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Valproic acid, trichostatin and their combination with hemin preferentially enhance γ-globin gene expression in human erythroid liquid cultures

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Background and Objectives. In addition to conventional therapy, current treatment of thalassemia and sickle cell anemia includes inducers of hemoglobin F synthesis (hydroxyurea, erythropoietin, azacytidine and butyrate). However, because of concerns about the dose-limiting myelotoxicity, potential carcinogenicity and high cost of the above agents, an intensive search for less toxic or more effective drugs is ongoing. In this study we tested the effect of valproic acid and trichostatin, alone or in combination with hemin, on γ chain synthesis in human erythroid liquid cultures.

Design and Methods. The agents were tested on erythroid human liquid cultures derived from normal peripheral blood, peripheral blood from β^s/β^{thal} patients, normal cord blood and normal bone marrow samples. The effect of the agents was expressed as increase of γ/γ+β m-RNA, measured with competitive reverse transcriptase-polymerase chain recation (RT-PCR), or as increase of HbF, measured by high performance liquid chromatography (HPLC).

Results. Addition of valproic acid or trichostatin to human erythroid cell cultures preferentially enhanced γmRNA synthesis in all blood samples (2.9 to 3.5-fold). The addition of hemin enhanced the effect up to 10-fold.

Interpretation and Conclusions. Valproic acid, trichostatin and their combination with hemin (all three FDA-approved drugs) preferentially increase γ-globin chain synthesis and may be helpful for the treatment of hemoglobinopathies.

Key words: fetal hemoglobin, trichostatin, valproic acid

Expression of the globin genes in man is characterized by two major *switches* during ontogeny: the transition from the embryonic to the two types of fetal hemoglobin (HbF) after the two first months of gestation, and the switch from HbF to adult hemoglobin (HbA) starting in mid-gestation and being completed six months after birth.[1] Increased HbF synthesis in postnatal life may occur in various conditions, of which the most frequent are globin chain disorders such as sickle cell anemia and β-thalassemia.[2] In the latter conditions, the increase of HbF has a clearly beneficial effect: in sickle cell anemia, HbF not only *dilutes* HbS thus decreasing molecular contact and polymerization, but it also inhibits the latter event through formation of $\alpha_2\beta^s\gamma$ hybrids.[3] In β-thalassemia, the presence of γ-chains neutralizes the noxious intracellular precipitation of the unbound α-chains thereby preventing premature cellular death. Moreover, it compensates for the relative shortage of β-chains by increasing cellular hemoglobin content.

Recently, several agents have been used in attempts to induce fetal hemoglobin pharmacologically in humans. These include: (a) erythropoietin and other hemopoetins which stimulate HbF production by inducing rapid erythroid regeneration but require prohibitive amounts to be effective;[4] (b) a series of cytotoxic drugs (5'-azacytidine, cytosine arabinoside, hydroxyurea, etc.) that induce HbF by altering the kinetics of erythropoiesis but are myelotoxic and potentially carcinogenic[5-7] and (c) butyric acid and its derivatives which promote γ gene transcription and inhibit histone deacetylase, which affects chromatin structure and interferes with the rate of transcription.[8-10] As these substances appear to be less toxic than the previous drugs, scientific interest over the last years has focused on identifying agents of this category.

Two substances whose structure closely resembles that of butyric acid and are already FDA-approved drugs are valproic acid and trichostatin.[11]

Valproic acid, an anticonvulsant drug, is an analog of pentanoic acid and contains a propyl side chain in the second carbon position (2-propylpentanoic acid). It is a safe drug which has already been widely used for the treatment of epilepsy. Trichostatin (TSA) has been used as an antifungal antibiotic.[11]

The present study examines the activity of these agents *in vitro*, using human erythroid cell cultures incubated in the presence of recombinant erythropoietin.[12] Our results show that valproic acid and TSA alone or in various combinations with hemin, preferentially accelerate fetal globin gene expression in liquid cultures of erythroid progenitors derived from normal adult individuals, compound heterozygotes for β-thalassemia and HbS β^s/β^{thal}, cord blood and normal bone marrow samples.

Design and Methods

Human adult erythroid cultures (hAEC)

Fresh blood samples from normal adults (400 mL) and from patients with $\beta^s\beta^{thal}$ (no more than 100 mL) were obtained from the Blood Bank following informed consent from the donors. Cord blood samples (of the order of 50 mL) were provided by the University Maternity Hospital, while normal bone marrow samples were obtained from aspirations done for other clinical purposes in this Department.

