Effects of \textit{N}-Acetylcysteine on Dense Cell Formation in Sickle Cell Disease

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The extent to which dense and irreversible sickle cells (ISCs) contribute to vaso-occlusive episodes in sickle cell disease remains unclear. \textit{N}-Acetylcysteine (NAC) inhibits dense cell and ISC formation in sickle erythrocytes in vitro and restores glutathione levels toward normal. A phase II double-blind randomized clinical trial was completed to determine the efficacy of NAC in decreasing dense cell and ISC formation, and vaso-occlusive episodes in sickle cell disease. Twenty-one subjects with a history of at least two vaso-occlusive episodes per year and 6\% dense cells were enrolled. Four treatment groups were analyzed; NAC at a dose of 2,400 mg per day decreased the percent dense cells from 20.1 ± 2.9 to 12.6 ± 2.1 ($P < 0.05$) and increased red cell glutathione levels from 292.8 ± 74.5 to 576.7 ± 155.1 ($P < 0.05$). In addition, we observed a decrease in vaso-occlusive episodes from 0.03 to 0.006 episodes per person-days and a decreased in relative risk to $R = 0.39$. Although NAC did not significantly decrease the number of ISCs, there was a downward trend at all doses tested. In summary, NAC inhibited dense cell formation, restored glutathione levels toward normal, and decreased vaso-occlusive episodes at a well-tolerated dose of 2,400 mg per day. To determine the long-term efficacy and safety of NAC, a multicenter phase III clinical trial is required. Am. J. Hematol. 73: 26–32, 2003. © 2003 Wiley-Liss, Inc.

Key words: sickle cell disease; vaso-occlusive episodes; \textit{N}-acetylcysteine; dense cells; glutathione

INTRODUCTION

Sickle cell disease (SCD) is a genetic disorder in which abnormal hemoglobin S polymerizes in erythrocytes, producing free radicals and oxidative damage to the red cell membrane. This leads to the formation of dense and irreversible sickle cells (ISCs) that contribute to the pathophysiology of vaso-occlusive (VOC) episodes. The complications encountered in SCD include recurrent pain episodes, infection, splenic and liver dysfunction, and central nervous system damage [1]. Hydroxyurea was demonstrated to decrease VOC episodes in a multicenter phase III clinical trial [2] and remains the only drug specifically indicated for treating sickle cell VOC complications. The potential long-term teratogenic risks of antitumor drugs such as hydroxyurea to sickle cell patients are presently being investigated. Additional concerns have been raised about the growth effects of treating young children with this agent. Recently, a pilot project demonstrated that hydroxyurea is safe and effective in infants on a short-term basis [3]. Therefore, a need remains to identify alternative effective therapeutic options.

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Studies by Gibson et al. [4] demonstrated that N-acetylcysteine (NAC), a clinically relevant antioxidant could effectively blocks dense cell and ISC formation in vitro. Over 2,500 patients ranging in age from 2 months to 88 years with respiratory conditions have participated in clinical trials with NAC [5,6] without significant side effects. Based on the in vitro studies and clinical safety of NAC, a phase II double-blind, placebo-controlled human trial was completed to ascertain whether NAC could decrease dense cell formation and acute VOC episodes in SCD. NAC significantly decreased the number of dense cells and increased red cell glutathione levels. The decrease in the number of ISCs did not reach statistical significance, however, there was a downward trend at all doses tested compared to placebo. NAC at a dose of 2,400 mg per day produced a decrease in VOC episodes. These data provide “proof of concept” for NAC as an anti-oxidant agent of potential therapeutic value.

MATERIALS AND METHODS

Subjects

Twenty-one subjects diagnosed with homozygous sickle cell anemia or hemoglobin S–β0-thalassemia, 15 years of age or greater, were enrolled in the study after approval by the Institutional Review Board at the University of South Alabama. Parents of subjects under 18 years old gave written consent, and children greater than 10 years old gave assent for participation. Patient recruitment was targeted to occur over a 6-month period with the entire study period lasting 1 year, but due to slow accrual rates the study lasted 18 months.

Eligibility Criteria

The primary eligibility criteria included individuals at least 15 years old, diagnosed with sickle cell anemia or hemoglobin S–β0-thalassemia, with dense cells greater than 6% and 2 or more VOC episodes per year for the 2 years prior to enrollment. Exclusion criteria included all other hemoglobinopathies not included in the eligibility criteria, pregnancy, narcotic addition, chronic transfusions, and history of stroke, HIV positive, investigational drug therapy, or known allergy to NAC.

