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RAPID COMMUNICATION

ZIDOVUDINE-INDUCED BLOCKADE OF THE EXPRESSION AND FUNCTION OF THE ERYTHROPOIETIN RECEPTOR

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Abstract - Treatment of bone marrow progenitor cells (BMPC) with zidovudine (AZT) at various concentrations (0.5 to 20 μM) *in vitro* for 24 hr caused a concentration-dependent decrease in erythropoietin (Epo) receptor expression. The decrease in Epo receptors correlated with a decline in mRNA levels of the receptor. These results suggest that AZT-induced down-regulation of Epo receptor expression followed by loss of Epo-receptor mediated signal transduction is a significant contributory factor to AZT-induced erythroid toxicity.

Anemia is the primary dose-related toxicity associated with the administration of zidovudine (AZT[++]) and remains a limiting factor in the clinical management of AIDS [1]. Recently, recombinant human erythropoietin (Epo) has been approved by the FDA for the treatment of AZT-induced anemia in AIDS patients. However, the beneficial response produced by this factor was limited to patients whose endogenous Epo levels were <500 IU/L [2]. The baseline serum levels of endogenous Epo upon AZT therapy alone in some AIDS patients were significantly higher (>3000 IU/L) [2,3], suggesting that the elevated levels of Epo in these anemic AIDS patients reflected relative resistance to the proliferative and differentiative action of Epo on bone marrow progenitor cells (BMPC).

Erythropoietin, a glycoprotein hormone produced by the kidney, is an obligatory growth factor which provides a proliferative signal to burst-forming unit-erythroid (BFU-E) and a differentiative signal to colony-forming unit-erythroid (CFU-E). These effects are mediated by the binding of Epo to specific receptors present on the surface of these cells [4,5]. The Epo receptor expression is dynamic and has been shown to increase as cells differentiate to early erythroblasts and to decrease during terminal erythroid maturation [6]. One potential mechanism for AZT-induced anemia and for the ineffectiveness of exogenously administered Epo in certain AIDS patients may be due to a decrease in Epo receptor expression or its function. Therefore, in this report we have investigated the effects of AZT and didanosine (ddI), the other FDA approved anti-HIV drug for patients intolerant to AZT, on Epo receptor expression and function.

MATERIALS AND METHODS

BMPC. The BMPC were obtained from femurs of 8-week-old CD-1 male mice as described previously [7]. The cells were counted with a hemocytometer and viability was assessed by the trypan blue dye exclusion method.

Biotinylation of Epo. Biotinylation of Epo was carried out as described by Wognum *et al.* [5]. Briefly, 500 U of Epo was diluted in sodium acetate buffer (0.1 M, pH 5.5), containing 0.02% Tween 20 and mixed with a solution of sodium

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++ Abbreviations: AZT, zidovudine (3'-azido-3'-deoxythymidine); ddI, didanosine (2',3'-dideoxyinosine); ddN, 2',3'-dideoxynucleosides; Epo, erythropoietin; b-Epo, biotinylated-erythropoietin; BMPC, bone marrow progenitor cells; and CFU-E, colony-forming unit-erythroid.

metaperiodate (10 mM). The mixture was incubated on ice for 20 min and excess periodate was removed by gel filtration through a PD-10 column (Pharmacia, Piscataway, NJ). The oxidized Epo was conjugated by incubating with a solution of 20 mM biotin-aminocaproylhydrazide (Sigma, St. Louis, MO) for 2 hr at 20°. The unbound biotin reagent was removed by passage through a PD-10 column. The biotinylated-erythropoietin (b-Epo) solution was stored at -70°. The biological activity of b-Epo was compared with Epo in murine BMPC and in spleen cells obtained from phenylhydrazine-treated mice [8] by measuring the stimulation of [^{3}H]thymidine (0.5 μCi/well) incorporation for 4 hr.

