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Inhibition of β-globin gene expression by 3′-azido-3′-deoxythymidine in human erythroid progenitor cells

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Abstract

3′-Azido-3′-deoxythymidine (AZT) treatment in HIV-infected patients is limited by bone marrow suppression including neutropenia and anemia. Previous studies had shown a direct effect of high concentrations of this drug on globin gene expression in K-562 erythroleukemia cells. To better define the mechanism(s) of AZT-induced bone marrow toxicity, the present study evaluates these effects in more relevant human erythroid progenitor liquid cultures, because AZT is 100 times more toxic to human bone marrow cells than K-562 cells. At a clinically relevant concentration of 1 μM, AZT inhibited specifically erythroid cell growth by ~58% as compared with untreated cells. The percentage of cells synthesizing hemoglobin was decreased also by 47% in AZT-treated cells with β-globin mRNA levels accounting for 0.27 pmol in treated cells as compared with 1.44 under control conditions while β-actin levels remained unchanged. Under the same conditions, AZT inhibited the β-globin chain synthesis by ~60% as compared with the control. Consistent with the data described above was the finding that a concentration as low as 0.1 μM of AZT decreased by almost 40% the binding level of the erythroid-specific transcription factor GATA-1. These findings demonstrate that AZT, at clinical relevant concentrations, specifically inhibits β-globin gene expression in human erythroid progenitor liquid cell culture.

Keywords: Drug; HIV; AIDS; Nucleoside; Toxicity

1. Introduction

Since 1983, when the human immunodeficiency virus type 1 (HIV-1) was first isolated in individuals affected by the Acquired Immunodeficiency

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Syndrome (AIDS), the main group of clinically active compounds utilized on a large scale to inhibit viral replication are nucleoside analogs (Mitsuya and Broder, 1986). This class of compounds are metabolized to their respective 5′-triphosphate (Furman et al., 1986) and acts as DNA chain terminator or as inhibitors of the viral reverse transcriptase.

Among the more prominent therapeutic nucleoside analogs is 3′-azido-3′-deoxythymidine (zidovudine or AZT), which was first discovered and has been used since 1987 (Mitsuya et al., 1985). Despite the blocking of HIV cytopathic effects, AZT treatment has been limited by its toxic side effect in bone marrow cells which are manifested by anemia and neutropenia in about 40% of patients (Richman et al., 1987). Although decreased dosage has been evaluated, the frequency of hematological toxicity is still observed in $\sim 30\%$ of patients (Fischl et al., 1990; Collier et al., 1990).

In an attempt to decrease AZT-induced hematopoietic toxicity, nucleoside treatment regimens have been combined with recombinant erythropoietin (Fischl et al., 1990), hemin (Levere et al., 1991; Loewy et al., 1992; Fowler et al., 1996b) and hemoglobin both in vitro (Fowler et al., 1996a) or in vivo (Moqattash et al., 1997). Substantial decreases in bone marrow toxicity have resulted from a combination of AZT with different hematopoietic growth factors and cytokines such as interleukin-1 (IL-1) plus granulocyte-macrophage colony stimulating factor (GM-CSF) (Castello et al., 1995), interleukin-3 (IL-3)/GM-CSF in immunodeficient mice (Gallicchio et al., 1995) or as a fusion protein in vitro (Gallicchio and Hughes, 1995), erythropoietin with IL-3 in anemic mice (Gogu et al., 1995). Moreover, other agents such as lithium carbonate (Gallicchio et al., 1993), zinc and *N*-acetylcysteine (Gogu and Agrawal, 1996) have also been shown to reduce the AZT induced anemia.

