Erythropoietin-Induced Metallothionein Gene Expression: Role in Proliferation of K562 Cells

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Recent evidence has demonstrated an appreciable expression of metallothionein (MT) in erythrocytes. However, the induction of the MT protein by hematopoietic growth factors and its subsequent functional significance on clonal expansion or differentiation of erythroid progenitor cells remain elusive. We therefore examined the effects of growth factors erythropoietin (EPO), granulocyte-monocyte colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3) on MT gene expression in erythroid progenitor K562 cell line. EPO, but not IL-3 or GM-CSF, induced a 3-fold increase in MT transcripts in K562 cells. MT induction was associated with EPO-induced cellular proliferation, suggesting a specific role for MT induction by EPO in erythroid progenitor cells. However, EPO- or sodium butyrate–induced differentiation as monitored by hemoglobin formation was inhibited in K562 cells stably transfected with an expression vector containing human MT-IIA gene. This inhibition of differentiation was partially reversed in these cells by an MT antisense phosphorothioate oligonucleotide. Furthermore, the MT-induced inhibition of differentiation was associated with downregulation of EPO receptor transcripts in K562 cells. These data suggest that, among growth factors required for erythropoiesis, EPO is a potent inducer of MT, and that MT may plays a significant role in EPO-induced proliferation, but not in the erythroid-specific differentiation of the progenitor cells. Exp Biol Med 228:1033–1039, 2003

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Metallothioneins (MTs) are a class of cysteine-rich, low-molecular-weight, heavy metal binding proteins (1). MTs are expressed in a variety of cell types, and regulation of their biosynthesis occurs mainly at the level of transcription. The metalloproteins are inducible at the transcriptional level by a vast array of hormones and cytokines in a tissue-specific manner. An increase in the expression of MT gene in the bone marrow cells has been shown to occur in response to zinc, but not upon exposure to interleukin (IL)-1 or -6 (2). We have also reported that the expression of MT mRNA was significantly enhanced by zinc in murine bone marrow cells under *in vitro* conditions (3). It has been reported that under conditions of induced erythropoiesis or anemia, the induction of MT in the rat bone marrow is enhanced, with predominant accumulation in erythroblasts; however, in nonanemic rats, the induction of MT in bone marrow by zinc requires prior treatment with erythropoietin (EPO) (4). These studies suggest that not only is MT synthesis increased in EPO-sensitive precursor cells, but it may also be involved in clonal expansion or erythroid terminal differentiation. These observations prompted us to hypothesize that enhanced synthesis of marrow MT is not exclusively confined to heavy metal induction but also to factors controlling growth and differentiation of erythroid lineage.

Detection of an appreciable quantity of MT proteins in mature erythrocytes (5, 6) suggests that the source of MT in red blood cells may be due to an induction by EPO in the marrow erythroid precursor cells. This may be attributable to the fact that exogenous ^{109}Cd-MT was not internalized by erythrocytes, whereas higher concentrations were detected in mature erythrocytes from ^{109}Cd-injected mice pre-treated with phenylhydrazine or EPO (7). Although these data in mature erythrocytes demonstrated enhanced MT synthesis ensuing from heavy metal treatment, the direct induction of MT in erythroid progenitor cells by hematopoietic growth factors, such as EPO, IL-3, or granulocyte-monocyte colony-stimulating factor (GM-CSF), has not been elucidated.

Perturbations of signal transduction pathways in erythroid precursor cells by various agonists can lead to growth and terminal maturation of these cells. In erythropoiesis, erythrocyte maturation is governed by a cascade of events controlled largely by cytokine receptor superfamily members GM-CSF, IL-3, and EPO. Of these factors EPO is regarded as the key regulatory element involved in growth and terminal maturation of late-stage erythroid progenitors

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by interacting on their surface with specific high-affinity receptors (8, 9). A number of hematopoietic cell systems have been developed to demonstrate the growth and differentiation-promoting ability of EPO. To this end, studies using colony assays of early erythroid progenitor cells (10) indicated that not only does EPO act as a mitogen, but it also acts as a survival factor of splenic erythroblasts in Friend virus–infected mice (11). Further studies demonstrated that ectopic expression of EPO receptor in the IL-3-dependent hematopoietic progenitor cell line Ba/F3 conferred EPO-dependent induction of β-globin transcripts, suggesting that EPO is required for terminal differentiation of erythroid cells (12). Although the differential effects of EPO on erythroid cells are thought to be mediated by EPO receptor, the signaling mechanisms through which mitogenic- and differentiation-specific domains act are still fragmentary. Furthermore, the role of MTs present in the bone marrow progenitor cells in modulating these mechanisms has not been explored.

