RAPID COMMUNICATION

Metabolic Persistence of Fetal Hemoglobin

By Jane A. Little, Nancy J. Dempsey, Mendel Tuchman, and Gordon D. Ginder

Hereditary persistence of fetal hemoglobin (HPFH) has typically been ascribed to mutations in the \( \beta \)-globin gene cluster. Pharmacologic agents, including the short-chain fatty acid butyrate, have been shown to upregulate fetal and embryonic globin gene expression. In this report we investigate the possibility that metabolic derangements characterized by an inability to metabolize another short-chain fatty acid, propionate, could be associated with a persistence of fetal hemoglobin unrelated to alterations in the \( \beta \)-globin cluster. Embryonic globin gene upregulation in a murine adult erythroid cell culture was shown by RNase protection after induction with three short-chain fatty acids (C\(_2\)-C\(_3\)). Chart reviews and measurement of fetal hemoglobin in five patients with abnormalities in propionate (C\(_3\)) metabolism were undertaken; SSCP/dideoxy fingerprint analysis of the \( \gamma \)-globin gene promoters was done in three of these five patients. Twelve patients with other metabolic derangements served as controls. Only the four patients with clinically severe abnormalities in propionate metabolism (ages 2 to 11), but without anemia, showed a sustained elevation in fetal hemoglobin (3% to 10%). The level of elevation of fetal hemoglobin in these patients, who lack erythropoietic stress, suggests that propionic acid and/or its metabolites are potent stimulators of fetal hemoglobin expression. Study of this group of patients should allow unique insights into the long-term effects of sustained exposure to elevations of short-chain fatty acid levels.

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Table 1. Clinical Summary

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age (yrs)</th>
<th>Diagnoses</th>
<th>Hb F (%)</th>
<th>Date</th>
<th>Complete Blood</th>
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<tbody>
<tr>
<td>A.L.*</td>
<td>3</td>
<td>Propionic acidemia</td>
<td>8</td>
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<td>WBC 7,300/µL</td>
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<td></td>
<td></td>
<td>Seizures, controlled</td>
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<td>Hb 13.1 g/dL</td>
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<td></td>
<td></td>
<td>Developmental delay, mild, stable to improved</td>
<td>4</td>
<td>5/94</td>
<td>WBC 4,900/µL</td>
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<td></td>
<td></td>
<td></td>
<td>Hb 12.9 g/dL</td>
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<td></td>
<td></td>
<td>Platelets 265,000/µL</td>
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<tr>
<td>K.F.*</td>
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<td>Propionic acidemia</td>
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<td>2/93</td>
<td>WBC 4,100/µL</td>
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<td>Precocious puberty (status post pulsatile</td>
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<td>Hb 12.6 g/dL</td>
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<tr>
<td></td>
<td></td>
<td>GnRH therapy)</td>
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<td>7/93</td>
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<td>Status postgastrostomy</td>
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<td></td>
<td></td>
<td></td>
<td>Hb 11.9 g/dL</td>
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<tr>
<td>M.S.*</td>
<td>5</td>
<td>Propionic acidemia</td>
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<td>1/93</td>
<td>WBC 4,700 (1,100 PMNs)</td>
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<td>Seizures, controlled</td>
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<td>Developmental delay, mild, stable</td>
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<td>3/93</td>
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<td>Intermittent neutropenia</td>
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<td></td>
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<td>Relapsing metabolic decompensation</td>
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<td>4/94†</td>
<td>WBC 7,000/µL</td>
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<tr>
<td>I.M.</td>
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<td>Methylmalonic acidemia</td>
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<td>M.D.</td>
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<td>Homocystinuria</td>
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<td>Citrullinemia</td>
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<td>Hospitalized at analysis with hyperammonemia</td>
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<td>T.C.§</td>
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<td>Ornithine transcarbamylase (OTC) deficiency</td>
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<td>1/93</td>
<td>Hb 14.4 g/dL</td>
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<tr>
<td>J.C.§</td>
<td>12</td>
<td>OTC deficiency</td>
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<td>1/93</td>
<td>WBC 4,200/µL</td>
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<td>B.B.§</td>
<td>45</td>
<td>Hyperammononemia, hyperornithinemia,</td>
<td>4/94†</td>
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<td>WBC 4,900/µL</td>
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<td></td>
<td>homocitrullinuria (&quot;HHH&quot;) syndrome</td>
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<td>Hb 13.6 g/dL</td>
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<td></td>
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<td></td>
<td>Platelets 264,000/µL</td>
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<tr>
<td>S.C.</td>
<td>4.5</td>
<td>Medium chain acyl-CoA dehydrogenase (MCAD)</td>
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<td>1/93</td>
<td>WBC 6,700/µL</td>
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<tr>
<td></td>
<td></td>
<td>deficiency</td>
<td></td>
<td></td>
<td>Hb 12.9 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Platelets 533,000/µL</td>
</tr>
<tr>
<td>D.R.</td>
<td>7</td>
<td>MCAD</td>
<td>&lt;1</td>
<td>1/93</td>
<td>WBC 5,200/µL</td>
</tr>
<tr>
<td>A.R.</td>
<td>5.5</td>
<td>MCAD</td>
<td>&lt;1</td>
<td>1/93</td>
<td>WBC 7,100/µL</td>
</tr>
</tbody>
</table>

