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ORIGINAL ARTICLE

α-THALASSEMIA-LIKE GLOBIN GENE EXPRESSION BY PRIMITIVE ERYTHROCYTES DERIVED FROM HUMAN EMBRYONIC STEM CELLS

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□ *Under culture conditions that promote hematopoietic differentiation, human embryonic stem cells (huESC) give rise to primitive erythroid cells that closely resemble the nucleated erythrocytes of early-stage human embryos. The globin chain distribution of these cells is similar to that seen during the embryonic and fetal stages of development. Here we show that huESC-derived erythroid cells produce substantial quantities of homotetrameric hemoglobin (Hb) composed exclusively of γ-globin-containing subunits. The globin synthesis of these erythroid cells was also significantly unbalanced, with a substantial decrease of α-like globin chain synthesis in relation to that of their β-like globins, a pattern characteristically associated with α-thalassemia (α-thal). This pattern of unbalanced globin synthesis appears to be an inherent feature of human erythroid cells that synthesize predominantly embryonic-stage globins.*

Keywords Human embryonic stem cells (huESC), Embryonic stem cells, Embryonic hemoglobins (Hbs), Hb γ_4 globin synthesis

INTRODUCTION

Recent methodological advances in the directed hematological differentiation of human embryonic stem cells (huESC) have provided efficient means for generating substantial quantities of human erythrocytes (1,2),

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sufficient to allow characterization of their cellular and functional properties (2). These erythroid cells exhibit features similar to those seen during the yolk sac stage of human embryogenesis: they are substantially larger than adult erythrocytes; they are nucleated; ζ- and ε-globins are their predominant globin chain types; and the β-globin chains of adult hemoglobin (Hb) are characteristically absent (1,2). In spite of these differences, within the physiological range the oxygen affinity, Bohr effect, Hill's n coefficient, and 2,3-diphosphoglycerate interaction of these primitive erythroid cells are similar to those of normal postnatal erythrocytes (2). Here we report further characterization of these huESC-derived erythroid cells, including their Hb composition and their pattern of globin chain synthesis.

MATERIALS AND METHODS

Procedures for the propagation and directed erythropoietic differentiation of huESC were as previously described (2). For Hb determinations, washed nucleated erythroid cells were lysed in three volumes of 3% saponin, and the supernatants were clarified by centrifugation at 20,000 × *g*. Electrophoresis was performed on cellulose acetate membranes in Tris/EDTA/borate buffer at pH 8.6. The individual Hb bands were cut out from the membranes, and the Hbs were eluted with distilled water. Globin chain identification of the eluted Hb bands, as well as from each of the chromatographic fractions recovered from the globin chain synthesis study, was by matrix-assisted laser desorption-time of flight (MALDI-TOF) protein mass spectrometry (MS) (2).

For the determination of the relative rates of synthesis of the individual globin chains, washed nucleated erythroid cells derived from huESC line MA-01 were incubated for 1 hour in medium (3) containing a mixture of amino acids from which methionine was excluded. ^{35}S-L-methionine (1175 Ci/mmol; MP Biochemicals, Solon, OH, USA) was added as a radio labeled tracer. Globin chain fractionation was by carboxymethylcellulose column chromatography (4).

RESULTS

Electrophoresis of Hb extracted from nucleated erythrocytes derived from huESC lines MA-01 (2), H-1 and H-7, each demonstrated virtually identical patterns. A prominent Hb band, migrating in a position slightly more cathodal than Hb C [β6(A3)Glu→Lys], comprised *ca.* 70% of the total (Figure 1); this Hb fraction was composed of ζ and ε subunits, and therefore represented Hb Gower-1 (5). The other major Hb band, which migrated in a more anodal position than Hb A, accounted for 12-18% of the total. This Hb was composed exclusively of γ subunits, and therefore was