Study Protocol

Baseline hematological tests were performed at visits 1–3, at a 2-week interval for the first month run-in period, during which time all subjects received placebo. Laboratory analysis included complete blood cell and reticulocyte counts, hemoglobin quantitation by high-pressure liquid chromatography, general chemistries, and C-reactive protein and urine analysis by standard methods. The Zambon Corporation obtained an IND for NAC from the Food and Drug Administration and provided NAC as odor-free capsules dispensed in blister cards, each containing a week supply of drug. The drug supply was secured in the hematology clinic during the study period. During the first month (visits 1–3) of the study period all subjects were treated with 4 placebo capsules by mouth 3 times a day and were taught to record in diaries to keep track of VOC episodes, other medications, and adverse side effects. Participants were not aware that everyone on study received placebo during the run-in period of 1 month. During follow-up clinic visits a capsule count was completed, diaries reviewed, and urine pregnancy tests were performed for female subjects before the subsequent month’s drug supply was dispensed. Blood samples for laboratory tests were drawn 1 hr after drug dosing in the clinic to obtain steady-state NAC levels.

Randomization into one of 4 treatment groups [0 mg (placebo), 600 mg, 1,200 mg, or 2,400 mg of NAC by mouth divided 3 times a day] took place at visit 3. Regardless of drug dose, all subjects received 4 capsules to maintain the double-blind status. Participants with less than 80% compliance on 2 follow-up visits were discharged from the study. During hospitalizations for acute VOC episodes, the study drug was continued. Four subjects were discharged from the study for transfusion therapy and noncompliance. Seventeen individuals were analyzed in detail. The study design consisted of a total of 9 visits over a 7-month period; not all participants completed the entire study period. Serious adverse events were reported promptly to our Institutional Review Board.

Dense and Irreversible Sickle Cells

The percent dense cells were measured by a Percoll step gradient as previously described [7]. Each density fraction within the Percoll layers was removed without cross contamination and the high-density sickle cells at 70% Percoll were counted and the percent dense cells was calculated as a percent of the total red cells. ISCs were determined from a 10-mL volume of oxygenated red blood cells treated with glutaraldehyde (290 µL of a 1.25% solution) and then placed on a glass slide to make a smear. After air-drying, the cells were stained with Leukostat Stain (Fisher Scientific, Pittsburgh, PA) and at least 500 cells were counted. Cells with a length/width ratio of ≥2 were counted as ISCs.

Glutathione and NAC Levels

Glutathione (GSH) levels were measured by a colorimetric assay [8,9]. Packed red blood cells were lysed in distilled water at 22°C, and then 10% trichloroacetic acid was mixed in the sample followed by mixing with 4 volumes of 0.5 M Tris, pH 8.2, with 0.2 mM 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) for 30 min. The DTNB absorbance was read at 412 nm. Molar concentrations of GSH was calculated based on the extinction
Positive coefficient $E = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$. Red cell NAC concentrations and plasma clearance were measured by chromatographic analysis [10] of blood samples drawn 1 hr after drug dosing in the hematology clinic during follow-up visits.

**Primary Outcome**

The primary efficacy variables analyzed included dense cell and ISC levels and the number of acute VOC episodes defined as a visit to a medical facility that lasted more than 4 hr for acute pain related to vaso-occlusion requiring parenteral narcotics. The occurrence of acute chest syndrome, priapism, splenic, or hepatic sequestration was also counted as a VOC episode. Acute chest syndrome included those subjects with chest wall pain and a new infiltrate on chest X-ray. Priapism was defined as a painful erection lasting more than 2 hr requiring medical intervention; splenic or hepatic sequestration was defined as a sudden increase in organ size and a concomitant drop in hemoglobin greater than 2 g/dL from average baseline values. The 4-hr period excluded time for registration at the medical facility and time spent waiting to be seen by a physician.

**Blinding**

The participants, investigators, and staff were not aware of the individual treatment group members. The hematologists reviewed all laboratory tests in a blinded fashion. The data collected was analyzed after study closure without interim analyses.

**Statistical Analysis**

Laboratory tests were analyzed using a chi-square test. The number of VOC episodes was analyzed using the Wilcoxon test. Age and sex were analyzed using one-way ANOVA, with paired comparisons using $t$-tests on contrasts. All tests were performed at the 5% level, $P < 0.05$ was considered significant.

