Erythropoietin-dependent Induction of Hemoglobin Synthesis in a Cytokine-dependent Cell Line M-TAT*

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Erythropoietin (EPO) is a cytokine that supports proliferation of erythroid progenitor cells (EPO) and granulocyte-macrophage colony-stimulating factor (GM-CSF), or stem cell factor (SCF). These long-term cultures are referred to as M-TAT/EPO, M-TAT/GM-CSF, and M-TAT/SCF cells, respectively. Hemoglobin concentration and γ-globin and erythroid δ-aminolevulinate synthase mRNA levels were significantly higher in M-TAT/EPO cells than in M-TAT/GM-CSF cells. When the supplemented cytokine was switched from GM-CSF to EPO, hemoglobin synthesis in M-TAT/GM-CSF cells increased rapidly (within 5 h), and the level of GATA-1 mRNA increased. In contrast, the addition of GM-CSF to the M-TAT/EPO cell culture decreased the amount of hemoglobin, even in the presence of EPO, indicating that the EPO signal for erythroid differentiation is suppressed by GM-CSF. Thus, erythroid development of M-TAT cells is promoted by EPO and suppressed by GM-CSF. These results support the hypothesis that EPO actively influences the programming of gene expression required for erythroid progenitor cell differentiation.

Since the discovery of cytokines, a significant amount of research has concentrated on the elucidation of cytokine functions. A number of cytokines are able to support the growth of hematopoietic cells, and the possibility of a common signaling pathway has been vigorously investigated (1-3). However, the fundamental question as to whether cytokines merely serve to salvage preprogrammed cells from apoptosis or actively influence the programming of gene expression for cellular differentiation remains to be clarified (4-7).

Erythropoietin (EPO) is a cytokine that supports proliferation of erythroid progenitor cells (8). EPO functions by binding to a specific cell surface receptor (9) (erythropoietin receptor, EPO-R) and has also been shown to promote the differentiation of erythroid progenitor cells at various stages (10-12). In fact, several EPO-dependent cell lines of murine and human origin have already been established and used as models for the characterization of EPO function in erythroid progenitor cells (7, 13-17).

To study the effect of cytokines on the growth and differentiation of human erythroid progenitor cells, we established a granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent cell line, M-TAT. As M-TAT cells also grow in media containing either EPO or stem cell factor (SCF), we cultured M-TAT cells in the presence of GM-CSF, EPO, or SCF for 1 year. These long-term cultured lines of M-TAT cells are tentatively named as M-TAT/GM-CSF, M-TAT/EPO, and M-TAT/SCF, respectively. We found that, when compared with the other two cytokine lines, M-TAT/EPO cells showed the highest level of erythroid-specific gene expression. Using these M-TAT cell cultures, we carried out cytokine-switching experiments. Switching from GM-CSF to EPO induced hemoglobin synthesis in M-TAT/GM-CSF cells within 48 h without any impairment of cell growth. In contrast, switching from EPO to GM-CSF, or adding GM-CSF to the M-TAT/EPO cell culture, markedly reduced the level of hemoglobin in M-TAT/EPO cells, indicating that the EPO signal for erythroid differentiation is suppressed and/or overridden by the GM-CSF signal. These results demonstrate that EPO/EPO-R system plays a specific role in the regulation of erythroid differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—M-TAT cells were cultured in suspension in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Cell Culture Laboratories, Cleveland, OH) in a 6% CO2 incubator. The original M-TAT cells were washed and divided into three cultures: 1) M-TAT/EPO′ cell culture, 2) M-TAT/GM-CSF cell culture, and 3) M-TAT/SCF cell culture. After 1 year, these M-TAT cell cultures were expanded. Six out of the 15 expanded subclones subsequently died. Two subclones were obtained (M-TAT/EPO′ and M-TAT/GM-CSF) and re-subcloned similarly by seeding 10 cells/96-well plate. Fifty M-TAT/EPO′ and M-TAT/GM-CSF were seeded into a 96-well microtiter plate, and both cultures were maintained in the same concentration of GM-CSF as the original M-TAT cells, so both lines are essentially the same. Dianisidine Staining—Hemoglobin concentration was examined by incubating cells in serum-free RPMI 1640 medium containing 0.5% 3,3′-dimethoxybenzidine, fast blue B (dianisidine, Sigma), 0.5% acetic acid, and 0.8% H2O2 for 30 min at room temperature (15).