The objective of this investigation was, therefore, to examine the differential induction of MT by growth factors IL-3, GM-CSF, and EPO in erythroid progenitor cells and to determine the role of MT in EPO-induced proliferation and erythroid-specific differentiation.

Materials and Methods

Cell Culture. Human erythroid progenitor cell line K562 were purchased from American Type Culture Collection (ATTC, Manassas, VA) and cultured in Rosewell Park Memorial Institute (RPMI) medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO BRL), penicillin (100 units/ml), and streptomycin (100 μg/ml). K562 cells were maintained in a humidified incubator with 5% CO_2 and 95% air at 37°C.

Induction of MT and Growth Stimulatory Studies. For MT induction experiments, cells were incubated in the absence or presence of various concentrations of recombinant human EPO (up to 120 mU/ml), GM-CSF (up to 200 U/ml), or IL-3 (up to 50 ng/ml) for 24 hr. Total RNA was isolated and analyzed by reverse transcription polymerase chain reaction (RT-PCR) (see below). The effect of EPO on cellular proliferation of K562 erythroid progenitor cells was monitored for 24 hr in the absence or presence of various concentrations of EPO up to 150 mU/ml. Cell growth was assessed by cell counting, in triplicates, and was expressed as percentage of control using analysis of variance (ANOVA) (InStat software, San Diego, CA) and Tukey's multiple comparison tests at $P < 0.05$ level. Significant differences between various groups were expressed as means ± SE.

Plasmid Vectors and Antisense Oligonucleotide. The eukaryotic expression plasmid pBAcNEO-sMT-IIA was a generous gift from Dr. J. Vilcek (New York University Medical Center, New York, NY). The vector contains human MT-IIA gene coding sequence immediately downstream from the β-globin promoter (13). In addition, the construct contains a polyadenylation signal, and the selectable markers, the ampicillin and neomycin resistance genes. A phosphorothioate antisense oligonucleotide (18-mer) with sequence 5′-GGCGCAGGAGCAGTTGGG-3′ (14, 15) complementary to the region 7 bases downstream from the AUG translational start site of the human MT-IIa gene was synthesized from commercial source (Oligo, Inc., Wilsonville, OR). An 18-mer random phosphorothioate oligonucleotide with sequence 5′-TGGATCCGACATGTCAGA-3′ was used as a control.

Transfection and Generation of Stable Cell Lines. Subconfluent cultures of K562 cells (3×10^6) were transfected by electroporation (Cell-Porator, GIBCO BRL) with a single pulse of 250 V/1980 μF using 10 μg of pBAcNEO-sMT-IIA or the empty control plasmid. Cells were then cultured in a complete medium for 48 hr and subsequently selected at low density in a medium containing G418 (1 mg/ml). Cells were replated at limiting dilutions in 96-well microtiter plates and colonies which expressed high neomycin titers were pooled and referred to as K652-MT for MT plasmid and K562-P for plasmid control. Transfection of K562-MT and K562-P with the MT antisense experiments was performed according to the procedure we have described earlier (16). The efficiency of the antisense to downregulate MT was monitored similar to the procedure described earlier (14).

Immunocytochemical Analysis of MT Protein. MT protein levels were determined in K562-MT and K562-P cells with a modified procedure as described previously (17). Briefly, cells were fixed onto Poly-Prep slides (poly-L-lysine coated) (Sigma-Aldrich, St. Louis, MO) and permeabilized using 100% ethanol for 1 min followed by rinsing in tris-buffered saline (TBS) (50 m*M* tromethamine and 150 m*M* NaCl, pH 7.6). After blocking with bovine serum albumin (3% in phosphate-buffered saline), slides were incubated with an optimal dilution of mouse anti-MT monoclonal antibody (E9) (DAKO, Carpinteria, CA) (1:500), rinsed in TBS, and incubated with horseradish peroxidase (HRP)-conjugated protein G, rinsed with TBS, and detected with chromogenic substrate (3,3′-diaminobenzidine, Sigma, St. Louis, MO). Samples were tested and compared for MT protein levels using light microscopy.

