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COMBINED EFFECTS OF ARGININE AND HYDROXYUREA ON BFU-E DERIVED COLONY GROWTH AND HbF SYNTHESIS IN ERYTHROID PROGENITORS ISOLATED FROM SICKLE CELL BLOOD

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Abstract – Hydroxyurea (HU) increases HbF synthesis in sickle cell disease (SCD). Recent studies suggest HU-induced HbF synthesis is mediated through a NO-cGMP pathway. Since arginine is the main precursor of NO, we investigated the effects of arginine and HU mixtures on HbF synthesis and burst forming unit erythroid (BFU-E) proliferation. Mixtures of HU (0, 15, 25, 100µM) and arginine (0, 25, 50, and 100µM) resulting in optimal HbF synthesis and minimal HU-induced cytotoxicity in erythroid progenitors were determined. HU dose-dependently attenuated growth of BFU-E colonies and stimulated HbF synthesis. In contrast, arginine dose-dependently increased BFU-E colonies without affecting HbF synthesis. Furthermore, arginine at concentrations >100µM in combination with varying concentrations of HU, decreased HbF synthesis compared to HU controls. HU, 15-25µM, in combination with 25-50µM arginine not only minimized cytotoxicity, but also increased HbF synthesis when compared with HU controls. N^G-nitro-L-arginine-methyl ester (L-NAME; 100µM), a nitric oxide synthase inhibitor, attenuated the effects of HU+arginine on HbF synthesis compared to HU and HU+arginine controls. These results suggest HU+arginine-induced HbF synthesis in human erythroid progenitors is NO dependent. The synergistic effect on HbF synthesis seen with combinations HU+arginine is an important observation in understanding potential therapeutic uses of HU and arginine.

Key words: Nitric oxide-cGMP pathway, N^G-nitro-L-arginine-methyl ester, cytostatic effect, proliferative effect.

INTRODUCTION

Arginine is one of the most metabolically versatile amino acids. In addition to its major role in protein synthesis, it serves as a precursor for the urea cycle, nitric oxide (NO) production, and synthesis of polyamines, proline, glutamate, creatine and agmatine (18).

In sickle cell disease (SCD), arginine deficiency is commonly seen in adults (7,8). In normal individuals, plasma arginine concentrations range between 54 to 100µM (15,21) as compared to 40-50µM in adults with SCD (14). In contrast, children with SCD have arginine levels comparable to those reported in normal children (15). Recently, it has been reported that individuals with SCD have higher red blood cell arginase activity than normal controls and this increase in arginase activity may be one of the reasons for lower serum arginine levels reported in this population (1,13,14,16).

In SCD, induction of fetal hemoglobin (HbF) synthesis by hydroxyurea ameliorates the frequency of pain crisis and acute chest syndrome (3,4). The mechanism by which hydroxyurea stimulates the production of HbF is not clearly understood. Several studies have suggested that NO production and hydroxyurea-induced HbF synthesis are inter-linked (5,6,11,12,17). Cokic, et al. have demonstrated that hydroxyurea can undergo oxidation by heme compounds to produce NO and increase c-GMP levels in human erythroid cells (5). The induction of HbF by hydroxyurea is blocked by specific inhibitors of NO synthase (NOS), and the c-GMP synthesizing

Abbreviations: **ANOVA:** analysis of variance; **ARG:** arginine; **BFU-E:** burst forming unit-erythroid; **cGMP:** cyclic GMP; **HbF:** fetal hemoglobin; **HU:** hydroxyurea; **L-NAME:** N^G-nitro-L-arginine-methyl ester; **NO:** nitric oxide; **NOS:** nitric oxide synthase; **SCD:** sickle cell disease

enzyme, guanylyl cyclase (5,6,11,12,17). Compilation of these independent findings suggest that the induction of HbF synthesis by hydroxyurea may be mediated through an arginine-NO-cGMP pathway.

Morris et. al. (17), studied the effects of hydroxyurea and arginine individually and in combination on NO production in sickle cell patients. They observed that arginine alone did not increase serum NO products at steady state. Similarly, Styles, et al. (20), reported that oral arginine alone did not provide clinical benefit to pediatric patients with sickle cell disease. Interestingly, in the study by Morris et. al. (17), serum NO products increased when arginine was given in combination with hydroxyurea. From these observations, they concluded that arginine and hydroxyurea in combination might augment NO responses in sickle cell patients.

