Vanadate Elevates Fetal Hemoglobin in Human Erythroid Precursors by Inhibiting Cell Maturation

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Increased fetal hemoglobin (HbF) in erythroid precursors of patients with β-hemoglobinopathies (sickle cell anemia and β-thalassemia), in which adult hemoglobin synthesis is defective, ameliorates the clinical symptoms of the underlying diseases. The production of erythroid precursors depends on the action of erythropoietin (EPO), which prevents their apoptosis and stimulates their proliferation. EPO binds to its surface receptor, induces its homodimerization, and initiates a cascade of phosphorylation and dephosphorylation of a series of proteins by kinases and phosphatases, respectively. Vanadate inhibits various phosphatases, including those that are involved in the EPO pathway, thereby intensifying the signal.

In this study, we investigated the effect of vanadate on the proliferation and maturation of human erythroid precursors in culture. When vanadate was added to cells derived from normal donors, cell maturation was delayed, as indicated by cell morphology, cell growth kinetics, the rate of appearance of hemoglobin-containing cells, and the expression of surface antigens (CD117, CD71, and glycophorin A). Analysis by high-performance liquid chromatography and flow cytometry of the hemoglobin profile of vanadate-treated normal cells revealed a higher proportion of HbF than was found in untreated cells. When vanadate was added to cells derived from patients with β-thalassemia, a significant increase in HbF was observed.

The results suggest that intensification of the EPO signal by vanadate results in maturation arrest and increased HbF production. Thus, inhibitors that are more specific and less toxic than vanadate may present a novel option for elevating HbF in patients with β-hemoglobinopathies, as well as for intensifying the EPO response in other forms of anemia. Exp Biol Med 232:654–661, 2007

Key words: erythropoiesis; fetal hemoglobin; maturation; vanadate; flow cytometry

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Introduction

Fetal hemoglobin (HbF; $\alpha_2\gamma_2$), the major hemoglobin (Hb) found during embryonic life, is replaced after birth by adult Hb (HbA; $\alpha_2\beta_2$; Ref. 1). Elevated levels of HbF in adults may be acquired, such as in juvenile myelomonocytic leukemia (2) or during acute erythropoietic stress (3), and are frequently observed in inherited blood disorders such as the β-hemoglobinopathies: sickle cell anemia and β-thalassemia. Increased HbF in these diseases ameliorates the clinical symptoms of the underlying disease (4, 5). In sickle cell anemia, not only do HbF-containing cells have a lower concentration of sickle Hb, but HbF inhibits polymerization of this Hb directly, accounting for the lower propensity of such cells to undergo sickling (6–8). In β-thalassemia, elevated HbF should compensate partially for the deficiency in β-chains and balance the excess of α-chains. Various agents have been shown to augment HbF production, and one of them, hydroxyurea, is currently in clinical use for treatment of these diseases (9).

The mechanisms by which drugs stimulate HbF are not known. Two broad hypotheses have been explored. One is based on drug-induced modifications of the DNA due to hypomethylation of globin promoter regions (10), inhibition of histone deacetylases (11), or activation of responsive regions, such as following binding of butyrates to a specific region of the $^{A}\gamma$-globin promoter (12). The other mechanism involves modification of the cell cycle kinetics (13) and the rate of differentiation of erythroid progenitors (14, 15). Observations of elevated HbF during erythropoietic stress (3) also suggest that the rate of erythroid maturation is involved in the modulation of HbF production.

Production of erythroid cells (erythropoiesis) depends on the glycoprotein hormone erythropoietin (EPO), which prevents apoptosis and stimulates proliferation of erythroid precursors (16). The activity of EPO is mediated through binding to its surface receptor (EPO-R; Ref. 17). EPO binding induces receptor homodimerization and the initiation of a stepwise signal transduction process (18). The EPO signal is regulated by several factors: (i) the concentration of EPO. EPO is produced by the kidneys in response to hypoxia (16), secreted into the blood stream, and interacts

with the erythroid progenitor/precursor cells in the bone marrow. (ii) The density of EPO-R on erythroid precursors that is downregulated during erythroid maturation (19). (iii) The stimulating activity of kinases that induce tyrosine phosphorylation of various proteins in the EPO signaling pathway (20). A single-base somatic mutation in the gene of one of these kinases, Janus kinase 2 (JAK2), was recently reported to be responsible for autostimulation of the pathway, causing EPO-independent growth of erythroid cells in polycythemia vera (21). (iv) The inhibitory activity of phosphatases that downregulates the signal, causing it to be transient and dependent on continuous EPO binding. The activity of various protein tyrosine phosphatases, including those involved in the EPO pathway, can be inhibited by sodium orthovanadate (Na_3VO_4, vanadate; Ref. 22).

