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In vitro maturation of erythroid progenitors from human umbilical cord blood and patterns of globin gene expression: Serum from different developmental stage plays important roles in liquid culture

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Abstract

To investigate the maturation of neonatal erythrocytes and kinetics of globin expression, we induced $CD34^+$ cells purified from human umbilical cord blood to erythroid differentiation in different suspension culture systems containing human cord serum (CHS) and adult serum (AHS), respectively. The ζ- to α-globin switching and the ε- to γ-globin switching were observed in CHS^+ cultures but not in AHS^+ cultures. A reduced proportion of F cell and two day postponed cell enucleation in AHS^+ cultures compared with in CHS^+ cultures was also found. However, predominant γ-globin expression compared with ε- and β-globin was always observed from days 5 in either CHS^+ or AHS^+ cultures. Our data showed that neonatal erythrocytes in CHS^+ cultures maintained their fetal characteristics which differed from their counterpart from adult. Serum from different developmental stage significantly affected the maturation and globin expression pattern of cultured neonatal erythrocytes. Obviously, some uncertain serum components play important roles in cultures, but they are not enough to fundamentally change the developmentally specific globin gene transcription program, suggesting that this program is mainly controlled by determinants within the erythroid progenitors.

Keywords: Erythroid progenitor; Cord blood; Liquid culture; Adult human serum; Human cord serum; Real-time PCR; Globins

As a model of gene regulation, erythroid cell maturation, known as erythropoiesis, has been extensively studied. During human ontogeny, the expression of α-like globin gene displays an embryonic (ζ-) to adult (α-) globin switch, whereas the β-like globin gene expression displays two major switches: the embryonic (ε-) to fetal globin (γ-) switch coinciding with the transition from embryonic (yolk sac) to definitive (fetal liver) hematopoiesis, and the fetal to adult (β-) globin switch, occurring near the perinatal period concomitantly with the establishment of the bone marrow as the main site of hematopoiesis [1]. The second switching did not complete until 6–12 months after birth [2,3]. The fetal hemoglobin (Hb F) comprises 60–80% of total hemoglobin in erythrocytes of umbilical cord blood (UCB) of newborn, whereas only 0.5–1% of total hemoglobin in human adult erythrocytes [4].

The erythropoiesis and globin gene expression may be controlled by both genetic and acquired conditions. Fetal hemoglobin (Hb F) reactivation in adult is observed under various pathological and physiological conditions, especially the stress erythropoiesis. Some chemical reagents and cytokines were also found to reactivate the γ-globin gene expression in adult erythroid cells. Most important, the increases in Hb F levels ameliorate the clinical symptoms in both sickle cell disease and β-thalassemia [5]. So the understanding of the

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mechanisms of hemoglobin switching is not only of biological significance but also relevant to development of rational design of therapies for a variety of hemoglobinopathies.

Along with the development of experimental technique, several approaches have been introduced for the investigation of globin gene switching. Especially, the establishment of liquid culture procedure provides us an important tool for studying the regulation of globin gene expression during the erythroid cell development in vitro [6,7]. It has been reported that with the liquid culture system, globin gene expression in the maturing erythrocyte from adult bone marrow or peripheral blood recapitulates expression patterns which occur in the fetal to adult hemoglobin switch during ontogeny [8].

To particularly investigate the in vitro maturation of erythroid progenitors from human umbilical cord blood (UCB) and kinetics of globin gene expression, we purified the $CD34^+$ hematopoietic progenitor cells from human UCB. Then cells were induced to erythroid differentiation in two kinds of liquid culture media containing human cord serum (CHS) and adult human AB-type serum (AHS), respectively. The α-like and β-like globin (including α-, ζ-, β-, γ-, and ε-globin) mRNA in the cultured maturing erythrocytes was directly measured by real-time quantitative PCR and the erythroid maturation was also observed. We found that the patterns of globin gene expression in maturing neonatal erythroid cells cultured in CHS-containing medium reflect the patterns of globin gene expression found in vivo. Data also showed that serum from different human ontogeny stages significantly affected the in vitro maturation and the patterns of globin gene expression of cultured neonatal erythroid cells.