Since hydroxyurea is a highly cytotoxic drug that depletes bone marrow erythroid stem cells during long periods of treatment (19), it is essential to explore novel treatment strategies employing hydroxyurea which could provide less cytotoxicity without compromising HbF synthesis in individuals with SCD.

In the present study, we used a mononuclear cell culture system containing erythroid progenitors from peripheral blood of individuals with sickle cell anemia. The effects of various combinations of hydroxyurea and arginine on the growth of BFU-E colonies and HbF synthesis were determined. We observed a synergistic effect on HbF synthesis when lower concentrations of hydroxyurea were used in combination with arginine as compared with hydroxyurea alone. When using lower concentrations of hydroxyurea in combination with arginine, the cytotoxicity seen with higher concentrations of hydroxyurea was not observed while achieving comparable HbF synthesis. This is the first study that directly assesses the synergistic effect(s) of lower concentrations of hydroxyurea and arginine in combination on HbF synthesis. The potential significance of our findings in the treatment of SCD is discussed.

MATERIALS AND METHODS

Blood samples

Whole blood from individuals with homozygous sickle cell anemia, not previously treated with hydroxyurea, was used in this study. All participants gave informed consent, and the Human Use Committee approved the protocol. During steady state, ten milliliters of peripheral blood was collected from each patient in a tube containing sodium heparin. All participants were individuals seen at the University of South Alabama Comprehensive Sickle Cell Center clinics. The mononuclear cells from whole blood samples were isolated by density centrifugation using Histopaque-1077 (Sigma Chemical Co., St. Louis, MO. FH:1.077 gm/cm^3) as described previously (23). After centrifugation, the buffy coat at the interface was collected and centrifuged for mononuclear cells. The pellet of mononuclear cells was washed three times with Iscove's Modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum, 2mM glutamine, and 10^{-4} M β-mercaptoethanol. Finally, cells were suspended in 2% of original IMDM media and counted using a Cell-DYN 900 Coulter counter.

Primary culture of mononuclear cells

Mononuclear cells were mixed at a concentration of 5 x 10^5 cells per 0.1 ml in 2% IMDM buffer containing 2% fetal bovine serum and 0.9 ml of Methocult media (GF-H4434, from Stem Cell Technologies, Vancouver, BC, Canada.) as recommend by the product information sheet provided by the Stem Cell Technologies Inc. The basal concentration of arginine in the Methocult media and cell fraction was 8μM. An aliquot of 0.5 ml of cells suspended in with Methocult media was transferred to each well in 12 wells tissue culture plates and incubated in a humidified atmosphere containing 5% CO_2 at 37^0 C for 14 days. BFU-E derived colonies start appearing by day 7-8 with the maximum number of BFU-E colonies seen by day 13-14 (2). Therefore, BFU-E derived colonies were counted on day 14 using an inverted microscope. Cells from BFU-E derived colonies were then harvested for HbF determination.

Fetal hemoglobin measurement

The measurement of HbF levels was carried out using an alkaline denaturation procedure as described previously (9,23) and also by ELISA using a kit purchased from Bethyl Laboratory, Montgomery, TX. After 14 days of incubation BFU-E derived colonies from each experiment in triplicate wells were pooled, centrifuged, and washed three times with PBS. The cells were lysed with sterile water and the sample was split in half for total hemoglobin and HbF measurements. The lysed sample for HbF measurement was subjected to alkaline treatment followed by ammonium sulfate precipitation to remove adult hemoglobin as described previously (9). The HbF in supernatant was measured by conventional colorimetric method using TMB (Tetramethyl benzidine) as a chromogen (Sigma Chemical Co., St. Louis, MO). For comparison, we also measured HbF levels by ELISA (10,12). The percentage HbF was calculated as a percent of total hemoglobin normalized by total protein. Thus, percent HbF is expressed as % HbF = (HbF/Total Hb) x 100.