RT-PCR Analyses. Due to the presence of a limited number of EPO receptors (EPO-R) in K562 cells, we opted to use RT-PCR analysis for detection of EPO-R and also for MT transcripts in these cells. Total RNA was isolated using a commercial kit (RNAzolB, Tel-TEST, Inc., Friendswood, TX). Stably transfected cells or their respective controls were tested by PCR for MT using [^{32}P]end-labeled universal human MT primers (5′-GCTCGCCATGG ACCCAACTG as a sense and 5′-TTGTCCGACGCCCCTTTG as antisense), as we described previously (14). These primers were designed within the coding region of MT gene and are capable of amplifying both hMT-I and hMT-II transcripts. Equal loading of RNA was judged by the housekeeping gene GADPH (glyceraldehyde-3-phosphate dehy-

drogenase); 5′-GAAGGTGAGGTCGGAGTCAAC G as the sense primer and 5′-TGCCATGGGTGGAATCATATTGG as an antisense primer.

The primers to determine EPO receptor gene expression in K562 cells and stable cells lines were 5′-GCACCGAGTGTGTGCTGACGAA for the sense and 5′-GGTCAGCAGCACCAGCATGAC for the antisense. The PCR conditions included an initial hold at 95°C for 90 sec, followed by 35 cycles of 94°C (denaturation), 55°C (annealing), and 72°C (extension). Blots were exposed to XAR-Kodak films (Rochester, NY), and autoradiographs were analyzed by densitometry (Bio-Rad, model GS700, Hercules, CA).

Induction of Differentiation in MT-Transfected K562 Cells. To examine the effects of MT on EPO-induced differentiation, K562, K562-P, or K562-MT cells were grown in the presence or absence of EPO (1 or 2 U/ml) in complete medium supplemented with 20% conditioned medium from erythroid-potentiating activity (EPA) secreting monocytic cell line U937, 100 units/ml penicillin, and 100 μg/ml streptomycin. EPA condition medium has been shown to increase the expression of EPO receptors in K562 cells (18).

Differentiation induced by sodium butyrate was determined at a final concentration of 0.4 m*M* for up to 48–72 hr. The formation of Hb was monitored by the standard methodology using benzidine staining. The cell suspension (250 μl) was mixed with an equal volume of 0.2% (wt/vol) benzidine dihydrochloride in 0.5*M* acetic acid solution containing 0.4% of a 30% hydrogen peroxide solution. After 10 min, the cells were scored for Hb production using a hemocytometer.

Effect of MT Antisense on Sodium Butyrate–Induced Differentiation. K562-MT and K562-P cells were transfected with an MT antisense or the random control oligonucleotide in accordance with the standard procedure described above. The cells were allowed to grow for 24 hr and subsequently cultured for an additional 48 hr in RPMI medium containing 10% fetal bovine serum in the presence or absence of 0.4 m*M* sodium butyrate. Induction of differentiation was assessed by benzidine staining as described above.

Statistical Analysis. Comparisons between controls and treatment groups was evaluated by one-way ANOVA using GraphPad InStat statistical analysis software (San Diego, CA). Significant differences between various treatment groups were determined using Tukey's multiple comparison test at $P < 0.05$ level.

Results

Induction of MT Gene by EPO in Hematopoietic Cells. The addition of EPO at the physiologic concentrations to human erytholeukemic cells K562 resulted in significant induction of MT gene (Fig. 1). Densitometric analysis demonstrated a 3-fold increase in relative MT mRNA expression in K562 cells treated with EPO (120 mU/ml) as

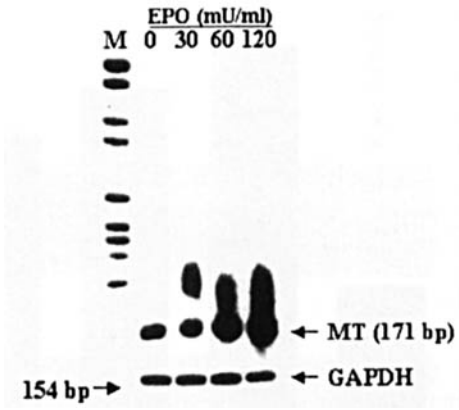

Figure 1. Induction of MT gene expression by EPO in K562 cells. Cells were grown in medium in the absence or presence of EPO as described in "Materials and Methods." Total RNA was extracted with RNAzol kit. RNA was initially reverse transcribed with oligo(dT) primers, amplified with Taq DNA polymerase in presence of MT primers, fractionated onto an agarose gel containing ethidium bromide, and analyzed under UV with densitometric scanning ($n = 3$). Values are presented as means ± SE. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

