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Genotoxicity Associated with Hydroxyurea Exposure in Infants with Sickle Cell Anemia: Results from the BABY-HUG Phase III Clinical Trial

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Abstract

Background—The laboratory and clinical benefits of hydroxyurea therapy for children with sickle cell anemia (SCA) are well recognized, but treatment in young patients is limited in part by concerns about long-term genotoxicity, and specifically possible carcinogenicity.

Procedure—The Pediatric Hydroxyurea Phase III Clinical Trial (BABY HUG) was a multi-center double-blinded placebo-controlled randomized clinical trial (NCT00006400) testing whether hydroxyurea could prevent chronic organ damage in very young patients with SCA. An important secondary objective was the measurement of acquired genotoxicity using 3 laboratory assays: chromosomal karyotype, illegitimate VDJ recombination events, and micronucleated reticulocyte formation.

Results—Our data indicate that hydroxyurea treatment was not associated with any significant increases in genotoxicity compared to placebo treatment.

Conclusions—These data provide additional support to the safety profile of hydroxyurea for young patients with SCA, and suggest that genotoxicity in this patient population is low.

Keywords

genotoxicity; hydroxyurea; mutagenicity; sickle cell anemia

INTRODUCTION

The clinical and laboratory benefits of hydroxyurea for both adults and children with sickle cell anemia (SCA) have been consistently demonstrated over the past 25 years, as recently reviewed [1, 2]. The evidence for hydroxyurea decreasing the frequency of painful events, episodes of acute chest syndrome, and the need for hospitalization in both adults and children with SCA due to its ability to increase fetal hemoglobin (HbF) is compelling [3, 4].

With prolonged treatment, sustained benefits have been documented without serious side effects [5, 6] and there is an expanding literature demonstrating long-term efficacy [7, 8] and safety [9, 10]. Despite this accumulated evidence and even encouragement to expand treatment [11], hydroxyurea has yet to become widely accepted as a universal treatment option for patients with SCA. Long-term safety concerns persist for hydroxyurea treatment in SCA, in part related to its mechanism of action as a potent inhibitor of ribonucleotide reductase [12] and prior reports of *in vitro* [13,14] and *in vivo* [15] genotoxicity.

BABY HUG was an NHLBI and NICHD-sponsored phase III randomized, double-blinded, placebo-controlled trial (NCT00006400) investigating the efficacy of hydroxyurea for preventing organ damage in very young patients with SCA. The BABY HUG main study results were recently published, documenting a failure to achieve statistical significance for either primary study endpoint but substantial benefits for a wide variety of secondary study endpoints [16]. One critical secondary endpoint was the *in vivo* genotoxicity of hydroxyurea in this very young and potentially susceptible population. The primary results paper briefly mentioned that genotoxicity results were not concerning but provided no data or discussion. We now provide a comprehensive description of these quantitative measures of genotoxicity in the BABY HUG trial, collected at baseline and after two years of hydroxyurea or placebo exposure.

METHODS

Samples

As previously described [17], BABY HUG was conducted across 14 centers and was approved by the local institutional review boards of all participating centers. A total of 193 infants (mean 13.6 months) with SCA (HbSS or HbS/ β^0 -thalassemia) were randomized to receive hydroxyurea (fixed dose of 20 mg/kg/day) or placebo for two years. There were 167 subjects who completed the study and a total of 374 patient-years of observation included in final study analyses [16]. Laboratory measurements of genotoxicity were collected before randomization to study treatment and at the end of study treatment.

