Induction of Hemoglobin Synthesis by Downregulation of MYB Protein With an Antisense Oligodeoxynucleotide

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Reduced expression of the proto-oncogene c-myb appears necessary for erythroid differentiation induced by chemical agents and by the natural regulator, erythropoietin (Epo). Treatment of Epo-responsive Rauscher erythroleukemia cells with an anti-sense oligodeoxynucleotide to c-myb in the absence of other inducers downregulated myb protein markedly and caused hemoglobinization of the cells within 48 hours. Epo treatment, which downregulates c-myb in these cells, induced hemoglobinization to the same degree. Epo also induced the appearance of anion transport protein on the plasma membrane, consistent with terminal differentiation. In contrast, antisense c-myb did not induce this erythroid marker. The results are consistent with a role for myb protein in the regulation of hemoglobin synthesis.

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MATERIALS AND METHODS

Cell growth and oligodeoxynucleotide treatment. Rauscher cells (clone EMSIII) were cultured in 96-well plates, 1,500 cells/well (100 μL) in the absence or presence of 10 U recombinant human Epo (rhEpo)/mL (Elanex Pharmaceuticals, Bothell, WA). Oligo (O-oligo or S-oligo), 40 μg/mL, was added followed by a second addition of 10 μg/mL after 4 hours. Hemoglobinized cells (Hb') were scored by benzidine staining after 48 hours (200 cells scored). Points represent mean ± SD. Oligos were synthesized using an Applied Biosystems (Foster City, CA) DNA Synthesizer 380A and were purified by ethanol precipitation.

Immunocytochemical identification of myb protein. After incubation with oligo, cells were harvested and centrifuged onto glass slides, fixed with acetone at room temperature, and stored at −20°C. Slides were thawed and fixed in acetone for 10 minutes at 4°C, and then stained with monospecific anti-myb monoclonal antibodies (MoAbs) generated against a synthetic peptide corresponding to amino acids 94-112 of v-myb (Microbiological Associates, Bethesda, MD) as described.5

RESULTS

We treated Epo-responsive Rauscher cells9,10,12,13,15,16 with an anti-sense oligo complimentary to codons 2 through 7 of mouse c-myb17 (Fig 1). After 48 hours of incubation, 17% of the cells were hemoglobinized (Hb'). Treatment of replicate cultures with Epo instead of the oligo also yielded 17% Hb' cells. Exposure of the cells to both anti-sense c-myb oligo and Epo simultaneously yielded 18% Hb' cells. The absence of an additive effect of anti-sense c-myb oligo and Epo strongly suggests that both agents were probably operating on the same responsive population of cells. Importantly, addition of antisense c-myec, anti-sense vesicular stomatitis virus M protein (vsv),18 or a nonsense sequence with the same base composition as anti-myb as control oligos did not induce Hb' cells. Addition of anti-vsv oligo plus Epo resulted in 10% Hb' cells. Similar results were obtained in two other experiments.

The ability of anti-sense c-myb to induce hemoglobin synthesis in these cells was concentration dependent and was also demonstrable with an S-oligo (Fig 2). Addition of...
was again without effect. Yielded 5% and were obtained with all three control oligos.

Rauscher murine erythroleukemia cells. Anti-myb, GTGTCGGGGTC-TCCGGGC. Controls: anti-myc, AACGTTGAGGGGCAT; anti-vsv, TTGGGATAACACTTA; and a nonsense oligo with the same base composition as anti-myb, GCGCGCTTGGCTGGGC. Similar results were obtained with all three control oligos.

We demonstrated that anti-sense c-myb oligo decreased myb protein using an antipeptide MoAb. A high concentration of the myb protein was found in the cytosol of Rauscher cells (Fig 3A). Although myb protein binds DNA and has been found concentrated in the nucleus of some cells,9,20 other cells, including normal T lymphocytes, exhibit higher cytosolic concentrations very similar to that shown here.19 Importantly, treatment of Rauscher cells with anti-sense c-myb drastically reduced the myb protein concentration in the cells (Fig 3B), confirming the efficacy of the treatment.

We considered the possibility that anti-sense c-myb oligo might trigger hemoglobin synthesis in a cell and simultaneously arrest further proliferation of that cell (see Discussion). To test this possibility, we treated cells in suspension culture for 18 hours and then transferred them to plasma clot culture13,14 to permit examination of Hb+ daughter cells arising in colonies. In the absence of inducer or in the presence of control oligo only 2.6% of colonies were Hb+. Most contained no Hb+ cells (Fig 4A, Table 1). However, anti-sense c-myb oligo or Epo resulted in 20% and 21% Hb+ colonies, respectively (Fig 4B, Table 1). Importantly, the plating efficiencies observed under all treatment conditions were identical (15% ± 2%), as were the sizes of the colonies. Thus, we found no evidence for a growth-inhibiting action of the anti-sense c-myb on Rauscher cells.

Although anti-sense c-myb oligo treatment clearly resulted in hemoglobin synthesis, it did not induce expression of all mature erythrocyte characteristics ("terminal differentiation"). We determined this by staining for the membrane anion transport protein, band 3.21 Epo treatment resulted in the appearance of band 3 on 19% of the cells. However, we did not detect the band 3 on cells treated with the anti-sense c-myb oligo (not shown).