All samples were obtained, except as noted, during clinic visits when patients were metabolically stable. No patients had signs/symptoms or family history suggestive of a concomitant hemoglobinopathy. All patients were supplemented with multivitamins, minerals, and trace elements.

* Neither SSCP nor sequence analysis indicated known HPFH point mutations.
† Nierhaus-Betke stain: 8% to 10% cells with Hb F.
§ On acylator agents.

Pholyte-imbedded polyacrylamide gel; densitometric Hb quantitations were made after Coomassie Brilliant Blue staining. The percentage of cells containing Hb F was estimated using a modified Nierhaus-Betke stain, as follows: air-dried blood smears from heparinized blood were fixed in 80% alcohol eluted in hematoxylin, ferric chloride, and alcohol at pH 1.5 and then counterstained with eosin. The percentage of bright pink to RBCs (containing fetal Hb) in 1,000 RBCs was obtained by manual count.

DNA analysis. The structural integrity of the immediate 5' region of the human γ-globin genes was assessed in all three patients.
cell mRNA was protected from RNase digestion with a radiolabeled erythroid differentiation, but only butyrate and pentanoate upregulated embryonic globin gene expression.

In the upper and lower panels, respectively. A probe for triose phosphoisomerase (TPI) served as an internal control. All agents induced erythroid differentiation, but only butyrate and pentanoate upregulated embryonic globin gene expression.

with propionic acidemia using the single-stranded conformational polymorphism (SSCP) technique on a DNA ladder generated through dideoxy nucleotide sequencing (dideoxy fingerprinting)\(^3\); the wild-type \(\gamma\)-globin gene promoter and two known \(\gamma\)-globin HPFH promoter point mutations served as controls. Using standard techniques, DNA was isolated from peripheral blood mononuclear cells7J and analyzed identically, except for a pneumonia before the March visit. She has had intermittent neutropenia, typically associated with metabolic decompensation, and was neutropenic of examination, and none were experiencing acute decompensation.

Fetal Hb was elevated in four of five patients with inherited abnormalities in the metabolism of branched-chain and other amino acids (ie, valine, isoleucine, methionine, and threonine); the clinical history of these patients is discussed below and outlined in Table 1. All five children were being managed with protein restriction and L-carnitine, and none had been treated with phenylactate or phenylbutyrate during this investigation. All evaluations were made at routine clinic follow-up, when the patients were metabolically stable. Serum propionate levels were not available in these children; the diagnoses were typically made shortly after birth through clinical and enzymatic analyses. Glycine levels were obtained intermittently, and serve as an indirect measure of disease severity. When drawn, glycine levels throughout these patients’ lives were typically two to three times normal and did not change markedly over the course of this study.

K.F., 11 years old, was diagnosed at 3 days of age with propionic acidemia when she presented with severe acidosis and hyperammonemia, hyperglycinemia, and characteristic excess urinary excretion of methylcitrate, propionylglycine, and 3-OH propionate. Fetal Hb has ranged from 3% to 10% (Table 1). Of note, K.F. had been hospitalized with metabolic decompensation 2 weeks before a clinic visit on February 21, 1993 (Hb F 5%) and again at 2 months before a February 9, 1994 visit (Hb F 10%). SSCP analysis of the \(\gamma\)-globin genes shows no evidence for a 5’ HPFH point mutation (Fig 2).

M.S. is a 5-year-old child who presented at 2 days of age with neonatal hyperammonemia, hypotonia, and acidosis. Analysis of urinary organic acids suggested propionic acidemia, and enzymatic analysis confirmed propionyl-CoA carboxylase deficiency. Fetal Hb has ranged from 8% to 12%. She has had a clinically difficult course characterized by frequent metabolic decompensations, but was stable at these analyses, except for a pneumonia before the March 1993 visit. She has had intermittent neutropenia, typically associated with metabolic decompensation, and was neutropenic.