Materials and methods

Purification of $CD34^+$ cells from human umbilical cord blood. After informed consent, four umbilical cord blood (UCB) samples (40–50 ml each) from normal full-term deliveries were collected with EDTA as anti-coagulant. All UCB samples were diluted 1:4 with phosphate-buffered saline (PBS) containing 2 mM EDTA and 0.5% bovine serum albumin (BSA, Sigma, St. Louis, MO) for further use. Light-density mononuclear cells (MNCs) were separated from the diluted UCB by centrifugation on a gradient of Ficoll–Hypaque (density 1.077 g/ml, Sigma, St. Louis, MO) [9]. Then $CD34^+$ hematopoietic progenitor cells were purified using Mini-MACS columns according to the manufacturer's protocol (Miltenyi, Auburn, CA) and the purity of $CD34^+$ cells was determined (>90%).

Serum. Adult human AB-type serum (AHS) was purchased from TBD (TJ, PR China). Human umbilical cord blood (UCB) was collected without anti-coagulant. After centrifugation at 3000 rpm for 20 min, the supernatant serum was collected, then the serum was filtered through 0.2 μm Millipore disc filter and stored at −20 °C for further use.

Cell culture and analysis. Two kinds of liquid culture media were used for induction of $CD34^+$ hematopoietic progenitor cells to erythroid differentiation for 3 weeks. Iscove's modified Dulbecco's medium (IMDM, Gibco, Invitrogen, Carlsbad, CA) containing 1% deionized bovine serum albumin (BSA, fraction V), 1×10^{-5} M dexamethasones, 1×10^{-5} β-mercaptoethanol (β-ME), 2 mM glutamine, 100 μg/ml transferring (all from Sigma, St. Louis, MO), 3 IU/ml Epo (R&D System, Minneapolis, MN), and very low dosage of GM-CSF (0.001 ng/ml) and IL-3 (0.01 U/ml, all from PeproTech, Rocky Hill, NJ) was supplied with 30% adult human AB-type serum and 30% human cord serum (CHS), respectively. $CD34^+$ cells were equally divided into two parts and seeded at 1×10^5 cells/ml in different media at 37 °C in 5% CO_2 humidified atmosphere. Cell samples were collected from cultures on day 1, 3, 5, 7, 9, 11, 13, and 15, respectively, and stored at −80 °C for purification of total RNA used in quantitative real-time PCR. Hemoglobin-containing erythroid cells were identified by benzidine staining [10]. And erythroid cell differentiation was monitored throughout culture until day 18 by morphological analysis of the cells after cytospin centrifugation and May–Grünwald–Giemsa staining.

Quantitative real-time PCR analysis of globin gene expression. Total RNA was extracted from cell samples harvested at different time points using TRIzol reagent (Invitrogen, Carlsbad, CA) and quantitated. Trace of DNA was removed by treatment with 5 U DNase I (Promega, Madison, WI) at 37 °C for 45 min followed by inactivation at 65 °C for 10 min. Then first strand of cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction and gene-specific primers for *GAPDH*, α-, β-, γ-, ε-, and ζ-globin gene were designed using Primer Express (Applied Biosystems, Foster City, CA) software Version 2.0 (Table 1) [11]. To exclude any possible amplification of contaminating genomic DNA, primers were designed to span intron–exon boundaries in mRNA. The expected fragment length lies between 119 and 190 bp. The PCR products were cloned into pMD18-T vector (Takara, Dalian, PR China) and sequenced to verify the cloned amplicons. Then the confirmed recombinant plasmids DNA were purified using silica cartridges (Qiagen, Hilden, Germany) and diluted ranging from $1{:}10^2$ to $1{:}10^7$ used as standards to generate standard curves for quantification. The mRNA expression level of target genes in cultured erythroid cells was quantified by real-time PCR analysis on ABI PRISM 7500 real-time PCR System (Applied Biosystems, Foster City, CA) with the SYBR *Premix Ex Taq* kit (Takara, Dalian, PR China). PCR amplification was performed in a 50 μl volume containing 1× SYBR Green PCR Master mix, 0.2 μM of forward and reverse primers and 2 μl cDNA (1:5 diluted). The following PCR cycle parameters were used: 95 °C 10 s, 45 cycles at 95 °C 5 s, 60 °C 34 s. Each PCR was performed in triplex tubes with *GAPDH* as an endogenous control to standardize the amount of sample RNA. The quantification data were analyzed with the sequence detection system (SDS) software (Applied Biosystems). After PCR, baseline subtraction was performed by the software, the log-linear portion of the fluorescence-versus-cycle plot was extended to determine a fractional cycle number at which a threshold fluorescence was obtained (threshold cycle, C_T) for each analyzed gene and *GAPDH* as the reference. Standard curves were prepared based on accurately determined dilutions of plasmids containing cDNA of globins as a template. For all standard curves, linear correlation coefficients $R^2 \geqslant 0.99$. The comparative C_T method was used for quantification of the target genes relative to GAPDH.

