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Induction of gamma-globin gene transcription by hydroxycarbamide in primary erythroid cell cultures from Lepore patients

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Summary

Increased expression of fetal haemoglobin (HbF) may ameliorate the clinical course of beta-thalassemia and sickle cell disease. Some pharmacological agents, such as hydroxycarbamide (HC), can increase fetal haemoglobin synthesis during adult life. Cellular selection and/or molecular mechanisms have been proposed to account for this increase. To explore the mechanism of action of HC we focused on homozygous Hb-Lepore patients that presented with high fetal haemoglobin levels and were good responders to HC treatment "*in vivo*". We performed primary erythroid cultures from peripheral blood of four homozygous Lepore patients. The increase in *HBG* (γ-globin) transcription levels and HbF content in these cultures, after HC treatment, were detected by quantitative real time polymerase chain reaction analysis and flow cytometric analysis. Primary transcript "*in-situ*" hybridization analysis showed a 2-fold increase in the number of cells expressing both *HBG* alleles in HC-treated erythroid cultures. These studies, demonstrating the larger number of biallelic *HBG* expressing cells, suggest that HC is able to stimulate the activation of *HBG* transcription. These observations provide evidences that the molecular mechanism of action is involved in the increase of fetal haemoglobin production by HC.

Keywords: hydroxycarbamide, fetal haemoglobin, primary erythroid cultures, RNA-fluorescence "*in situ*" hybridization, Lepore patients.

High levels of fetal haemoglobin (HbF) may ameliorate the clinical course of β-thalassemia and sickle cell disease (Olivieri, 1996; Charache *et al*, 1996). Studies "*in vitro*" and in animal models have shown that some pharmacological agents, such as 5-azacytidine, hydroxycarbamide (HC) and butyrate, can increase HbF synthesis during adult life (De Simone *et al*, 1982; Kruh, 1982; Letvin *et al*, 1984). Among the various drugs recently proposed to improve the clinical course in such diseases, HC seems to be the most effective, is genotype dependent and has been tested in clinical trials for patients with haemoglobinopathies (Olivieri, 1996; Bradai *et al*, 2003; Yavarian *et al*, 2004; Dixit *et al*, 2005; Rigano *et al*, 1997; Charache *et al*, 1995; Maier-Redelsperger *et al*, 1998; Steinberg *et al*, 1997). Hydroxycarbamide, a ribonucleotide-reductase inhibitor, is a cytostatic agent that has been used to treat myeloproliferative disorders (Streiff *et al*, 2002).

Different mechanisms have been proposed to account for the induction of HbF by HC. Because HC kills late erythroid progenitor cells and triggers rapid erythroid regeneration, it has been assumed to promote HbF production by perturbing the kinetics of erythropoiesis (Veith *et al*, 1985). Accelerated erythropoiesis increases the chance of premature lineage commitment and the induction of F-cell formation (termed "cellular mechanism") (Blau *et al*, 1993; Stamatoyannopoulos, 2005). The finding that the highest level of HbF is achieved with myelosuppressive doses of this drug is compatible with this mechanism (Charache *et al*, 1996; Steinberg *et al*, 1997; Charache, 1997).

An alternative mode of action could involve the activation of the fetal gene transcription that results in the increase of HbF level in all or the majority of the cell population (termed "molecular mechanism").

More recently, experimental findings supporting the molecular mechanism theory have emerged.

It has been reported that up-regulation of GATA2 and down regulation of GATA1, trans-acting factors important for

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controlling globin gene expression, could be involved in the induction of HbF by HC (Wang *et al*, 2002; Ikonomi *et al*, 2000). Moreover, it has been recently shown that increases in *HBG* m-RNA and protein production strongly correlate with NO-radical donation by HC acting through the sGC/cGMP pathway: activation of sGC raises the production of cGMP leading to increased fetal haemoglobin synthesis (Cokic *et al*, 2003). There are several transcription factors regulated by this biochemical pathway, such as AP1 (Moi & Kan, 1990; Safaya *et al*, 1994; Haby *et al*, 1994; Idriss *et al*, 1999) and SP1 (Fischer *et al*, 1993; Wang *et al*, 1999) that might affect *HBG* expression.