Subcloning of M-TAT′/GM-CSF and M-TAT′/EPO′ Cells—Fifty M-TAT′/GM-CSF cells were seeded into a 96-well microtiter plate, and cultured in RPMI 1640 medium supplemented with 10 ng/ml of GM-CSF. Twenty-one days later, 15 wells contained proliferating cells and were expanded. Six out of the 15 expanded subclones subsequently died. Two subclones were obtained (M-TAT′/EPO′ and M-TAT′/H-9) and re-subcloned similarly by seeding 10 cells/96-well plate. Fifty M-TAT′/EPO′ cells were seeded into a 96-well microtiter plate, and cultured in the presence of 50 ng/ml of EPO for 3 weeks. Eight wells...
RESULTS

M-TAT Cells Are Dependent on Cytokines for Viability and M-TAT/EPO Cells Express Erythroid-specific Genes at High Levels—M-TAT is a new GM-CSF-dependent cell line established from peripheral blood cells of a leukemia patient. M-TAT cells are also capable of proliferating with EPO, interleukin-3, or SCF, so we cultured M-TAT cells long term (>12 months) in the continuous presence of GM-CSF, EPO, or SCF. These long term cell cultures were tentatively named as M-TAT/GM-CSF, M-TAT/EPO, and M-TAT/SCF, respectively. All three cell lines are dependent on cytokines for viability as demonstrated in Fig. 1. In the presence of each cytokine, cells proliferate normally, but they stop proliferating and eventually die when the cytokines are omitted from the culture medium (Fig. 1). We had established and maintained the original M-TAT cells in the presence of GM-CSF, so the properties of M-TAT/GM-CSF cells are similar to those of the original M-TAT cells.

Original M-TAT cells stained positively with anti-glycophorin A antibody, indicating that they are committed to the erythroid lineage. To determine whether the three M-TAT long term cultures had maintained their erythroid properties or changed them, we stained the cells for hemoglobin with diaminobenzidine. We found that the frequency of diaminobenzidine-positive cells was unexpectedly high (30–50%) in M-TAT/EPO cells, whereas those in M-TAT/GM-CSF cells was less than 10% (data not shown). This finding suggests that long term culture of M-TAT cells with EPO promotes the maintenance of an erythroid-differentiated state of the M-TAT cells.

We also analyzed the expression of mRNAs encoding erythroid-specific 6-aminolevulinate synthase, the first regulatory enzyme of the erythroid heme biosynthetic pathway, and γ-globin by RNA blot analysis. Erythroid-specific 6-aminolevulinate synthase and γ-globin mRNAs were much higher in M-TAT/EPO cells (Fig. 2, lane 2) than in M-TAT/GM-CSF (lane 4) cells. Levels of erythroid-specific 6-aminolevulinate synthase mRNA in M-TAT/EPO cells are comparable with levels found in K562 cells, which express erythroid-specific 6-aminolevulinate synthase mRNA, the highest among tested human leukemia cell lines.

EPO Stimulates the Expression of Erythroid Genes in M-TAT/GM-CSF Cells—We hypothesized that, if EPO actively induces the erythroid differentiation program, it may induce erythroid differentiation in M-TAT/GM-CSF cells in much less than 12 months. To test whether EPO can induce the expression of late erythroid genes in M-TAT cells, we carried out a series of cytokine-switching experiments in which the supplemented cytokine was switched from GM-CSF to EPO and vice versa.