Statistical analysis

All results are presented as means ± SEM. Statistical analyses were performed using one way analysis of variance (ANOVA) in figures 1-2 and table 3. The Tukey test and Dunnett test (24) were used for multiple comparisons when ANOVA indicated statistically significant differences between or within groups. To analyze the combined effects of various concentrations of HU and arginine, the data is arranged in a factorial design format and the two-way ANOVA is performed. For the data depicted in table 1 and table 2, with different sample sizes, a generalized linear model is applied and the two-way ANOVA is performed

using SAS 9.1. To compare the significance of the responses with respect to the control, we use the two sample t-test as depicted in table 1 and table 2. Differences were considered to be significant when $p<0.05$.

RESULTS

The number of BFU-E derived colonies from erythroid progenitors and HbF synthesis in the culture medium were measured on day 14. The number of BFU-E derived colonies in the culture medium reflects the cytostatic effect of hydroxyurea on cell growth. An attenuation of BFU-E colony growth is essential in the induction of HbF synthesis (2,9,23). Most experiments in this study compare the number of BFU-E derived colonies and percent HbF seen in culture medium in response to treatment with varying concentrations of hydroxyurea, arginine or combinations of hydroxyurea and arginine.

Effects of hydroxyurea and arginine individually on BFU-E derived colony growth and fetal hemoglobin synthesis

Prior to establishing the conditions required for optimum HbF synthesis using hydroxyurea and arginine in combination, the individual effects of hydroxyurea and arginine on BFU-E derived colony growth and HbF synthesis in cultured erythroid cells were assessed. As shown in Fig.1a, arginine (25µM-1000µM) caused a dose-dependent increase in the number of BFU-E derived colonies compared to control (no arginine). Maximum BFU-E derived colony growth was seen with arginine, 500µM. Arginine concentrations at 25-50µM did not significantly increase the number of BFU-E colonies above control (no arginine). Compared with control cells and cells treated with 25-50µM arginine, a significant increase in the number of BFU-E derived colonies was seen when cells were treated with arginine 100-1000µM ($p<0.001$). There was not a significant difference in the number of BFU-E derived colonies when comparing cells that were treated with arginine 100, 500, and 1000µM. In contrast to arginine, hydroxyurea (25-1000µM) dose-dependently decreased the number of BFU-E derived colonies (Fig. 1a). The maximum cytostatic effect of hydroxyurea was seen at 100µM. Hydroxyurea was cytotoxic at a dose of >100µM and caused cell death as demonstrated with trypan blue exclusion technique and also by MTT test procedure.

