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Decitabine Increases Fetal Hemoglobin in *P. Anubis* by Increasing γ-globin Gene Transcription

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Abstract

1) Objective—The mechanism responsible for increased fetal hemoglobin (HbF) levels following decitabine treatment remains controversial. These experiments were performed to evaluate the role of transcriptional versus translational mechanisms in the ability of decitabine to increase HbF levels in vivo.

2) Methods—Three normal, nonanemic baboons were treated with decitabine subcutaneously (0.5mg/kg/d) for 10 days. The effect of decitabine on globin chain synthesis and globin mRNA levels was measured in pre- and post-treatment bone marrow (BM) aspirates by biosynthetic radiolabelling with [^{3}H] leucine followed by separation of globin chains by HPLC, and real time PCR, respectively. The effect on DNA methylation of the ε- and γ-globin gene promoters was determined by bisulfite sequence analysis.

3) Results—Decitabine treatment of normal, nonanemic baboons induced similar increases in the γ/γ+β chain synthetic ratio and the γ/total β-like globin RNA ratio and also increased expression of ε-globin transcripts. Increased expression of ε- and γ-globin was associated with decreased DNA methylation of the ε- and γ-globin gene promoters.

4) Conclusion—Decitabine increases HbF in vivo by transcriptional activation of the γ-globin gene.

Introduction

Increased fetal hemoglobin (HbF) alleviates the symptoms associated with sickle cell disease and β-thalassemia increases the life span of patients (1, 2). The ability of decitabine (5-aza-2′-deoxycytidine) to increase HbF levels (>20% HbF) and F cell numbers (>80%) in

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patients with sickle cell disease suggests that this drug may be a useful pharmacological agent in the treatment of this disease (3).

Although the ability of pharmacological inhibitors of DNA methyltransferase (DNA MTase) such as decitabine and 5-azacytidine to increase HbF has been known for nearly thirty years, the mechanism remains controversial. Evidence from multiple studies has implicated DNA methylation of the γ-globin gene promoter in repression of γ-globin gene expression. These include 1) a correlation between DNA hypomethylation of the γ-globin promoter and γ-globin gene expression during development (4, 5, 6), 2) phylogenetic footprinting studies showing acquisition of CpG residues in the 5′ γ-globin promoter region coincident with the recruitment of the γ-globin gene to a fetal-stage expression pattern during the evolution of simian primates (7), and 3) the ability of DNA MTase inhibitors to increase HbF levels in experimental primates and patients with β-thalassemia and sickle cell disease (3, 8-14). The mechanism responsible for DNA methyltransferase (DNA MTase) inhibitors to increase HbF was therefore hypothesized to involve a reduction in the level of DNA methylation of the γ-globin gene promoter, thus alleviating DNA methylation-mediated gene repression and allowing high levels of γ-globin transcription.

The effects of DNA MTase inhibitors are not limited to DNA methylation, however, and other mechanisms have been proposed to explain the ability of DNA MTase inhibitors to increase HbF. These drugs can alter the kinetics of erythroid differentiation by inducing terminal erythroid differentiation in a population of more primitive progenitors capable of expressing higher levels of HbF (15). Transcription factors such as CREB and ATF2 that bind to sequences near the γ-globin gene promoter (20) can be activated by DNA damage and stress signaling pathways following incorporation of DNA MTase inhibitors into DNA (16-19). While stress signaling has been proposed as a general mechanism for HbF induction by a variety of agents (21), the dual mechanism model states that drugs such as histone deacetylase inhibitors and DNA MTase inhibitors increase γ-globin transcription by alleviating the repressive chromatin state of the γ-globin promoter to allowing access to transcription factors activated by stress signaling pathways (22). Recent data from cell culture systems has suggested the hypothesis that decitabine increases γ-globin expression by post-transcriptional and/or translational mechanisms associated with activation of stress-signal transduction pathways rather than through direct transcriptional activation (21, 23, 24). The objective of our studies was to investigate the role of transcriptional and translational control mechanisms in the ability of decitabine to increase HbF in vivo in order to address differences between these proposed mechanisms.