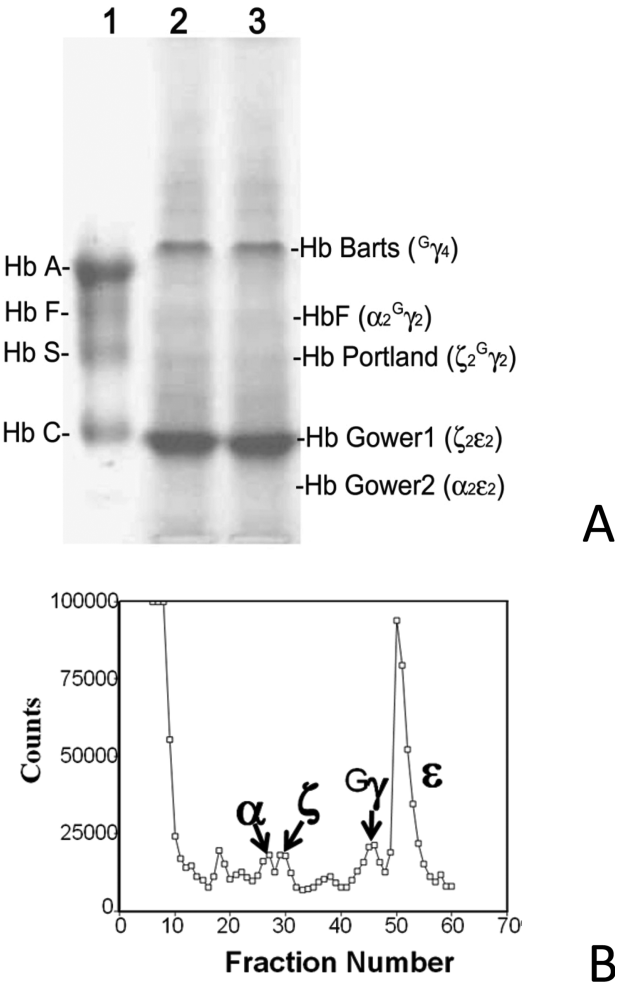

FIGURE 1 Hemoglobin electrophoresis and globin chain synthesis by nucleated erythroid cells derived from huESC line MA-01. A) Electrophoresis in EDTA-Tris buffer, pH 8.6, on cellulose acetate. Lane 1: Hbs A, F, S and C; lanes 2 and 3: Hb extracted from nucleated erythroid cells from huESC line MA-01. B) Incorporation of ^{35}S-L-methionine into globin chains by MA-01-derived erythroid cells. The identification of the globin chains from each of the fractions isolated from both the electrophoresis and the globin synthesis studies was by MALDI-TOF protein MS (2).

identical to Hb Bart's (γ_4), a homotetramer characteristically present in measurable amounts in erythrocytes of neonates with α-thalassemia (α-thal) (6). The other Hb bands that could be identified included Hb F ($\alpha_2\gamma_2$), Hb Portland ($\zeta_2\gamma_2$) and Hb Gower-2 ($\alpha_2\varepsilon_2$), each representing less than 5% of the total.

To examine the relative rates of synthesis of the individual globin chains, we incubated nucleated erythroid cells derived from huESC line MA-01 in a medium containing ^{35}S-L-methionine. The incorporation of

radioactivity into the individual globin chains is shown in Figure 1. After correction for the numbers of methionine residues in each of the globin chains (α = 2, ζ = 1, γ = 2, ε = 3)the calculated ratio of $(\alpha + \zeta)/(\gamma + \varepsilon)$ was 0.53, typical of that seen in reticulocytes of individuals with Hb H disease ($--/-\alpha$) (7,8).