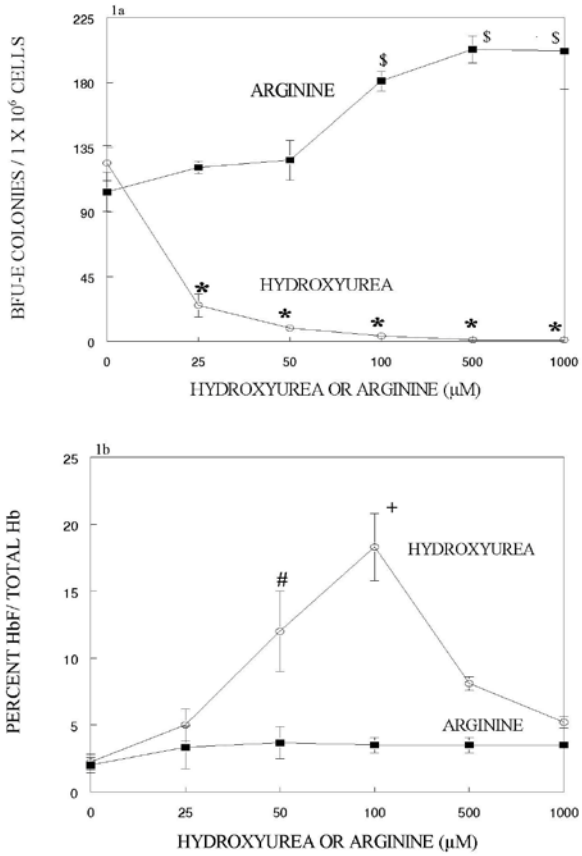

Figure 1a and 1b. Effects of hydroxyurea and arginine on BFU-E derived colony growth and HbF synthesis in human erythroid progenitors. The cell culture system contained erythroid progenitors from peripheral blood of individuals with homozygous HbS. Cell culture conditions for growing BFU-E derived colonies are described in methods. BFU-E derived colonies were counted after 14 days. Fig. 1(a) shows the number of BFU-E derived colonies in the presence of varying concentrations of hydroxyurea and arginine. BFU-E derived colonies were expressed as the number per $1x10^6$ mononuclear cells. Cells from BFU-E derived colonies were isolated and used to measure HbF. The percent HbF is expressed as % HbF= (HbF/Total Hb) x 100. $^{\$}$ $p<0.001$ compared to arginine 0-50µM, and *$p<0.001$ compared to cells untreated with hydroxyurea. Fig. 1 (b) shows the percent HbF in hydroxyurea and arginine treated cells isolated from BFU-E derived colonies. $^{+}p<0.001$, and $^{\#}p<0.01$ compared to cells untreated with hydroxyurea. The results are expressed as mean ±SEM of 4 experiments done in triplicate.

In contrast to the cytostatic effect of hydroxyurea on BFU-E derived colony growth (Fig. 1b), hydroxyurea significantly ($p<0.001$) increased HbF synthesis in erythroid progenitors in a dose-dependent manner (25-100µM), Fig. 1b. The percent HbF ranged from 2.23% ± 0.58 at base line to 18.3% ± 2.52 ($p<0.001$) with hydroxyurea, 100 µM. Hydroxyurea concentrations above 100 µM attenuated HbF synthesis. Independent of the doses studied, arginine had no

effects on HbF synthesis in this cell culture system (Fig. 1b). These data further confirm previous observations (9,23) that HbF synthesis can occur only after cessation of growth of BFU-E derived colonies in erythroid progenitor cell culture systems.

Effects of hydroxyurea and arginine in combination on BFU-E derived colony growth and hemoglobin F synthesis

To study further the effects of arginine and hydroxyurea combinations on BFU-E derived colony growth and HbF synthesis, cultured erythroid progenitor cells isolated from individuals homozygous for hemoglobin S were treated with varying combinations of arginine (100, 500, and 1000μM) and hydroxyurea (25, 50, and 100μM) shown in Fig. 2a, and 2b. As shown in Fig. 2a, arginine dose-dependently reverses the cytostatic effect of hydroxyurea on growth of BFU-E derived colonies. The increased number of BFU-E colonies is most likely related to the proliferative effects of arginine on BFU-E derived colony growth. The effect(s) of varying concentrations of arginine on hydroxyurea induced HbF synthesis was next examined. Fig. 2b indicates that hydroxyurea dose-dependently increases HbF synthesis. When compared to hydroxyurea controls (50-100μM), hydroxyurea-induced HbF synthesis in cells treated with arginine, 100μM + hydroxyurea, 50-100μM, was not different. Interestingly, arginine at 100μM concentration significantly increased HbF synthesis induced by hydroxyurea at 25μM ($p<0.001$). In contrast, arginine at 500-1000μM significantly attenuated hydroxyurea-induced HbF synthesis. These results suggest that high concentrations of arginine (>100μM) inhibit hydroxyurea-induced HbF synthesis.

The increased stimulation of HbF synthesis seen with 25μM hydroxyurea in combination with 100μM arginine, prompted us to further examine the validity of enhanced HbF synthesis by hydroxyurea at lower concentrations when combined with arginine 100μM. Since the experiments in Fig. 2 were performed using blood from only four patients, additional experiments were performed using blood from larger numbers of patients as shown in Tables 1 and 2.

