Globin Gene Silencing in Primary Erythroid Cultures

AN INHIBITORY ROLE FOR INTERLEUKIN-6*

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There are numerous similarities between the erythroid and megakaryocytic lineages which suggest that commitment to either lineage occurs relatively late in hematopoiesis. Commitment toward megakaryocytic development requires obligatory silencing of erythroid-specific genes. Therefore, we investigated the effects of interleukin-6, a known inducer of thrombocyte production, on globin gene expression during erythroid differentiation. Studies in K562 cells demonstrated inhibition of γ globin gene mRNA production and chain biosynthesis in the presence of exogenous interleukin-6 which was abrogated by anti-interleukin-6 monoclonal antibody. Similar studies in primary erythroid progenitors showed inhibition of burst-forming unit-erythroid colony formation when interleukin-6 was added late in cultures with decreased γ and β globin gene mRNA production. Protein binding studies demonstrated an increase in activator protein-1 binding to its consensus sequence by 24 h of interleukin-6 treatment. Inhibition of activator protein-1 gene activity had no effect on γ gene silencing by interleukin-6. A potential interleukin-6 response element was identified in the γ globin gene. Interleukin-6 treatment led to a rapid increase in protein binding to the target DNA sequence. These results suggest that interleukin-6 may play an important role in globin gene silencing during megakaryocytic lineage commitment.

The hematopoietic pathways for megakaryocytic (MK) and erythroid (E) differentiation are closely related. These lineages share common hematopoietic-specific trans-acting factors including GATA-1 and nuclear factor-erythroid 2 (Nf-E2) (1). GATA-1 is absolutely required for differentiation of the E lineage (2); and although it is not required for MK differentiation, cell lines with the highest concentrations of GATA-1 differentiate into megakaryocytes (3). Nf-E2 is required for both high level enhancer activity of the β globin locus control region (LCR) (4) and production of platelets (3). The regulation of E- and MK-specific genes shares many features (5, 6), suggesting that restriction toward either pathway occurs relatively late in hematopoietic hierarchy. Evidence for a common E/MK progenitor is demonstrated by expression of both E and MK markers in erythroleukemic cell lines (1, 7). Recently, a bipotent burst forming unit (BFU)-E/MK progenitor has been identified in human bone marrow (7). The factors that determine whether commitment to the E or MK lineage will occur are largely unknown. Optimal development of BFU-E/MK requires a combination of several cytokines including erythropoietin, stem cell factor, interleukin (IL)-3, and megakaryocyte growth and development factor, which are proposed to act sequentially on progenitors at different stages of maturation (7). Therefore, at some stage during maturation of the putative bipotential BFU-E/MK progenitor cell an additional level of regulation responsible for the proper silencing of globin genes (which are not normally expressed in megakaryocytes) must occur.

In vivo studies in mice and in vitro studies in low density bone marrow cells demonstrated stimulation of megakaryocytopenesis and platelet production by IL-6 (8–12). IL-6 works synergistically with IL-3 to stimulate early megakaryocyte development and proliferation of hematopoietic progenitors (8–10, 13). In this study we investigated the possible role of IL-6 in globin gene silencing during megakaryocytic lineage commitment. Studies were conducted using K562 cells, a human erythroleukemic cell line that expresses both erythroid and megakaryocytic-specific genes in the uninduced state and has the capacity to differentiate along the E or MK lineage (14, 15). Levels of γ globin mRNA and protein biosynthesis in K562 cells were analyzed after treatment with IL-6. We observed an IL-6 concentration-dependent decrease in both γ globin mRNA production and protein biosynthesis. This inhibition was abrogated by the addition of anti-IL-6 monoclonal antibody. K562 cells treated with IL-6 responded with increased glycoprotein Ib (GpIb) mRNA levels. Similar studies were performed using mononuclear cells isolated from human peripheral blood. IL-6 inhibited BFU-E colony formation when added late in cultures and decreased γ and β globin mRNA production. Gel mobility shift assays (GMSAs) showed increased protein binding to a potential IL-6 response element (IL-6RE) after IL-6 treatment. These studies collectively suggest that IL-6 serves as a negative regulator of globin gene expression during megakaryocytic lineage commitment.