In the present study we investigated the effect of vanadate on primary cultures of human erythroid cells. For this purpose we used the two-phase liquid culture protocol (23). In the first phase of this protocol, which is EPO independent, peripheral blood mononuclear cells are cultured for 1 week with various growth factors but in the absence of EPO. During this phase, early erythroid committed progenitors—erythroid burst-forming units—proliferate and differentiate into late, erythroid colony-forming unit–like, EPO-dependent progenitors. In the second phase, the latter cells, cultured in an EPO-supplemented medium, continue to proliferate and differentiate, eventually maturing into Hb-containing orthochromatic normoblasts and enucleated erythrocytes.

When erythroid precursors derived from normal donors were exposed to vanadate in the second phase of the culture, their maturation was delayed, as indicated by their morphology, their Hb content, and the expression of surface antigens. Analysis by high-performance liquid chromatography (HPLC) and flow cytometry of the Hb profile of normal vanadate-treated cells showed a higher proportion of HbF compared with untreated cells. Addition of vanadate to cultures of cells derived from patients with β-thalassemia increased HbF as well. Since the presumed molecular target of vanadate is known, the finding that it delayed maturation and increased the proportion of HbF may help to elucidate the mechanism of HbF modulation in adult erythroid cells. This is of basic scientific interest as well as of clinical significance in patients with β-hemoglobinopathies.

Materials and Methods

Vanadate Preparation. Sodium orthovanadate (Sigma, St. Louis, MO) at 100 m*M* was prepared in phosphate-buffered-saline (PBS). The pH was adjusted to 10.0 with 1 *N* NaOH or 1 *N* HCl, and the solution was stored until use at 4°C.

Cultures of Human Erythroid Cells and Their Analysis. Peripheral blood was obtained from healthy adult blood donors and six patients with β-thalassemia. The patients had various mutations: four had β-thalassemia major and two had intermedia. The patients with β-thalassemia major were polytransfused and on iron chelation therapy; their blood was drawn prior to blood transfusion. Informed consent was obtained in all cases. The blood mononuclear cells were cultured according to the two-phase protocol as described previously (23). After 5 to 7 days of incubation in phase I, the nonadherent cells were harvested and recultured in fresh medium composed of alpha medium: 30% fetal calf serum, 1% deionized bovine serum albumin, 1×10^{-5} *M* β-mercaptoethanol, 1.5 m*M* glutamine, 1×10^{-6} *M* dexamethasone, and 1 U/ml human recombinant EPO. On various days in phase II, cells were harvested and analyzed: the percentage and absolute number of Hb-containing cells were determined microscopically in a hemocytometer following staining with benzidine dihydrochloride (Sigma), as previously described (24). Cell morphology was assessed microscopically on cytocentrifuge-prepared slides stained with alkaline 3,3-dimethoxybenzidine (Sigma) followed by Giemsa, as previously described (24). Intracellular Hb was separated and quantified in the cell lysates by HPLC as previously described (25).

Surface markers were analyzed by flow cytometry after staining with fluorescein isothiocyanate (FITC)–conjugated monoclonal antibodies directed against CD36 or CD71 or phycoerythrin (PE)–conjugated monoclonal antibodies directed against CD117 or GPA, as previously described (26). The percentage of HbF-containing cells was determined as follows (27): cells were fixed with 1 ml of 4% paraformaldehyde for 10 mins at room temperature and centrifuged. The pellet was permeabilized with 1 ml of a ratio of methanol to acetone (1:4 vol/vol) for 1 min and was washed twice with PBS. The cells were resuspended in 100 μl of 1% BSA and incubated for 45 mins at room temperature with PE-conjugated mouse anti-human HbF. The cells were washed once with PBS and analyzed by flow cytometry.

Cell cycle kinetics were quantified by flow cytometry of propidium iodide–stained cells as previously described (28).

Flow Cytometry. Cells were analyzed with the aid of a fluorescence-activated cell sorter (FACS-calibur; Becton Dickinson, Mountain View, CA). The cells were passed at a rate of ~1000/sec, using saline as the sheath fluid. A 488-nm argon laser beam was used for excitation. The FACS was calibrated and set using CaliBRITE-3 beads (Becton Dickinson). To exclude nonerythroid cells from analysis, erythroid cells were gated according to their size (forward light scatter) and granularity (side light scatter). The fluorescence of the erythroid cells was measured using either linear (for propidium iodide–stained cells) or logarithmic (for FITC- and PE-stained cells) amplification. Cells stained with isotype control antibodies served to set the background, nonspecific staining of the cells. The percentage of positive cells was calculated using the FACS-equipped CellQuest software.