To date very few molecular studies have attempted to understand the precise mechanisms of AZT-induced anemia, and thus far they have not been clearly delineated. This fact led our group to thoroughly investigate all the possible molecular mechanisms involved in AZT toxicity. Initially, we demonstrated that AZT (Weidner and Sommadossi, 1990), as well as its metabolite 3′-amino-3′-deoxythymidine (Weidner et al., 1992), decreased steady state levels of globin mRNA in butyric acid-induced differentiating human K-562 leukemia cells. The reduction in globin mRNA levels was associated with decreased globin transcription in vitro, suggesting that AZT and its metabolite may effect regulatory processes directly involved in the control of gene expression and therefore alter the development and differentiation of erythroid cells. We provided further support for the hypothesis that AZT directed affected gene expression regulatory processes by showing that K-562 cells cultured in the presence of AZT, exhibited a decrease in the binding levels of two major transcription factors of erythroid differentiation, GATA-1 and NFE-2 (Bridges et al., 1996). It is important to take into consideration that the K-562 leukemic cell line is a cellular model, which only after induction with various chemical agent such as butyric acid is able to synthesize embryonic and fetal hemoglobins. However, these cells are at least two orders of magnitude less sensitive to AZT than human bone marrow cells, necessitating the use of non-clinically relevant concentrations of AZT (100 μM) (Weidner and Sommadossi, 1990; Bridges et al., 1996).

The goal of the present study was to evaluate AZT toxicity in primary liquid culture of erythroid progenitor cells (BFU-E), which more closely represents a model to study the bone marrow toxicity at clinical relevant concentrations. In this report, we show the effect of AZT (0.1–1 μM) on mRNA steady-state levels, in vitro transcription of the β-globin gene, and binding levels of the erythroid-specific transcription factor GATA-1 in BFU-E cells.

2. Materials and methods

2.1. Materials

AZT, 2′,3′-dideoxycytidine (DDC), benzidine dihydrochloride, β-mercaptoethylamine, pyronine Y hystopaque 1770 and human recombinant stem colony factor were purchased from Sigma, St.

Louis, MO. Guanidine thiocyanate was obtained from USB, Cleveland, OH. Human recombinant IL-3 and anti-CD34-PE were purchased from Becton Dickinson, Bedford, MA. Human recombinant erythropoietin (EPO) was purchased from Connaught labs, Willowdale, Ont., Canada. Hank's balanced salt solution, fetal bovine serum, McCoy's 5A medium, reverse transcriptase and ribonuclease inhibitor were obtained from Gibco/BRL, Grand Island, NY. CD 34^+ isolation kit and magnetic separation columns were obtained from Miltenyi Biotech, Inc., Sunnyvale, CA. Taq polymerase and dNTPs were obtained from Perkin Elmer, Foster City, CA. β-Globin primers were obtained from Oligos Etc., Wilsonville, OR. All other chemicals and reagents were of the highest analytical grade available.

2.2. Enrichment and purification of CD 34^+ cells

Human bone marrow cells were obtained from a rib following thoracic surgery as described previously (Sato et al., 1991). Cells were flushed using a 10 cc syringe filled with Hanks balanced salt solution (HBSS) supplemented with 10% fetal bovine serum (FBS) and 2% heparin (Sato et al., 1991). Bone marrow samples where then layered on Ficoll–Hystopaque and centrifuged at $400 \times g$ for 30 min to isolate mononuclear cells (MNCs). The MNCs were then washed twice in HBSS/10% FBS. Between 35 and 50×10^6 MNCs were incubated overnight in a T-25 flask containing 6 ml minimum medium (McCoy's 5A medium and nutrients) at 37°C with 5% CO_2 to adhere the monocytes and macrophages. Enrichment of hematopoietic progenitor cells CD 34^+, which represents the 0.5–3% of MNCs, was obtained using the Magnetic Cell Sorting kit (MACS) (Lansdorp et al., 1990). This immunomagnetic system allows isolation of CD 34^+ cells using colloidal superparamagnetic microbeads recognizing modified CD 34 antibodies that specifically attach to antigens present in cells surface of targeted cells. Therefore, passing all MNCs trough a column attached to a magnetic device, only targeted cells were retained. When the column was removed from the magnetic separator, CD 34^+ cells were eluted simply washing the column with MACS buffer (PBS/0.5% BSA). Cells were then incubated for 1 h with anti-CD34-PE (HPCA 2) monoclonal antibody before being sorted by fluorescence activated cell sorting (FACS) as described previously (Faraj et al., 1996). Cells were then counted using a hemocytometer and a viability of more than 96% was measured following trypan blue staining.