EXPERIMENTAL PROCEDURES

Cell Culture—K562 cells were maintained in suspension cultures in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cells were grown in IMDM without fetal bovine serum for 24 h prior to beginning analyses. Experiments were conducted with 6.6 × 10⁵ tiate into megakaryocytes (3). Nf-E2 is required for both high level enhancer activity of the β globin locus control region (LCR) (4) and production of platelets (3). The regulation of E- and MK-specific genes shares many features (5, 6), suggesting that restriction toward either pathway occurs relatively late in hematopoietic hierarchy. Evidence for a common E/MK progenitor is demonstrated by expression of both E and MK markers in erythroleukemic cell lines (1, 7). Recently, a bipotent burst forming unit (BFU)-E/MK progenitor has been identified in human bone marrow (7). The factors that determine whether commitment to the E or MK lineage will occur are largely unknown. Optimal development of BFU-E/MK requires a combination of several cytokines including erythropoietin, stem cell factor, interleukin (IL)-3, and megakaryocyte growth and development factor, which are proposed to act sequentially on progenitors at different stages of maturation (7). Therefore, at some stage during maturation of the putative bipotential BFU-E/MK progenitor cell an additional level of regulation responsible for the proper silencing of globin genes (which are not normally expressed in megakaryocytes) must occur.

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cells/ml in IMDM containing hemin (100 µM), sodium butyrate (2 mM), or exogenous IL-6 (10, 25, 50, 100 ng/ml) and collected at 0, 24, 48, and 72-h increments for analysis by RT-PCR and RNase protection, described below. Antibody studies were performed by incubating anti-IL-6 monoclonal antibody (5 or 10 µg/ml) with IL-6 protein (50 ng/ml) for 1 h at 4 °C. To determine the relative contribution of IL-6-induced ΔIII to give a 205-bp protected fragment. Mononuclear cells were isolated from the peripheral blood of each patient studied by density gradient centrifugation using the Histopaque 1077 (Sigma). The cells were washed with IMDM containing 30% fetal bovine serum, 2 mM glutamine, and 10−4 M β-mercaptoethanol, then were mixed at a concentration of 3 × 10^6 cells/ml in MethoCult GF H4434 medium (StemCell Technologies Inc., Vancouver, B.C.). The mononuclear cells were incubated in multiwell tissue culture plates in a humidified atmosphere containing 5% CO2, at 37 °C. The following experimental conditions were analyzed in triplicate wells in the culture plates: an untreated control, IL-6 added on day 0, and IL-6 added on day 7 of the study period (IL-6 concentrations were 5, 25, or 50 ng/ml). The concentration with the greatest inhibitory effect on colony growth was determined to be 50 ng/ml IL-6, therefore subsequent experiments were performed at this concentration. BFU-E colonies were counted on day 14 using an inverted microscope and harvested for RNA analysis by an RNase protection assay as described above.

**RESULTS**

**Effects of Exogenous IL-6 on γ Globin Gene Expression**—Phorbol esters have been shown to induce MK differentiation while simultaneously inhibiting E differentiation in erythroleukemia cells (19, 20–22). The majority of work in this area has been accomplished through the use of tetradecanoylphorbol acetate and phorbol myristate acetate in K562 cells. During induction of MK development by phorbol esters there is decreased γ globin gene expression which occurs through negative regulation of both the rate of production and the stability of γ globin mRNA (21). In addition, erythroleukemia cells induced with tetradecanoylphorbol acetate become committed to the MK lineage (23). Similar results have been observed with phorbol myristate acetate, which also induces the expression of IL-6 and IL-6R genes in K562 cells (24). These studies suggest that the changes associated with phorbol ester treatment may be mediated by an IL-6 autocrine loop mechanism. Initial experiments were performed to determine whether the K562 cell line would be maintained in culture, and then cytotoxicity was examined for IL-6 and its receptor endogenously. Primer pairs for IL-6 and IL-6R were used to screen for mRNA production by RT-PCR (Table 1). The ratios of mRNA production of IL-6 and IL-6R to that of GAPD were found to be 0.49 and 1.0 respectively (data not shown). Subsequently, we began investigating the role of IL-6 in erythroid versus megakaryocytic lineage
commitment by analyzing its effects on \( \gamma \) globin gene expression. K562 cells were grown in increasing concentrations of IL-6 (0, 10, 25, 50, and 100 ng/ml) in culture for 72 h followed by \( \gamma \) globin mRNA quantitation by RT-PCR. The level of detectable \( \gamma \) globin gene expression decreased in a concentration-dependent manner with a maximal (70%) decrease at 100 ng/ml IL-6 (Fig. 1). This response was determined to be highly significant using a multiple regression model with a \( p \) value of 0.002 and \( R^2 \) of 0.86. This inhibition was significant at 25 ng/ml and maximal at 100 ng/ml, therefore subsequent experiments were performed at the 50 ng/ml concentration of IL-6. The same RNA samples obtained from K562 cells treated with increasing concentrations of IL-6 were analyzed for expression of GATA-1, an E-specific nuclear trans-acting factor present in erythrocytes and megakaryocytes (2). The expression of GATA-1 was unaffected by increasing concentrations of IL-6 (data not shown) which suggests that the down-regulation of \( \gamma \) globin gene expression by exogenous IL-6 is independent of significant changes in GATA-1 gene activity levels.