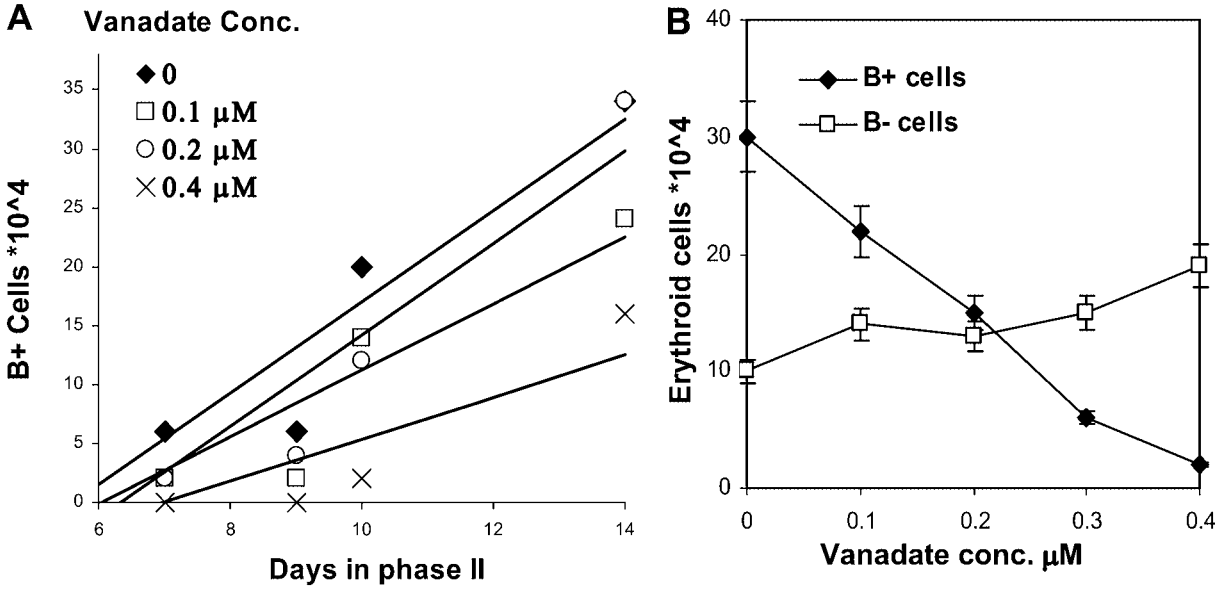

Figure 1. Effect of vanadate on accumulation of hemoglobin-containing erythroid cells. Cells derived from normal donors were cultured according to the two-phase liquid procedure. On Day 6 of phase II, vanadate was added at the indicated concentrations. Hb-containing cells were scored by staining with benzidine. (A) The number of B^+ cells on different days of phase II. The results of one representative experiment of four experiments carried out with cells from different donors are shown. (B) The number of Hb-containing (B^+) and non–Hb-containing erythroid cells (B^-) on Day 12 of phase II. The results show the average ± SD of four experiments using cells from different donors.

Results

Effect of Vanadate on Erythroid Cell Maturation. To observe the effects of vanadate on the kinetics of erythroid cell maturation, different concentrations of vanadate were added to phase II cultures. Hb-containing (benzidine positive; B^+) cells first appeared on Day 6 and continued to accumulate until Day 14. Addition of vanadate inhibited the accumulation of B^+ cells in a dose-dependent manner. Figure 1A depicts the kinetics of B^+ accumulation when vanadate was added on Day 6. The results of four experiments using cells from different healthy donors indicated inhibitions of 14.3%, 37.1%, and 65.7% with 0.1 μ*M*, 0.2 μ*M*, and 0.4 μ*M*, respectively. Maturation of erythroid precursors in the culture was not synchronous—both B^+ and B^- erythroid cells were present in the cultures. Figure 1B depicts the effects of different concentrations of vanadate on the number of B^+ and B^- erythroid cells on Day 12. The results of four experiments showed that in the absence of vanadate, 75% of the erythroid population were B^+ cells, compared with 9.5% in the presence of 0.4 μ*M* vanadate, indicating that vanadate inhibited cell hemoglobinization. This point is further illustrated in Figure 6, which shows the total amount and the proportion of various hemoglobins in cell lysates of cultures that had been treated or nontreated with vanadate. Note that the total Hb of the treated culture was 27% of that in the untreated culture.