Laboratory Analyses

Peripheral blood was collected in EDTA and peripheral blood mononuclear cells (PBMC) were isolated and purified using Ficoll-Hypaque density centrifugation as previously described [18]. For karyotype analysis to measure clastogenicity, PBMC were stimulated with phytohemagglutinin (Sigma-Aldrich Corp., St. Louis, MO) and incubated for 72 hours at 37°C. At the end of incubation, the cells were arrested in metaphase by the addition of Colcemid (Invitrogen Corp., Carlsbad, CA) and after incubation, were centrifuged and suspended in 10 mL of hypotonic 0.075 M KCl solution. The hypotonic supernatant was discarded and the cell pellet was fixed by the slow addition of 10 mL of freshly prepared 3:1 methanol- acetic acid Carnoy's fixative. The suspension was centrifuged and the supernatant discarded. The fixative step was repeated two to three times until a clear cell pellet was visible [19]. The metaphase spreads were prepared as previously described using the Harleco Giemsa stain (GTG Banding) protocol [20, 21]. Chromosome spreads were visualized with an Olympus BX60 microscope using brightfield microscopy at 1000x. For each sample 100 metaphase cells were evaluated for chromosome/chromatid breaks and any other structural chromosome aberration such as translocations, inversions, rings and markers. Photographs were taken with an Olympus U-CAMD3 digital camera on a CytoVision (Genetix Corp., San Jose, CA) karyotype imaging system.

For quantitation of interlocus (illegitimate) VDJ recombination events representing genomic damage, a 2-step nested polymerase chain reaction (PCR) reaction was used to amplify these

rare genomic rearrangements between T-cell receptor V γ and J β 1 segments as previously described [22]. Recombination events were tested at serial DNA dilutions and results are reported as the number of events per microgram of genomic DNA.

The frequency of micronucleated reticulocytes is a validated endpoint of cytogenetic damage. Rodent-based micronucleus tests have become an important component of regulatory safety assessment [23, 24]. More recently, studies involving human blood samples have indicated its effectiveness at detecting chromosomal damage caused by a variety of exposure situations, including a variety of anti-neoplastic chemo- and radiotherapies [25, 26], tobacco use [27], and Zidovudine for the prevention of HIV transmission from infected mothers to their infants [28]. To enumerate micronucleated reticulocytes, whole blood was collected and fixed with -80°C methanol. Fixed blood samples were maintained at ultracold temperatures until the day of analysis, at which time they were washed out of fixative and incubated with anti-CD71-FITC, anti-CD61-PE, RNase, and propidium iodide as previously described (9, 29). Flow cytometric analysis proceeded until at least 2×10^4 CD71 $^{+}$ reticulocytes and 1×10^6 total erythrocytes were acquired per sample. These data were used to calculate the frequency of micronucleated cells among CD71 $^{+}$ reticulocytes (i.e., recent genotoxicity), and the frequency of CD71 $^{+}$ reticulocytes among total erythrocytes (i.e., erythropoietic drive) [9, 29].

Statistical Analyses

Statistical analyses were performed by Clinical Trials & Surveys Corp. (C-TASC, Owings Mills, Maryland) using SAS as the chosen analytical software. Descriptive statistics for each mutation variable were calculated and reported using means and standard deviations.

Comparisons were performed assuming that the data were Poisson in origin and were correlated within a patient between the baseline and exit evaluations. Specifically, a Generalized Estimating Equation Model [30] with a Poisson link function was used to analyze these data. Two specific comparisons were performed: 1) a statistical test was performed to determine if the number of mutation events in the hydroxyurea group was equal at exit and baseline, and 2) a statistical test was performed to determine if the number of mutation events was the same between the hydroxyurea and placebo treatment groups at exit.

These results are presented in Table I. Statistical significance was defined by a p-value less than 0.05

RESULTS

Karyotype

Subjects in both the hydroxyurea and placebo groups had significantly increased number of total chromosome breaks at study exit compared to study entry (Table I). At study exit, subjects treated with hydroxyurea had similar numbers of chromosome breaks as subjects receiving placebo (0.5 ± 1.4 breaks per 100 metaphases vs. 0.4 ± 2.5 , $p=\text{NS}$). In contrast, there were no significant changes in the number of observed chromatid breaks at study entry and exit for either the hydroxyurea or placebo group. At study exit, there were no differences in the number of chromatid breaks between these two treatment groups (0.6 ± 1.1 breaks per 100 metaphases for hydroxyurea group vs. 0.8 ± 3.4 for placebo, $p=\text{NS}$, Table I).