Similar experiments were performed to determine the effects of exogenous IL-6 on \( \gamma \) globin chain biosynthesis. K562 cells were treated with IL-6 (100 ng/ml) for 72 h, and the total cellular protein was isolated for HPLC analysis. \( \gamma \) Globin chain biosynthesis (expressed as the percent \( \gamma \) globin chains of the total protein produced) was 3.2% in uninduced K562 cells (Fig. 2A). In contrast, the level of \( \gamma \) globin protein produced after treatment with IL-6 was below the level of detection (Fig. 2B). The protein analysis indicates that IL-6 inhibits the production of \( \gamma \) globin chains in K562 cells which correlates with the results obtained for \( \gamma \) globin mRNA production.

To gain additional evidence that IL-6 was directly responsible for the observed down-regulation of \( \gamma \) globin gene expression in our experimental system, anti-IL-6 monoclonal antibody (5 and 10 \( \mu \)g/ml) was incubated with IL-6 protein (50 ng/ml) for 1 h at 37°C prior to addition to K562 cells. Anti-IL-6 antibody treatment restored \( \gamma \) globin mRNA levels to 80% (5 \( \mu \)g/ml) and 57% (10 \( \mu \)g/ml) of untreated levels, demonstrating an abrogation of the inhibitory effects of IL-6 (Fig. 1B). Thus, exogenous IL-6 treatment in K562 cells resulted in decreased \( \gamma \) globin gene expression, which was abrogated by monoclonal antibodies to IL-6, and decreased \( \gamma \) globin chain biosynthesis.

A second method, RNase protection assay, was used to analyze the inhibitory effects of IL-6 on \( \gamma \) gene expression in K562 cells. \( \gamma \) mRNA was quantitated as a ratio to GAPD (an internal control). Similar to the findings obtained by RT-PCR, \( \gamma \) mRNA production was decreased by 68% in the presence of 50 ng/ml IL-6 (Fig. 3). Likewise, pretreatment with anti-IL-6 antibodies resulted in \( \gamma \) mRNA at 63% of base-line levels. These findings are comparable to those obtained by RT-PCR analysis.

To determine whether the down-regulation of \( \gamma \) globin gene expression by IL-6 was accompanied by an increase in MK-specific markers, studies were performed to analyze the level of GpIIb gene expression, an early marker for commitment to the MK lineage (25). GpIIb mRNA levels increased 17% (\( p = 0.01 \)) by RT-PCR analysis after 6 days of IL-6 (50 ng/ml) treatment (data not shown). The sequential down-regulation of \( \gamma \) globin mRNA production and the up-regulation of GpIIb mRNA are similar to the results obtained in K562 cells treated with tetradecanoylphorbol acetate (26). Together these results suggest that IL-6 may be involved in E versus MK lineage commitment.

**Decreased IL-6 Gene Expression in the Presence of Fetal Hemoglobin-inducing Agents**—Previous studies have demonstrated the induction of \( \gamma \) globin gene expression in K562 cells treated with hemin and sodium butyrate (27). Based on these data, we hypothesize that IL-6 gene activity may be decreased when \( \gamma \) globin gene expression is induced by either of these two agents. Levels of IL-6 mRNA were analyzed at 0, 24, 48, and 72 h after the addition of hemin (100 \( \mu \)M) or sodium butyrate (2 \( \mu \)M) in cultures of K562 cells. A 99% and 50% decrease in IL-6 mRNA was observed in sodium butyrate- and hemin-treated cells, respectively (Fig. 4). These results suggest that induction of \( \gamma \) gene activity is associated with suppression of IL-6 gene expression and indirectly support a role for IL-6 in \( \gamma \) globin gene silencing during MK lineage commitment.