opposed to parental cells. The relatively lower magnitude of induction of MT gene by EPO in K562 cells may be related to fewer number of EPO receptors per cell. This was confirmed by a 30-fold induction of MT gene (data not shown) using similar concentrations of EPO in the growth factor–dependent TF-1 cells, a cell line that has a higher number of EPO receptors. Furthermore, RT-PCR analysis demonstrated that addition of IL-3 or GM-CSF at various concentrations up to 50 ng/ml or 200 U/ml, respectively, for 24 hr, did not change the basal level of MT mRNA expression in either growth factor–dependent TF-1 or K562 cells (data not shown).

MT Expression Correlates with EPO-Induced Proliferation. The addition of EPO to the culture medium enhanced the proliferative response of K562 cells in a concentration-dependent manner ($P < 0.001$), with a peak induction of 2-fold at 60 mU/ml after 24 hr (Fig. 2), which plateaued at 120 mU/ml ($P < 0.001$). The EPO-induced mitogenic responses in TF-1 and K562 cells correlated with an increase in the MT mRNA expression (Fig. 1), suggesting a role for MT in mediating the EPO-induced erythroid proliferation.

MT Overexpression Inhibits EPO- and Butyrate-Induced Erythroid Differentiation. The effect of MT overexpression on EPO-induced differentiation in progenitor cells was determined in parental K562, K562-P, or K562-MT cells. The relative expression of MT transcripts and protein levels in various cell lines was determined by RT-PCR analysis (Fig. 3A) and immunocytochemical analysis (Fig. 3B), respectively. MT transcripts increased by 5-fold in K562-MT cells in comparison with K562-P and parental cells ($P < 0.02$). Likewise, MT protein levels were also increased as demonstrated by enhanced immunostaining in K562-MT cells in comparison with K562-P (Fig. 3B). To further increase the EPO-R expression, K562 cells were

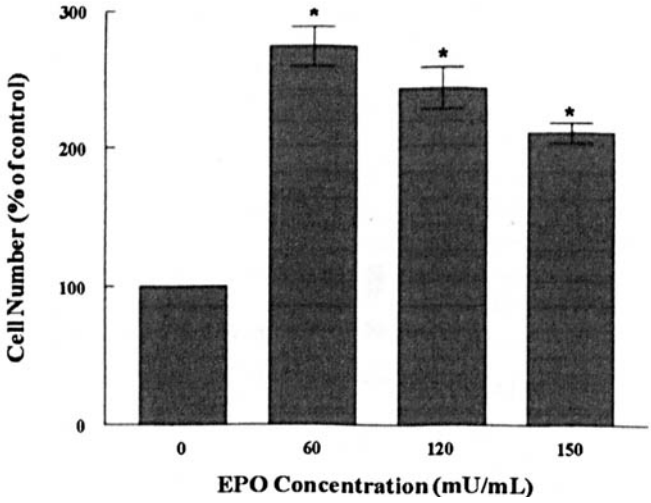

Figure 2. Proliferative response of EPO in K562 cells. Cells were grown in RPMI medium in the absence or presence of various concentrations of EPO for 24 hr. Cells number was determined by cell counting using a hemocytometer and was expressed as percent of control using ANOVA and Tukey's multiple comparison test at $P < 0.05$. Values are presented as means ± SE. *Statistical significance ($P < 0.001$) ($n = 3$).