Flow cytometry. Cells harvested on day 18 were washed twice with PBS and re-suspended. 2×10^5 cells were fixed with 80% cold acetone at 4 °C for 15 min. Cells were subsequently washed twice and pre-blocked with 2% BSA for 15 min. Then cells were re-suspended in PBS containing sheep anti-human HbF antibodies (Bethyl Laboratories, Montgomery, TX), incubated for 1 h at 37 °C, and washed three times with PBS. Then the secondary FITC-conjugated mouse anti-sheep IgG (Bethyl Laboratories, Montgomery, TX) was applied in 1:100 dilutions for 30 min at room temperature and washed twice with PBS. At least 10,000 cells were analyzed using an Elite cytometer (Coulter, Miami, FL). Green fluorescence derived from FITC was measured using a

Table 1
Oligonucleotide primer sets for real-time PCR

cDNA target	Primer squence (5′–3′)	Product size (bp)
γ-globin	TGG GTC ATT TCA CAG AGG AGT TG ATG GCA GAG GCA GAG GA	166
β-globin	GTC TAC CCT TGG ACC CAG AGG TTC TGA GCC AGG CCA TCA CTA AAG	131
ε-globin	CAG CTG CAA TCA CTA GCA AGC AGA CGA CAG GTT TCC AAA GC	190
α-globin	GGT CAA CTT CAA GCT CCT AAG C GCT CAC AGA AGC CAG GAA CTT G	116
ζ-globin	TGA GCG AGC TGC ACG CCT AC GTA CTT CTC GGT CAG GAC AGA	173
GAPDH (H)	TCA ACG ACC ACT TTG TCA AGC TCA GCT GGT GGT CCA GGG GTC TTA CT	119

525 nm bandpass filter. Erythrocytes from human UCB were used as positive control.

Results

Comparison of erythroid maturation in different liquid cultures

$CD34^+$ cells from human UCB were induced to erythroid differentiation in vitro for 3 weeks in CHS-containing and AHS-containing medium, respectively. During the culture period, the erythroid maturation was observed after Giemsa staining. We found that erythroid progenitors proliferated and differentiated into erythroid precursors. Then, proerythroblasts, basophilic normoblasts, polychromatic normoblasts, and orthochromatic normoblasts were sequentially identified as predominant population on day 7, 9, 11, and 13, respectively, in both kinds of cultures (Fig. 1A). Hemoglobin-containing erythroid cells at different time points were verified by benzidine staining and scored in a hemocytometer. The results showed that hemoglobin-containing cells were observed on day 5 either in CHS^+ cultures or AHS^+ cultures. The percentage of positive cells in two kinds of cultures increased from 8–10.1% on day 5 up to 91.3–94.7% on day 15 (Fig. 1B). In addition, we also found that CHS was more effective than AHS in stimulating the growth of erythroid cell expansion (data not shown).

Notably, enucleated erythrocytes were observed on day 15–16 in CHS^+ cultures, whereas they were identified on day 17–18 in AHS^+ cultures. That is to say, the cell enucleation in CHS^+ cultures was advanced by about 2 days compared with AHS^+ cultures.

Kinetics of α-like globin gene expression

The transcripts of α-like globin gene in cultured erythroid cells at different time points were measured by quantitative real-time PCR (Fig. 2). In CHS^+ cultures, moderate level of embryonic ζ-globin mRNA was detected at the early phase and the mRNA level rapidly decreased from day 3 and became undetectable after day 5. In contrast, the α-globin mRNA level was very low at the beginning of culture period, steadily increased from day 3, and reached a plateau on day 13 (Fig. 2A). That is, the kinetics of α-like globin mRNA expression in CHS^+ cultures displayed an embryonic (ζ-) to adult (α-) globin switching.

In AHS^+ cultures, the low level of α-globin mRNA persisted for 6–7 days and then a rapid rise of the mRNA level was observed from days 7 to days 11, while the ζ-globin mRNA was always undetectable during the erythroid maturation (Fig. 2B).