The results summarized for our studies in K562 cells include the following: 1) endogenous expression of both IL-6 and IL-6R genes; 2) simultaneous decrease in \( \gamma \) globin mRNA production and protein biosynthesis in the presence of exogenous IL-6; 3) abrogation of the inhibitory effects of IL-6 by anti-IL-6 monoclonal antibody; and 4) stimulation of GpIIb mRNA production.

**Inhibition of BFU-E Colony Growth by Exogenous IL-6**—Although erythroleukemia cell lines can serve as a paradigm for investigating the process of hematopoietic differentiation, it is desirable to verify findings in primary E cultures. Therefore, we performed parallel experiments in primary cultures to cor-

### Table I

**Synthetic PCR primers and GMSA oligonucleotides**

| RT-PCR primer pairs*  | H\(y\)(352) | GATA-1(470) | IL-6(516) | IL-6R(567) | GAPD(417) | GpIIb(448) | Gel shift oligonucleotides* |
|-----------------------|------------|-------------|--------|-----------|-----------|------------|-----------------|
| s                     | 5'−TGGATGGCAGAAGC−3' | 5'−GGAACCCCTCTACGCTCAG−3' | 5'−CCACACAGACCCCATCAC−3' | 5'−CTACATTGCCCAGAGCGTCC−3' | 5'−GTGATGTTGCGAGGCGAT−3' | 5'−GAAGATGCCAGGAC−3' | 5'−GTCATTTCACAGAG−3' |
| a                     | 3'−CGCTTGAATGTCATAGTGG−3' | 3'−TGTGATGTTGCGAGGCGAT−3' | 3'−GTCATTTCACAGAG−3' | 3'−CACACTCCTCTGGAAGCTG−3' |

* Parenthetical material indicates primer size, in bp; s, sense; a, antisense.

* Sense strand.
Globin Gene Silencing Induced by Interleukin-6

Fig. 1. Effects of exogenous IL-6 on γ globin expression in K562 cells. Increasing concentrations of IL-6 were added to K562 cells and analyzed by RT-PCR. Panel A, agarose gel stained with ethidium bromide. The top panel contains the PCR product for the constitutively expressed GAPD gene. The bottom panel contains the γ globin PCR product from cells treated with increasing concentrations of IL-6 (lanes 1–5) or IL-6 preincubated with anti-IL-6 monoclonal antibody (lanes 6 and 7). Panel B, graphical representation of the densitometric analysis of fluorescence intensity observed for the PCR products in panel A. Results are reported as a ratio of the fluorescence intensity of Aγ to GAPD. The concentration-dependent decrease of Aγ mRNA with IL-6 treatment reached a maximal 70% inhibition at 100 ng/ml (●). The greatest abrogation of this inhibition (80% of untreated levels) was observed with 5 μg/ml anti-IL-6 antibody (■).

The total cytoplasmic RNA was isolated from the BFU-E colonies and the levels of γ and β globin mRNA production were determined by an RNase protection assay. Although IL-6 added on day 0 had a stimulatory effect on total BFU-E colony number, mRNA data demonstrated an inhibitory effect on γ and β globin gene activity for both day 0 and day 7 conditions (Fig. 6). There was a 63% and 80% decrease in γ globin mRNA levels in BFU-E colonies treated with IL-6 on day 0 and day 7, respectively (p < 0.05). Likewise, a 79% (p < 0.05) decrease in β globin mRNA was also observed when IL-6 was added on day 7 (Table II). These experiments give additional support for a role of IL-6 in globin gene silencing and expand its inhibitory effects to both γ and β globin gene expression.

Fig. 2. Effect of IL-6 on γ globin chain biosynthesis. K562 cells grown in IL-6 (100 ng/ml) were harvested at 72 h and prepared for HPLC analysis. The tracing shows human α and γ globin chain biosynthesis for untreated K562 cells (panel A) and IL-6-treated K562 cells (panel B). γ Globin was reduced to undetectable levels in the IL-6-treated samples.