To explore the mechanism of action of HbF-inducing HC at the cellular and molecular levels, we used the two phase liquid erythroid culture "*in vitro*" system from homozygous Lepore patients. Homozygous Lepore patients have elevated production of fetal haemoglobin and very low expression of the hybrid *HBD-HBB* (Lepore gene). Lepore patients, both homozygous and compound heterozygous for beta-thalassemia, are reported to be high responders to "*in vivo*" HC treatment (Rigano *et al*, 1997; Olivieri *et al*, 1997). To mimic the haematological changes that are observed in patients "*in vivo*", we performed various molecular analysis of erythroid cells after exposure of cultures to HC treatment.

Patients and methods

Buffy coat cells from a normal individual were obtained from a local blood bank. After informed consent was obtained, 20 ml of peripheral blood were withdrawn from four homozygous Lepore patients (Lepore-Boston) (Baird *et al*, 1981). One of these patients was under "*in vivo*" HC treatment at 20 mg/kg per day. The haematological profiles of all patients are listed in Table I. The percentages of Hb Lepore and HbF were determined using Variant V-II (USA Bio-Rad Laboratories, Hercules, CA, USA).

Two phase liquid primary erythroid cultures

Primary cell cultures were performed as previously described (Di Marzo *et al*, 2005).

Briefly, mononuclear cells from peripheral blood were isolated by centrifugation over Ficoll-Hypaque (1·077 g/ml; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 1200 ***g*** for 15 min. The nucleated cells were first cultured in alpha-minimal essential medium supplemented with 1 μg/ml cyclosporine A (Novartis Basilea, Switzerland), 10% fetal calf serum (FCS, Invitrogen, Carlsbad, CA, USA) and 10% conditioned medium collected from cultures of the human bladder carcinoma 5637 cell line as described elsewhere (Quentmeier *et al*, 1997). After 6 d of incubation in phase I culture, non-adherent cells were harvested, washed, and resuspended in phase II medium composed of alpha-medium, 30% FCS, 0·1% deionized bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 10 mM 2-mercapto-ethanol (Sigma), 1·5 mM glutamine (Euroclone, Life Sciences Division, Pero, MI, Italy), 1 mM dexamethasone (Laboratorio Farmacologico Milanese, MI, Italy), 1 U/ml recombinant erythropoietin (Janssen-Cilag, Leiden, The Netherlands), 10 ng/ml Stem Cell Factor (Sigma), and 0·3 mg/ml human holo-transferrin (Sigma). Cells were harvested at day 10 of phase II culture.

Table I. Haematology profiles of patients and normal individual.

Patient no.	Total Hb (g/l)	% Hb Lepore	% HbF	HbF (g/l)
1	91	21·2	76·8	70
2	89	18·1	80·1	71
3	87	20·8	77	67
4 (before HC)	84	20	78·1	66
4 (during HC)	107	18·7	80·3	86
Normal control	145	86·7 (% HbA)	0·4	0·6

HC treatment

At day 6 of phase II, cells were washed with alpha-medium and the culture was split. One half of the culture was exposed to 100 μM HC (Teofarma Srl, Pavia, Italy) and the other half was grown without the drug. At day 10 the cells were harvested and analyzed.

Benzidine staining

Staining with a mixture of benzidine di-hydrochloride (Sigma) and hydrogen peroxide was performed as described elsewhere (Fibach, 1998). Briefly, after staining with benzidine, blue cells were scored in a haemocytometer.

Flow cytometric analysis

The phase II cultured cells were monitored for erythroid differentiation by measuring transferrin receptor (CD71) and glycophorin A antigen (GPA). Cells were washed twice in phosphate-buffered saline (PBS) 1× and stained with phycoerythrin (PE)-conjugated anti-human CD71 antibody (Immunotech, Marseille Cedex 9, France) and with fluorescein isothiocyanate (FITC)-conjugated anti-human GPA antibody (Immunotech).

For F-cell detection, 5×10^5 cells were washed twice with PBS 1×, fixed and permeabilised with FIX & PERM (Caltag, Burlingame, CA, USA), according to the manufacturer's instructions, and labelled using a PE-conjugated anti-human HbF antibody (Caltag). Cells were analyzed using the Cytomics FC 500 (Beckman Coulter Inc., Fullerton, CA, USA).