Kinetics of β-like globin gene expression

The expression of three β-like globin genes was detected in two kinds of cultures. We found that the γ-globin in CHS^+ and AHS^+ cultures shares a similar pattern of expression (Fig. 3). The γ-globin mRNA levels in CHS^+ and AHS^+ cultures increased from the beginning of culture period and reached their highest level on days 7 and 9, respectively. In CHS^+ cultures, the β-globin mRNA levels remained relatively low until day 9, while a rapid rise in the expression of β-globin was detected between day 9 and day 13. But in AHS^+ cultures, a drastic fluctuation of β-globin mRNA was not observed during the days of culture. An abrupt rise in embryonic ε-globin mRNA was measured between day 1 and day 3 in CHS^+ cultures and then, the ε-globin mRNA levels slowly decreased and remained at a moderately high level. In contrast, the ε-globin expression in AHS^+ cultures stabilized at low level.

On the whole, an embryonic (ε-) to fetal (γ-) globin switching on day 5 and decrease of γ-globin expression from day 7 accompanied by an increase of β-globin expression from day 9 were observed in CHS^+ cultures, whereas these changes were not clearly observed in AHS^+ cultures. Obviously, the expression patterns of globin genes were significantly affected by serum.

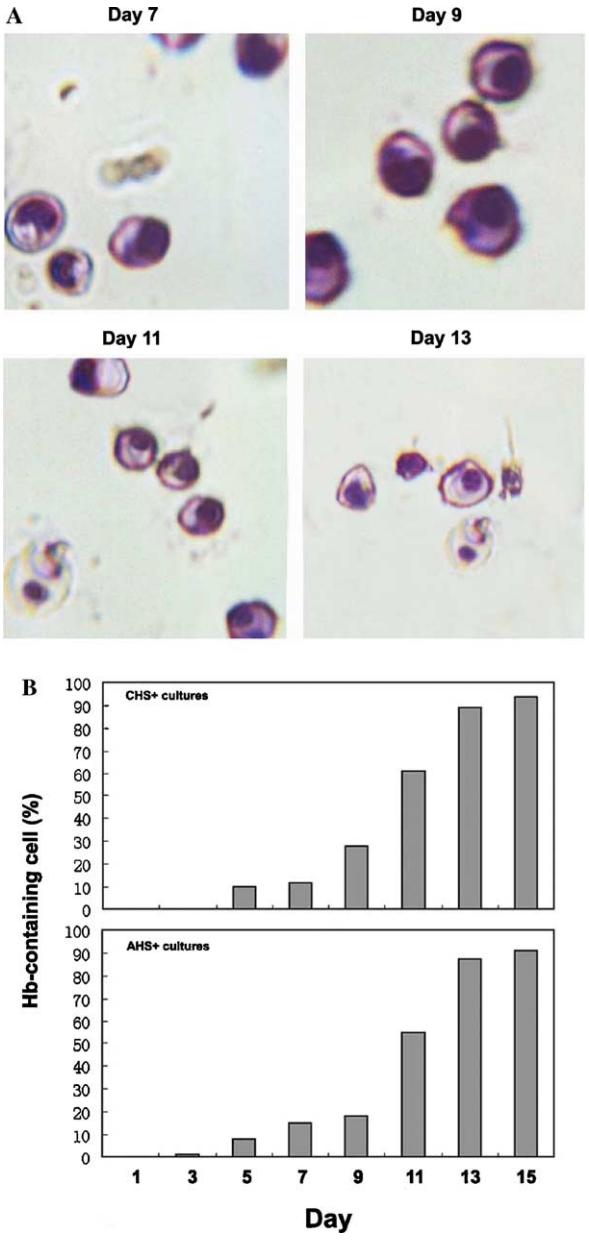

Fig. 1. Erythroid maturation and hemoglobin-containing cells in maturing neonatal erythrocytes at different time points in culture. (A) Giemsa staining of maturing erythroid cells on day 7, 9, 11, and 13, which were sequentially identified as proerythroblasts, basophilic normoblasts, polychromatic normoblasts, and orthochromatic normoblasts. Very similar results were observed in either CHS^+ or AHS^+ cultures. (B) The percentage of benzidine-positive cells in the CHS^+ and AHS^+ cultures. The upper panel shows the percentage of positive cells in CHS^+ cultures and the bottom panel shows that in AHS^+ cultures determined by benzidine staining. Hb-containing cells were observed on day 5 in either CHS^+ or AHS^+ cultures and the percentages of benzidine-positive cells in the two kinds of cultures all peaked on day 15 and the ultimate percentage reached 94.7% in CHS^+ and 91.3% in AHS^+ cultures, respectively.