Molecular Mechanisms Involved in the Down-regulation of γ Globin Gene Expression by IL-6—The 5’ hypersensitive sites (5’HS) of the β globin LCR are required for high level expression of the globin genes (29–32). Studies in K562 cells have demonstrated regulation of the enhancer activity of the LCR, by proteins that bind to the NF-E2/Ap-1 tandem repeat within 5’HS2 (33, 34). Therefore, we examined the effects of IL-6 on protein binding to this tandem repeat. Initially GMSA was performed with two oligonucleotide probes, one specific for Ap-1 and a second containing the NF-E2/Ap-1 tandem repeat within the β globin 5’HS2 (HS2 NF-E2). To determine specific binding patterns for the Ap-1 and HS2 NF-E2 oligonucleotides, GMSA was performed with extracts from untreated K562 cells. Equal amounts of nuclear proteins (4 μg) were added to all GMSA reactions. The protein-DNA complexes observed without competitor are shown in Fig. 7A, lanes 1 and 5. The experiments with the Ap-1 probe (Fig. 7A, lanes 1–4) resulted in the formation of a single specific protein-DNA complex. Specificity of the bands produced with each probe was confirmed by cold competition reactions with the Sp-1 (Fig. 7A, lanes 4 and 8) and Oct-1 (data not shown) oligonucleotides. In addition, studies performed with a polyclonal anti-Ap-1-specific antibody resulted in a complete loss of the single protein-DNA complex (data not shown), thus confirming the protein in this complex to be Ap-1. Four specific protein-DNA complexes were observed with the HS2 NF-E2 probe demonstrated by the cold competition reaction shown (Fig. 7A, lane 6). The B2 complex was specifically competed by the Ap-1 oligonucleotide (Fig. 7A, lane 7). Andrews et al. (4) previously identified a single protein-DNA complex as NF-E2 with nuclear extracts from mouse erythroleukemia cells and the NF-E2/Ap-1 tandem repeat, using p45 NF-E2 anti-
serum. In our study, a single complex formed in experiments performed with the HS2 NF-E2 probe and mouse erythroleukemia cell extract which migrated at the same rate as the B4 complex produced in K562 cells (data not shown).

Experiments were completed to determine whether IL-6 had an immediate effect on the proteins bound to the Ap-1 or HS2 NF-E2 oligonucleotide probes. K562 cells were treated with IL-6 (50 ng/ml) for 0, 5, 15, 30, and 60 min and 4, 24, 48, and 72 h. There was an increase in the Ap-1 protein-DNA complex observed at 24 h with both the Ap-1 and HS2 NF-E2 oligonucleotides compared with uninduced extract (data not shown). The increased binding observed with the Ap-1 oligonucleotide was sustained in K562 cell nuclear extracts isolated after 48 and 72 h of IL-6 treatment (data not shown). These results suggest that Ap-1 may be a nuclear trans-acting factor that mediates the inhibitory effects of IL-6 on globin gene expression.

Additional experiments were performed with curcumin, a known inhibitor of Ap-1 gene expression, to confirm the role of Ap-1 as a possible mediator of the silencing effects of IL-6 on globin gene expression (35). Various concentrations of curcumin were analyzed (5–50 μM) with maximum inhibition of Ap-1 and least toxicity to the cells at a concentration of 20 μM (data not shown). Subsequently, K562 cells were incubated in the presence of curcumin (20 μM) for 12 h prior to the addition of IL-6. RNase protection assays showed no significant change in g-globin mRNA production either in the absence or presence of curcumin. We conclude from these results that IL-6 increases protein binding to the Ap-1 consensus sequence; however, the observed increase in Ap-1 is not responsible for the inhibitory effects of IL-6 on g-globin mRNA production.

Cytokines are known to activate gene expression through the Janus kinase/signal transducers and activators of transcription (STAT) pathway via the STAT3 and STAT1 nuclear trans-factors (36). The sequences of the natural palindromic STAT-responsive elements vary considerably, but they conform to the general structure T(T(N))AA, where N is any DNA base (37). The motif with a spacing of 4 bp selectively binds to complexes.
containing STAT3. Although the majority of experimental data support an activator role for the STAT proteins, there has been interest to determine at which stage of lineage commitment and transcription factor hierarchies and networks of this type are absolutely required for erythropoiesis (2). The coordinate expression of GATA-1 and stem cell leukemia may be associated with crucial factor-1 and nuclear factor κB (data not shown). In the presence of IL-6 (50 ng/ml) there was, by PhosphorImager quantitation of the acrylamide gels, a 30% increased protein binding at 30 min and 4 h of treatment with a further increased 45% by 24 h. Further studies are being conducted to define the role of nuclear trans-factors that bind to the Ay IL-6RE in response to IL-6, in regulating globin gene expression.