2.3. Liquid suspension culture of BFU-E

Cells were cultured in supplemented McCoy's 5A medium enriched with 10% FBS in the presence of 0.1 mM 2-mercaptoethanol, 50 U/ml human recombinant IL-3, 2 U/ml human recombinant EPO and 7.14 ng/ml stem cell factor (SCF). Cells were then incubated in a 24-well tissue culture dish with or without drugs. After 14 days of incubation, viability was $> 96\%$, as measured by trypan blue staining. The synthesis of heme or hemoglobin was determined by a benzidine staining methodology (Gopalakrishnan and Anderson, 1979). Briefly, after pipetting up and down to ensure homogeneous distribution of cells, an aliquot (100 μl) was placed in a 1.5 ml microcentrifuge tube to which was added 25 μl of 0.2% (w/v) benzidine dihydrochloride in 0.5 M acetic acid solution containing 0.4% of a 30% hydrogen peroxide solution. After a 10-min incubation at room temperature, cells were scored for hemoglobin production using a hemacytometer.

2.4. Isolation and reverse transcription of total RNA

BFU-E cells were pelleted and total cellular RNA was isolated by acid guanidium thiocyanate/phenol/chloroform extraction (Chomcynsky and Sacchi, 1987). Total RNA was then dissolved in 30 μl DEPC treated water and stored at -70°C until use. RNA concentration and purity was assessed by UV absorbance of 5 μl of sample at 260/280 nm (ratio > 1.8). Equal volume (100 ng) of each total RNA sample was reverse transcribed in a total volume of 20 μl, each containing 5 mM $MgCl_2$, $1 \times$ PCR buffer, 1 mM each dNTP, 1 U/μl Rnase inhibitor, 2.5 M-MLV reverse transcriptase and 2.5 μM of ran-

dom primers. Incubation was performed in a Perkin Elmer GeneAmp PCR system 9600 for 15 min at 42°C, followed by 5 min at 99°C and 5 min at 5°C.

2.5. Quantitation of mRNA by competitive RT-PCR

A competitive PCR was performed following Gilliland's procedure to measure quantitatively the amount of β-globin mRNA, assuming that the small intron present in the competitor genomic fragment would not affect the efficiency of PCR amplification. A standard competitive PCR was used to establish range of amplification for a known competitor concentration which allowed for greater quantitation accuracy for β-globin in samples of unknown concentrations (Gilliland et al., 1990). A puC19 plasmid with a genomic β-globin gene insertion, kindly provided by Dr Tim Townes, was used as a competitor template. Appropriate oligonucleotide primers were selected in different exons, separated by a 130-bp intron. Therefore, the co-amplification of the competitor that uses the same primers as the target, allowed distinction of products by size. Sense primers GR1 was 5′ CACTAGCAACCTCAAACAGACACC 3′ from the first exon; antisense primer GR2 was 5′ TGCAGCTTGTCACAGTGCAGCTCACT 3′ from the second exon of the β-globin gene. PCR was performed in a total volume of 50 μl, each containing 2 μl of RT reaction, 5 μl of a serial dilution of competitor, 1 × reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 27 pM of each primer and 2.5 U of AmpliTaq DNA polymerase. The cycles profile was as follow: 1 cycle of denaturation at 94°C, 35 cycles of three setpoints: denaturation for 1 min at 94°C, annealing for 1 min at 72°C and extension of primers for 1 min at 60°C; final extension at 72°C for 7 min. For the analysis of the control β actin gene the genomic DNA fragment was 252 bp, between the fifth and the sixth exon, with a small 112 bp introne. Primers were 20 bp long as it follows: left 5′ TGGCACCCAGCACAATGAAG 3′ and right 5′ GCCGGACTCGTCATACTCCT 3′. Amplification was performed in the same volume and concentration detailed above, but the cycle profile was different: 30 cycles of three setpoint, 1 min at 94°C followed by 1 min at 58.2°C and 1 min at 72°C. The final extension was as usual for 7 min at 72°C.

2.6. Densitometric scan analysis

Fifteen microliters of PCR reaction products were resolved by 10% polyacrylamide gel electrophoresis, visualized by ethidium bromide staining and photographed (Polaroid 665) under UV irradiation. The PCR product band densities were analyzed on a GS670 imaging densitometer (Biorad, Hercules, CA). To correct for differences in molecular weight, the band densities of the competitor were multiplied by 1.44 (423:293 bp). Each target value was plotted against its competitor. At the competition equivalent point (ratio = 1), the original quantity of target mRNAs corresponds to the initial quantity of competitor used.

