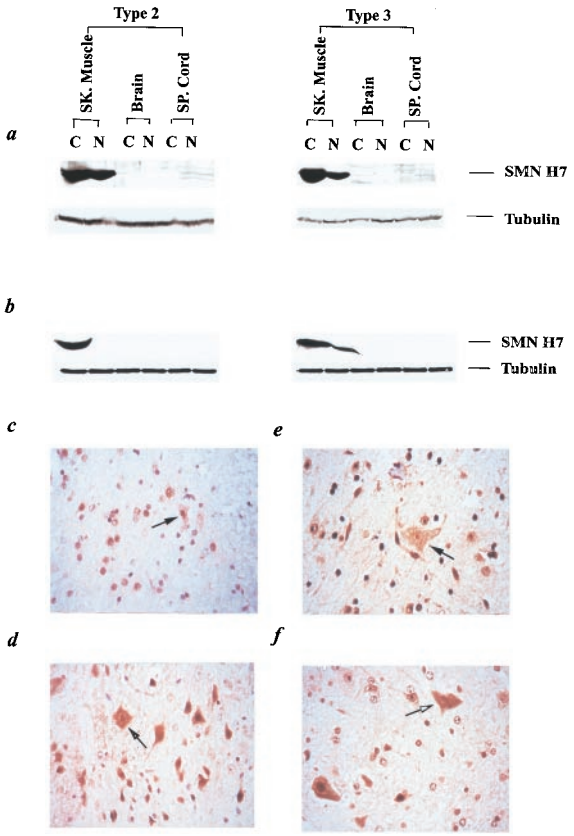

Fig. 6. Expression of human exon 7-containing SMN protein in types 2 and 3 SMA mice after sodium butyrate treatment. (*a*) Western blot analysis of exon 7-containing SMN protein (detected by SMN H7 antibody) in cytosolic (C) and nuclear (N) fractions from different tissues. The exon 7-containing SMN protein in treated mice was increased in skeletal muscle (SK. Muscle) and spinal cord (SP. Cord) after sodium butyrate treatment, especially in the nuclear fraction, compared with untreated types 2 and 3 mice. (*b*) Control incubation was performed with an anti-α-tubulin antibody to determine the relative amount of SMN protein (Tubulin). (*c–f*) Immunohistochemical staining of anterior horn cells of the spinal cord showed an increase of SMN protein in motor neurons of type 2 SMA-like mice after sodium butyrate treatment. (*c*) Untreated (H2 antibody). (*d*) Treated (H2 antibody). (e) Untreated (H7 antibody). (*f*) Treated (H7 antibody). Arrow: motor neuron.

from the untreated group (Table 1). These results show that treatment with sodium butyrate from day 15 of pregnancy significantly ameliorated the clinical symptoms of the severe SMA phenotype, leading to milder types of SMA in offspring (Table 1). In addition, fewer SMA-like mice were born in the untreated group, which may have been due to some severe-type mice being aborted in the fetal stage or eaten by their mothers after birth, and thus remaining uncounted.

Table 1. Phenotype comparison and statistical analysis between pups from sodium butyrate-treated and nontreated mothers (control)

Mouse	Sodium butyrate	Control
Litters	32	46
Total pups	294	364
Type 1	21	35*
Type 2	22	17
Type 3	48	38

*, $P < 0.05$ for statistical analysis of type 1 and type 2 + 3 between treated and untreated groups.

Discussion

The amount of exon 7-containing SMN protein has been shown to be an inverse indicator of disease severity in SMA patients and mice (6, 7, 15, 16). Therefore, increasing the expression of intact SMN protein may have clinically therapeutic effects on SMA patients. In this study, we found that sodium butyrate treatment of human SMA lymphoid cell lines increased the expression of exon 7-containing SMN protein from the *SMN2* gene. The mechanism by which sodium butyrate affects SMN protein expression of the *SMN2* gene involves a change in its RNA splicing pattern of the gene. After sodium butyrate stimulation *in vitro* and *in vivo*, the transcription pattern of *SMN2* changed to an *SMN1*-like transcription pattern, which was nearly identical to the *SMN* pattern in healthy individuals. These findings may have important implications regarding the treatment of SMA patients.