**DISCUSSION**

The molecular mechanisms that govern E lineage commitment are largely undetermined. Utilization of human cell lines has served to increase our understanding of globin gene regulation during differentiation and the cellular signals necessary for silencing E-specific genes during MK lineage commitment. Mixed E/MK colonies have been described in both humans (3) and mice (39, 40). In humans, almost all leukemic cell lines described as erythroleukemic or megakaryoblastic express E and MK-specific genes, and this dual expression is found in the same cell (41, 42). Both K562 and human erythroleukemia cells retain the ability to differentiate down the MK or E lineage when exposed to specific inducers (26, 43). Recently, Debili et al. (7) characterized a normal bipotential BFU-EMK progenitor. This cell is found in the CD34⁺/CD38⁻ cell fraction isolated from bone marrow samples by flow cytometry methods. Further evidence to support a common bipotential E/MK progenitor is the shared expression of the genes for the transcription factors GATA-1 (44), GATA-2 (5), stem cell leukemia (45), and NF-E2 (22, 27). These genes are expressed in multipotential progenitor, erythroblasts and megakaryocytes. GATA-1 is a major activator of hematopoietic gene expression and is absolutely required for erythropoiesis (2). The coordinate expression of GATA-1 and stem cell leukemia may be associated with the presence of a conserved GATA motif in the stem cell leukemia promoter that binds GATA-1 and mediates trans-activation of the stem cell leukemia promoter (46). It is likely that transcription factor hierarchies and networks of this type are important for hematopoietic differentiation. It is clearly of interest to determine at which stage of lineage commitment and differentiation the genes for particular transcription factors are themselves active.

As E maturation progresses from embryonic to fetal to adult stage erythroblast, there is a switch in globin chain production from e to γ to β globin, respectively (47). The expression of the individual globin genes reflects the interactions of proximal and distal cis-active regulatory elements with trans-acting factors (47). An important distal cis-active regulatory element is T(N)(N)AA with the Ay globin gene and promoter. At nucleotide +11 relative to the cap site a potential IL-6RE TTCTGGAA (Ay IL-6RE) was identified which, by the palindromic spacing, would be predicted to bind STAT3. Protein binding to this region in response to IL-6 treatment in K562 cells was analyzed by GMSA (Table I). In untreated K562 cells there was a single specific protein-DNA complex (Fig. 8, lanes 1 and 3) which was not disrupted by cold competition reactions with Sp-1 (Fig. 8, lane 2), Oct-1, transcription factor IID, glucocorticoid response element, GATA-1, Ap-1, NF-E2, HS2 NF-E2, cyclic AMP response element-binding protein, CAAT-binding transcription factor/nuclear factor-κB (data not shown). In the presence of IL-6 (50 ng/ml) there was, by PhosphorImager quantitation of the acrylamide gels, a 30% increased protein binding at 30 min and 4 h of treatment with a further increased 45% by 24 h. Further studies are being conducted to define the role of nuclear trans-factors that bind to the Ay IL-6RE in response to IL-6, in regulating globin gene expression.
Globin Gene Silencing Induced by Interleukin-6