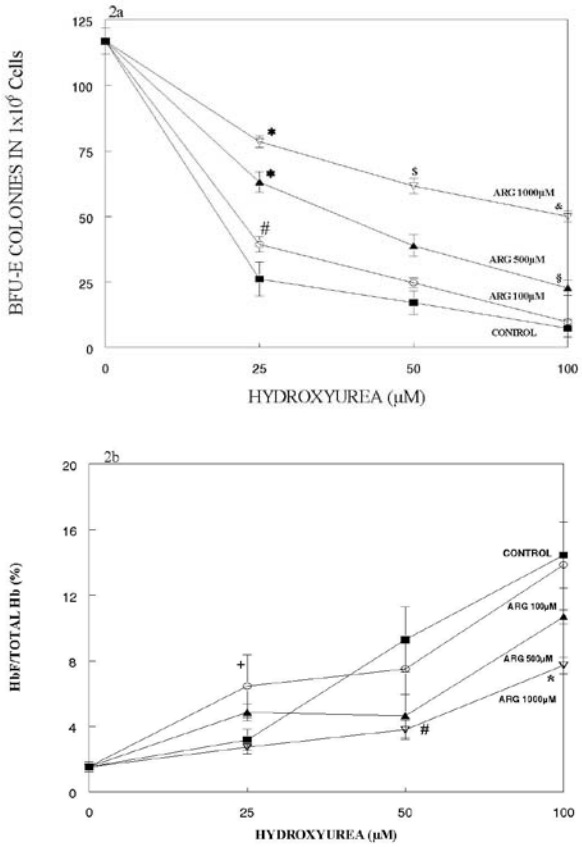

Figure 2a and 2b. Effects of combined mixtures of varying concentrations of hydroxyurea and arginine on BFU-E derived colonies and HbF synthesis in a mononuclear cell culture system. The conditions for cell culture are described in Fig. 1. The effects of varying doses of hydroxyurea and arginine mixtures on the number of BFU-E derived colonies are presented in Fig. 2 (a). *$p<0.001$ compared to hydroxyurea 25μM control; $^{\#}p<0.05$ compared to hydroxyurea 25μM control; $^{\$}p<0.001$ compared to hydroxyurea 50μM control; $^{\&}p<0.001$ compared to hydroxyurea 100μM control; $^{\S}p<0.01$ compared to hydroxyurea 100μM control. The percent of HbF is presented in Fig. 2 (b). $^{+}p < 0.05$ compared to hydroxyurea 25μM control; $^{\#}p<0.05$ compared to hydroxyurea 50μM control; * $p< 0.01$ compared to hydroxyurea 100μM control. The results are expressed as mean ±SEM of 4 experiments done in triplicate.

Effects of lower concentrations of hydroxyurea and arginine combinations on hemoglobin F synthesis

In erythroid progenitors, the concentration of hydroxyurea required to induce maximum HbF synthesis is between 50-100μM. Hydroxyurea concentrations above 100μM is cytotoxic to cell growth and concurrently decrease HbF synthesis. The IC_{50} for hydroxyurea has been reported as approximately

Table 1. Effects of hydroxyurea and arginine combinations on HbF synthesis

		FETAL HEMOGLOBIN/ TOTAL HEMOGLOBIN (%)		
HU	CONTROL (no Arginine)	ARGININE 25 μM	ARGININE 50 μM	ARGININE 100 μM
CONTROL (no HU)	1.53 ± 0.13 (n=12)	0.9 ± 0.09 (n=12)	0.5 ± 0.09 (n=12)	none detected (n=12)
HU 15 μM	2.32 ± 0.16 (n=12)	6.16 ± 0.24* (n=5)	5.14 ± 0.62* (n=5)	6.31 ± 0.39* (n=12)
HU 25 μM	4.08 ± 0.19 (n=16)	6.89 ± 0.38+ (n=12)	8.08 ± 0.33+▪ (n=12)	6.52 ± 0.25+ (n=16)
HU 100 μM	13.24 ± 0.85 (n=12)	10.63 ± 0.81¶ (n=12)	9.67 ± 0.8¶ (n=12)	9.01 ± 0.78¶ (n=12)

* p<0.05 c/w HU 15μM; + p<0.05 c/w HU 25μM; ¶ p<0.05 c/w HU100μM; ▪ p<0.05 c/w HU 25μM/Arg 25 μM; HbF = hemoglobin F; Data are expressed as mean ± SEM. Number of experiments as indicated in triplicates.

Table 2. Effects of hydroxyurea and arginine combinations on BFU-E derived colony growth.

		FETAL HEMOGLOBIN/ TOTAL HEMOGLOBIN (%)		
HU	CONTROL (no Arginine)	ARGININE 25 μM	ARGININE 50 μM	ARGININE 100 μM
CONTROL (no HU)	101.33 ± 2.49	113.58 ± 3.98β	121.67 ± 2.59β	136.25 ± 2.07β
HU 15 μM	74.06 ± 3.5 (n=12)	92.40 ± 2.73+ (n=5)	111.0 ±5.31+ (n=5)	116.7 ± 4.0+ (n=12)
HU 25 μM	37.25 ± 1.70 (n=16)	36.90 ± 0.92* (n=12)	47.58 ± 2.21* (n=12)	79.80 ± 3.36* (n=16)
HU 100 μM	7.17 ± 0.8 (n=12)	6.11 ± 0.86¶ (n=12)	14.83 ± 1.91¶ (n=12)	28.75 ± 2.21¶ (n=12)

β p< 0.05 c/w Control; + p< 0.05 c/w HU 15μM; *p<0.05 c/w HU 25μM; ¶ p< 0.05 c/w HU 100μM; Data are expressed as mean ± SEM of experiments as indicated in triplicates.