Real-time quantitative polymerase chain reaction

RNA was isolated with TRIzol® reagent (Invitrogen) according to manufacturer's instructions. 200 U Moloney murine leukemia virus (M-MLV; Invitrogen) was used to synthesize cDNA

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from 1 μg of total RNA after priming with random examers (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) assay of transcripts was carried out with the use of gene-specific double fluorescently labelled probes in a 7300 Sequence Detector (Applied Biosystems, Norwalk, CT, USA). All samples were assayed using TaqMan Universal PCR Master Mix (Applied Biosystems Foster City, CA, USA). The following primer and probe sequences were used:

HBB forward primer, 5′-CTCATGGCAAGAAAGTGCTCG-3′;

HBB reverse primer, 5′-AATTCTTTGCCAAAGTGATGGG-3′;

HBB probe 5′-FAM-CGTGGATCCTGAGAACTTCAGGC-TCCT-TAMRA-3′;

HBG forward primer, 5′-GGCAACCTGTCCTCTGCCTC-3′;

HBG reverse primer, 5′-GAAATGGATTGCCAAAACGG-3′;

HBG probe, 5′-FAM-CAAGCTCCTGGGAAATGTGCTGG-TG-TAMRA-3′ (Smith *et al*, 2000). As an endogenous control, the human glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) (Pre-Developed Taqman assay control kit, Applied Biosystem) was used. The comparative Ct (threshold cycle) method was used to determine the difference (ΔCt) between the Ct of treated samples and the Ct of the untreated samples. Before subtraction the Ct was normalized by the Ct of *GAPDH*. The experiments were performed in triplicate to ensure the reproducibility of the results.

Slide preparation and primary transcript "in situ" hybridization

Cells were resuspended in PBS at a density of 10^5 to 10^6 cells/ml. Single cell suspension (15 μl) was settled as a spot in a poly-L-lysine coated slide (Sigma). Cells were fixed in 4% formaldehyde/5% acetic acid for 18 min at room temperature. The cells were subsequently washed in PBS three times for 5 min and stored in 70% ethanol at −20°C.

Primary transcript "*in situ*" hybridization was performed as previously described (Di Marzo *et al*, 2005). The slides were pretreated for hybridization with a 0·01% pepsin digestion for 5 min at 37°C in 0·01 M HCl, followed by a short wash in water and a 5-min fixation in 3·7% formaldehyde at room temperature. The slides were washed in PBS, dehydrated in 70%, 90% and 100% ethanol steps and then air-dried. Hybridization mixture (40 μl) was applied per 24 × 60 mm coverslip and incubated overnight at 37°C in a chamber humidified with 25% formamide, 2× saline sodium citrate (SSC). The hybridization mixture contained 1 ng/μl of each oligonucleotide probe in 50% formamide, 2× SSC, 200 μg/ml salmon sperm DNA, 5× Denhardt's, 50 mM sodium phosphate pH 7 and 1 mM ethylenediamine-tetraacetic acid (EDTA). The coverslip was removed by dipping in 2× SSC and the cells were washed three times for 10 min in 2× SSC at 37°C, followed by a 5-min wash in 0·1 M Tris pH 7·5, 0·15 M NaCl, and 0·05% Tween-20 at room temperature. Antibody detection of the label was essentially as previously described (Dirks *et al*, 1993), with three amplification steps. Mounting was in 4′-6-Diamidino-2-phenylindole (DAPI)/1,4-diazobicyclo[2.2.2]octane (DABCO):Vectashield (1:1) in glycerol (90%) and stored at 4°C in the dark. Fluorescence was detected by epifluorescence microscopy (Zeiss, Germany). A minimum of 200 cells were counted per data point. A mixture of four probes (oligonucleotides 30 bases long) was used for specific hybridization of each gene. Each oligonucleotide was triple-labelled with digoxygenine (HBG-IVS II-specific probe) or dinitrophenol (HBB-IVS II specific probe) (Di Marzo *et al*, 2005). *HBG*-specific probes were homologous to both HBG2 and HBG1 IVS II. FITC fluorochrome was used for *HBG* transcription detection (green signal), while Texas Red fluorochrome was used for *HBB* transcription detection (red signal). A yellow signal indicated simultaneous *HBG* and *HBB* transcription on the same allele.