**RESULTS**

A Federal Drug Administration approved, phase II double-blind, placebo-controlled, clinical trial was initiated at the University of South Alabama to determine the efficacy of NAC in SCD. The primary objectives of the trial were to determine the ability of NAC (1) to block dense cell and ISC formation, (2) to increase red cell GSH levels, and (3) decrease the number of VOC episodes in SCD. In addition, we determined the safety of oral NAC in this patient population. We screened 47 individuals with sickle cell anemia and hemoglobin S–β0-thalassemia as potential participants. A total of 23 subjects met the eligibility criteria with greater than 6% dense cells; 2 refused to participate due to the experimental nature of NAC. As illustrated in Table I the groups were comparable with regard to age, sex, male to female ratio, blood counts, and hemoglobin and reticulocyte levels. The average age for the placebo group was increased insignificantly to 26.1 years due to one participant who was 48 years old. Likewise the groups have comparable fetal hemoglobin, dense cells, ISCs, and GSH levels at baseline. The data were analyzed for sex effects, and no significant difference was observed for the different treatment groups. All participants were African American, therefore difference in ethnic groups was not an issue for this study. Two patients were dropped from the study for noncompliance, which was defined as taking less than 80% of the prescribed medication on two follow-up visits, and two others were dropped due to transfusion requirements during an acute vaso-occlusive episode. A total of 17 out of the 21 enrolled participants were included in our analysis. Results for the last visit. Our laboratory tests analysis for the 17 patients is shown in Table I. There were no significant changes during the study period in the white blood cell, hemoglobin, or reticulocyte counts, blood chemistries, or fetal hemoglobin levels for any of the treatment groups (data not shown). NAC was well tolerated by all participants with no adverse effects related to drug therapy encountered during the study.

**NAC Decreases Dense Cell Formation**

All laboratory analyses were completed on blood samples drawn 1 hr after NAC dosing in the hematology clinic. According to the two-step model [11], NAC was predicted to decrease the number of dense cells and ISCs. The data analysis was completed from visit 4 (1 month after randomization) through visit 8, since all 17 participants had completed the study to that time point. As shown in Figure 2A, although the dense cell levels decreased for the 1,200- and 2,400-mg treatment groups, this effect reached significance for the 2,400-mg NAC dose alone. The dense cell percent decrease from 20.1 ± 3.2 to 12.6 ± 2.1 ($P < 0.05$). Figure 2B shows the detailed course for dense cell response for the 2,400-mg dose. We observed a steady decrease in the dense cell level up to visit 8. In contrast, the dense cell level increased in the placebo group (Fig. 2B, top graph). This observation is consistent with the in vitro data, where NAC protects the K+ channel from oxidative damage [11] and prevents dense cell formation.
NAC Restores Red Cell Glutathione Levels Toward Normal

We next analyzed whether NAC would also decrease ISC formation in SCD by either preventing or reversing β-actin from oxidative damage and restoring red cell GSH levels toward normal. The ISC level trended downward at all treatment doses but did not reach significance due to great variability (Fig. 3A), suggesting that ISC levels might not be a reliable marker of treatment response.

As anticipated due to the rapidity in which NAC enters the red blood cell after absorption, the free NAC level and plasma clearance was comparable for all four treatment groups (data not shown). In contrast, red cell GSH levels increased significantly from 292.8 ± 74.5 to 576.7 g/mL due to the formation of GSH-Px from GSH (Fig. 3B). This increase in GSH-Px activity restores red cell GSH levels toward normal.

TABLE I. Study Group Characteristics at Baseline

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo (N = 5)</th>
<th>600 mg (N = 5)</th>
<th>1,200 mg (N = 5)</th>
<th>2,400 mg (N = 6)</th>
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<tr>
<td>Average age (years)</td>
<td>26.1 ± 12.9</td>
<td>18.1 ± 2.9</td>
<td>17.9 ± 1.2</td>
<td>20.1 ± 4.9</td>
</tr>
<tr>
<td>Average VOC episodes*</td>
<td>5.0 ± 2.0</td>
<td>6.8 ± 2.9</td>
<td>6.4 ± 3.7</td>
<td>4.3 ± 1.0</td>
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<tr>
<td>Male/female ratio</td>
<td>3:2</td>
<td>3:2</td>
<td>2:3</td>
<td>3:3</td>
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<tr>
<td>White cells (10^9/mm)</td>
<td>11.5 ± 2.8</td>
<td>14.2 ± 3.2</td>
<td>13.2 ± 3.4</td>
<td>12.2 ± 3.5</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.4 ± 1.6</td>
<td>8.4 ± 1.2</td>
<td>8.3 ± 1.4</td>
<td>8.4 ± 1.1</td>
</tr>
<tr>
<td>Platelets (10^9/mm)</td>
<td>404 ± 84.7</td>
<td>330 ± 53.6</td>
<td>641 ± 455.4</td>
<td>354 ± 105.8</td>
</tr>
<tr>
<td>Reticulocytes (10^9/mm)</td>
<td>6.1 ± 1.2</td>
<td>10.4 ± 3.2</td>
<td>8.8 ± 6.6</td>
<td>9.6 ± 2.5</td>
</tr>
<tr>
<td>Fetal hemoglobin (%)</td>
<td>6.6 ± 6.2</td>
<td>8.6 ± 7.9</td>
<td>7.8 ± 3.9</td>
<td>8.1 ± 7.6</td>
</tr>
<tr>
<td>Dense cells (%)</td>
<td>15.7 ± 4.4</td>
<td>21.4 ± 11.2</td>
<td>20.5 ± 11.9</td>
<td>16.9 ± 6.6</td>
</tr>
<tr>
<td>Irreversible sickle cells (%)</td>
<td>14.7 ± 5.3</td>
<td>13.9 ± 7.3</td>
<td>16.1 ± 8.6</td>
<td>13.3 ± 8.6</td>
</tr>
<tr>
<td>Glutathione (μg/mL)</td>
<td>184.0 ± 90.4</td>
<td>245.8 ± 89.3</td>
<td>383.3 ± 144.0</td>
<td>351.5 ± 77.9</td>
</tr>
</tbody>
</table>