VDJ recombination events

There were no changes in the number of illegitimate VDJ recombination events observed, comparing study entry and exit samples either in the hydroxyurea or the placebo treatment group (Table I). At study exit, subjects treated with hydroxyurea had similar numbers of illegitimate VDJ recombination events as subjects receiving placebo (0.7 ± 0.5 events per μg of DNA versus 0.7 ± 0.4 events, $p=\text{NS}$, Table I).

Micronucleated reticulocytes

Subjects with hydroxyurea exposure had a significantly decreased number of CD71⁺ reticulocytes at study exit compared to study entry, reflecting the expected myelosuppressive effects of study treatment on total reticulocyte count (data not shown). In contrast, subjects receiving placebo had an increased number of immature CD71⁺ reticulocytes at study exit compared to study entry, reflecting the development of more severe hemolytic anemia with increasing age (data not shown). Comparing study treatments, subjects treated with hydroxyurea had a similar number of early reticulocytes containing micronuclei (MN-CD71⁺) at study exit compared to subjects receiving placebo ($0.3 \pm 0.2\%$ versus $0.3 \pm 0.2\%$, $p=\text{NS}$, Table I).

DISCUSSION

Although the benefits of hydroxyurea for children with SCA are well documented and compelling to most hematologists, treatment of very young patients and long-term exposure may be limited by concerns about genotoxicity and potential carcinogenicity. Unfortunately, there are no accurate *in vitro* measures of drug-related carcinogenicity. Cell-based experiments and animal models typically rely on high-dose short-term exposures and endpoints of genotoxicity as opposed to tumorigenicity per se. Laboratory-based assays using human samples after hydroxyurea exposure represent the best available means to measure DNA damage and thereby infer risks of treatment-related genotoxicity and possible carcinogenicity. For example, VDJ recombinase-mediated gene rearrangements in non-malignant human lymphocytes have shown significant biologic, epidemiologic and genetic overlap with the development of childhood lymphoid malignancies [31], serving as an *in vivo* biomarker of more widespread genotoxicity and potential leukemogenesis.

Given the lack of a definitive genotoxicity assay, a multifaceted approach to the investigation of genotoxicity was employed in the BABY HUG study in an effort to capture various forms of chromosome damage, measured as chromosome breaks and chromatid breaks on standard karyotype analysis, as well as double-stranded DNA breaks observed as micronucleated reticulocytes and specific DNA damage manifest as illegitimate VDJ recombination events. Although none of these assays is itself an accurate predictor of carcinogenicity, each is a measure of acquired genomic damage and their combined use represents a comprehensive assessment of cytogenetic damage. Because the young subjects in BABY HUG had increasing age during the study with additional host factors beyond study treatment, it was important to measure and analyze the frequency of each event at study entry and exit in both hydroxyurea and placebo groups. Together, these assays provided a comprehensive assessment of the potential impact of continuous *in vivo* hydroxyurea exposure on genomic integrity in this very young population.

Prior *in vivo* reports investigating chromosome aberrations in patients with SCA receiving hydroxyurea have produced mixed results, but overall have been reassuring. A recent report by Friedrisch et al. [15] demonstrated increased levels of DNA damage using the comet assay in 28 patients receiving hydroxyurea for SCA compared to 28 normal controls. This increase in DNA damage among patients treated with hydroxyurea was observed in damage