Of note, K.F. had been hospitalized with metabolic decompensation 2 weeks before a clinic visit on February 21, 1993 (Hb F 5%) and again at 2 months before a February 9, 1994 visit (Hb F 10%). SSCP analysis of the \(\gamma\)-globin genes shows no evidence for a 5’ HPFH point mutation (Fig 2).

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**RESULTS**

**In vitro culture assay.** Expression of the embryonic murine \(\beta\)-globin gene \((c^\beta)\) in an adult phenotype murine erythro-leukemia cell line was used to screen for agents that could upregulate embryonic globin gene expression in an adult phenotype erythroid cell. Both 1 mmol/L sodium butyrate and 5 mmol/L sodium propionate upregulated embryonic globin gene expression in vitro (Fig 1). Pentanoate was active in this assay system, whereas methylmalonate and acetate, at concentrations in the range of 1 to 20 mmol/L, were not (data not shown).

**Patient analysis.** Fetal Hb was quantitated in 17 blood samples obtained from 12 patients with metabolic abnormalities; only one patient, as noted, was hospitalized at the time
(1,100 neutrophils/mm$^3$) during the first analysis of fetal Hb, but not subsequently. A Nierhaus-Betke stain from April 20, 1994, when Hb F was 8%, showed that approximately 8% to 10% of peripheral blood cells contained acid-insoluble Hb F (Fig 3B). SSCP analysis of genomic DNA showed a wild-type pattern only (Fig 2).

A.L. is 3 years old. She was diagnosed with propionotic acidemia when she presented with neonatal hyperammonemia and acidosis. At 11 months of age she was diagnosed with infantile spasms for which she receives phenobarbital.

DISCUSSION

We report here a persistence of fetal Hb found in four of five patients with an abnormality in amino acid metabolism that is characterized by increased metabolites of propionate, but without an associated molecular alteration in the $\beta$-globin gene cluster itself. To our knowledge, this is the first report of a persistence of fetal Hb beyond infancy that is of a genetic metabolic origin. A modified Nierhaus-Betke stain with a value of approximately 8% to 10% in a 5-year-old patient suggests an heterocellular persistence of fetal Hb. Hb F levels in these children were consistently elevated and varied over time but were consistently higher after metabolic decompensation (ie, K.F. February 1994) or frequent exacerbations (ie, M.S.). Propionate levels were not available retrospectively because these children were managed clinically, so the exact relationship between levels of propionate and fetal Hb is not yet available. Of note, all propionate patients had sustained twofold to threefold elevations of glycine throughout this study, implying ongoing metabolic derangements even when these patients were clinically well. The two children with methylmalonic acidemia are additionally enlightening, as M.D., with a relatively mild, chronic 10-fold elevation in methylmalonate, showed no increased fetal Hb at 5 years of age, whereas I.M., with a sustained 1,000-fold elevation in methylmalonate, had 6% fetal Hb at 2 years of age.

We also evaluated patients with two other types of metabolic abnormalities but with normal fetal Hb levels. Children with MCAD deficiency would be expected to have excessive accumulation of C-6- and C-8-CoA derivatives during exacerbations but not when metabolically stable. At the time of Hb F analysis, these children were in excellent metabolic control and it is possible that results would differ during exacerbations, or at diagnosis. Patients with enzymatic abnormalities in the urea cycle who were treated with nitrogen scavenging agents, such as phenylacetate or phenylbutyrate, that had been associated with an increase in F cells, showed no detectable elevations in fetal Hb in our study. However, those earlier reports that showed an elevation in F cells used a highly sensitive antibody technique, whereas this study used less sensitive but potentially more physiologically relevant methods, ie, isoelectric focusing and Nierhaus-Betke staining. These results highlight the potency of propionate metabolites in stimulating fetal Hb synthesis.

Fetal Hb is 50% to 90% of total Hb at birth and decreases, by mechanisms not yet fully characterized, to less than 1% at 6 to 12 months postpartum, as Hb A predominates.
persistence of fetal Hb beyond infancy has been ascribed to mutations within the β-globin cluster itself, i.e., deletions downstream of the γ-globin genes and point mutations in the γ-globin gene promoters. These observations helped to identify regions of potential developmental regulatory importance, including cis-acting sites, trans-acting factors, and DNA secondary structure(s) which likely play a role in γ-globin gene regulation.

Patients with HPFH mutations have likewise been clinically illuminating, as the observation that elevated Hb F in patients with concomitant β-thalassemia or sickle cell disease ameliorates the typical clinical sequelae of these diseases has led to ongoing pharmacologic trials in an attempt to upregulate fetal Hb.