Figure 2 shows the effect of vanadate on cell morphology. Cells grown from Day 1 of phase II in the presence (0.02 μ*M*) or absence of vanadate were harvested on Day 12 and stained with 3′-3′-dimethoxybenzidine and Giemsa. In untreated cultures (Fig. 2A) most of the cells were small, with little cytoplasm and a small nucleus with coarse chromatin. Benzidine staining was intense, indicating the presence of Hb, particularly in the smallest cells. Cells grown with vanadate (Fig. 2B) were larger, with abundant cytoplasm and a large, round nucleus with fine chromatin. The majority of the cells stained lightly with benzidine or proved negative. In contrast to untreated cultures, cultures treated with vanadate contained many mitotic cells.

The size of the erythroid cells was further analyzed by flow cytometry of their forward light scatter, which is proportionate to cell size. The mean fluorescence channel of cells treated with vanadate was 27% higher than that of untreated cells. Since maturation of erythroid cells is associated with a decrease in cell size, the results suggest inhibition of maturation by vanadate.

The effect of vanadate on cell cycle kinetics was quantified by flow cytometry of propidium iodide staining of cells on Day 12 of phase II (Fig. 3). The results of four such experiments using cells from different donors show that in the absence of vanadate, 81.3% of the erythroid cells were in G_1, indicating terminal maturation and loss of mitotic potential. In the presence of vanadate, 49% of the cells were in G_1, 12% were in S phase and 39% were in G_2 + M phases. The presence of cycling cells indicated a delay in terminal maturation induced by vanadate.

The effect of vanadate on cell maturation also was studied by determining the expression of surface markers. In the experiments summarized in Figure 4, different concentrations of vanadate were added on Day 6 of phase II, and the cells were analyzed on Day 10 for expression of the stem cell factor receptor (CD117), the transferrin receptor

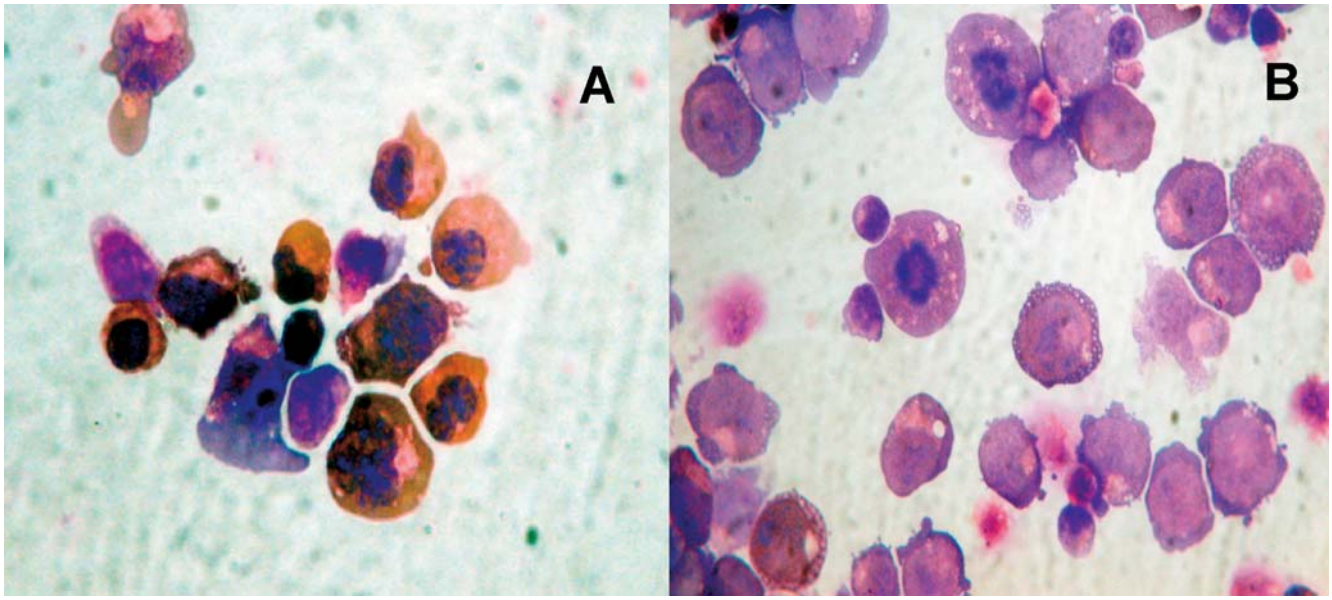

Figure 2. Effects of vanadate on morphologic maturation of erythroid cells. Vanadate (0.02 μ*M*) was added on Day 1 of phase II to cultures of cells derived from healthy donors. The cells were harvested on Day 12 and stained with 3-dimethxybenzidine and Giemsa. (A) Without vanadate. (B) With vanadate.