However, predominant γ-globin expression was observed from day 5 to day 15 in either CHS^+ or AHS^+ cultures.

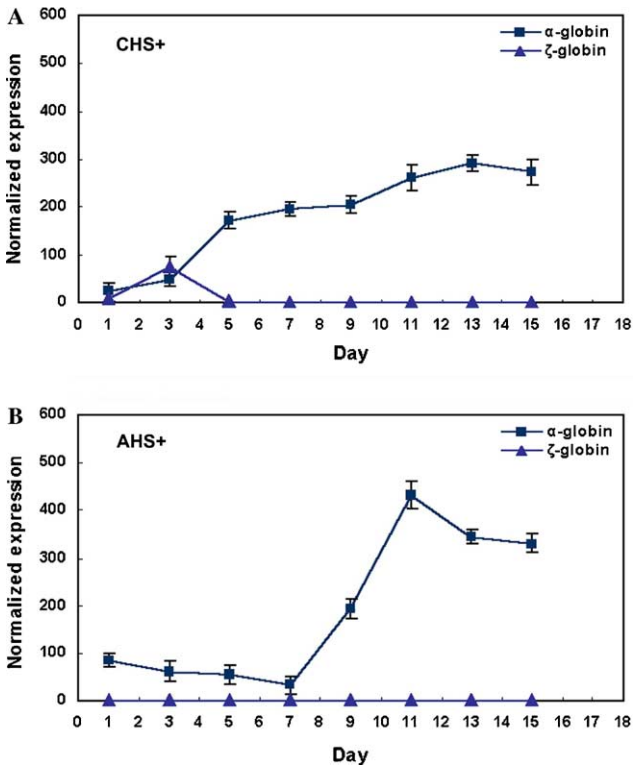

Fig. 2. Kinetics of α-like globin gene expression during the erythroid maturation in CHS^+ cultures (A) and AHS^+ cultures (B). Patterns of α-like globin gene expression were characterized by quantitative real-time PCR. All the expression level of each target PCR product was normalized to that of GAPDH, used as an endogenous control. The ζ-globin mRNA was undetectable in AHS^+ cultures.

Analysis of F cells in terminal erythrocytes by flow cytometry

The cellular distribution of Hb F was analyzed by flow cytometry on day 18 using monoclonal antibodies directed against Hb F and the erythrocytes from umbilical cord blood were used as positive control. The results showed that the proportion of Hb F-containing cells of the matured erythrocytes cultured in CHS-containing medium (89.7%) is similar to that in the UCB control (85.2%). In contrast, the percent of F cells was 57.6% in AHS^+cultures, which is significantly lower than that in CHS^+ cultures and control UCB sample (Fig. 4).

Discussion

As the basis for development of genetic and cellular therapies for hemoglobinopathies, the regulation of globin gene expression has been extensively investigated. However, our understanding of the mechanisms that control hemoglobin switching remains limited upto now. In humans, two globin gene switches occurred during development and there are differences between the patterns of globin gene expression of erythroid cells from various