M-TAT/GM-CSF cells were washed and replated with media supplemented with EPO. This switch of cytokines resulted in a marked increase of diaminobenzidine-positive cells (Fig. 3A). The percent of diaminobenzidine-positive cells increased from 6 to 30% within 72 h after switching to EPO. The levels of γ-globin and erythroid-specific 6-aminolevulinate synthase mRNAs in M-TAT/GM-CSF cells also increased significantly within 48 h of the switch to EPO (Fig. 3, B and C). In contrast, switching from EPO to GM-CSF, which was performed with the M-TAT/EPO cells, resulted in a decrease of γ-globin and erythroid-specific 6-aminolevulinate synthase mRNAs within 24 h (Fig. 3, B and C). The percentage of diaminobenzidine-positive cells decreased from 44 to 8% within 48 h after the switch to GM-CSF (Fig. 3A).

The growth of M-TAT cells was not affected by these cytokine switches (Fig. 3D), and no apparent morphological changes were observed.
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**Fig. 3.** Cytokine-switching experiment with M-TAT cells. M-TAT/GM-CSF cells were washed twice and placed in RPMI 1640 medium containing EPO (○). M-TAT/EPO cells were washed and placed in the same medium but with GM-CSF (□). A, changes in hemoglobin production after cytokine-switching was determined by dianisidine staining. B and C, RNA was extracted from the cells at various time points, and the levels of γ-globin (B) and erythroid-specific δ-aminolevulinate synthase (C) mRNAs were determined. Relative changes of radioactivity were determined using 28S ribosomal RNA as standard. D, viable cell count in the culture. Three independent experiments of each switching showed similar results.

were observed during the experiments (data not shown). These results are consistent with the hypothesis that signals mediated by the EPO/EPO-R system actively induce the expression of erythroid-specific genes.

Cytokine-switching Experiments with Monoclonal Populations of M-TAT/GM-CSF and M-TAT/EPO Cells—We assumed that the original M-TAT cells and each serially transferred M-TAT cell culture achieved homogeneity during continuous passage of the cells. However, if the M-TAT/GM-CSF and M-TAT/EPO cells were not monoclonal, that is, some cells in the culture were responsive to EPO and others were responsive to GM-CSF, this heterogeneity could underlie the observed EPO-mediated erythroid gene expression. To eliminate this possibility, we generated subclones of the M-TAT/GM-CSF and M-TAT/EPO cell lines by the limiting dilution method. In this experiment, 50 M-TAT/GM-CSF cells were seeded into a 96-well microtiter plate. Fifteen wells were later found to contain proliferating cells, and these were expanded. Six subclones ceased to grow within several weeks, so the cytokine-switching experiment was carried out using the remaining nine subclones. In agreement with the result of the original M-TAT/GM-CSF cells, the percentage of dianisidine-positive staining cells was markedly increased when the cytokine was switched from GM-CSF to EPO for all nine subclones (Fig. 4A, column 1, solid bar and data not shown).

We then resubcloned M-TAT/H-9 and M-TAT/E-9 (two of the nine M-TAT/GM-CSF subclones) by seeding 10 cells/96-well microtiter plate, and three (H-9-1, H-9-2, and E-9-1) subclones were obtained. The probability that these subclones would be of polyclonal origin was less than 1%. Cytokine-switching experiments using these subclones confirmed our previous results that EPO induces hemoglobin synthesis (Fig. 4B).

Similarly, 50 M-TAT/EPO cells were seeded into a 96-well microtiter plate, and eight wells were obtained with proliferating cells. Of these M-TAT/EPO subclones, four continued to proliferate in the presence of EPO. When the cytokine was switched to GM-CSF for one of the M-TAT/EPO subclones (M-TAT/EPO-6), the culture showed a substantial decrease in the percentage of dianisidine-positive staining cells (Fig. 4A, column 5, diagonally-lined box) and in the levels of γ-globin and erythroid-specific δ-aminolevulinate synthase mRNAs (data not shown). These results clearly exclude the possibility that the observed effect of adding EPO (the induction of erythroid-specific gene expression) is due to clonal expansion of a heterogeneous population of cells in the M-TAT/GM-CSF serially transferred cultures.