(CD71), CD36, and GPA. These surface proteins have been demonstrated to provide flow cytometric correlates with the developmental stage of erythroid precursors (29). The results show that treatment with vanadate caused an increase in the expression of early markers (CD117, CD71, and CD36) and a decrease in a late marker (GPA).

Effect of Vanadate on Fetal Hemoglobin Production. The effect of vanadate on HbF was measured by two techniques: flow cytometry, to quantify the percentage of HbF-containing cells (%F-cells), and HPLC, to measure the amount of HbF and its representation in the total Hb (%HbF). Vanadate was added on Day 1 of phase II, and cells were analyzed on Day 14. Figure 5 shows a vanadate dose-dependent increase in both the %F-cells (maximal 5.7-fold) and in the %HbF in the lysates of the treated cells. HPLC analysis of lysates of 5-ml samples of untreated cultures and cultures treated with vanadate (0.05 μ*M*) is shown in Figure 6. The areas under the peaks of the chromatograms indicate the relative amount of each type of Hb, and the total area under the peaks indicates the total Hb produced. The insets show enlargement of the HbF region. The results show that the total Hb was lower in vanadate-treated cells, but that the proportion of HbF (peaks HbF1 and HbF0) was higher compared with that in untreated cells.

The effect of vanadate on HbF production also was tested on β-thalassemia cells. Figure 7 shows the mean %F-cells in cultures treated from Day 6 of phase II with various concentrations of vanadate and analyzed on Day 10. In the absence of vanadate, the mean %F-cells in six cultures from different patients was 12% ± 2% (range: 6%–30%). It increased with vanadate in a dose-dependent manner up to 55% ± 8% (range: 11%–72%).

Discussion

EPO is a crucial cytokine for erythropoiesis; it modulates the proliferation, maturation, and apoptosis of late progenitors and precursors of the erythroid lineage (16). Its signaling pathway is modulated by phosphorylation and dephosphorylation of a series of proteins by kinases and phosphatases, respectively (20). Vanadate inhibits various phosphatases, including those involved in the EPO pathway (22), and thereby presumably causes overexpression of the signal. Previous studies showed that treatment with vanadate of the EPO-dependent HCD57 murine cell line resulted in increased tyrosine protein phosphorylation. Vanadate acted synergistically with EPO to stimulate DNA synthesis and prevented apoptosis following EPO withdrawal without promoting proliferation (30, 31). It also delayed apoptosis in primary human erythroid progenitors (31). Vanadate was also shown to act on normal erythroid progenitors as a phosphatase inhibitor that potentiates the kinase activity induced by EPO and stem cell factor (SCF). This function was, however, reduced in polycythemia vera cells (32).

In our study, using a two-phase liquid culture protocol we found that vanadate delays maturation of human erythroid precursors. When vanadate was added together with EPO on the first day of phase II, it reduced the output of erythroid cells on Day 14. This is most probably because vanadate is a nonspecific inhibitor of various phosphatases, including those involved in cellular metabolism and proliferation (33). When vanadate was added on Day 6 of phase II, the number of erythroid cells was only modestly reduced, but cell maturation was postponed significantly. This resulted in a delay in the appearance of Hb-containing