52μM in erythroid cell culture (12).The plasma concentration of hydroxyurea in patients taking the drug is approximately150-300μM (3). Since our preliminary studies demonstrated that concentrations of arginine >100μM inhibit HbF synthesis induced by hydroxyurea, and that hydroxyurea >100μM was cytotoxic, subsequent studies were performed using hydroxyurea, 15-100μM and arginine, 25-100μM. Furthermore, arginine concentrations used in this range closely mimic physiologic concentrations of arginine in normal individuals and arginine deficient patients with SCD (14,15,21) as previously discussed. Based on our earlier data, these concentrations were chosen in an attempt to assess whether or not lower concentrations of hydroxyurea + arginine would result in less cytotoxicity while sustaining maximum synthesis of HbF. In Table 1, hydroxyurea, 15μM, in combination with arginine, 25-100μM, resulted in a 2.2 to 2.7 fold increase in hemoglobin F synthesis when compared to the hydroxyurea 15μM control, $p<0.01$. The maximum stimulation of HbF synthesis was seen in the hydroxyurea 15μM + arginine 25μM group as compared with the hydroxyurea 15μM control. Higher arginine (50-100μM) concentrations did not further increase hydroxyurea induced HbF synthesis at the 15μM concentration. Mixtures containing hydroxyurea, 25μM, and arginine, 25μM, 50μM, or 100μM, significantly ($p<0.01$) stimulated HbF synthesis 1.7 fold, 2 fold and 1.6 fold, respectively, as compared to the hydroxyurea 25μM control. When compared to the hydroxyurea 25μM control, maximum stimulation of HbF synthesis was seen in the hydroxyurea 25μM + arginine 50μM group. In the hydroxyurea 100μM groups, maximum stimulation of HbF synthesis was observed with hydroxyurea alone. When comparing the hydroxyurea 100μM control to mixtures containing arginine 25-100μM, reduced HbF synthesis was seen in the hydroxyurea 100μM + arginine 50μM ($p<0.05$) and hydroxyurea 100μM + arginine 100μM ($p<0.01$). This suggests that no additional increase in HbF synthesis occurs when arginine is added to hydroxyurea 100μM (a dose shown to cause maximum stimulation of HbF synthesis).

BFU-E derived colonies were enumerated in each well after 14 days in culture and treatment with various combinations of hydroxyurea and arginine, Table 2. These results suggest that hydroxyurea, even at lower concentrations, must exert a cytostatic effect on the growth of BFU-E derived colonies in order to induce HbF synthesis. The addition of arginine dose-dependently reverses the cytostatic effect of hydroxyurea on BFU-E derived colonies. As expected, hydroxyurea dose-dependently decreases BFU-E colonies in the absence of arginine.

The addition of arginine (25-100μM) resulted in a gradual increase in the number of BFU-E colonies independent of the hydroxyurea concentration (15-100μM) used. The cytostatic effects of hydroxyurea and proliferative effects of arginine on BFU-E derived colony growth were seen with lower concentrations of hydroxyurea and arginine combinations also.