2.7. In vitro translation of RNA and gel analysis

Total RNA at a concentration of 100 ng/μl was translated with an in vitro wheat germ translation system (Boehringer Mannheim) at 30°C for 1 h, in the presence of 15 μCi of [^{3}H]leucine (S.A. 153 Ci/mmol) in a total volume of 50 μl. Tritiated leucine incorporation was determined through a standard TCA precipitation. Twenty microliters of translation products were analyzed on a 0.1% SDS, 10% polyacrylamide gel with a discontinuous buffer system, the gel was stained with Coomassie blue, processed for fluorography and scanned to determine the amount of ^{3}H-leucine incorporated.

2.8. Nuclear extracts and mobility shift assay

Total nuclear protein was isolated in the presence of aprotinin, leupeptin, benzamidin, peptastatin A, phenylmethylsulfonyl fluoride and *o*-phenanthroline (Schreiber et al., 1989). Protein concentrations were calculated following the Bradford method (Biorad Kit, Richmond, CA). In vitro binding of nuclear extract to DNA and electrophoretic mobility shift assay were performed as described previously (Mantovani et al., 1987; Trentesaux et al., 1993; Bridges et al., 1996).

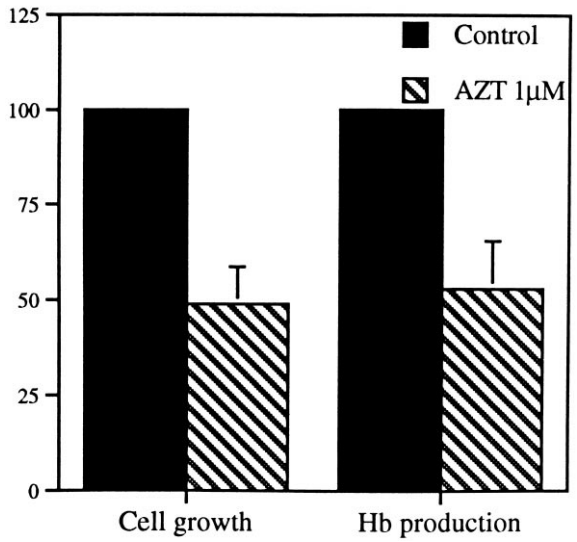

Fig. 1. The effect of AZT on cell growth and hemoglobin production. Human erythroid progenitor cells were cultured in the presence or absence of 1 μM AZT as described in Section 2. Cell number and hemoglobin production were determined after 14 days in culture. Each value is the mean ± S.D. of the value obtained from at least three different experiments.

3. Results

3.1. Effects of AZT on cell growth and hemoglobin production

The growth proliferation of human erythroid progenitor cells was inhibited by AZT at a pharmacologically relevant concentration of 1 μM. Treated cells were inhibited by ~58% as compared with untreated cells (Fig. 1). Viability of cells as assessed by trypan blue exclusion was >96% in treated and control cells. Effects of AZT on hemoglobin synthesis were monitored by a benzidine staining method. The cell Hb synthesis decreased by 53% in AZT treated cells as compared to control (Fig. 1).

3.2. Inhibition of β-globin mRNA expression by AZT

To define if the inhibition of hemoglobin synthesis was due to a decrease in the steady-state level of β-globin mRNA, total RNA was isolated from BFU-E cells after 14 days in culture and β-globin mRNA levels were quantitated by competitive RT-PCR. The PCR products were resolved by acrylamide gel electrophoresis stained with ethidium bromide, photographed (Fig. 2A and B) and picture were scanned for quantitation (Fig. 2C). The effect of AZT on the mRNA globin level were compared to the control group of untreated cells. Results of this analysis showed that AZT treatment reduced steady-state level of β-globin mRNA to 0.27 pmol in BFU-E cells, while under control conditions β-globin mRNA levels were 1.44 picomoles (Fig. 4). We also quantified the mRNA steady-state level of a housekeeping gene, β-actin (Fig. 3A–C), used as an internal control because its gene is highly conserved and expressed in most cell types at high levels. As shown in Fig. 4, the level of β-actin mRNA was essentially unchanged between control and AZT-treated cells accounting for 0.20 and 0.23 pmol, respectively. These results were obtained after at least three different experiments with human bone marrow cells obtained from three different volunteer donors and data where statistically significant (unpaired Student's *t*-test).