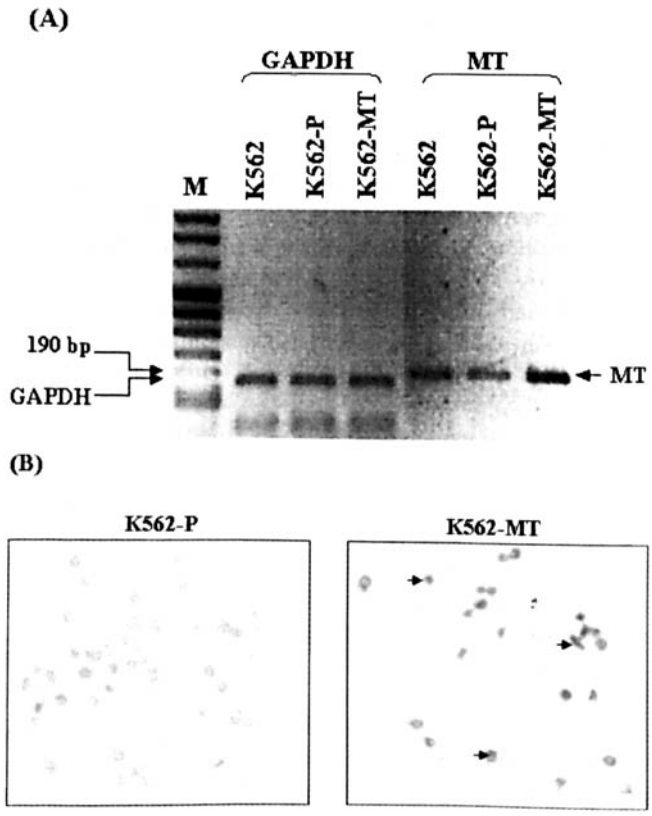

Figure 3. Assessment of MT gene expression in K562 cells. (A K562 cells stably transfected with pBAcNEO-sMT-IIA (K562-MT), empty control plasmids (K562-P), and parental cells (K562). Cells were selected in G418-containing medium as described in "Materials and Methods." Total RNA was isolated from K562-MT, K562-P, or parental cells and essentially analyzed by RT-PCR for MT gene expression ($n = 2$). (B) Immunocytochemical staining of MT protein in K562-P and K562-MT cells as described in "Materials and Methods." Arrow denotes higher expression levels in MT-expressing cells in comparison with controls.

initially cultured in an RPMI medium containing 20% U937 cell conditioned medium prior to addition of EPO at 1 or 2 U/ml for 48 hr. At these concentrations of EPO, both K562 and K652-P cells showed an EPO-induced differentiation of approximately 25%. Conversely, the EPO-induced differentiation in K563-MT cells was significantly decreased by nearly 2-fold ($P < 0.001$) (Fig. 4A). Likewise, the effect of MT overexpression on the induction of differentiation by sodium butyrate was also examined. Whereas sodium butyrate induced differentiation in parental and K562-P cells ($P < 0.001$) in comparison with the untreated control cells, no significant difference was observed in sodium butyrate–treated K562-MT cells in comparison with the parental K652-P or K562-MT untreated control cells (Fig. 4B). The results suggested that MT overexpression inhibits EPO- or sodium butyrate–induced erythroid differentiation.

Reversal of MT Inhibition of EPO-Induced Differentiation by MT Antisense. To determine the specificity of MT's inhibitory effect on erythroid differentiation, we tested whether sodium butyrate–induced differentiation in K562-MT cells can be reversed by an 18-mer MT antisense phosphorothioate oligonucleotide (MT-AS). Initially, we examined whether MT transcripts in K562-MT cells will be downregulated by an MT-AS oligomer. RT-PCR analysis revealed significant (3-fold) reduction in MT transcript levels in cells transfected with MT-AS in comparison with those transfected with random oligomer (RO) or parental control cells (Fig. 5A). Furthermore, transfection of K562-MT cells with an MT-AS resulted in reversal of MT-induced blockade of sodium butyrate–induced differentiation ($P < 0.001$) when compared with cells transfected with the control RO (Fig. 5B).

MT Overexpression Downregulates EPO-R Transcripts. To determine whether the inhibitory effects of MT overexpression on EPO-induced differentiation are mediated by downregulation of EPO-R, total RNA from K562-MT, K562-P, or parental cells was analyzed by RT-PCR using specific amplimer sets for EPO-R and the housekeeping gene G3PDH. As opposed to parental cells or K562-P cells, the relative EPO-R expression was decreased by 3- to 5-fold, whereas the expression of the housekeeping gene remained unchanged in the three cell lines (Fig. 6). These results suggest that the downregulation of EPO-R by MT overexpression may explain inhibitory effects of MT on EPO-induced differentiation in this cell line.