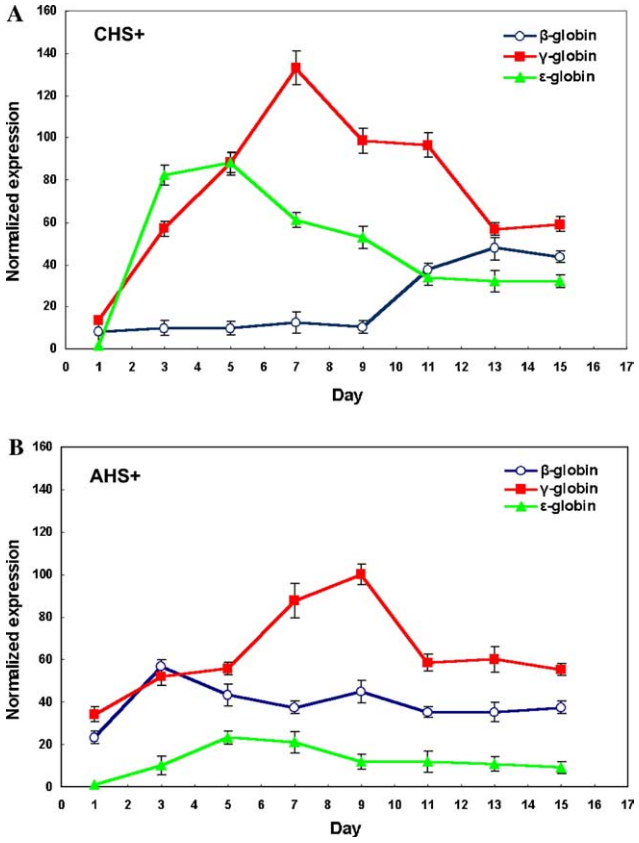

Fig. 3. Kinetics of β-like globin gene expression during the erythroid maturation in culture determined by quantitative real-time PCR. (A) The expressions of three β-like globin genes (including β-, γ-, and ε-globin gene) were detected in CHS^+ cultures differed from that in AHS^+ cultures. (B) The expressions of three β-like globin genes were also detected in AHS^+ cultures.

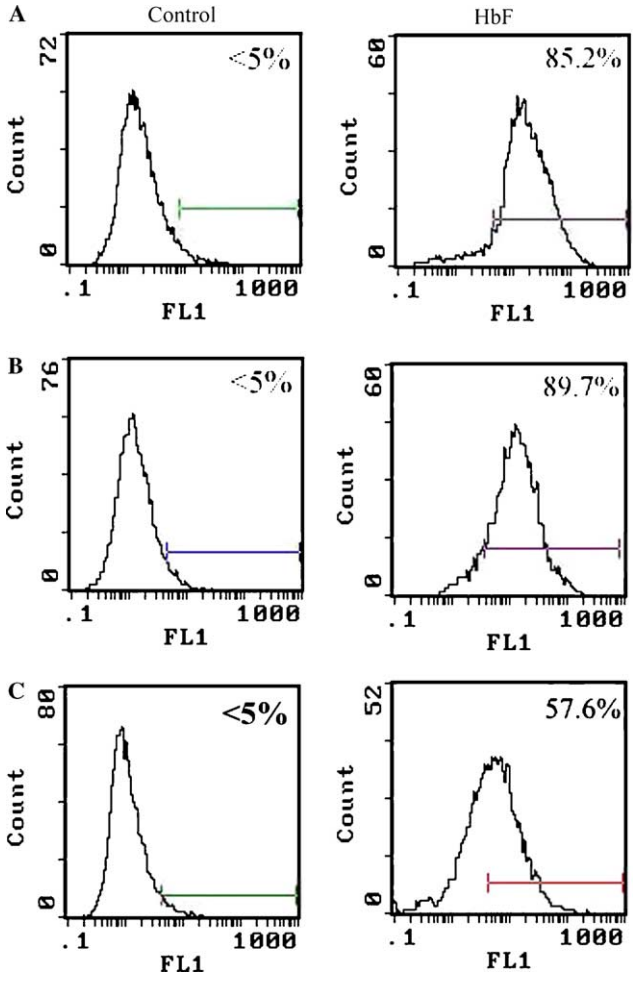

Fig. 4. Flow cytometric analysis of Hb F-containing cultured erythroid cells. The cellular distribution of Hb F was analyzed by flow cytometry on day 18 using monoclonal antibodies directed against Hb F. All the left panels represent the samples only labeled with the second FITC-conjugated mouse anti-sheep IgG and used as background. (A) The erythrocytes from umbilical cord blood were used as positive control. (B) The proportion of F cells in matured erythrocytes cultured in CHS-containing medium. (C) The proportion of F cells in matured erythrocytes cultured in AHS-containing medium. The results shows that the proportion of F cells in matured erythrocytes cultured in CHS-containing is similar to that in the control UCB sample and AHS dramatically decreased the proportion of F cells.

ontogenic stages. It is believed now that erythroid progenitors encode developmental programs of globin gene expression that are characteristic for each developmental stage, suggesting that switching is an intrinsic property of hematopoietic cells. And data also suggested that hemoglobin switching does not represent a change in stem cell population carrying fixed globin gene transcription program, but a change in transcription program taking place in the cells of the same stem cell lineage [12]. However, how these changes of transcription programs happened is still unknown. Differences in the anatomic and ultrastructural characteristics of hematopoietic microenvironment in hematopoietic tissues of different developmental stages have been reported [13–16]. It is supposed that specific components of microenvironment and serum may play roles in the change of the patterns of globin gene expression.