Results

Peripheral blood mononuclear cells from four homozygote Lepore patients were cultured according to the two-phase liquid erythroid culture protocol. For each patient two different erythroid cultures were analyzed.

On day 6 of phase II, cells were exposed to 100 μM HC, which corresponded to the "*in vivo*" serum concentration after administration of 20 mg/kg per day. The phase II cultures were monitored for erythroid differentiation by measuring transferrin receptor (CD 71) and glycophorin A antigen (GPA) by flow cytometric analysis. Similar expression profiles were found in treated and untreated erythroid cells (data not shown) and more than 90% of cells were positive for both markers at the end of the cultures (day 10).

The cytostatic effect of HC was evaluated by the decrease in the number of the benzidine-positive cells (Hb-containing cells). After 4 d of treatment, HC-treated cells had approximately half the number of cells as the non-HC-treated cells.

Effects of HC on HbF production and HBG mRNA levels

The effect of HC on the production of HbF was assessed by determination of the F-cell percentages in treated and untreated erythroid cultures at day 10. Flow cytometric analysis using PE-conjugated anti-human HbF antibodies was performed in fixed and permeabilized cells.

The results demonstrated that HC treatment increased the F-cell percentage from 61·9 ± 3·2% to 74·9 ± 2·8% (Table II). The F cell percentages measured were slightly lower than expected for Lepore patients and this could be due to the heterogeneous distribution of fetal haemoglobin (Efremov, 1978), to the sensitivity of the detecting method and also to the presence of non-erythroid cells in the two-phase cultures (even though this was low).

The HbF content/cell was inferred from the fluorescence intensity of cells as determined by MFC (mean fluorescence channel). Although a direct quantitative relationship between MFC and HbF content was not available, the MFC provided a good semi-quantitative estimation of HbF content. The

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Table II. Percentages of F cells in HC-untreated and -treated cells.

	Untreated cells		Treated cells	
Patient no.	% F cells	MFC*	% F cells	MFC*
1	65	9·2	71·3	12·7
2	57·5	13·1	77·5	16·6
3	62	12·2	74	19·8
4	63·3	10·2	76·7	15·4
Average	61·9 ± 3·2	11·2 ± 1·8	74·9 ± 2·8	16·1 ± 2·9

*MFC, mean fluorescence channel.

analysis revealed a MFC increase from 11·2 ± 1·8 to 16·1 ± 2·9 (Table II).

The effect of HC on *HBG* mRNA level was studied by relative quantification using a fluorescence-based real time PCR assay, normalizing for *GAPDH* expression. The results revealed a 1·97 ± 0·26-fold increase of *HBG*-mRNA, following HC treatment. As expected, we found a very low level of *HBD-HBB* mRNA that was unchanged by HC (data not shown).

Effect of HC on transcription of HBG alleles

To explore the mechanism of action of HC that is responsible of *HBG* mRNA and fetal haemoglobin increases, erythroid cultures were analyzed by primary transcript fluorescence "*in situ*" hybridization (RNA FISH). This technique can detect primary transcripts in single cell nuclei and visualize each allele separately. RNA FISH was performed on treated and untreated cultures from patients, starting from day 6 to day 10 of phase II, using *HBG* and *HBB* intron-specific probes and a minimum of 200 cells was counted for each time point. Cells of a normal individual were used as hybridization control. In the normal individual, the number of *HBB* alleles transcribed increased progressively while the number of *HBG* alleles reciprocally decreased (Fig 1). Similar data at day 10 have already been reported (Di Marzo *et al*, 2005; Chakalova *et al*, 2005). Under our hybridization conditions, in untreated cultures from all Lepore patients, the analysis revealed a peculiar pattern of *HBG* expression in that the majority of the cells expressed one *HBG* allele only (γ:) in both early (day 6) and late erythroid precursors (day 10). A representative picture of RNA FISH analysis performed at day 10 is shown in Fig 2. In particular, 78 ± 2·3%, 76·2 ± 3·8%, 76·5 ± 2·5%, 76 ± 0·5% and 74·3 ± 2·5% of cells expressed one *HBG* allele and 20 ± 4%, 19·4 ± 3·5%, 20·5 ± 3%, 18·8 ± 4·5% and 21·5 ± 4·6% of cells expressed both *HBG* alleles (γ:γ) on days 6, 7, 8, 9 and 10 of culture, respectively. In order to exclude the possibility that *HPFH* point mutations could account for the *HBG* mono-allelic expression observed in all patients, about 700 bp of both *HBG*1 and *HBG*2 promoters were sequenced and no point