Materials and Methods

Animals

Three normal, unbled baboons (*P. anubis*) were treated with decitabine (0.5mg/kg/d; sc) for ten days. Pre-treatment BM aspirates were obtained prior to treatment and post-treatment BM aspirates were obtained on the day following the last decitabine injection for analysis of globin chain synthesis and globin mRNA levels. All baboon treatments were performed with approval of the Animal Care Committee of the University of Illinois at Chicago.

Purification of BM Erythroid Precursor Cells

Low-density mononuclear cells were enriched from BM aspirates by Percoll gradient sedimentation. Erythroid cells were purified by magnetic column separation using a mouse monoclonal antibody to baboon erythrocytes (BD Bioscience) in combination with anti-mouse IgG1 microbeads (Miltenyi) as previously described (6). Purity of cell purifications were evaluated by microscopic examination of Wright's stained cytospin preparations.

Measurement of Globin Chain Synthesis

Globin chain synthesis in pre-treatment and post-treatment BM erythroid cells was determined by biosynthetic radiolabelling of globin chains in the presence of [^{3}H] leucine (25). Purified BM erythroid precursor cells (1×10^6) were incubated overnight at 37°C in 1ml leucine-free αMEM (Invitrogen) containing 20% dialyzed fetal bovine serum and 50μCi L-[4, 5-^{3}H] leucine. Cells were lysed by multiple freeze-thaw cycles in dry ice methanol baths. Globin chain separation was achieved by HPLC using a LithoCART 250-4 column (VWR) and a Spectra System HPLC (Thermo-Finnegan with acetonitrile-methanol gradients (26). Quantitation of radioactivity in collected fractions was determined by liquid scintillation counting using a Packard Tricarb 1600TR liquid scintillation analyzer. Results are expressed as γ/γ+β chain ratios.

Real Time PCR Analysis of Globin mRNA

RNA was purified from BM erythroid precursor cells using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. RNA was treated with DNase I (Ambion) and used to prepare cDNA using kits according to the instructions of the manufacturers (Fermentas). Custom designed primer-probe combinations from Applied Biosystems were used for the analysis of ε- γ-β- and α-globin gene expression. The sequences of these are also listed in Supplemental Data. Absolute numbers of globin transcripts were determined by extrapolation from standard curves prepared from the cloned amplicons. Results were expressed as a ratio of total β-like (ε+γ+β)-globin mRNA. Statistical significance was assessed using a two-tailed T test for either paired or unpaired observations.

Bisulfite Sequence Analysis

Genomic DNA was extracted using the QIAmp DNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. Bisulfite conversion was performed using the EpiTect Bisulfite kit (Qiagen). PCR amplification of the baboon ε- and γ-globin promoter regions from bisulfite converted DNA was performed as previously described (6). The PCR products were cloned in the pCR4-TOPO vector using a TOPO-TA cloning kit (Invitrogen) and E.coli TOP10 cells for transformation. Clones were selected on LB plates containing ampicillin and minilysate DNA from individual clones was sequenced using an ABI PRISM 3100 genetic analyzer at the University of Illinois at Chicago Core Genomic Facility. At least ten clones were sequenced for each sample.

Results

Effect of decitabine on globin chain synthesis

Three normal nonanemic baboons were treated with decitabine (0.5mg/kg/d; 10d; sc). The effect on globin chain synthesis was measured by biosynthetic radiolabelling of purified erythroid precursor cells isolated from pre-and post-treatment BM aspirates. The globin chains in radiolabelled lysates were separated by HPLC and radioactivity in the separated fractions determined. The γ/γ+β chain ratio was low in the pre-treatment samples (0.04±0.05; mean±SD; Table 1). One animal, PA 7470, did exhibit an unexpectedly high γ/γ +β ratio (0.10) in the pretreatment sample, while γ/γ+β chain ratios in the other two animals reflected the normal low level of HbF synthesis. The γ/γ+β chain ratio was increased in all three animals following the ten day course of decitabine treatment (0.41±0.15; mean±SD; Table 1).