**EPO/EPO-R Signals for Erythroid Differentiation Are Suppressed by the Presence of GM-CSF**—The results obtained in the previous section indicate that, while signals from the GM-CSF/GM-CSF-R system can support the proliferation of M-TAT cells, they do not promote the differentiation of M-TAT cells along the erythroid lineage. To test whether EPO can stimulate the expression of erythroid-specific genes even in the presence of GM-CSF, we added EPO to the M-TAT/E-9 (subclone of M-TAT/GM-CSF) cell culture (Fig. 4A, solid bar). As described above, hemoglobin synthesis in M-TAT/E9 cells could be induced by switching from GM-CSF to EPO. This increase in hemoglobin synthesis was suppressed by the simultaneous addition of as little as 0.1 ng/ml of GM-CSF (Fig. 4A). The growth of the original M-TAT cells was dependent on the concentration of supplemented GM-CSF, and maximum growth of the cells was obtained by the addition of 1 ng/ml of GM-CSF. Thus, a suboptimal concentration of GM-CSF was enough to cause inhibition of the hemoglobin synthesis in M-TAT/E9 cells in
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EPO. Similarly, GM-CSF suppressed EPO-induced hemoglobin synthesis of other M-TAT/GM-CSF subclones, resubcloned cells, and the original M-TAT/GM-CSF cells (Fig. 4B, column 3 and data not shown).

Subcloned M-TAT/EPO-6 cells showed a high percentage of dianisidine-positive cells when the cells were maintained in medium containing EPO (Fig. 4A, column 1, diagonally-lined box). The addition of GM-CSF at the concentration of 0.01–10 ng/ml decreased the percentage of the dianisidine-positive M-TAT/EPO-6 cells (Fig. 4A). The concentration of EPO (50 ng/ml (1.67 nM)) used for the maintenance of M-TAT/EPO-6 cell growth easily saturates all of the EPO-R on the surface of M-TAT cells (as estimated by receptor binding analysis), whereas the 0.1 ng/ml (4.5 × 10^{-3} nM) of GM-CSF does not saturate all of the GM-CSF-R. Similar results were obtained using the original M-TAT/EPO cells (data not shown). The results of the cytokine-switching experiment and the receptor binding analysis, taken together, imply an interesting possibility. Both the GM-CSF/GM-CSF-R system and the EPO/EPO-R system are functional and signaling in M-TAT cells, and the former overrides or suppresses the latter. That is, the GM-CSF system dictates the direction of differentiation in M-TAT cells.

Expression of GATA-1 mRNA Increases When the Cytokine Is Changed to EPO—GATA transcription factors are believed to be important in the regulation of genes specifically expressed in erythroid cells (29, 30). Functionally important GATA sites have been identified in the regulatory regions of many erythroid-specific genes, including EPO-R, erythroid-specific δ-amino levulinate synthase, GATA-1, and γ-globin (9, 29–34). We found that GATA-1 and GATA-2 proteins were both expressed in M-TAT/GM-CSF and M-TAT/EPO cells by immunohistochemical analysis (data not shown). When the level of GATA-1 mRNA in M-TAT/GM-CSF cells was examined after switching the cytokine to EPO, it was found to have increased (Fig. 5A and B). In contrast, the GATA-1 mRNA level decreased in M-TAT/EPO cells after switching from EPO to GM-CSF. Changes in the GATA-1 mRNA level in the cytokine-switching experiment correlate very well with that of the γ-globin and erythroid-specific δ-amino levulinate synthase mRNAs, suggesting that GATA-1 may play an important role in the lineage-specific gene expression induced by EPO in erythroid cells.