Sodium butyrate has been shown to induce differentiation and apoptosis (23, 24). There is evidence that sodium butyrate may act at the transcription level by increasing the acetylation of histones, thereby releasing constraints on the DNA template and reactivating a number of genes (25, 26). Sodium butyrate also increases the expression of fetal-globin genes in adult baboons, humans, and other animals (27–29). *In utero* infusions of butyrate delay the developmental switch from γ- to β-globin gene expression in sheep fetuses (29). These effects of butyrate may occur through the inhibition of histone deacetylase (25, 26, 29, 30). In the case of *SMN*, sodium butyrate may acetylate nucleosomal DNA and release other factors that control alternating splicing of exon 7 of the *SMN2* gene.

We demonstrated that sodium butyrate induced two specific SR proteins involved in inclusion of exon 7 for full-length *SMN* expression of the *SMN2* gene. These reactions were blocked by either the mitogen-activated protein kinase inhibitor or a phosphatase inhibitor. Our results strongly support that SR proteins are involved in *SMN2* exon 7 inclusion after sodium butyrate treatment.

Approximately 15% of all mutations that cause genetic diseases result from the defective splicing of pre-mRNA (31). A number of these mutations do not alter consensus splice sites or generate missense or nonsense mutations, yet do affect splice site selection (32, 33). These mutations may cause skipping of exon(s) by disrupting the splicing enhancer(s). Our findings suggest that an approach similar to that used in our study may be effective in treating these kinds of genetic diseases as well.

Most SMA patients gradually develop clinical symptoms after birth. We previously demonstrated that the *SMN2* in SMA-like mice expressed only a decreased or nearly normal amount of intact SMN protein in most tissues, except in motor neurons (15). This is why SMA is a disease that directly affects only the motor neurons. The motor neuron-specific splicing factors regulating the inclusion/exclusion of exon 7 in the fetal stage, which are shut down in the spinal cord after birth, may account for the specific defect present in SMA. These factors also may play an important role in genotypic and phenotypic discrepancies. Sodium butyrate inhibits the deacetylation of these phenotype-related genes, modifying the clinical symptoms of SMA in a fashion similar to the mechanism involved in fetal hemoglobin gene expression (25, 26, 30). However, there is a major difference between modification of the γ-globin gene and the *SMN2* gene after butyrate reaction. The transcription of the *SMN2* gene is modified through the alternative splicing of exon 7 rather than directly through the inhibition of histone deacetylation. Gene modification after sodium butyrate treatment not only increased the transcription of *SMN2*, but also changed the splicing pattern of exon 7 of *SMN2* whereas the splicing pattern of exons 3 or 5 remained unchanged. This may be caused by the influence on

exon 7 inclusion of specific SR proteins that are induced by sodium butyrate treatment.

Sodium butyrate and related compounds have been used clinically to treat patients with sickle cell anemia and thalassemia for several years (34, 35). The pharmacokinetics and toxicities of sodium butyrate are well documented; its toxicity is low and has been well tolerated in both human and animal studies (34–37). Our findings suggest that sodium butyrate is an excellent candidate for the treatment of human SMA. In the present study, although sodium butyrate had a therapeutic effect on SMA symptoms, a number of severe types of SMA mice were born to sodium butyrate-treated pregnant mice and a few type 2 mice showed poor response after sodium butyrate treatment. This may have been due to incomplete treatment, or because the increase in the amount of intact SMN protein after treatment was unable to sufficiently compensate, to provide the minimal requirement for motor neuron survival. It is also possible that the timing of treatment was too late after day 15 of pregnancy.

In summary, SMA lymphoid cell lines and SMA-like mice were used to explore possible medication for the treatment of human SMA in this study. We found that sodium butyrate can effectively treat SMA-like mice by changing the expression pattern of *SMN2* and increasing the amount of full-length mRNA of *SMN2* both *in vitro* and *in vivo*. The methods developed in this study may be useful in screening other candidate drugs for SMA treatment. We also demonstrated that the mechanism of action of sodium butyrate involves a modification of the splicing of exon 7 of the *SMN2* gene under the regulation of SR proteins. This study shows that a deacetylase inhibitor can specifically modulate a disease-related defect gene to change its expression pattern, resulting in amelioration of the related symptoms. Our methods also may provide a useful approach for the treatment of other splicing defect-related diseases (31, 38).

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