To look for evidence that one or more genetic determinants of α-thal might have been present in this huESC line, the α2- and α1-globin genes were amplified by polymerase chain reaction (PCR), the amplicons were purified, and their nucleotide sequences were determined from 60 nts (nucleotides) 5′ to the ATG initiation codon through 50 nt 3′ to the polyadenylation signal (poly A) site. No mutation or evidence of a single nt polymorphism (SNP) heterozygosity was detected in these sequences. Gap-PCR tests designed to detect single α-globin gene deletions of the rightward ($-\alpha^{3.7}$) and leftward ($-\alpha^{4.2}$) types, and also deletions of both α-globin genes *in cis* of the ($--^{MED}$), $-(\alpha)^{20.5}$, ($--^{SA}$), ($--^{SEA}$), ($--^{FIL}$) and ($--^{THAI}$) types were done. None of these deletions was found. Polymerase chain reaction-based testing to detect α-globin gene triplications of both the anti-3.7 and anti-4.2 types was also performed (9) and neither abnormality was detected.

The ζ2-globin gene of huESC line MA-01 was also examined for evidence of a thalassemia mutation. The sequences from nt 13449 to 13890 (GenBank NT_000006.1) and from 15125 to 15476, corresponding to exons 1 and 3 of the gene, were mutation-free. However, repeated attempts to sequence the remainder of the sequences were not successful. To look for evidence of a large deletion involving the HS-40 regulatory sequences, multiplex ligation probe amplification (10) of the α-globin gene cluster was also performed, from which no deletion was detected.

Although the α- and β-like globin chains are encoded by genes that reside on separate chromosomes, in mature erythroid cell lineages the synthesis of these complementary globins proceeds in a highly coordinated manner (11). The characterizing feature common to all of the various pathological forms of thalassemia is an unbalanced pattern of α/β chain synthesis, which appears to play a causative role in the disease manifestations of these syndromes (12).

However, several lines of evidence now suggest that a significant imbalance between the complementary globins, with a relative excess in the representation of β-like chains, may be the normal pattern of globin chain synthesis at the embryonic yolk sac stage of erythropoiesis. Studies of the Hb composition of erythrocytes from early-stage human embryos (13,14) demonstrated in virtually every case, the presence of significant quantities of Hb Bart's, an indication of a relative deficiency of α-like chains. Hb Bart's was seen most prominently in the earliest-stage embryos and diminished in

embryos of increasing size and maturation of Hb expression, in which embryonic Hb types were also declining.

A similar pattern has also been observed from studies of K562 cells, a line of human myeloid leukemia cells that can be induced to synthesize Hb. Their Hb composition is composed of embryonic and fetal types, with a characteristic absence of adult Hb A. These cells have been shown to produce measurable quantities of Hb Bart's (15), and correspondingly, K562 cells have also been observed to express an α-thal-like pattern of globin chain synthesis (16).

Our observations from the present studies indicate that nucleated erythroid cells derived from huESC, in addition to producing a pattern of globin chain expression typical of the yolk sac stage of development, demonstrate an unbalanced ratio of complementary α- and β-like globin chains, comparable to that seen in individuals with moderately severe α-thal. These findings, taken together with the reported observations from studies of human embryonic erythrocytes and K562 cells, suggest that this thalassemia-like pattern of globin synthesis may be a characteristic common to all erythroid cells of human origin that synthesize predominantly embryonic Hbs. Moreover, in light of the more extreme expression of this imbalance we observed in this study, nucleated red cells derived from the differentiation *in vitro* of huESCs would appear to correspond to a very early stage of erythropoietic development.

We have previously demonstrated that nucleated erythroid cells produced from huESC possess functional properties similar to those of erythrocytes of normal adult blood (2). The directed erythroid differentiation of human embryonic stem cells could therefore potentially be capable of generating an inexhaustible and donorless supply of transfusable red cells, suitable for clinical applications. However, in light of the adverse consequences known to be associated with thalassemia-like globin chain synthesis imbalance, a further maturation process to advance the developmental stage of their globin production might well be essential if these erythroid cells are to prove clinically useful.

Although culture conditions that promote the enucleation of huESC-derived erythroid cells also appear to stimulate the synthesis of Hb A in these cells (2); thus far this effect has been observed only to a minimal degree. Consequently, culture conditions that promote this transition with considerably greater efficiency will be needed if this aim is to be achieved.

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Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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