*VOC, vaso-occlusive.

Fig. 1. Compliance rates for the different treatment regimens. During each follow-up visit the number of capsules returned, was recorded and compliance rating calculated as a percent based on the number of capsules dispensed at the previous visit. The numbers of capsules taken for the 4 doses of NAC, 0 mg (white bars), 600 mg (black bars), 1,200 mg (gray bars), and 2,400 mg (striped bars) were comparable for the run-in (visits 1–3) and post-randomization periods (visits 4–8).

Fig. 2. N-Acetylcysteine decreased dense cell formation. The dense cell levels were measured by density gradient centrifugation using Percoll separation. The percent dense cell was calculated as a fraction of the total red cells in the blood samples. (A) Comparison between the average numbers of dense cells for visits 1–3 (white bars) and at visit 8 (black bars) for the 4 treatment groups. (B) Detailed analysis of the dense cell levels for the placebo (●) and 2,400-mg (■) treatment groups for visits 4–8.
± 155.1 (P < 0.05) for the 2,400-mg treatment group by visit 8 compared to the average pre-randomization levels (Fig. 3B). Whether the decrease in dense cells and increase in red cell GSH levels at the 2,400-mg NAC dose would translate into a clinical effect on VOC rates was analyzed for our study cohort.

Fig. 3. Effects of N-acetylcysteine on irreversible sickle cell and glutathione levels. (A) Irreversible sickle cells (ISCs) levels were determined from oxygenated red blood cells treated with a 1.25% glutaraldehyde solution. After being air-dried on a glass slide, the cells were stained and counted. The percent ISCs was calculated as a fraction of the total red cells counted. A comparison was made between the average numbers of dense cells for visits 1–3 (white bars) and at visit 8 (black bars) for the 4 treatment groups. (B) Glutathione (GSH) levels were measured in red cells lysed in distilled water followed by the addition of 10% trichloroacetic acid. The supernatant was incubated with 5,5'-dithiobis-(2-nitrobenzoate) and the absorbance read at 412 nm. The GSH level at visit 8 (black bars) was compared to the average level for visits 1–3 for the four treatment groups (white bars).

NAC Decreased the Number of VOC Episodes in Study Participants

The most important clinical parameter analyzed was the impact of NAC on acute VOC episodes. Study participants were given diaries to record the number of VOC episodes between clinic visits at home and those requiring an emergency room or doctor visit. Emergency room encounters were verified using hospital medical records. NAC was continued if possible during hospitalizations for uncomplicated VOC episodes. As shown in Table IIA for the pre-randomization period of 574 person-days, a total of 21 VOC episodes occurred for the four treatment
groups. Tests for homogeneity showed no significant difference for the treatment groups in VOC rates ($P = 0.380$). Table IIB contains a summary of the VOC rates for the 17 subjects that completed at least 8 follow-up visits, for a total of 2,667 person-days of follow-up. The VOC rate decreased from 0.03 to 0.006 episodes per person-days, and the relative risk decreased to $R = 0.39$ for the 2,400 mg of NAC treatment group. The preliminary data provides “proof of concept” that NAC has the ability to decrease acute VOC episodes in sickle cell patients. A larger cohort of subjects and extended study period will clarify the efficacy of NAC in treating VOC episodes on a long-term basis.