The process of normal megakaryocyte maturation is stimulated by IL-6, which synergizes with IL-3 to produce MK colonies from progenitor cells, but IL-6 has no colony stimulating activity of its own. IL-6 is a pluripotent cytokine with proinflammatory (48), hematopoietic (28), and immunomodulatory (8) effects. Different human hematopoietic cell lines produce IL-6 constitutively; these include K562, HEL, KU812, Meg01, and Dami (49). Experimental data support the ordered silencing of E-specific genes expressed in early progenitors when MK commitment occurs. A role for IL-6 in the alternate lineage commitment process is supported by the following experimental data. First, IL-6 has been shown to inhibit the production of erythropoietin in perfused rat kidneys (50). Second, induction of K562 and HEL cells with tetradecanoylphorbol acetate results in silencing of E-specific genes (γ globin) (21) and stimulation of MK-specific genes (GpIIb/IIIa) (51). Finally, tetradecanoylphorbol acetate has been shown to induce IL-6 and IL-6R gene expression in erythroleukemia cell lines (52). Based on these experimental data we performed studies to analyze the direct effects of IL-6 on γ globin gene expression in K562 and primary human progenitor cells. We demonstrated a direct concentration-dependent suppression of γ globin gene expression in K562 cells with a concomitant increase in GpIIb mRNA production, suggesting that IL-6 has the ability to divert K562 cell differentiation toward the MK pathway. Conversely, when K562 cells were grown in the two E differentiating agents, sodium butyrate or hemin, endogenous IL-6 mRNA production was decreased to barely detectable levels. We next extended our studies to primary cultures of human progenitor cells and demonstrated the ability of IL-6 to inhibit BFU-E colony growth significantly when added on day 7 in cultures. One might speculate that on day 7 the multipotential progenitors present have acquired the signals necessary for inhibition of E maturation by IL-6. Maturation along the MK lineage did not occur in our culture system because of a lack of the growth factors, thrombopoietin or IL-11, required to support terminal MK maturation. Recent evidence suggests that there may be factors in fetal bovine serum which inhibit MK development in vitro (7). Navarro et al. (49) demonstrated the expression of both the IL-6 and IL-6R genes during normal megakaryocytogenesis, suggesting that megakaryocyte maturation may be regulated in part by an IL-6 autocrine loop. Clinical trials to examine the efficacy of recombinant IL-6 have shown both increased platelet counts and production of a rapid onset anemia (53, 54). Therefore, collectively the in vitro and in vivo data suggest an inhibitory role for IL-6 toward erythroid cell commitment and maturation. The observation made in our primary culture system has important implications for E maturation in vitro. Whereas IL-6 has known proliferative effects toward early progenitor cells, it may ultimately inhibit late E maturation.

The level of globin gene expression in E progenitor cells during lineage commitment is modulated directly by cell-specific transcription factors that bind to specific motifs in the locus control region and proximal globin gene promoters. The LCR becomes active in multipotential hematopoietic cells (55) and is required to establish an active chromatin domain and expression of genes located in the β globin locus (30). An important motif is the NF-E2/αp-1 tandem repeat that binds the nuclear trans-acting factor NF-E2, a heterodimer of p45NF-E2, and the small Maf proteins p18 (MafF, MafG, and MafK) which lack canonical trans-activation domains but have dimerization and DNA binding domains (56). NF-E2 binds to the NF-E2/αp-1 tandem repeat and activates globin gene activity. Expression of p45 NF-E2 is restricted to erythroid and mast cells, megakaryocytes (2), and multipotential hematopoietic progenitors. A third ubiquitous transcription factor Ap-1, a heterodimer of the two proto-oncogenes c-fos and c-jun, binds to the Ap-1 site in the NF-E2/αp-1 tandem repeat, resulting in either transcription activation or repression (57). Taken together these data suggest that a p45 NF-E2/Maf/c-fos concentration-dependent switch may regulate globin gene expression through the NF-E2/αp-1 binding site.

Experiments were completed to investigate possible molecular mechanisms for inhibition of globin gene expression by IL-6. Protein binding to the NF-E2/αp-1 tandem repeat was performed using GMSAs. Nuclear extracts from IL-6-treated K562 cells produced increased binding to the Ap-1 consensus sequence by 24 h in culture (Fig. 7B). In other systems IL-6 has been shown to induce the expression of Ap-1 via an IL-6/RE (58). A view of the experimental data to support a possible inhibitory role for homodimerization of Ap-1 at the tandem NF-E2/αp-1 site in the LCR, this observation suggested a possible inhibitory role for Ap-1 in this setting. Additional studies with curcumin, which specifically blocks Ap-1 gene expression, produced no effect on the down-regulation of γ globin gene...
mRNA production in the presence of IL-6. Therefore, the increase in Ap-1 binding may not be involved in the mechanism for globin gene silencing by IL-6.

Gene activation by IL-6 occurs through the Janus kinase/STAT signal transduction pathway via the STAT3 or STAT1 nuclear trans-factors binding to specific DNA sequences defined as IL-6REs, which activate gene expression. These target genes can then go on either to activate or to suppress secondary genes. Such indirect mechanisms for inhibiting gene activity have been defined for the cyclin-dependent kinase inhibitor, p21 

STAT signal transduction pathway via the STAT3 or STAT1 increase in Ap-1 binding may not be involved in the mechanism mRNA production in the presence of IL-6. Therefore, the sequence is identical to an IL-6RE identified in the promoter of the IL-6-responsive 10. Carrington, P. A., Hill, R. J., Stenberg, P. E., Levin, J., Corash, L., Schreurs, M. M. (1976) Anal. Biochem. 71, 899–906

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