Discussion

In this report, we present evidence that MT gene is inducible by EPO in an erythroleukemic cell line, K562 cells. Zinc, a known inducer of MT, has been shown to regulate MT gene expression in bone marrow progenitor cells and other cell types, including hepatocytes (1–4). Although it has been shown that MT concentrations in erythrocytes correlate with zinc plasma concentrations (5), the

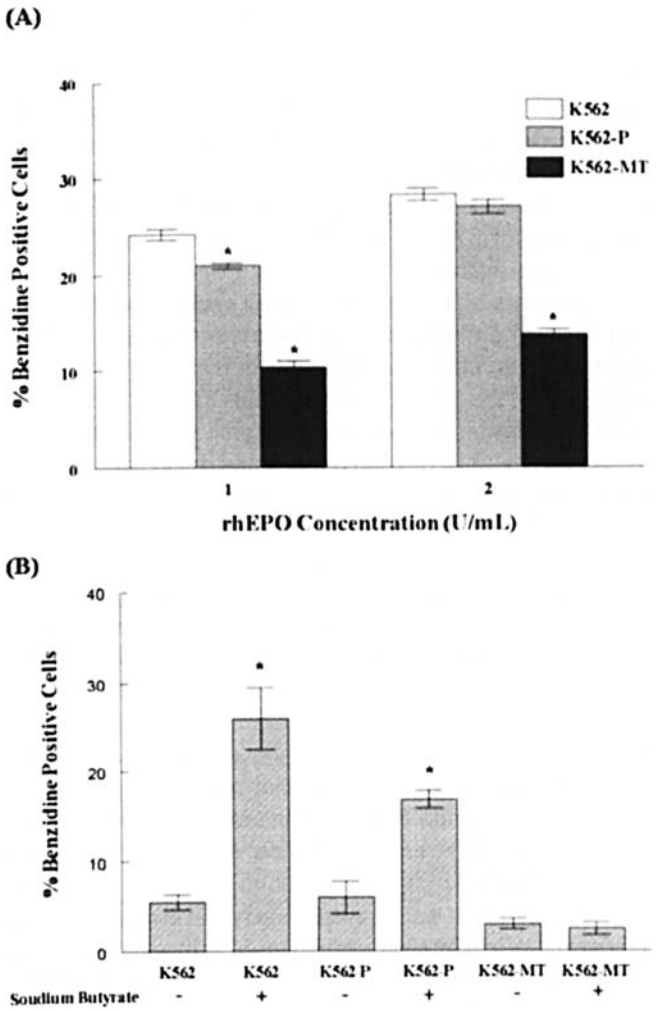

Figure 4. Induction of differentiation in MT-transfected K562 cells. K562-MT, K562-P, or parental cell line using recombinant human EPO (rhEPO) (A) or sodium butyrate (B). The cells were cultured in EPA-conditioned medium in the presence of EPO (1 or 2 U/ml) or sodium butyrate (0.4 m*M*) for 48 hr as described in "Materials and Methods." Cells were then assessed for differentiation using benzidine staining method. The data were compared by using one-way ANOVA and Tukey's multiple comparison test at $P < 0.05$ level. Values are presented as mean ± SE. *Significant difference at $P < 0.001$ ($n = 3$).