Modulation of Epo receptor expression. Modulation of Epo receptor expression by 2',3'-dideoxynucleosides (ddN) was investigated by incubating the BMPC (1 x 10^6 cells/mL) in the presence or absence of various concentrations of AZT or ddI (0.5 to 20 μM). Fluorescence staining of the drug-treated or untreated BMPC was achieved by incubating the cells with b-Epo (7.5 U/mL) for 1 hr at 37° in Hanks' balanced salt solution (300 μL) containing 1% bovine serum albumin and 0.05% sodium azide followed by incubation with streptavidin-R phycoerythrin (Molecular Probes, Eugene, OR) for 1 hr at room temperature [5]. The cells were washed and analyzed by flow cytometry. Quantitative fluorescence analyses were performed using an Epics Profile I flow cytometer (Coulter Electronics, Hialeah, FL). For most of the experiments >25,000 events were collected. The BMPC were identified by forward-versus right-angle light scatter analysis followed by selective gating and Epo receptor analysis of erythroid progenitor cells.

Determination of Epo receptor mRNA. Total RNA was extracted from the control and AZT-treated murine BMPC (1 x 10^7 cells) by the RNAzol method [9,10]. The Epo receptor mRNA was monitored in 5 μg of the cellular RNA by the slot blot hybridization technique. A 39-mer (583-621) cDNA probe was synthesized in our laboratory based upon the published Epo receptor cDNA sequence [11]. The DNA probe was end-labeled with [α-^{32}P]dCTP. A ß-actin probe was used to monitor the actin mRNA by hybridization of the same blot after stripping the Epo receptor probe. The density of the hybridization signals of the Epo receptor mRNA was measured with a GS300 transmittance/reflective scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA).

CFU-E assay. The proliferative capacity of BMPC treated with AZT or ddI was measured by the CFU-E assay as described earlier [7].

RESULTS AND DISCUSSION

Prior to the use of b-Epo to quantitate Epo receptor expression, we first established that the b-Epo conjugate would induce an appropriate biological response as measured by the uptake of [^{3}H] thymidine in murine BMPC and in spleen cells obtained from phenylhydrazine-treated mice [8]. Both Epo and b-Epo equally stimulated [^{3}H]thymidine incorporation in BMPC and spleen cells in a concentration-dependent manner (data not shown). We further demonstrated that the binding of b-Epo to the Epo receptor in BMPC was blocked significantly by a 10-fold excess of Epo (data not shown). These results demonstrated that biotinylation had not altered the biological activity of Epo or its ability to bind to the Epo receptor.

Treatment of BMPC with AZT (0.5 to 20 μM) for 24 hr caused a significant decrease in the cell surface expression of Epo receptors in a concentration-dependent manner (Fig. 1A). At 10 μM AZT, there was an approximately 70% reduction in Epo receptors of BMPC in this assay. The proliferative response of AZT-treated BMPC, simultaneously measured by the CFU-E assay, correlated with the decrease in Epo receptor expression as shown in Fig. 1A. These data suggest a cause-and-effect relationship between AZT treatment and Epo receptor expression and that the loss of Epo receptor-mediated signal transduction may contribute to AZT-induced erythroid toxicity. In contrast, a similar treatment of BMPC with ddI up to 20 μM did not decrease Epo receptor expression significantly as compared to untreated controls (Fig. 1B). However, a concentration-dependent decrease in CFU-E derived colonies was observed upon incubating ddI-treated cells in the CFU-E assay, but to a lesser extent than the AZT-treated cells. Thus, the data with ddI support the previously published results that ddI is relatively less toxic to BMPC.

The effects of AZT at various concentrations (0.5 to 10 μM) on the levels of Epo receptor mRNA in BMPC after a 24-hr exposure are shown in Fig. 2. At this time interval, AZT caused a concentration-dependent decrease in the receptor mRNA levels which declined by 20% at 5 μM and 66% at 10 μM as measured by densitometry. There was a further reduction in the mRNA levels as a function of time declining by 80 and >95% of the control by 48 and 72 hr, respectively.