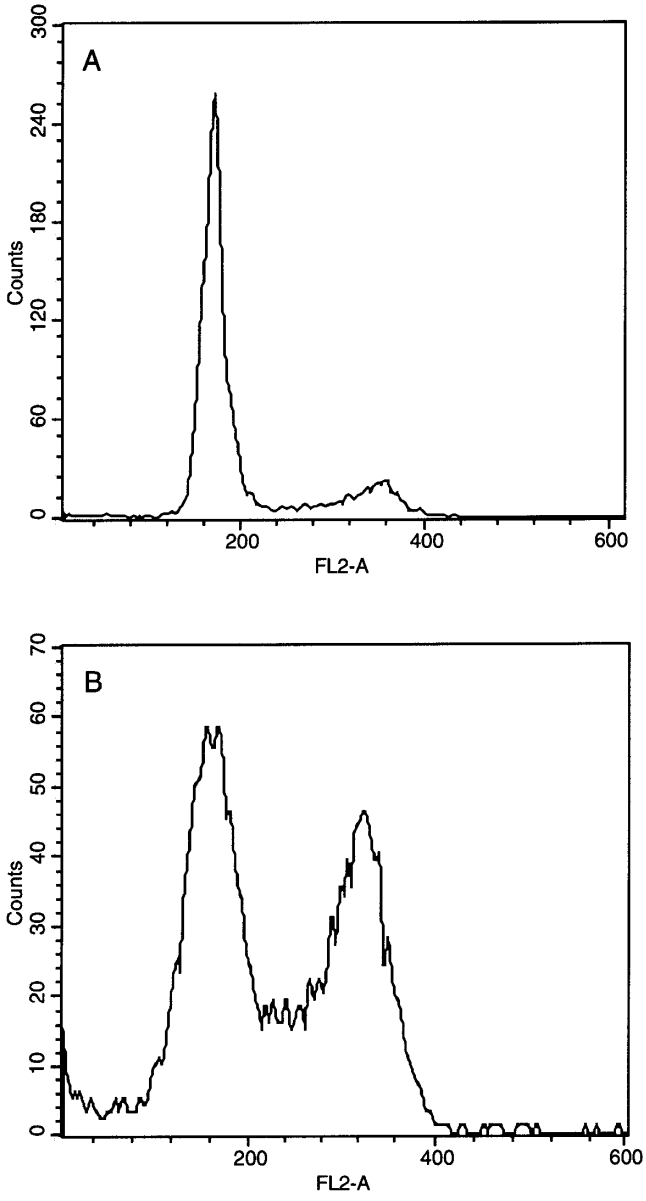

Figure 3. Effect of vanadate on the cell cycle. Vanadate (0.02 μM) was added on Day 1 of phase II to cultures of cells derived from healthy donors. The cells were harvested on Day 12, stained with propidium iodide, and analyzed for their distribution in the cell cycle. (A) Without vanadate. (B) With vanadate. The results of one representative experiment of four experiments carried out with cells from different donors are shown.

cells and in Hb accumulation, as reflected by benzidine staining and HPLC, respectively. Cells treated with vanadate had a morphology characteristic of immature erythroid precursors, relative to untreated cells of the same age. Analyses of cell size, cell cycle, and Hb content substantiated this conclusion. In addition, cells treated with vanadate maintained expression of early erythroid surface markers and a delay in the expression of the late marker. The delay in cell maturation caused by vanadate was accompanied by an increase in the proportion of HbF produced, as shown by HPLC analysis and flow cytometry.

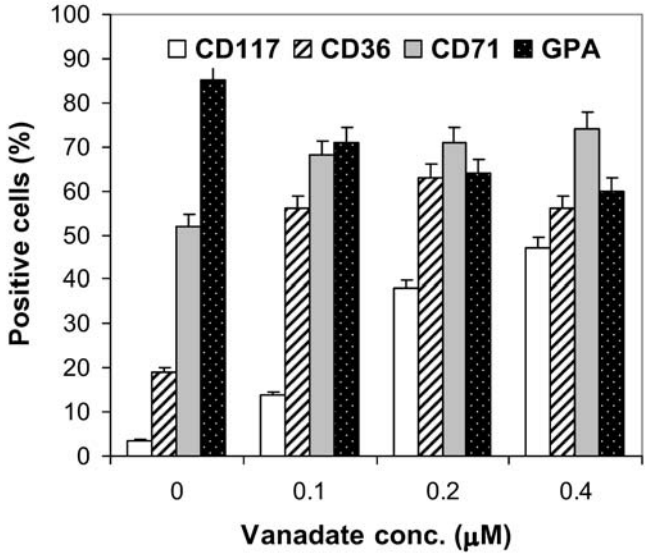

Figure 4. Effects of vanadate on surface marker expression by erythroid cells. Vanadate was added at the indicated concentrations on Day 1 of phase II to cultures of cells derived from healthy donors. The cells were harvested on Day 12, washed, and stained with fluorescent antibodies to CD117, CD36, CD71, and GPA. The cells were analyzed by flow cytometry, and the percentages of cells positive for each marker were determined. The results show the average ± SD of four experiments using cells from different donors.