3.3. Inhibition of β-globin gene transcription by AZT

The decrease in β-globin mRNA levels could be due to decreased gene expression and/or decreased mRNA stability. To test this hypothesis, we analyzed the effect of AZT in the expression of other mRNA species. Total RNA was translated in an in vitro translation system, composed of wheat germ extract, and products were analyzed in a SDS electrophoresis gel. As shown in Fig. 5, AZT has a minor influence on the expression of other genes as compared to globin genes with the total β-globin mRNA product decreasing in a dose dependent manner as assessed by densitometric scanning.

3.4. Effect of AZT in the binding level of erythroid-specific transcription factor GATA-1

Induction of human erythroid progenitor cells towards erythroid differentiation in the presence

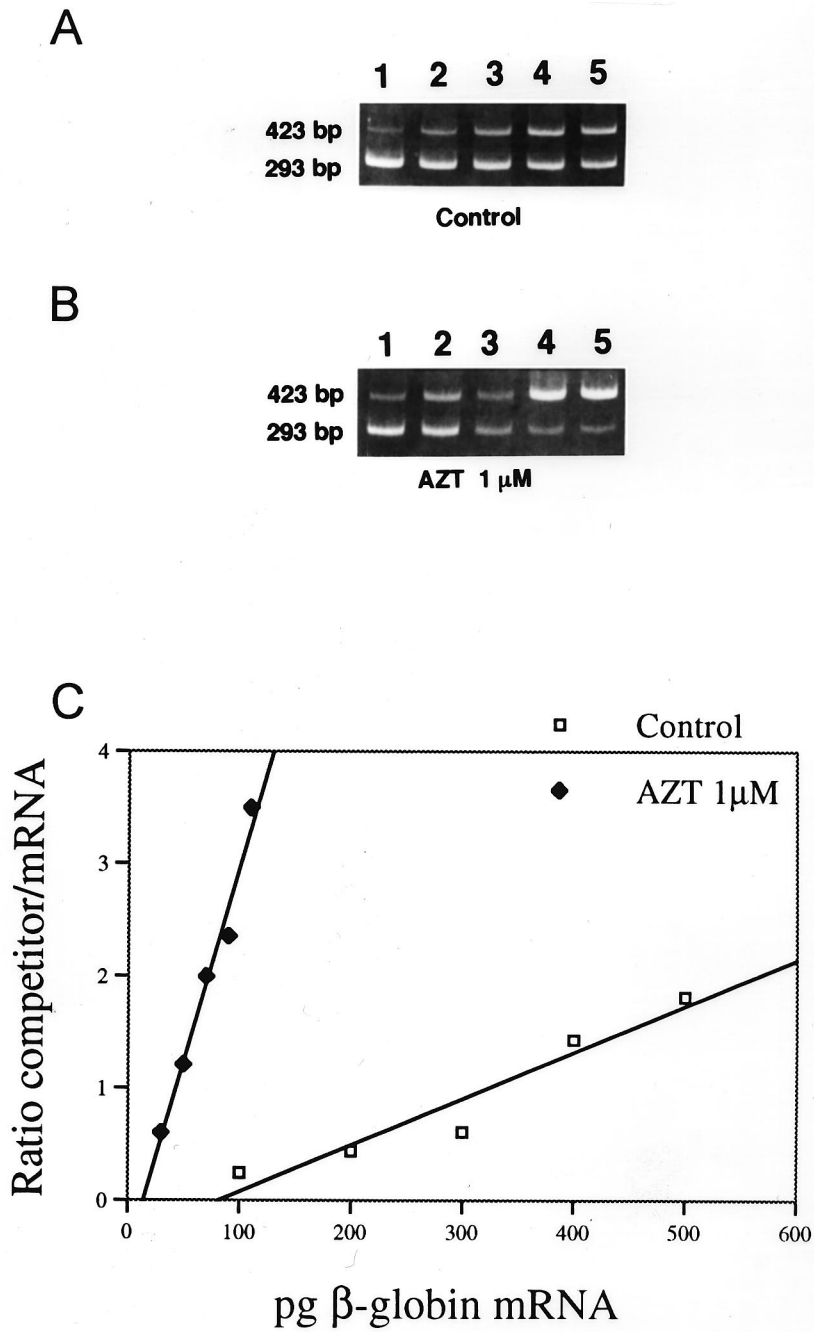

Fig. 2. β-Globin mRNA quantitation by competitive RT-PCR. A constant amount of total RNA (200 ng) isolated from untreated BFU-E cells (A) or treated with 1 μM AZT (B) was reverse transcribed and 1/10 of the product was amplified with a serial dilution of the intron containing competitor fragment: in (A) lane 1, 100 pg; lane 3, 70 pg; lane 4, 90 pg; lane 5, 500 pg; in (B) lane 1, 30 pg; lane 2, 50 pg; lane 3, 70 pg; lane 4, 90 pg; lane 5, 110 pg. A gel electrophoresis separation of the larger competitor fragment (423 bp) and the smaller mRNA target fragment (293 bp) is shown. (C) A graphic representation of the above pictures after scanning of each band. When the ratio competitor/target is equal to 1, the known concentration of the competitor fragments is equal to the target mRNA concentration. In this case 43 pg for the control and 322 pg for the AZT treated sample.