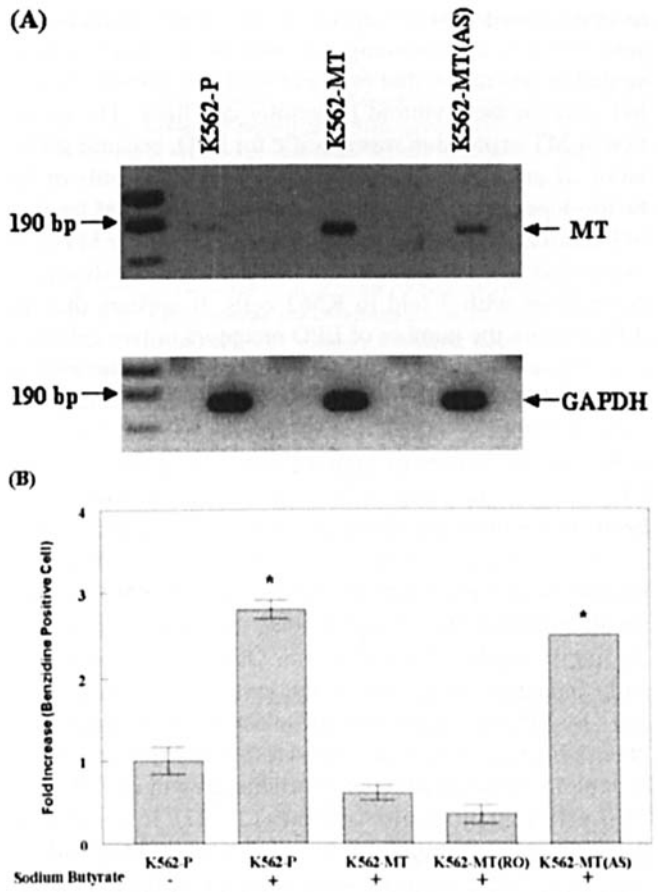

Figure 5. MT overexpression inhibits induced erythroid differentiation. (A) Assessment of MT transcript downregulation in MT antisense transfected K562-MT cells. Transfection of K562-MT with an MT antisense was performed using electroporation and total RNA was isolated and analyzed by RT-PCR as described in "Materials and Methods." (B) Partial reversal of MT inhibition of butyrate differentiation in MT antisense transfected cells. K562-MT cells were initially transfected with an MT antisense [K562-MT(AS)] or random control [K562-MT(RO)] oligomer (see "Materials and Methods") and subsequently subjected to 0.4 m*M* sodium butyrate for 72 hr. Induction of differentiation was monitored by benzidine staining method. Results are expressed as a "fold" increase of blue cells (benzidine +) relative to control. *Statistical significance at $P < 0.001$ level ($n = 3$).

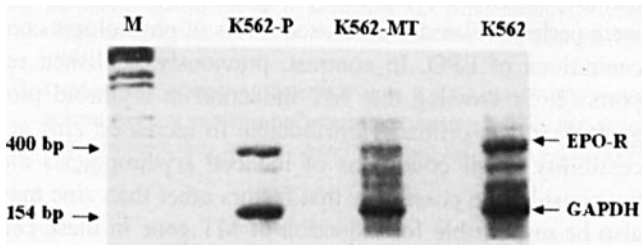

Figure 6. MT overexpression downregulates EPO-R transcripts in K562 cells. K562 -MT, K562-P, or parental cells were monitored for EPO-R mRNA expression using RT-PCR. Details for conditions and primers used for PCR are described in "Materials and Methods." Results are expressed relative to a housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) ($n = 3$).

functional significance of MT in erythrocytes and bone marrow has not been fully delineated. However, it has been proposed that MT may be required for metabolic response to increased zinc demands during proliferation and differentiation of blood cells (2) or to protect DNA against hydroxyl-radical damage (19).

The apparent regulation of MT levels in the bone marrow is of particular interest, as it relates to the proliferation and differentiation of the progenitor cells. To delineate the role of MT in erythropoiesis, we hypothesized that certain members of the cytokine receptor superfamily, particularly EPO, GM-CSF, and IL-3, play a significant role in the regulation of MT synthesis in erythroid progenitor cells. These growth factors share structurally similar extracellular ligand- binding domains, which upon binding by a ligand confer a unique transmembrane signaling. It has long been shown that growth and terminal differentiation of erythroid progenitor cells is mediated by complex interactions between the glycoprotein hormone EPO (20), IL-3 (21), and GM-CSF (22) and their receptors. Although many of these

receptor-ligand systems appear to be largely interchangeable (23, 24) in promoting cell growth, we have demonstrated in this report that only EPO induces transcription of MT gene in the erythroid progenitor cell lines. The induction of MT expression was specific for EPO, because stimulation of growth by IL-3 or GM-CSF of K562 cells or the factor-dependent TF-1 cells did not alter the basal level of MT transcripts. The magnitude of induction of the MT gene expression by EPO was 30-fold in TF-1 (data not shown) in comparison with 3-fold in K562 cells. It appears that the difference in the number of EPO receptors in two cell lines probably accounts for the distinctive variation in the level of induced MT gene expression by EPO in TF-1 and K562 cells. It has been shown that K562 cells express a significantly lower number of high-affinity surface receptors for EPO (18, 25) in comparison with TF-1 cells, which have been shown to express high levels of EPO receptors (26).