To set up the cultures, mononuclear cells were isolated by centrifugation on a Ficoll-Hypaque gradient and cultured in α-minimal essential medium supplemented with 10% fetal calf serum (FCS), 1 mg/mL cyclosporin A and 10% conditioned medium from the K5637 bladder-carcinoma cell line. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air with extra humidity (phase I). After one week, the non-adherent cells were washed and recultured in fresh medium containing α-medium, 30% FCS, 1% bovine serum albumin, 1×10^{-5} M mercaptoethanol, 1×10^{-6} M dexamethasone and 1 U/mL human recombinant erythropoietin (phase II). Hemoglobin-containing cells were identified by the acetic acid-benzidine peroxide procedure. Cell viability was determined by trypan-blue exclusion. Erythroid cell maturation was assessed microscopically on cytocentrifuge slides stained with alkaline benzidine and Giemsa.

Valproic acid was added in the form of its sodium salt (Sigma P4543). TSA was obtained from Sigma (T8552). Hemin was obtained in its chloride form and was dissolved according to the instructions of the providers (Sigma H1652). The optimal concentrations and times for the addition of the above agents in the cultures were identified by a series of experiments which are shown in the results.

Globin chain identification

Cultured cells were washed 3 times in cold 154 mM NaCl or 50 mM Bis-tris/140 mM NaCl pH 7.40 solution; following centrifugation the final pellet was lysed by addition of 1 mL solution containing 7 mM KCN, 24 mM potassium phosphate and 2 mL/L saponin pH 5.50. Membranes were removed by centrifugation at 13,000g for 15 min. Finally, 20 µL of hemolysate were chromatographed on a HPLC column (Bio-Rad Laboratories, Hercules CA, USA).

Globin RNA analysis

Cytoplasmic RNA was extracted from hAEC by the vanadyl ribonucleoside complex method. cDNA was synthesized using 1 mg RNA as template, 200 units Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Gibco, BRL) and 10 pg of primers specific for each globin gene. DNA amplification was carried out by the polymerase chain reaction (PCR) using 10 µL cDNA as template and specific oligonucleotide primers chosen from sequences within each globin gene. The sequences of the primers have been described elsewhere.[13]

Quantification of RNA by competitive PCR

Each DNA sample was co-amplified with increasing concentrations of specifically prepared standard DNA in a series of reaction tubes. Each standard was designed so that the 5' and 3' ends were complementary with the primers used to amplify the cDNA sample; however the PCR product they yielded was significantly shorter. The number of cycles for the amplification was based on preliminary experiments to ensure that the reaction was within the exponential phase. In each PCR reaction the gene of actin was co-amplified in order to ensure comparable amounts of cDNA in each reaction. The product of the latter was analyzed by electrophoresis on 2% low melting agarose, stained with ethidium bromide, photographed and quantified by scan pack (Bio-Rad) The concentration of the cDNA was determined as the amount of standard which could provide PCR products of equal intensity (Figure 1).

Results

Evaluation of the method

Addition of valproic acid in the cultures on the first days of phase II caused a significant decrease

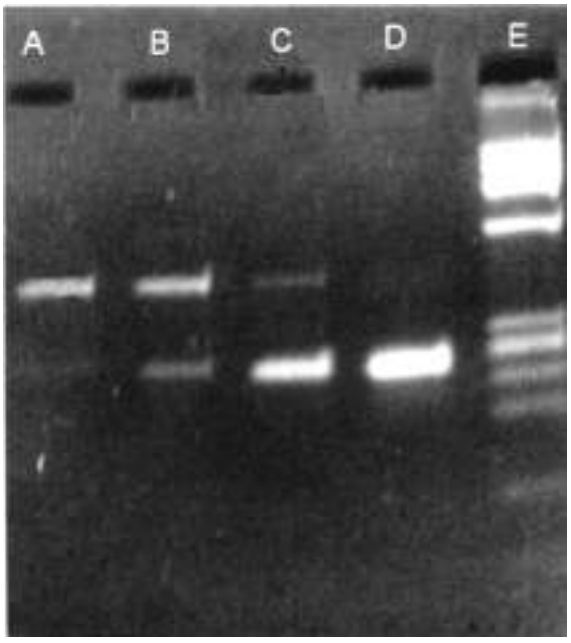

Figure 1. Competitive RT–PCR. The γ-c DNA (upper band) is co-amplified with increasing concentrations of the competitor (lower band). Lane A: 0.001 pg. Lane B: 0.01 pg. Lane C: 0.1 pg. Lane D: 1 pg. Lane E: Size marker phiX Hae I.