In order to test whether the increase in HbF synthesis seen in cells treated with mixtures of low dose (15-25μM) hydroxyurea and arginine (25-50μM) is dependent on NOS activity, similar experiments depicted in Tables 1-2 were repeated in the presence of the NOS inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME, Sigma Biochemical, Co.). In the experiments depicted in Tables 3-4, the maximum hydroxyurea concentration used was 50μM which approximates the IC_{50} of hydroxyurea in erythroid cell culture (12). In Table 3, the percent HbF in cells treated with varying concentrations of hydroxyurea and arginine mixtures $\pm$ L-NAME were compared. In these experiments, L-NAME, 100μM, was added to the incubation mixtures containing three concentrations of hydroxyurea (15μM, 25μM or 50μM) and two arginine concentrations (25μm or 50μM). In these studies, arginine (25-50μM) $\pm$ L-NAME had no effect on the induction of HbF synthesis compared to control (no arginine or hydroxyurea). Hydroxyurea alone, (15-50μM), increased HbF synthesis in a dose-dependent manner as previously reported. All combination mixtures of arginine + hydroxyurea increased HbF synthesis compared to their hydroxyurea control. Interestingly, L-NAME significantly inhibited (>80%) HbF synthesis seen in cells treated with mixtures of hydroxyurea (15-50μM) and arginine (25-50μM). This suggested that NOS is involved in the induction of HbF synthesis observed in cells treated with arginine and hydroxyurea mixtures.

The effects of L-NAME on the growth of BFU-E derived colonies treated with arginine and hydroxyurea mixtures were also assessed. These studies were performed in parallel to HbF estimations reported in Table 3. No difference in BFU-E colony growth was observed in cells

Table 3. Effects of L-NAME on HbF synthesis in mononuclear cells treated with varying concentrations of hydroxyurea and arginine

FETAL HEMOGLOBIN / TOTAL HEMOGLOBIN (%)

HU	CONTROL (no Arginine)	ARGININE 25µM - L-NAME	ARGININE 25µM + L-NAME	ARGININE 50µM - L-NAME	ARGININE 50µM + L-NAME
CONTROL (no HU)	1.18 ± 0.12	1.25 ± 0.08	1.33 ± 0.11	1.55 ± 0.18	1.25 ± 0.1
15µM	2.35 ± 0.18 ¶	4.7 ± 0.17*	2.12 ± 0.13 #	6.6 ± 0.11*	3.18 ± 0.13 #
25µM	2.83 ± 0.15 ¶	7.7 ± 0.19*	3.30 ± 0.29 #	8.33 ± 0.18*	3.35 ± 0.48 #
50µM	8.33 ± 0.24 ¶	9.45 ± 0.26*	8.43 ± 0.24 #	9.17 ± 0.15*	8.23 ± 0.22 #

HU = hydroxyurea; L-NAME = N^G-nitro-L-arginine-methyl ester; (-) no L-NAME; (+) L-NAME added 100µM; Arg = arginine. *p<0.05 c/w HU (15µM, 25µM, 50µM) control; #p<0.05 c/w the appropriate HU & Arg control; ¶p<0.05 c/w control (no HU, no Arg) The results are expressed as mean ±SEM of 4 experiments done in triplicate.

treated with arginine (25 and 50µM) + L-NAME (100µM) + hydroxyurea (15, 25, and 100µM) as compared to the appropriate arginine (25 and 50µM) + hydroxyurea (15, 25, and 100µM) control.

DISCUSSION

Hydroxyurea is the only drug approved by the FDA that attenuates vaso-occlusive crisis and acute chest syndrome in SCD. The importance of hydroxyurea treatment in SCD is largely attributed to its ability to induce HbF synthesis and improve cellular hydration. HbF has a higher affinity for oxygen than hemoglobin S. Thus when mixtures of these hemoglobins are present in hydrated sickle erythrocytes, polymerization kinetics of hemoglobin S are altered in such a way that favors a slower rate of hemoglobin S polymerization and thus a reduction in the number circulating sickled erythrocytes. Thus the stimulation of HbF synthesis is clearly advantageous in many individuals with sickle hemoglobinopathies. Recent studies (5,6,11,12,17) have implicated the arginine-NO-cGMP pathway is mechanistically involved in hydroxyurea-induced HbF synthesis. In addition, other studies have implicated that individuals with sickle cell anemia have decreased NO bioavailability as a result of increased arginase activity and decreased arginine level (7, 8, 14, 21). While the affinity of arginine for purified NOS is much higher than for arginase, the activity of arginase is more than 1000 fold that seen with NOS which accounts for similar rates of arginine utilization at physiological concentrations (18, 22). These observations suggest that by increasing the availability of arginine for NO synthesis, an increase HbF synthesis might be seen with hydroxyurea treatment, particularly in those that are arginine deficient.