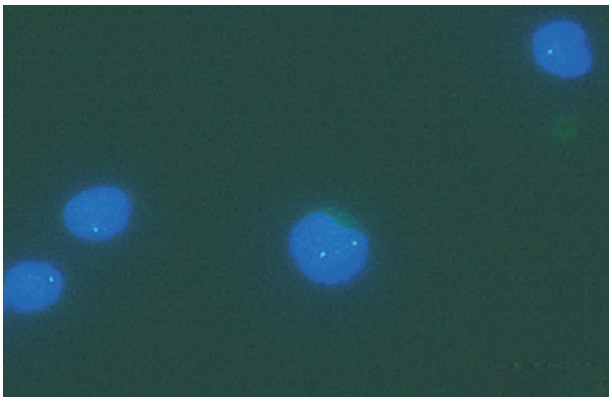

Fig 2. Typical pattern of primary transcript *in situ* hybridization performed on homozygous Lepore erythroid cultures at day 10 using *HBG*-(green) and *HBB*-(red) intron-specific probes.

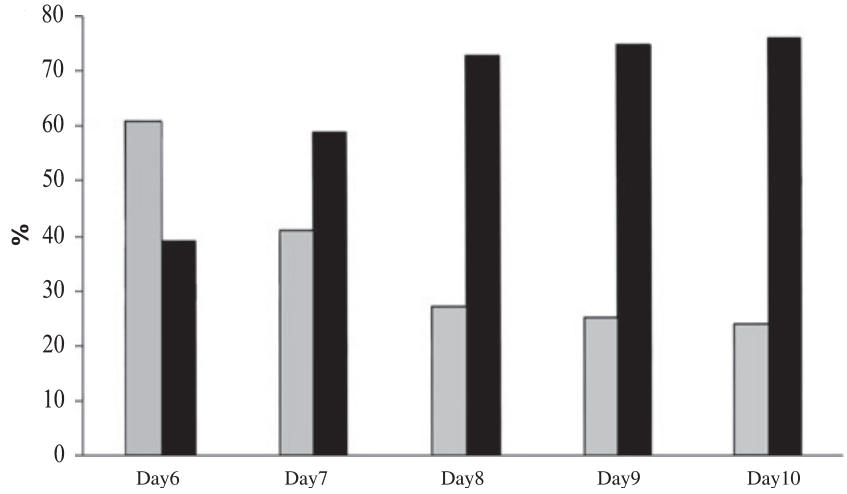

Fig 1. The percentages of total *HBG* and *HBB* signals at day 6, 7, 8, 9 and 10 of phase II of erythroid cultures in normal individual. Grey columns, γ transcription; black columns, β transcription.