DISCUSSION

To date, a number of EPO-dependent cell lines of murine and human origin have been reported, and EPO-dependent growth and differentiation of some of these cell lines has been extensively investigated (6, 13, 16, 35). Transcription of erythroid-specific genes was found to be induced by EPO in the EPO-dependent cell lines of murine origin (6, 7, 13), but this was not the case for the human EPO-dependent cell lines (15, 36, 37). To address the question as to whether EPO can influence the programming of gene expression in erythroid progenitor cells, we cultured M-TAT cells with GM-CSF, EPO, or SCF for 1 year and prepared three lines of serially transferred cultures. Of the three cell lines, M-TAT/EPO cells were unique in that the cells show markedly increased levels of mRNAs encoding GATA-1, erythroid-specific δ-amino levulinate synthase, and γ-globin and a high percentage of dianisidine-positive cells as compared with other M-TAT cell cultures. We then performed cytokine-switching experiments, and found that the expression of erythroid-specific genes was induced in the M-TAT/GM-CSF cells within 3 days after the switch from GM-CSF to EPO. In contrast, the EPO signal for erythroid differentiation was suppressed by the addition of GM-CSF. Thus, cytokines actively influence the programming of gene expression in M-TAT cells.

To our knowledge, three human EPO-dependent cell lines (TF-1 (15), F-36 (36), and UT-7 (17, 38)) have been reported, and all of these cell lines exhibit erythroid/megakaryocytic properties. In TF-1 cells, EPO can sustain short-term growth, but can not induce erythroid differentiation (15). Hemoglobin is constitutively synthesized in F36E cells, but hemoglobin synthesis is not affected by changing the cytokine; replacement of EPO by GM-CSF did not change the hemoglobin level in F36E cells (36). By maintaining UT-7 cells in media containing EPO for more than 6 months, a subline in which growth is totally dependent on the presence of EPO was established (UT-7/EPO) (17). Although the UT-7/EPO cells have progressed further in erythroid development than original UT-7 cells, erythroid development of UT-7/EPO cells by EPO progressed slowly, and at least 3 months were required to establish the UT-7/EPO phenotype after the start of the cell culture with EPO. This is in contrast to the M-TAT/GM-CSF cells, in which EPO induces the expression of erythroid-specific genes within 48 h. Additionally, UT-7/EPO lost the ability to proliferate in response to GM-CSF or interleukin-3 (17).

As compared with other cell lines of human leukemia origin, M-TAT cells are unique in that 30–50% of M-TAT/EPO cells...
produce hemoglobin as judged by dianisidine staining, while less than 10% of M-TAT/GM-CSF cells were dianisidine-positive. Replacement of GM-CSF by EPO caused rapid and significant increases in the percentage of dianisidine-positive cells, \( \gamma \)-globin, and erythroid-specific \( \delta \)-aminolevulinate synthase mRNA levels within 48 h (Fig. 3) This 5-fold-plus increase cannot be explained solely by the clonal expansion of hemoglobin-producing cells, because subcloned M-TAT/GM-CSF cells possess the same ability to increase the level of hemoglobin in response to EPO. This finding clearly excludes the possibility of clonal expansion of EPO-sensitive M-TAT cells during EPO treatment. Thus, we conclude that EPO can induce the transcription of genes important for erythroid development in M-TAT cells.

We found that both GATA-1 and GATA-2 factors are expressed in the nuclei of M-TAT cells by immunohistochemistry (28). The level of GATA-1 mRNA increases significantly in response to the cytokine-switching to EPO in M-TAT/GM-CSF cells, and this change in GATA-1 mRNA level correlates with the presence of \( \gamma \)-globin and erythroid-specific \( \delta \)-aminolevulinate synthase mRNAs (Fig. 5). A similar increase of GATA-1 expression was also observed in a mouse erythroid cell line, SKT-6, in cells along the erythroid lineage, the molecular mechanisms of this pathway remain to be clarified. "TAT cells may serve as a useful model system to examine the molecular mechanisms through which EPO stimulates, and GM-CSF represses, erythroid differentiation.

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