Perrine et al described a persistence of fetal Hb in infants of diabetic mothers which was felt to be metabolic in origin and was subsequently ascribed to elevated levels of butyric acid derivatives. More recently, Dover et al reported that phenylacetate and phenylbutyrate upregulated F cells in patients with urea cycle disorders, for whom these agents serve as ammonia scavengers. Neither phenylacetate nor phenylbutyrate is metabolized to butyrate or its analogues, however, both butyrate and phenylacetate are metabolized in the mitochondria via thiol-esters with acetyl-CoA, to acetate/acyetyl-CoA and phenylacetylglutamine, respectively. This common metabolic pathway for phenylacetate and butyrate led us to investigate the possibility that propionate and other short-chain fatty acids could upregulate embryonic/fetal globin genes in vitro. Propionate, although derived predominantly from branched-chain and other amino acids rather than odd-chain fatty acids, is also metabolized through an acetyl-CoA ester, propionyl-CoA, to succinyl-CoA, and the tricarboxylic acid cycle. Our studies showed that 5 mmol/L propionate was capable of upregulating murine embryonic β-globin gene expression in cell culture (Fig 1), as were butyrate and pentanoate, (data not shown) at, respectively, 1 mmol/L and 5 mmol/L concentrations. As had previously been shown for the butyrate-induced upregulation of a transfected avian embryonic β-type globin in MEL cells, this effect was not caused by terminal erythroid differentiation induced by such compounds, because DMSO, which also induces differentiation of the MEL cells to an adult erythroid phenotype, did not upregulate ε expression.

The mechanism by which propionate, butyrate, or phenylacetate and phenylbutyrate cause elevated expression of fetal and embryonic globin genes is not known. We and others have shown cis-acting sites for butyrate responsiveness in globin genes, but the specific mechanisms involved have not yet been elucidated. It is possible that butyrate and propionate affect the level or modification of one or more sequence-specific trans-acting factors, perhaps by altering acetyl-CoA flux in the mitochondria.

It is expected that ongoing detailed studies of these children with metabolically mediated upregulation of fetal Hb will offer insight into both underlying pathophysiologic mechanisms and potential long-term clinical benefits and sequelae of exposure to short-chain fatty acids, such as butyrate, which are currently under study. In addition, these findings strongly support the notion that ongoing therapeutic trials of SCFAs hold considerable promise, as the children in this study had sustained Hb F elevations of 3- to 10-fold over controls without obvious hematologic stress; it seems likely that similar agents, applied therapeutically, would be much more active in anemic patients with ongoing stress erythropoiesis. This possibility is supported by the observation that none of the urea cycle disorder patients who received high doses of phenylbutyrate had detectable elevations of fetal Hb in our studies, whereas treatment with similar doses has been associated with elevated Hb F in patients with stress erythropoiesis caused by β-globin gene disorders.

None of the children with propionic acidemia or methylmalonic acidemia have had obvious progressive neurologic deterioration beyond those changes that likely arise from a difficult perinatal course and subsequent intermittent metabolic decompensation and protein restriction. Nonetheless, despite reports in the literature of adolescent children with propionic acidemia who, at diagnosis or with careful management, were of normal intelligence and without neurologic sequela, this is not typical. The presence of seizures and developmental delay in our patients, coupled with reports by Blau et al regarding neurologic abnormalities in baboons infused with massive doses of sodium butyrate, do suggest the possibility that very high serum levels of short-chain fatty acid compounds and their metabolites could result in neurologic toxicity.

A number of other clinical sequelae have been associated with propionic acidemia, including cardiomyopathy, developmental delay, transient neutropenia, etc., but similar syndromes have not developed in patients with β-globin gene disorders who are participating in short-term trials to upregulate fetal Hb by treatment with short-chain fatty acids. Nonetheless, future trials need to be structured so as to carefully define the therapeutic index of these compounds.

In summary, this report describes a metabolic persistence of fetal Hb in patients with inherited derangements in amino acid metabolism, as predicted in a tissue culture model. The uniformity of this effect at the level of total fetal Hb in nonanemic children suggests that propionate may be a potent agent for pharmacologic stimulation of fetal Hb in patients with β-globin gene disorders. Further study of individuals with elevated levels of short-chain fatty acid metabolites should afford insights into new pharmacologic therapies for β-globin disorders that may be ameliorated by upregulation of fetal Hb, and offer a unique opportunity to examine potential long-term sequelae from therapy with such compounds.

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