**DISCUSSION**

With limited success at developing therapeutic options for SCD, new agents are being sought aggressively. The two major abnormalities in SCD are a chronic hemolytic anemia and microvascular occlusive episodes. During the course of vaso-occlusion, the highest density class of sickled red blood cells is selectively trapped in the microvasculature [13,14]. The dense cell population appears to block the narrowed lumen of vessels lined primarily with the more adherent lower density reversible sickle cells [15]. This is thought to be the reason why dense cells are elevated just prior to and at the early stages of an acute VOC episode but decrease in the latter stages [16]. Despite these important findings, there also exist data indicating a negative correlation between the occurrence of painful crises and the presence of dense cells and ISCs [17,18]. Hemolytic rates have also been correlated with the dense cell population, which contains red blood cells that have the greatest propensity to form polymers and are most susceptible to shear stress. While the literature supports a probable role for dense cells in the pathophysiology of sickle cell disease, most likely the process involves multiple events that culminate in clinically significant VOC episodes.

NAC is an antioxidant that has been utilized clinically for the past 30 years as a mucolytic. Over 2,500 subjects with respiratory conditions have participated in NAC clinical trials [19–21]. Other persons have been treated with NAC for meconium ileus as a complication of cystic fibrosis [22], to prevent hepatotoxicity in acetaminophen overdose [23], to reduce lipoproteins in hyperlipidemia [24], and for treatment of HIV infections [25]. In the latter study, subjects received 9.6 g/day for 1 year; NAC was safe and well tolerated. The reported side effects for NAC in 1–2% of subjects are gastrointestinal (nausea, vomiting, and diarrhea) and urticaria. Individuals involved in NAC clinical trials for treatment for acute respiratory infections or other pathology range in age from 2 months to 88 years.

NAC is converted in the cytoplasm to L-cysteine, which is a precursor for reduced GSH. As a result NAC blocks dense cell formation by protecting the K+ channels from reversible oxidative damage and restores GSH levels toward normal. As demonstrated in our two-step model (Fig. 4), dehydration is necessary but not sufficient for the formation of high-density sickle cells. A second step in the process, involving oxidation of β-actin or possibly abnormal spectrin ubiquitination in the membrane skeleton, is required to establish irreversibility. We speculated that the combined activities of NAC as an anti-oxidant and precursor for GSH (Fig. 4) are the basis for the clinical improvement we observed in our 2,400-mg treatment group.

Our data suggest that NAC may have an accumulative effect, first altering the total number of dense cells by returning GSH levels toward normal. We observed decreased ISCs at the doses studied, although these changes did not reach statistical significance. These findings suggest that either higher NAC levels are required to reverse the damage to β-actin in vivo or that a larger study population will be required to demonstrate significance. We have also demonstrated recently that spectrin is an E2/E3 ubiquitin conjugating enzyme [26] but that it cannot act as such in sickle red cells [27] because the cysteine sites in actin are blocked due to high intracellular GSSH/GSH ratios [28]. This leads to 80–90% reduction in sickle cell spectrin ubiquitination. Therefore, it is also possible that we have not raised the GSH level sufficiently to correct the spectrin ubiquitination abnormality observed in sickle red blood cells. A lack of spectrin ubiquitination slows the dissociation of the spectrin–4.1–actin ternary complex in vitro (Steven Goodman, personal communication). These findings support the prophylactic

<table>
<thead>
<tr>
<th>Study group</th>
<th>Follow-up time (person-days)</th>
<th>No. of VOC episodes</th>
<th>VOC rate (per person-days)</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg (N = 3)</td>
<td>98</td>
<td>6</td>
<td>0.05</td>
<td>1.00</td>
</tr>
<tr>
<td>600 mg (N = 5)</td>
<td>147</td>
<td>5</td>
<td>0.03</td>
<td>1.24</td>
</tr>
<tr>
<td>1200 mg (N = 5)</td>
<td>140</td>
<td>5</td>
<td>0.04</td>
<td>0.76</td>
</tr>
<tr>
<td>2400 mg (N = 6)</td>
<td>189</td>
<td>5</td>
<td>0.03</td>
<td>0.39</td>
</tr>
</tbody>
</table>

**TABLE II. Number of Vaso-occlusive Episodes During Study Period**

**A. Pre-Randomization**

**B. Post-Randomization**

*NAC, N-acetylcysteine.*
use of NAC to prevent cellular dehydration and β-actin oxidation, with the goal being the prevention of VOC complications in SCD. NAC is a well-tolerated and inexpensive drug that might provide a viable alternative drug treatment strategy. A multi-center double-blind study to guard against systematic biases such as over reporting pain episodes will be necessary to determine the efficacy of NAC in sickle cell disease. Whether NAC produces a decrease in VOC episodes long-term remains to be demonstrated.

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