class 1 (class 0= no migration/damage; class 4=maximal migration/damage) as measured relatively subjectively in the comet assay. Also of note, there was a negative correlation between the duration of hydroxyurea exposure and damage index. An early phase I/II trial of hydroxyurea in adults with SCA examined karyotypes at the beginning and end of the study and found no increase in the number of chromosome abnormalities identified [32]. A small cohort of 8 children with SCA (age 7-20 years) treated with hydroxyurea were monitored at two-month intervals over the course of one year, and no differences were observed in the frequency of chromosome aberrations or mitotic index in peripheral blood lymphocytes [33]. A recent report from our laboratory documented no increase in chromosome aberrations among 51 school-aged children with SCA and 3-12 years of hydroxyurea exposure, compared to 28 children with SCA but no prior hydroxyurea exposure and to 11 healthy controls [10]. In BABY HUG, chromosome breaks increased with age for both study treatment arms, but there were no differences in subjects treated with hydroxyurea compared to placebo (Table I). Similarly, previous reports have documented no abnormal increases in illegitimate VDJ gene rearrangements among children with SCA receiving hydroxyurea [22]. In BABY HUG, both treatment groups had similar numbers of VDJ events at baseline and at study exit (Table I).

Flanagan et al. [9] recently reported that school-aged children with SCA and hydroxyurea exposure had an increased number of micronucleated CD71⁺ reticulocytes compared to levels before treatment, and while the effect was sustained, it did not increase with longer drug exposure. In BABY HUG, although study treatment affected the total number of reticulocytes, the number of early CD71⁺ reticulocytes with micronuclei was not statistically different for children who received hydroxyurea treatment compared to placebo (Table I). The differences in the frequency of micronucleated CD71⁺ reticulocytes observed in the BABY HUG study in comparison to the study by Flanagan and colleagues [9] could potentially be explained by differences in either or both of the following: age (infants versus older children) or hydroxyurea dose (20 mg/kg/day in BABY HUG versus escalation to MTD at 25-30 mg/kg/day).

Childhood acute lymphoblastic leukemia (ALL) has a peak incidence of 2-5 years of age, but fewer than 5% of cases are associated with a known predisposing genetic syndrome (e.g., Bloom's syndrome, ataxia-telangiectasia). Although the pathogenesis of leukemia is likely multi-factorial, this clearly increased incidence in young children appears to be associated with the increased sensitivity to potentially genotoxic environmental and medical exposures in this population [34-36]. These results from BABY HUG provide evidence of cytogenetic stability in this susceptible population of young children and contribute to a growing body of evidence to suggest that *in vivo* genotoxicity of hydroxyurea in patients with SCA is low. It is important to realize that although reassuring, these data represent laboratory assays serving as harbingers of future carcinogenicity and do not rule out undocumented genotoxicity; further laboratory and clinical vigilance in this population is critical. These data represent genotoxicity measures over a short two-year timespan and must be repeated as treatment spans a longer range of time. It will be important to continue to monitor the BABY HUG population in carefully designed follow-up studies to monitor the potential genotoxicity of hydroxyurea over a longer period of time and over a range of dose levels, since they represent a unique population for whom hydroxyurea may truly become a lifelong therapy.

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S.D.D. is employed by Litron Laboratories, a company that owns patents regarding flow cytometry-based scoring of micronucleated erythrocytes. Litron sells assay kits related to these patented methods that are designed for several preclinical animal models; note that human blood-based analyses as described herein are not approved, cleared, validated or intended for clinical diagnostic use.

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Table 1**Genotoxicity measurements in BABY HUG**

Genotoxicity Assay	Hydroxyurea		Placebo		P-value *	P-value ^
	Baseline	Exit	Baseline	Exit		
Number of Chromosome Breaks	0.1 (0.4)	0.5 (1.4)	0.1 (0.4)	0.4 (2.5)	0.0003	0.6990
Number of Chromatid Breaks	0.9 (1.5)	0.6 (1.1)	0.7 (1.0)	0.8 (3.4)	0.1333	0.6029
% Micronucleated CD71 ⁺ Reticulocytes	0.2 (0.2)	0.3 (0.2)	0.2 (0.1)	0.3 (0.2)	<0.0001	0.4267
VDI events per microgram DNA	0.8 (0.5)	0.7 (0.5)	0.8 (0.6)	0.7 (0.4)	0.0780	0.8235

Results are shown as the mean (standard deviation).

* P-value of comparing average of baseline and exit values within the Hydroxyurea group;

^ P-value of comparing average of Hydroxyurea and placebo group at exit visit.