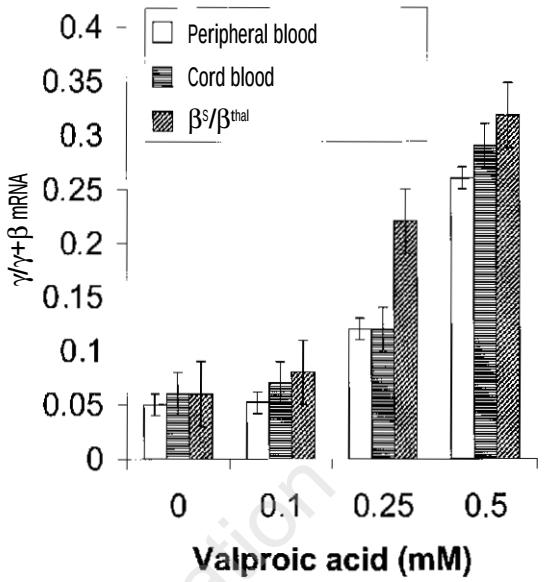

Figure 2. Effect of valproic acid on γ–chain synthesis expressed as increase of γ/γ+β mRNA in cultures from peripheral blood, cord blood and β^S/β^{thal} blood samples.

of the viability of the erythroid precursors, as assessed by trypan blue exclusion; the decrease proved to be dose- and time-dependent , i.e. 90% decrease when the addition was done on day 2 vs 50% when it was done on day 5. To obtain sufficient cells for analysis , addition of the drugs in all virtual experiments was done on days 5 and 7 and the cells were harvested for analysis on day 13. The concentrations of valproic acid ranged from 0.1-5.0 mM. Valproic acid increased γ-mRNA expression up to the concentration of 1 mM. Higher concentrations had no further effect .

Trichostatin was added on days 5 and 7 of phase II of the liquid culture system and the cells were harvested for analysis on day 13. The concentrations of TSA ranged from 0.01 mM to 0.1 mM. Higher concentrations proved cytotoxic, leading to a significant decrease of cell viability.

Hemin was added at the concentration of 100 mM on day 1 of phase II together with EPO. These conditions had been proven to produce the maximal heme effect in earlier experiments.

Effect of valproic acid

Competitive RT-PCR analysis showed a dose-dependent increase in the levels of γ-mRNA in all samples, expressed as the ratio of γ/γ+β m RNA; in contrast, there was no change in the amount of β transcripts and the β/β+γ mRNA ratio remained unchanged (Figure 2). All data represent the mean values of 4 experiments. The increase of γ/γ+β mRNA in the cultures from normal individuals was 2.8±1.8 SD fold; it was 3.5±1.71 SD fold in the cultures from β^S/β^{thal} patients, and 3.4±1.68 SD fold in the cord blood samples (Figures 2 and 3). The increase of γ/γ+β m-RNA in cultures from different cell sources did not differ significantly (t-test).

Effect of TSA

The increase of γ/γ+β mRNA upon addition of TSA to erythroid cultures from normal individuals was 2.8 ±1.1 SD fold that of the control values. In contrast, the β/β+γ mRNA ratio did not change significantly. The increase of γ/γ+β m RNA in cord blood cultures was 3.1±1.2 SD fold that of the control values (Figure 4).

Effect of valproic acid and TSA in combination with hemin

In these experiments the effect of the above agents on γ-chain synthesis was determined as amount of HbF. We examined two samples of erythroid precursors from normal bone marrow and two samples from normal peripheral blood. Addition of valproic acid alone resulted in a 2.9 fold

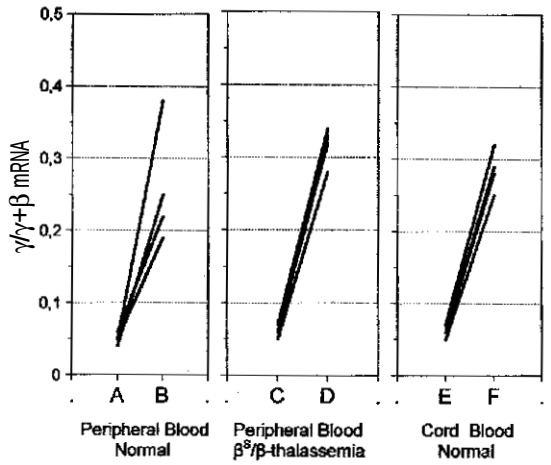

Figure 3. Influence of valproic acid (0.5mM) on γ/γ+β mRNA expression in erythroid cultures from peripheral blood, cord blood and β^S/β^{thal} blood samples. The values of four samples from each category are depicted.

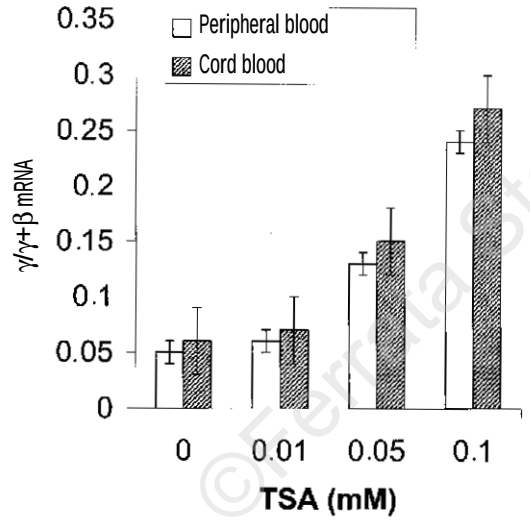

Figure 4. Effect of TSA on γ-chain synthesis expressed as increase of γ/γ+β mRNA in cultures from peripheral and cord blood.