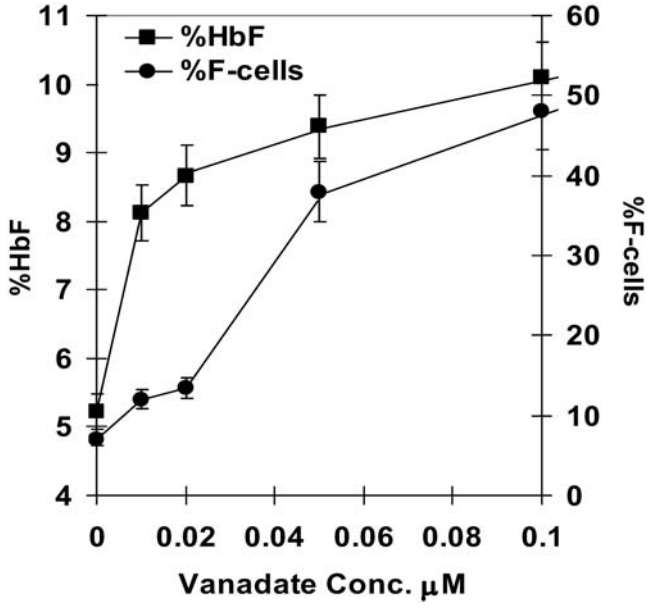

Figure 5. Effect of vanadate on HbF production. Vanadate was added at the indicated concentrations on Day 1 of phase II to cultures of cells derived from healthy donors. The cells were harvested on Day 14 and analyzed for both %F-cells (black circles), which was determined by flow cytometry using fluorescence-conjugated antibody to human HbF, and intracellular Hb content (black squares), which was analyzed by HPLC and for which is shown the %HbF. Average ± SD of four experiments using cells from different donors is shown.

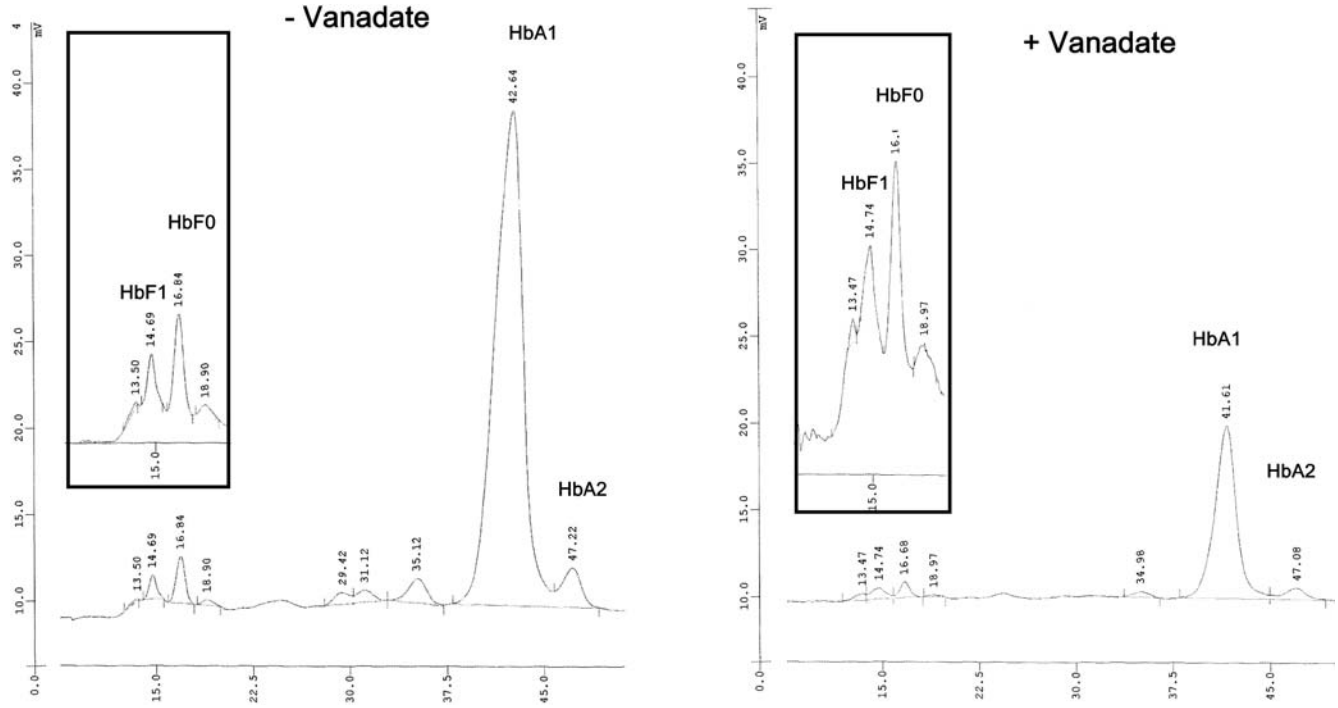

Figure 6. HPLC analysis of Hb in vanadate-treated and untreated cultures. Vanadate (0.05 μ*M*) was added on Day 1 of phase II to cultures of cells derived from normal donors. Five-milliliter cultures were harvested on Day 14, their cells lysed and Hb separated by HPLC, and heme-containing compounds were quantified at 415 nm. The chromatograms show the various peaks; HbF1, HbF0, HbA1, and HbA2 are labeled. The numbers indicate retention times.