Effect of decitabine on globin gene transcription

The effect of decitabine on globin gene expression was determined by real time PCR analysis of RNA isolated from pre- and post-treatment purified BM erythroid precursor cells. The ratio of ε-globin RNA / total β-like globin RNA was increased following decitabine treatment (.014±.0007; mean ±SD; p<.01, Table 2) compared to pre-treatment (. 0006±.0002). Likewise, the γ-globin / total β-like globin ratio was increased following decitabine treatment (0.378±.173; p<.05, Table 2) compared to pre-treatment (0.065±.081), while the β-globin / total β-like globin RNA ratio was decreased following decitabine treatment (0.505±.054; p<.01, Table 2) compared to the pre-treatment level (0.934±.081). No significant difference in the ratio of α-globin/ total β-like globin RNA was observed between the pre-treatment and post-treatment samples.

The similarity of the γ/γ+β chain ratio (0.41±0.15) and the γ-globin / total β-like globin RNA ratio (0.378±.173) of the three animals following decitabine treatment shows that increased γ-globin chain synthesis results entirely from increased levels of γ-globin mRNA.

Effect of decitabine on DNA methylation of the γ-globin gene promoter

The effect of decitabine on DNA methylation of both the ε- and γ-globin gene promoter regions was analyzed by bisulfite sequencing. Three CpG residues in the 5′ ε-globin promoter region and five CpG sites in the 5′ γ-globin promoter region were analyzed in pre and post-treatment purified BM erythroid precursor cells in 2 of the 3 animals treated. Decreased levels of DNA methylation of the γ-globin promoter were observed in post-treatment samples (23.4, 36.2 % dmC) compared to pretreatment samples (81.8, 74.4% dmC), respectively (Figure 1A). Similar decreases in DNA methylation of the ε-globin gene promoter were also observed in post-treatment (38.9, 60.6% dmC) compared to pre-treatment samples (87.8, 83.3% dmC, respectively (Figure 1B).

Discussion

Decitabine treatment of nonanemic baboons produced similar increases in the γ/ γ+β synthetic chain ratio and the γ-globin/total β-like globin RNA ratio. Therefore, we conclude

that decitabine increases HbF levels in vivo by increasing the level of γ-globin mRNA. Our data does not support a role for additional translational effects. These results are consistent with a previous report showing that 5-azacytdine increased γ-globin mRNA 4-6 fold in the BM cells of 8 of 9 patients (27). Previous analysis of the effect of decitabine on association of RNA polymerase II, histone acetyl H3, and H3 trimethyl (lys4) throughout the β-globin gene locus clearly showed that decitabine increased association of RNA polymerase II, acetyl histone H3, and histone H3 trimethyl (lys4) with the γ-globin gene promoter (28). Taken together, these results allow us to conclude that increased γ-globin gene transcription is the primary mechanism responsible for increased HbF levels by decitabine in vivo.

The exact mechanism whereby decitabine increases γ-globin transcription remains unclear. According to the dual mechanism model, activation of stress-signal transduction pathways plays a major role in increased γ-globin transcription (20, 22). Effects of the drug on the kinetics of erythroid differentiation could also play a role in the increased level of γ-globin transcription. Because the capacity to reactivate γ-globin is dependent on the differentiation state of erythroid progenitors, increased γ-globin transcription could thus result from induction of terminal differentiation in more primitive erythroid progenitors that contain a mix of positive and negative transcriptional regulatory proteins favoring γ- over β-globin transcription (29).