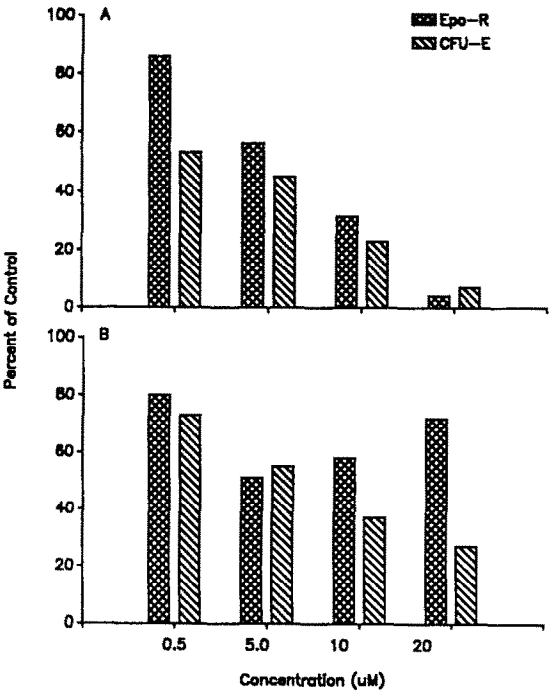

Fig. 1. Effects of AZT (A) and ddI (B) on Epo receptor (Epo-R) expression and proliferation of BMPC. BMPC (1 x10^6/mL) were treated in vitro with increasing concentrations (0.5 to 20 μM) of AZT or ddI for 24 hr and were then assayed for Epo receptor expression by flow cytometry and for proliferation by the CFU-E assay. The experiments were performed in duplicate, and each value is the mean of values obtained from at least two experiments. On an average, 10% of the control BMPC were stained with the red fluorescence. The control value for CFU-E derived colonies was 276 $\pm$ 30.

There was also an overall decline in the receptor mRNA levels of the untreated cells at 48 and 72 hr as compared to the 24-hr control which may be related to the differentiation process of the erythroid progenitor cells [9]. The decline in mRNA levels in the cells treated with AZT correlated with the decrease in Epo receptors (Fig. 1A). Since the half-life of Epo receptor mRNA in BMPC has been shown to be approximately 75 min [9], maintenance of this message for extended periods of time must involve continuous transcription. The results in this communication therefore suggest that AZT may be affecting the transcription process.

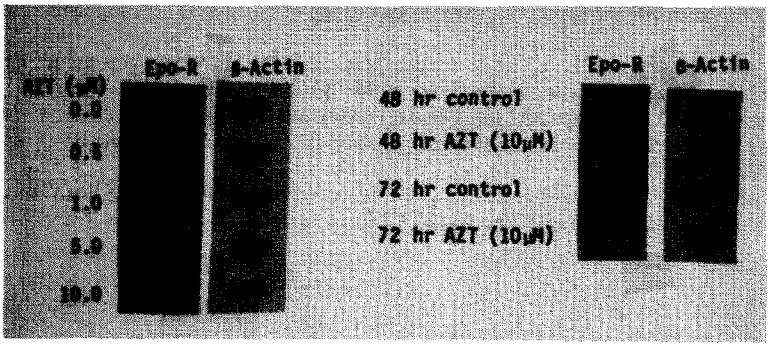

Fig. 2. Levels of Epo receptor (Epo-R) mRNA in murine BMPC treated with various concentrations of AZT for 24 hr and with 10 μM AZT for 48- and 72-hr time intervals. Slot blot analysis was carried out with 5 μg of the cellular RNA as described in Materials and Methods. A ß-actin probe was used to monitor the actin mRNA after stripping the same blot.

Recently, Weidner and Sommadossi [12] reported that the inhibition of globin gene expression may play a role in AZT-induced anemia as assessed by a decrease in globin mRNA levels in the erythroleukemia cell line K562. However, the inhibition of globin mRNA accumulation in these experiments required at least 25 μM or higher concentration of AZT, suggesting that AZT-induced toxicity to erythroid progenitor cells at lower therapeutic levels must also involve other mechanisms. It is also conceivable that K562 cells may be less sensitive to AZT than BMPC. Alternately, inhibition of mitochondrial DNA replication has also been suggested as a possible mechanism of ddN-induced toxicity [13,14]. The present study, however, demonstrates that AZT down-regulated Epo receptor transcription in murine BMPC in a concentration-dependent manner at levels of less than 10 μM. Furthermore, the decrease in Epo receptors correlated with a decline in Epo receptor mRNA levels. It is likely that this blockade of Epo receptor expression and function may precede eventual inhibition of the globin gene expression. These results also indicate that AIDS patients treated with AZT may be divided into Epo-responsive and Epo-nonresponsive groups based upon the presence of a normal or deficient number of Epo receptors in BMPC. Lack of Epo receptors would suggest that Epo alone may not be beneficial in alleviating AZT-induced anemia. This model could also be useful to determine if other pharmacological interventions may restore Epo receptor expression followed by erythroid progenitor cell proliferation, thereby alleviating AZT-induced anemia in patients with AIDS.

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