The rate of proliferation and maturation of erythroid cells is regulated by the intensity (and duration) of the ERO signal, which depends on the density of the receptors and on the concentration of EPO. At early stages of maturation the signal is intense due to an abundance of EPO-R (19). As the density of the receptors drops, the intensity of the signal weakens, and maturation occurs. The concentration of EPO, as well as SCF, also affects the intensity of the signal and, thereby, the rate of maturation. High concentrations of the cytokines delay maturation, increase proliferation/amplification, and result in a high cell yield. In addition, the intensity of the signals may depend on the balance between the activities of kinases and phosphatases in the EPO pathway. Thus, our finding of maturation arrest by vanadate might be the result of inhibition of phosphatases, which causes overphosphorylation of key proteins and intensification of the EPO/SCF signals.

Several previously reported observations suggest that the rate of maturation and the intensity and duration of the EPO signal affect HbF production. Indeed, HbF is elevated during acute erythropoietic stress (3). HbF production is relatively abundant in early precursors and, as the cells mature, progressively decreases due to rapid synthesis of HbA (34), suggesting that a strong EPO signal favors HbF production. Some studies (35, 36), but not all (37–39), showed that high-dose EPO treatment of primates (40, 41) and patients with β-hemoglobinopathies caused an elevation in HbF. We have previously shown that culture of erythroid

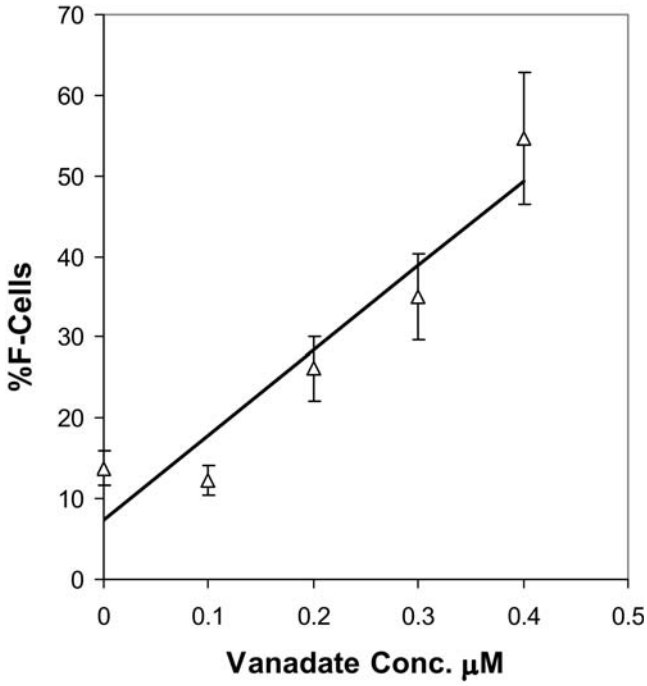

Figure 7. Effect of vanadate on HbF production by β-thalassemia cells. Vanadate was added at the indicated concentrations on Day 6 to phase II cultures of cells derived from β-thalassemia patients. The %F-cells was determined on Day 10 by flow cytometry. The average ± SD results of six cultures from different patients are shown.

cells in the continuous presence of low EPO reduced cell yield but did not affect the proportion of HbF. However, reducing EPO levels midway through the culture period, lowered cell yield, accelerated maturation, shortened the period of HbA production and, consequently, increased the proportion of HbF. In another study, we found that supplying early erythroid cultures with exogenous hemin resulted in high HbF in the mature cells. The effect of hemin, which is a rate-limiting factor for hemoglobinization in early precursors, may be related to the finding that when supplied with exogenous hemin, the precursors initiated Hb production earlier with HbF predominating. SCF, which delays cell maturation, also was shown to enhance HbF production in cultures of erythroid cells (42–45).

In summary, the potentiating effect of vanadate on HbF may be related to its ability to delay erythroid maturation. Vanadate, in the form of sodium metavanadate, has been tested in clinical trials (for treating both insulin- and non–insulin-dependent diabetes mellitus (46, 47). In our *in vitro* study vanadate was shown to reduce the number and hemoglobin content of erythroid cells, and therefore is not suitable for treatment of anemic patients. However, other more specific and less toxic inhibitors of phosphatases may be considered as a new therapeutic modality for elevating HbF in patients with β-hemoglobinopathies as well as intensifying the EPO response in other forms of anemia.

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