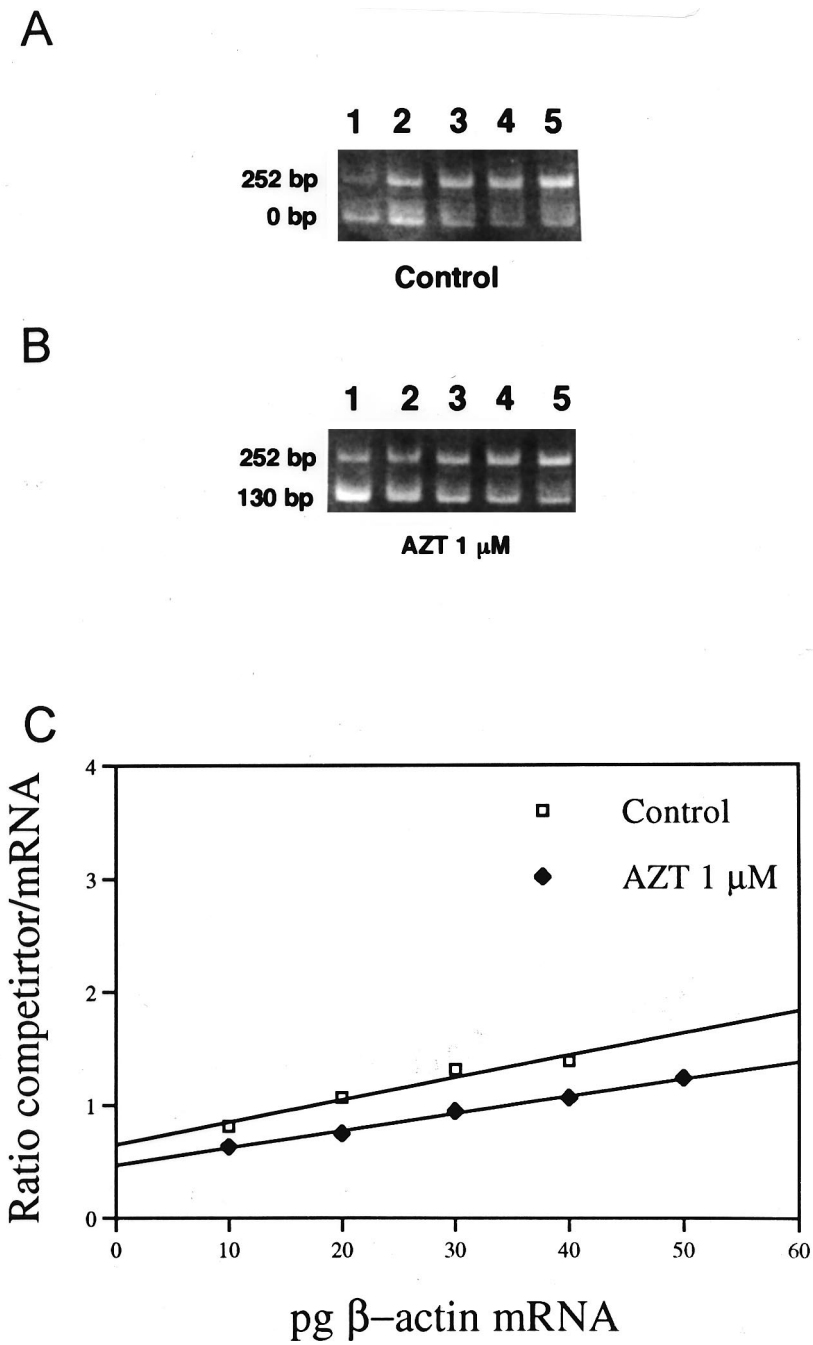

Fig. 3. β-Actin mRNA quantitation by competitive RT-PCR. A constant amount of total RNA (200 ng) isolated from untreated BFU-E cells (A) or treated with 1 μM AZT (B) was reverse transcribed and 1/10 of the product was amplified with a serial dilution of the intron containing competitor fragment; in both (A) and (B) lane 1, 10 pg; lane 2, 20 pg; lane 3, 30 pg; lane 4, 40 pg; lane 5, 50 pg. A gel electrophoresis separation of the larger competitor fragment (252 bp) and the smaller mRNA target fragment (130 bp) is shown. (C) A graphic representation of the above pictures after scanning of each band. When the ratio competitor/target is equal to 1, the known concentration of the competitor fragments is equal to the target mRNA concentration of the competitor fragments is equal to the target mRNA concentration. In this case 18 pg for the control and 34 pg for the AZT treated sample.

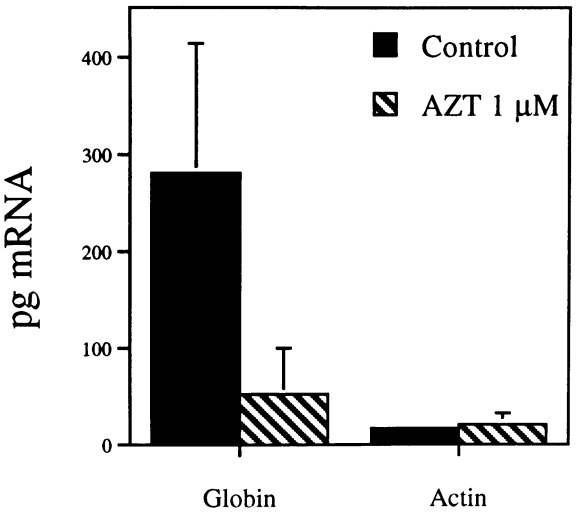

Fig. 4. Inhibition of β-globin mRNA steady state levels by AZT. This graph summarizes the results obtained in at least three experiments. The values represent the mean ± S.D.

or absence of 0.1 μM of AZT over 14 days, showed that AZT reduced GATA-1 binding. Specifically, the level of labeled oligonucleotides encompassing the GATA-1 binding site in nuclear protein extracts from AZT-treated accounted for 62.0 ± 4.0% (S.D.) in AZT-treated cells as compared to control when assessed by gel mobility shift assay. Values were normalized to the ubiquitous transcription factor OTF-1. Values represent the average of at least three different experiments.

4. Discussion

Extensive research has focused on AZT-induced anemia, however, very few studies provide an explanation of the molecular basis of AZT-induced cellular toxicity. Our research has focused on elucidating the molecular mechanism(s) of AZT-induced anemia. Previously, we have shown that AZT is incorporated into the nuclear DNA of BFU-E and CFU-GM in vitro (Sommadossi et al., 1989). In addition, the result of our studies showed that AZT and its in vivo metabolite 3′-amino-3′-deoxythymidine (AMT) (i) are directly involved in the regulation of globin gene expression (Weidner and Sommadossi, 1990), (ii) AZT decreases steady state levels of globin mRNA in butyric acid induced K-562 cells, which is due to a reduction in globin gene transcription and (iii) subsequently, we had shown that AZT reduces the binding level of two major transcription factors, GATA-1 and NFE-2, involved in globin gene expression (Bridges et al., 1996). These findings suggested a major cytotoxicity effect of AZT to the erythroid cell. However, these data were obtained in a K562 erythroleukemia model that has the disadvantage of being at least one hundred-fold less sensitive than human bone marrow cells in culture.