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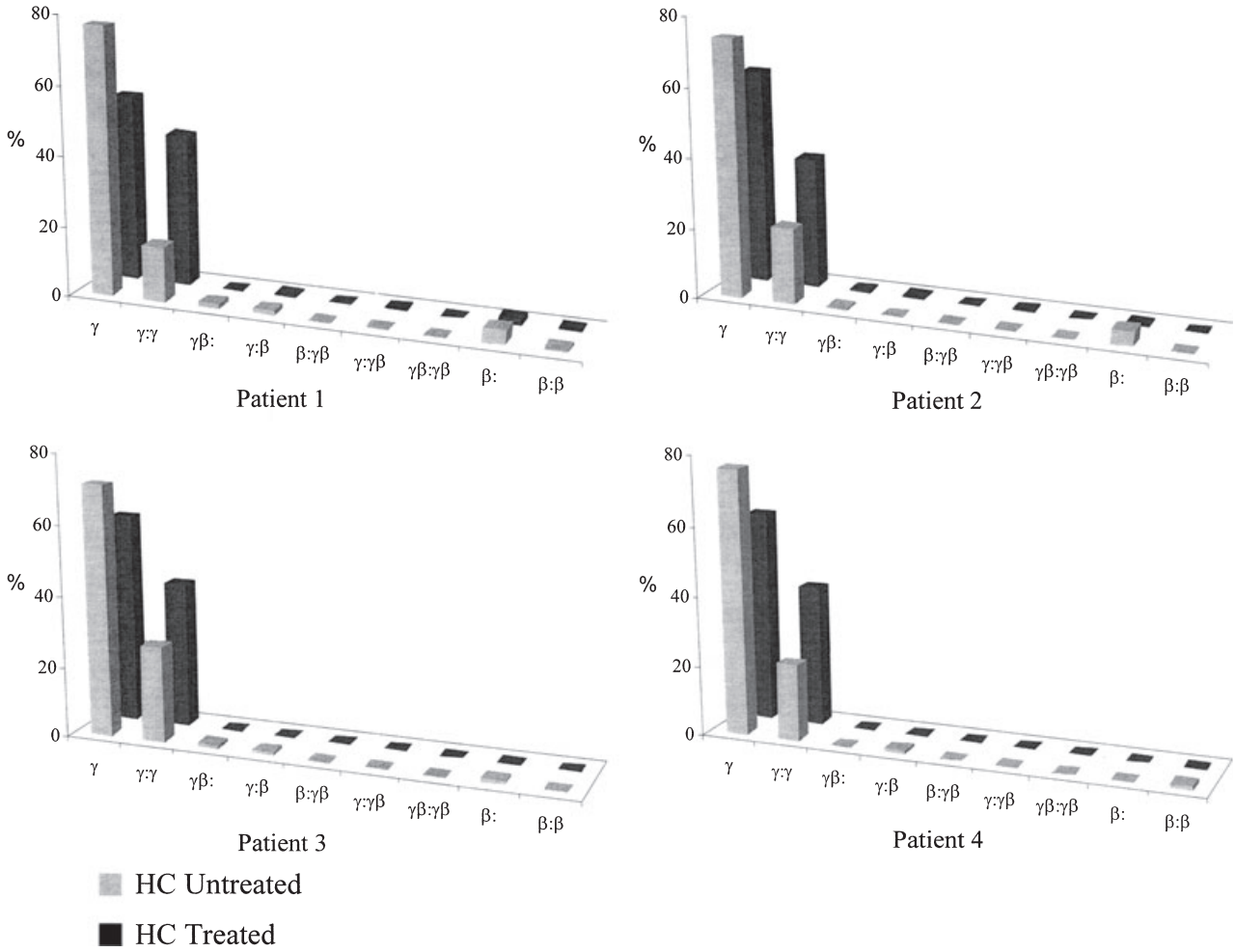

Fig 3. The percentage distribution of different transcriptional cell types by RNA FISH at day 10 in phase II of untreated (grey) and treated (black) primary erythroid cell cultures from four homozygous Lepore patients. Combinations of *HBG* and *HBB* allele expression in a single cell: γ:, expression of one *HBG* allele; γ:γ, expression of two *HBG* alleles; γβ:, expression of one *HBG* allele and one *HBB* allele on the same chromosome; γ:β, expression of one *HBG* allele and one *HBB* allele on different chromosomes; β:γβ, expression of one *HBG* allele and two *HBB* alleles; γ:γβ, expression of two *HBG* alleles and one *HBB* allele; γβ:γβ, expression of two *HBG* alleles and two *HBB* alleles; β:, expression of one *HBB* allele; β:β, expression of two *HBB* alleles.

mutations were found. This monoallelic expression made these cells a suitable model for the analysis of *HBG* expression in the presence of HC.

RNA fluorescence "*in situ*" hybridization analysis performed in HC-treated cultures showed a different pattern of expression in all patients during cell differentiation. In particular, if we consider day 10 of culture, the results showed a clear increase of cells expressing both *HBG* alleles from 21·5 ± 4·6% to 40·3 ± 2·6% and the relative decrease of cells expressing one *HBG* allele from 74·3 ± 2·5% to 58·3 ± 3·4% in the presence of the drug. The percentages of γ: (one allele) and γ:γ (two alleles) cell distribution for each patient are reported in Fig 3. Similar data showing the increase of percentage of γ:γ cells in respect to γ: cells were obtained also at the other time points of the culture; a representative sample is shown in Table III. As expected, a small percentage of cells showed β signals due to

Table III. Percentages of cells with different combinations of *HBG* and *HBB* allele expression in Lepore Patient 2.