increase of HbF in the bone marrow cultures and a 2.2 fold increase in the cultures of peripheral blood. When hemin was added at the initiation of phase II, the increase was 6.9 and 10.0 fold, respectively. Addition of TSA alone resulted in a 2.7 fold increase of HbF in the bone marrow cultures and a 2.6 fold increase in the cultures of peripheral blood. When

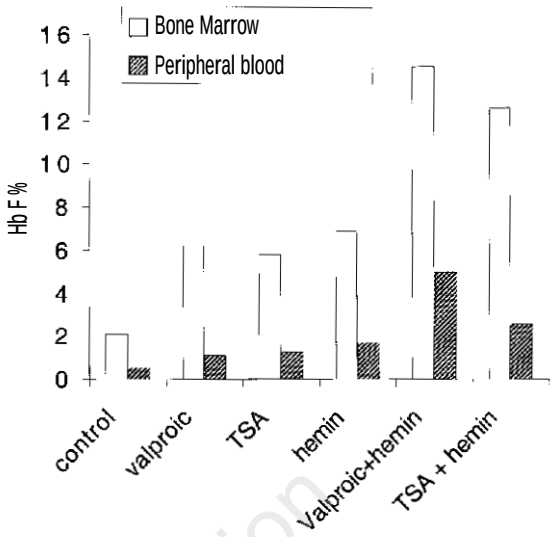

Figure 5. Effect of valproic acid and TSA alone or in combination with hemin in peripheral and bone marrow samples expressed as amount of HbF (%).

hemin was added, the expression of HbF was greatly enhanced,i.e. 6.0 and 5.2 fold, respectively. The amount of Hb A remained unchanged in all experiments (Figure 5).

Discussion

Human adult erythroid cultures provide a model system for the investigation of agents that may affect globin gene expression. This model is considered to be superior to other similar systems such as erythroleukemia cell lines and semisolid cultures because it allows terminal differentiation of the erythroid cells. Furthermore, the liquid hAEC allow the effect of various drugs, added at varying stages of erythroid maturation, to be tested.[14] hAEC have already been used for the assessment of several putative inducers of HbF (hemin, hydroxyurea, acetate) and for the investigation of the mechanism of their action.[15-17] As a rule results are expressed as increase of the γ over γ+β-mRNA ratio. Using this approach we studied the effect of valproic acid and trichostatin, alone or in combination with hemin, on γ gene expression in cultures derived from normal bone marrow, normal and β^S/β^{thal} peripheral blood and cord blood samples. Results showed that the above agents promote γ-chain synthesis in all cell types. Valproic acid and TSA, drugs that have already been approved for human use, can be useful for the

treatment of hemoglobinopathies. Their effects were potentiated when these agents were combined with another HbF inducer, hemin, which is also FDA-approved for the treatment of intermittent porphyria. We, therefore, suggest that the combination of two agents that act on different stages of erythroid maturation may have a synergistic effect, while, at the same time, obviating the cytotoxicity of single drugs administered in high doses. In fact, valproic acid and TSA are considered to act by promoting transcription and inhibiting histone deacetylase, while hemin acts by stimulating erythroid progenitor cell growth and affecting globin synthesis at transcriptional and post-transcriptional levels.[18] Valproic acid has already been used in clinical trials. Adults with sickle cell disease who were treated with valproic acid showed a three-fold increase of their HbF, although there was no evidence that the frequency of their vaso-occlusive crises decreased.[19]

In conclusion, our data show that the combination of valproic acid or trichostatin with hemin may significantly increase HbF synthesis in erythroid cell cultures. This observation calls for further exploration, because *in vitro* results cannot always predict *in vivo* response with regards to induction of HbF, while the concentrations used in the culture systems may not be attained *in vivo* due to toxic effects of the drugs. Clinical studies with TSA have not yet been reported; however, the *in vitro* effectiveness shown in the present work warrants further exploration.

Contributions and Acknowledgments

MP and DL planned the project. MP and SA carried out the experiments. PK supervised the execution. YP and AS carried out the HPLC. MP collected the data and prepared the draft. DL revised the draft critically and was responsible for the final editing.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Vittorio Rosti, who acted as an Associate Editor. The final decision to accept this paper was taken jointly by Dr. Rosti and the Editors. Manuscript received November 23, 2000; accepted May 24, 2001.

Potential implications for clinical practice

Hemin, valproic acid and trichostatin (all three FDA-approved drugs) preferentially enhance γ globin gene expression and their combination could be useful for the treatment of thalassemia syndromes.

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