**DISCUSSION**

Myb protein is a DNA binding protein that regulates transcription either positively or negatively.22 Positive regulation appears to occur by two different mechanisms, involving either sequence-specific or sequence-nonspecific binding of the protein.23 Studies of the role of c-myb in hematopoiesis using anti-sense oligos have shown an arrest or reduction of hematopoietic growth accompanying reduction of c-myb.24-27 However, we found no effect of anti-sense c-myb oligo on the growth of Rauscher cells. There are at least two possible explanations for this apparent contradiction. Firstly, most of the previous studies have examined the effect of c-myb reduction on the growth and differentiation of normal hematopoietic cells using colony assays requiring many days. As a result, these experiments are likely to encounter multiple effects of c-myb reduction that almost certainly must occur during progenitor differentiation. In addition, the study of normal progenitors requires the inclusion of appropriate growth factor(s) (eg, Epo), adding further complexity to the analysis. In contrast, in the present study we have used an erythroleukemia cell line that replicates independently and does not require Epo for growth. Therefore, we have been able to study the role of c-myb in erythroid differentiation separately from a potential role in growth factor-regulated cell proliferation and from Epo or other inducers. The use of a homogeneous cell population here has avoided the complexities inherent in the study of heterogeneous populations of normal cells, an approach that is made even more difficult by the possibility of pleiotropic effects of anti-sense oligo treatment on a variety of interacting cell types.

Although Epo regulates c-myc as well as c-myb expression,28-30 we observed no effect of antisense c-myc oligo either in the absence or presence of Epo. However, this finding does not rule out a role for c-myc in erythropoiesis. It may suggest that c-myc participates in Epo-induced cell proliferation rather than in differentiation. If so, our use of an independently proliferating Epo-sensitive cell line in these studies might be expected to result in an absence of

**Fig 1.** Anti-sense c-myb oligo or Epo induce hemoglobinization of Rauscher murine erythroleukemia cells. Anti-myb, GTGTCGGGGTC-TCCGGGC. Controls: anti-myc, AACGTTGAGGGGCAT; anti-vsv, TTGGGATAACACTTA; and a nonsense oligo with the same base composition as anti-myb, GCGCGCTTGGCTGGGC. Similar results were obtained with all three control oligos.

**Fig 2.** Induction of Hb+ by anti-sense c-myb is concentration dependent. Cells were cultured as in Fig 1. Anti-sense c-myb S-oligo (sulfurized form)13,26 was used.
Fig 3. Downregulation of myb protein in Rauscher cells treated with anti-sense c-myb oligo. (A) Cells incubated in the absence of oligo stained with anti-myb antibody. The red stain is interpreted as positive. (B) Cells grown in the presence of anti-sense c-myb oligo and stained with anti-myb antibody. Note the drastic reduction in myb protein. We verified that the addition of excess peptide antigen blocked the staining of myb protein and that control oligo had no effect (not shown).

anti-sense c-myc effect. We have not been able to test whether the effects of reduction of myb protein on erythroid differentiation seen here are found also in non-transformed cells, due to the requirement of Epo for the cells’ proliferation.

It should be noted that both Epo and anti-sense c-myb oligo induced hemoglobinization of individual cells (Fig 1) and of colonies (Table 1) to the same degree. We have taken this as evidence that the hormone and oligo are operating on the same population of responsive cells. Nonetheless, although myb protein appeared to be reduced in all cells treated with oligo, not all of the cells hemoglobinized. Such fractional response to induction appears to be an intrinsic property of erythroleukemia cells and is highly variable among different clones of the same line. This phenomenon was documented for dimethyl sulfoxide (DMSO) treatment of Friend erythroleukemia cells by Gusella et al39 and for both Epo and DMSO treatment of
Rauscher cells by ourselves. Its molecular basis is unclear. It appears to be related to the process of “commitment” that occurs with a certain time-dependent random probability (stochastically) during each cell cycle while in the presence of inducer. This concept arose from the classical studies of Till et al., who proposed that normal hematopoietic stem cell growth was governed by a stochastic process. Clearly, therefore, other factors besides inducer (“permissive factors”) are involved in determining whether a cell will differentiate, and it is highly likely that some of these factors are intrinsic to the cell. We speculate that differential expression of other genes required for the cell to respond to inducer (Epo, DMSO, oligo) occurs among individual cells in a population and that the presence and relative abundance of the gene products (permissive factors) helps determine the cell’s response to inducer. In this regard, we have found that Friend cells, which heretofore have been considered “Epo-nonresponsive,” exhibit several responses to Epo identical to Rauscher cells, including specific membrane protein (pp43) dephosphorylation and

Fig 4. Anti-sense c-myb oligo induces hemoglobin synthesis in Rauscher cells grown in plasma clot culture without altering cell growth. (A) Cells treated with anti-sense vsv oligo. No Hb⁺ cells are seen in these colonies. (B) Cells treated with anti-sense c-myb oligo. Note the Hb⁺ cells.
changes in c-myc and c-myb expression. Yet, Friend cells do not differentiate upon Epo treatment. This Friend cell "defect" may be due to the complete absence of one or more such permissive factors (gene products).

Our data support a role for c-myb in Epo-induced hemoglobin synthesis. However, reduction of myb protein did not trigger terminal differentiation. Rather, it was found to result in expression of a more limited repertoire of erythroid-specific genes. The possibility of regulation of a segment of the erythropoietic program by a single proto-oncogene product may be important in the design of specific therapies and suggests that the identification of other Epo early response genes should prove fruitful.

ACKNOWLEDGMENT
We would like to thank Drs A.M. Gewirtz, C. Civin, M.B. Kastan, and S. Bailey for helpful discussions, Dr S. Alper for the generous gift of antisera to murine band 3, D. Zurbich for oligo synthesis, Elanex Pharmaceuticals for their gift of rhEpo, and Rosemary Kilduff for her editorial expertise.

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