	Day 6	Day 7		Day 8		Day 9		Day 10	
	U	U	T	U	T	U	T	U	T
γ:	82·2	80	67	79	61·5	75·5	49·6	73·6	61
γ:γ	14	14·8	32·3	16·1	37	22	43	21·4	37
γβ:	0·8	0·8	–	–	–	0·6	1·5	0·6	0·5
γ:β	1·3	0·8	–	–	–	0·6	2·3	–	0·5
β:γβ	–	–	–	–	–	–	–	–	–
γ:γβ	–	–	–	–	–	–	2·9	–	0·5
β:γβ	–	–	–	–	–	0·6	–	–	–
β:	1·7	3·6	0·7	4·9	0·8	0·7	0·7	4·4	0·5
β:β	–	–	–	–	0·7	–	–	–	–

U, HC-untreated culture; T, HC-treated culture.

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the very low expression level of *HBD-HBB*. Our findings demonstrated that, on average, HC treatment caused a 2-fold increase of cells expressing both *HBG* alleles.

Discussion

Hydroxycarbamide (HC) is an approved therapeutic agent for treatment of β-thalassemia and sickle-cell disease (Rodgers *et al*, 1990; Charache *et al*, 1995; Rodgers & Rachmilewitz, 1995; Olivieri *et al*, 1998). Despite being used to treat a number of haematological malignancies for nearly 30 years, the mechanism of action of HC in increasing HbF levels remains unclear. Cytotoxicity resulting from HC therapy has been correlated with increased production of F-cells: it may involve a selection of a minor pre-existing subpopulation of F-cells that has a growth and/or survival advantage (Dover *et al*, 1986). It has been shown however that cytotoxicity alone is not sufficient for HbF induction (Ho *et al*, 2003). Moreover, recent data suggest that both *HBG* and *HBB* synthesis are regulated with proliferation during erythropoiesis with no evidence for globin chain switching (Wojda *et al*, 2002). This evidence suggests that other mechanisms may also be involved in the HbF increase by HC.

An alternative mechanism could involve stimulation of HbF in all or the majority of the population by direct induction of *HBG* gene transcription.

Our findings confirmed the increase of *HBG* mRNA and HbF in primary erythroid cell cultures of Lepore patients in agreement with results reported by others (Amoyal *et al*, 2003; Watanapokasin *et al*, 2005; Dixit *et al*, 2005). The increased percentage of F-cells, the HbF content/cell and the *HBG* mRNA level by HC treatment fails to suggest the mechanism of action; in fact both survival advantages of pre-existing F cell subset or activation of *HBG* genes would cause an elevation of these parameters.

The peculiar pattern of *HBG* expression, that is, only one *HBG*-allele expressed in the majority of cells in both early and late erythroid precursors from all Lepore patients, provided the opportunity to investigate whether the activation of fetal genes by HC is involved in HbF increase. RNA FISH analysis in the presence of the drug at day 10 showed an increase of cells expressing both *HBG* alleles. Similar data were obtained at the other time points of culture. These results demonstrate that more *HBG* alleles are expressed in HC-treated erythroid cells. HC had a cytostatic effect in our culture conditions, as inferred from the decreased cell counts at day 10 in treated cultures, and this could be evidence of a selective process on early precursors. Here we report that a monoallelic pattern of *HBG* expression was represented during differentiation in erythroid cultures and was more evident in early precursors (82·2% of γ: cells at day 6) than in late precursors (73·6% of γ: cells at day 10). If the increase of transcribing *HBG* alleles in the presence of HC were due only to cellular selection, one would expect, in treated cells, the same monoallelic pattern of *HBG* expression displayed by early precursors at day 6. The increase of cells expressing both *HBG* alleles, even within only 24 h of treatment, demonstrates the activation of *HBG* transcription, indicating that the molecular mechanism is also involved in HC action.

The RNA FISH procedure, which visualized allele transcription in single nuclei, enabled the demonstration of the increased number of active *HBG* alleles in HC-treated cells.

This study represents the first evidence of biallelic *HBG* activation by HC, confirming that the molecular mechanism is involved in the mode of action of this drug.

Better understanding of HC action provides a basis for further research into new drugs for HbF stimulation that do not have the cytotoxic effect of HC and therefore would provide safer and possibly more effective therapies for sickle cell disease and β-thalassemia.

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