The results of the present study focused on toxicity induced by AZT in an in vitro liquid

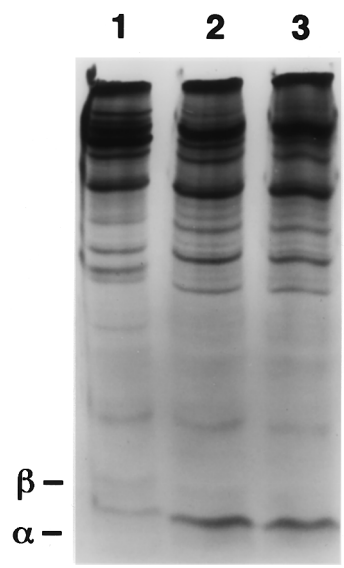

Fig. 5. Inhibition of β-globin gene expression by AZT in an in vitro translation system. Autoradiography of a SDS gel electrophoresis of the products obtained after translational of total RNA isolated from BFU-E cells in the presence of ^{3}H-leucine. Among all different proteins translated, AZT inhibits specifically β-globin expression as shown after 13 days exposure. Bands show the radioactive leucine incorporated: untreated cells (lane 1) show no inhibition of β-globin as compared with cells treated with 0.1 μM (lane 2) or 1 μM AZT (lane 3).

culture system for CD 34^+ human bone marrow cells. This liquid suspension culture, developed in our laboratory, generates cultures of highly purified hematopoietic progenitor cells that, after induction with appropriate hematopoietic growth factors, such as IL-3, SCF, and EPO, differentiate in the erythroid pathway (Faraj et al., 1996). Thus, we have a model system for investigating the effects of AZT that more closely approximates in vivo conditions. The results using this system shows that AZT, at clinical relevant concentration of 0.1 and 1 μM, inhibited cell growth and hemoglobin production by BFU-E. To determine if this effect was due to an inhibition on globin mRNA synthesis, the steady-state levels of β-globin mRNA were analyzed. The result of these studies showed that AZT reduces the β-globin mRNA levels, confirming our previous hypothesis. The effects of AZT were specific because similar quantitation of a housekeeping gene, β-actin, showed that in AZT-treated cells, β-actin levels were essentially identical to control. Further analysis on other mRNA products by in vitro translation system showed that AZT specifically inhibited the β-globin product, while the relative intensity of other mRNA products were essentially unchanged. Collectively, the results of our experiments indicate that changes in β-globin gene expression in AZT-treated BFU-E cells is due to decreased gene expression. The results of our studies showing decreased binding activity of the erythroid specific transcription factor GATA-1 in AZT-treated cells suggest that at the molecular level, the mechanism of AZT mediated effects are directly on β-globin transcriptional activation. Other hypotheses on the mechanism of toxicity of AZT have included a concentration dependent down-regulation of Epo receptor expression, with a reduction in the Epo receptor numbers correlating with a decrease in the mRNA levels of the receptor (Gogu et al., 1992), reversed by a combination of Epo and IL-3 (Gogu et al., 1995). However, these data were obtained treating murine bone marrow progenitor cells. Other groups have formulated different mechanism of AZT erythroid toxicity such as inhibition of mitochondrial DNA replication in a murine erythroleukemia cell model (Hobbs et al., 1992) with a direct evidence for an impairment of oxidative phosphorylation in the presence of 5 μM AZT (Hobbs et al., 1994) or a depression in the pool of available heme with a reduction in the rate-limit ing enzyme α-aminolevulinic acid synthase (Lutton et al., 1990). In addition, a recent study on J2E cells has shown that EPO exerts both a transcriptional and translational control over globin synthesis (Busfield et al., 1995). Other reports indicate that erythropoietin also induces β-globin mRNA in Ba/F3 murine cell line (Carroll et al., 1995) and the erythroid transcription factors GATA-1 and SCL, followed by accumulation of both alpha- and beta-globin chains (Chiba et al., 1993). Thus, our findings are consistent with the EPO inhibition demonstrated by Agrawal's group and with our previous results, suggesting a multifactorial mechanism(s) by which AZT may effect regulatory processes involved in the control of gene expression and development of human erythroid progenitor cells.

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