Morris, et al (17) previously investigated the effects of arginine and hydroxyurea in combination on their ability to produce metabolites of NO such as nitrates or nitrites in SCD patients. Neither arginine nor hydroxyurea alone increased serum NO products indicating that both arginine and hydroxyurea were required to enhance production of NO metabolites in SCD patients. It remains unclear whether or not the production of NO metabolites can be used as an index of efficacy in the treatment of SCD. While the arginine-NO-cGMP pathway is involved in HbF synthesis (5, 6, 11), NO metabolites such as nitrates or nitrites may not directly represent the amount of HbF produced. The nitrates and nitrites may be largely detoxified products of excess and unused NO generated by arginine.

In the present study, erythroid progenitors isolated from individuals with sickle cell anemia were used to study the effects of hydroxyurea and arginine individually and in combination on BFU-E derived colony growth and HbF synthesis. The primary objective was to determine if lower doses of hydroxyurea in

combination with arginine would stimulate HbF synthesis at a rate observed with higher dose(s) of hydroxyurea administered alone. Should this be the case, strategies employing combination therapy could lend to less cytotoxicity/bone marrow suppression associated with hydroxyurea in the clinical setting. The major findings in this study are: 1) arginine at doses of >100 μM, increase BFU-E derived colony growth and attenuate HbF synthesis induced by hydroxyurea at 50-100 μM; 2) arginine alone has no effect on HbF synthesis; 3) arginine (25-100μM) acts synergistically with low dose hydroxyurea (15-25μM) to stimulate HbF synthesis; and 4) the NOS inhibitor, L-NAME, inhibits HbF synthesis in erythroid progenitors treated with lower doses of arginine and hydroxyurea mixtures.

The inhibitory effect seen with arginine doses of >100μM on hydroxyurea-induced HbF synthesis has not been reported previously. (4,16,17) Although speculative, arginine administered in doses that result in a plasma concentration in excess of 100μM, which is the upper limit of normal plasma arginine, could actually diminish the efficacy of hydroxyurea when compared to hydroxyurea alone as a result of enhanced BFU-E derived colony growth. A second key observation is depicted in Fig. 2b which demonstrates no difference in the amount of HbF synthesis seen with hydroxyurea 100μM when compared to hydroxyurea in combination with arginine at 100μM. This suggests that arginine may not be utilized as a NO donor when hydroxyurea is administered at a dose achieving maximal stimulation of HbF synthesis. This dosing relationship could potentially lead to equivocal results as related to efficacy associated with HbF synthesis.

Furthermore, this study lends clarity to the importance of dosing when using combination therapies. In contrast to higher doses of arginine and hydroxyurea combinations, arginine at 25-100μM, acted synergistically with low dose hydroxyurea (15-25μM) to stimulate HbF synthesis. In Table 1, the arginine + hydroxyurea treatments of 50μM and 25μM, respectively, resulted in the stimulation of HbF synthesis at ~61% of that seen with hydroxyurea, 100μM, while the cytostatic effect of the combination treatment was ~6.6-fold less than that seen with hydroxyurea, 100μM. This observation supports the postulate of synergism between arginine and lower doses of hydroxyurea which potentially should result in less hydroxyurea-mediated cytotoxicity.

Lastly this extends the observation that hydroxyurea induction of HbF synthesis involves the arginine-NO-cGMP pathway. (5,6,11,12,17) In this study, inhibition of NOS with L-NAME attenuated the increase in HbF synthesis observed in the low dose arginine + hydroxyurea groups thus supporting a role for NO production in hydroxyurea induction of HbF synthesis. While others have suggested this (17), this is the first study directly linking arginine + hydroxyurea and HbF synthesis to NO metabolism.

Our findings provide a potentially useful strategy for understanding and designing the experimental conditions required to investigate the effects of combination therapy with arginine and hydroxyurea in the treatment of SCD. Using lower doses of hydroxyurea in combination with arginine may prove equally effective as higher doses of hydroxyurea alone with a lower probability for bone marrow suppression.

In conclusion, the novel and important findings of the present study are that high dose arginine (500-1000μM) antagonizes hydroxyurea – induced HbF synthesis. In contrast, arginine at a physiological concentration (100μM) acts synergistically with low doses of hydroxyurea to stimulate HbF synthesis in human, sickle erythroid cells in culture. Since in vitro studies may not produce the same effect(s) in vivo, the clinical utility of these findings will require further investigation.

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