The significant early induction by EPO of MT gene (within 24 hr) prompted us to speculate that MT plays a pivotal role in EPO-activated signal transduction pathways leading to erythroid proliferation. Our data demonstrating early induction of growth of the erythroid progenitor cell lines by EPO are consistent with this notion. Reports from other laboratories have also shown that addition of EPO to the culture medium stimulated cellular growth of K562 and TF-1 erythroid progenitor cell lines (25–27). In our studies, the EPO-induced proliferative response correlated with an increase in MT transcripts, suggesting a role for MT induction and stimulation of cell proliferation. These results strengthen the hypothesis that MT promotes cellular proliferation. By employing another cell line, the human breast adenocarcinoma MCF-7 cells, we have shown that the downregulation of MT by an antisense oligonucleotide resulted in cellular growth arrest (14). Another study demonstrated an 80-fold increase in MT protein levels in regenerating liver 24 hr after partial hepatectomy (28). Additionally, our results also support other studies (5, 6) in which the increased levels of MT in erythrocyte and late-stage erythroid progenitors, especially under anemic conditions, were perhaps related to increased levels of physiologic concentrations of EPO. In contrast, previously published reports (2, 5) showing that MT induction in erythroid progenitor cells is primarily attributable to increased zinc accessibility under conditions of induced erythropoiesis did not consider the possibility that factors other than zinc may also be responsible for induction of MT gene in these cell lines. Because cellular proliferation is a prerequisite for marrow MT induction (2, 3) and EPO induces proliferation of progenitor cells, particularly under anemic conditions, the induction of MT in bone marrow cells may be primarily ascribed to the presence of EPO.

It has been shown that EPO activates p21 *ras* via stimulation of tyrosine kinase (29). In another study, it has been shown that cells transformed with *ras* oncogene express atypically high basal levels of both hMT-I_E and hMT-II_A transcripts (30). These studies suggest that EPO-induced *ras* activation may contribute to MT induction. Alternatively, because protein kinase C (PKC) has been shown to contribute to signal transduction pathways of EPO (31), we were able to block the EPO-dependent increases of MT transcripts by PKC inhibitor H-7 (Abdel-Mageed and Agrawal, unpublished observation). Thus, whether the EPO-induced increase in expression of MT is mediated via stimulation of p21 *ras* or PKC activation certainly awaits further investigation.

The central question of how EPO mediates proliferation and terminal differentiation of erythroid progenitor cells remains unclear. Like other members of the cytokine receptor superfamily, the EPO receptor contains a conserved domain in its cytoplasmic region, which may be required for mitogenic activity (12). Recently, one study demonstrated a differentiation domain within the EPO-R (32), suggesting that the EPO-R transmits signals important for both proliferation and differentiation along the erythroid lineage. In the present study, we have shown that there is an inhibition of differentiation signal by EPO or sodium butyrate in the K562-MT cells. Furthermore, RT-PCR analysis of K562-MT cells showed inhibition of EPO receptor mRNA expression, suggesting that downregulation of EPO receptor may partially explain the MT inhibition of EPO-induced erythroid differentiation. The fact that transient MT overexpression had no impact on γ-globin gene expression (Abdel-Mageed and Agrawal, unpublished observation) suggests both the blockade of differentiation signal by EPO and downregulation of EPO receptor may have contributed to inhibition of differentiation when MT is overexpressed. However, MT inhibitory effect was also observed in butyrate-induced differentiation, suggesting that other unknown mechanisms may also be involved. This was further confirmed when MT inhibitory effects of EPO-induced differentiation were partially ameliorated by an MT antisense oligonucleotide.

It has been reported that EPO receptor contains both growth-promoting activity and differentiation-promoting activity (33). Thus it would be appropriate to suggest that EPO-induced MT gene expression plays a significant role during the growth-promoting activity phase of the interaction of EPO with EPO-R. In contrast, MT transcripts are downregulated before the cells are committed to differentiate upon EPO- and EPO-R-induced differentiation-promoting activity. Alternatively, EPO-R transcripts are downregulated and the differentiation of the erythroid progenitor cells is inhibited upon MT overexpression. Overall, these data suggest that, among growth factors required for erythropoiesis, EPO is a potent inducer of MT, and that MT plays a significant role in EPO-induced proliferation, but not in the erythroid-specific differentiation of the progenitor cells.

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