The establishment of erythroid progenitor suspension culture procedure makes it possible for us to obtain large cultures of a relatively pure erythroid cell population and it has been reported that globin gene expression in the maturing adult erythrocyte in two-phase liquid culture system recapitulates fetal-to-adult hemoglobin switching during human ontogeny [8]. However, the number of detectable $CD34^+$ hematopoietic progenitor cells is very low in healthy donors; in fact, the CD34 antigen is expressed on $0.84 \pm 0.83\%$ of human umbilical cord blood mononuclear cells (MNCs) [17]. The presence of nonerythroid cell populations in cultures, mainly T and B lymphocytes, will influence the measurement of globin mRNA level and F cell proportion.

In the present study, $CD34^+$ cells were purified from human UCB and cultured in two kinds of liquid culture systems containing CHS and AHS, respectively. The erythroid maturation and kinetics of globin gene

expression in cultures were particularly observed and measured. With benzidine staining procedure [10], hemoglobin-producing cells were detected and the results indicated that serum from different developmental stages (CHS and AHS) showed in difference on total hemoglobin accumulation. Positive cells were detected on day 5 in either CHS^+ cultures or AHS^+ cultures and the kinetics of hemoglobin-containing cell proportion were almost the same, and the final proportion of Hb-producing cells was also consistent with the previous studies before, ranging from 94.4% to 97.2% [7,8]. Our data obtained with Giemsa staining showed that the CHS advanced the enucleating time by about 2 days compared to AHS, suggesting that some uncertain components of serum affected the terminal differentiation and enucleation of erythroid cultures. However, the components responsible for the serum effects need to be identified.

The quantitative real-time PCR assay provides us a more sensitive and specific assay of DNA templates and a convenient assay of changes in gene expression under various conditions and varying time points [18,19]. Here, the kinetics of globin gene expression in CHS^+ and AHS^+ cultures were directly measured and characterized by quantitative real-time PCR. An embryonic (ζ-) to adult (α-) globin switching was observed in CHS^+ cultures, while ζ-globin mRNA was always undetectable in AHS^+ cultures. Moreover, an embryonic (ε-) to fetal (γ-) globin switching at day 5 and decrease of γ-globin expression from day 7 accompanied by an increase of β-globin on day 9 were observed in CHS^+ cultures, whereas these changes were not in AHS^+ cultures. These results suggested that sera from differential development stages have important effects on the patterns of individual globin gene expression. However, predominant γ-globin expression compared with ε-and γ-globin was always observed from days 5 in either CHS^+ or AHS^+ cultures.

The cellular distribution of Hb F in cultured erythroid cells was analyzed by flow cytometry after immunofluorescence staining. In contrast to the high and similar F cell percentage in CHS^+ cultures (89.7%) and control UCB samples (85.2%), the proportion of F cell in AHS^+ cultures was significantly low (57.6%), suggesting that serum from human adult significantly decreased the Hb F-containing cells. Nonetheless, compared to the cultured human adult erythroid cells (approximately 3–7% of erythrocytes are F cells) [3,20,21], the F cell percentages of cultured neonatal erythroid cells in our study were all very high.

Taken together, our data showed the patterns of globin gene expression and maturation in erythroid cells from UCB cultured in CHS-containing medium basically mimicked those in vivo. The sera from various developmental stages have differential effects on patterns of globin gene expression and maturation in cultured erythroid progenitors from UCB, suggesting that some uncertain serum components play important roles in liquid culture. However, these components are not enough to fundamentally change the developmentally specific globin gene transcription program in erythroid progenitors, suggesting that this program may be mainly controlled by determinants within the erythroid progenitors.

Just like the liquid culture procedure for adult erythroid progenitor cells established before, the suspension culture method we used here also provides an important experimental tool for studying the aspects of erythropoiesis. In humans, the switches from ε- to γ- and γ- to β-globin gene expression are controlled exclusively at the transcription level, and the ζ- to α-globin switch is controlled predominantly at transcriptional level, although post-transcriptional mechanisms also play a role [22]. Up to now, the transcriptional factors that specifically activate the γ-globin gene and control decisively the development switching of globin gene expression have not been validated. We hope that the comparison of cultured erythroid cells from adult bone marrow and UCB could be helpful for the identification of those factors and ultimately contributed to our understanding of the mechanisms of globin gene switching.

Acknowledgments

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