The large increases in γ-globin expression in these normal, nonanemic baboons are only slightly less than HbF levels observed in bled baboons treated with decitabine. Therefore, our results also clearly show that erythropoietic stress is not required to reactivate high levels of HbF synthesis in response to decitabine, confirming a previous report from this laboratory (8). Significant increases in HbF have been observed in cancer patients treated with azacytidine and decitabine confirm that erythropoietic stress is not required to achieve increased HbF synthesis in man (30, 31).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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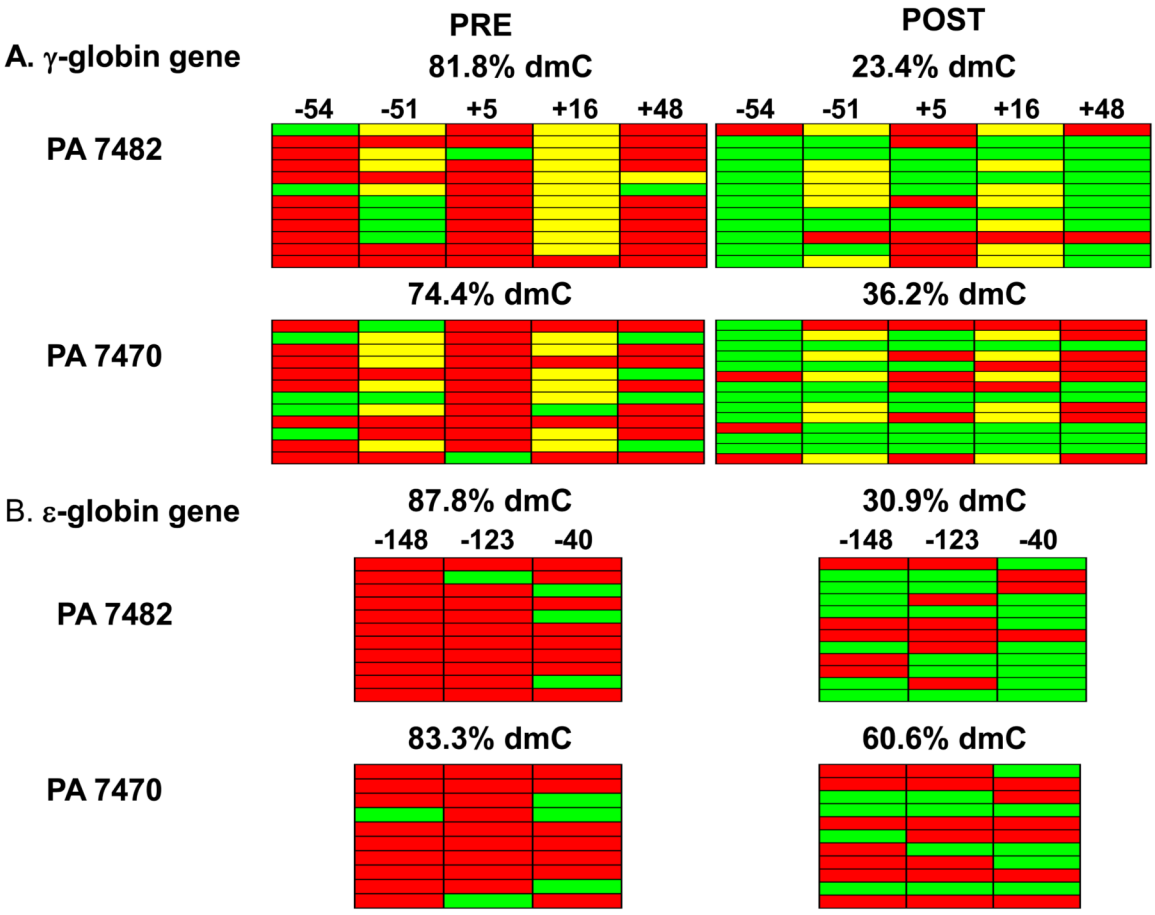

Figure 1.

Effect of decitabine on DNA methylation of ε- and γ-globin promoter regions. Results of bisulfite sequence analysis of DNA methylation of CpG residues within the 5′ ε- and γ-globin promoter regions in the BM erythroid precursor cells isolated from two baboons pre- and post-treatment. Each row depicts the results of sequence analysis of a PCR amplicon cloned in pCR4. Methylated residues (Red), unmethylated residues (green), polymorphic sites in the baboon γ-globin promoter where the CpG site is absent (yellow).

Table 1

Effect of decitabine on globin chain synthesis in vivo

	Globin chain synthesis (γ/γ+β)	
Animal	**Pre**	**Post**
PA 7482	0.01	0.51
PA7470	0.10	0.49
PA 7472	0.01	0.24
Mean±SD	0.04±0.01	0.41±0.15

Table 2

Effect of decitabine on globin mRNA expression in vivo

	Globin mRNA expression (mRNA/total β-like globin mRNA)					
Animal	**ε**		**γ**		**β**	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
PA 7482	.0008	.0141	.026	.526	.972	.457
PA 7470	.0005	.0135	.158	.421	.841	.564
PA 7472	.0005	.0149	.011	.187	.988	.493
Mean±SD	.0006±.0002	.014±.0007	.065±.081	.378±.173	.934±.081	.505±.054