Biosynthesis of Hemoglobin F Malta-I in Culture by Adult Circulating Erythropoietic Precursors

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ECLIDATION of the mechanisms controlling human globin gene expression is being actively pursued for potential application to the treatment of patients with β-chain hemoglobinopathies. Recent progress in the semisolid cell culture methods has made it possible to examine hemoglobin (Hb) synthesis by human erythropoietic precursors under controlled conditions. Synthesis of fetal hemoglobin (HbF) is significantly augmented in culture of adult erythropoietic precursors termed burst-forming units (BFU-E). For example, we have observed that between 8% and 60% of the Hb synthesized in culture was HbF, when we cultured marrow and peripheral blood erythropoietic precursors from adult normal individuals or patients with sickle cell anemia. Recently, Farquhar et al. reported concordance of γ-mRNA content and γ-chain synthesis in culture using hybridization techniques with cDNA probes. Their results indicated that Hb switching in culture is regulated at transcriptional levels.

Normal human γ-chain consists of two molecular species: one with glycine (Gγ) and the other with alanine (Aγ) at position 136. Synthesis of the two species is directed by two nonallelic structural genes, and the gene order for human non-α globin loci is Gγ·Aγ·δ·β. The ratio of Gγ·Aγ at birth (newborn ratio) and 2:3 in the small amount of HbF in adults (adult ratio). However, a number of exceptions to this rule have been detected in hemoglobinopathic and nonhemoglobinopathic families. The most recently recognized variant of γ-chains, namely Tγ-chain which contains threonine instead of isoleucine at position 75, is thought to be linked to the Aγ-locus. It would be important to examine which of the γ-chains are synthesized in culture of adult erythropoietic precursors. We report our preliminary attempts at the understanding of the regulation of the γ gene selection in culture.

We had an opportunity to study a family that carried a gene for HbF Malta-I (γ117 His→Arg), a Gγ variant with distinctive electrophoretic properties. Biosynthetic studies identified the father as the carrier of the Gγ gene mutant. An analysis of Hb biosynthesis in individual bursts revealed heterogeneous distribution of HbA, HbF, and HbF Malta-I/HbF in individual bursts.

MATERIALS AND METHODS

Erythropoietic Cell Culture

Mononuclear cells were harvested from the blood of the parents by using slight modifications of the Ficoll-Isopaque technique described by Bouy. Cell culture was carried out for 14 days using slight modifications of the methylcellulose assay developed by Iscove et al. We used human urinary erythropoietin (Ep) with a specific activity of 40 U/mg, which was supplied by the Division of Blood Diseases and Resources, NHLBI, Bethesda, Md. One milliliter of culture mixture, containing 3 x 10⁷ mononuclear cells, α-medium (Flow Laboratories, Inc., Rockville, Md.), 0.8% methylcellulose (Fisher Scientific Co., Norcross, Ga.), 30% fetal bovine serum (Flow Laboratories, Inc.), 1% bovine serum albumin (Calbiochem, San Diego, Calif.) and 1.0 U Ep, was plated in 35-mm Lux standard nontissue culture dishes (Flow Laboratories, Inc.) and incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air.

Analysis of Hb Biosynthetic Rates

Measurements of rates of Hb synthesis were carried out during the last 2 days of culture. On day 12 of incubation, 2 μCi of uniformly ¹⁴C-labeled amino acid mixture (Catalog NEC-445, New England Nuclear, Boston, Mass.) in 0.3 ml phosphate-buffered saline (PBS) was layered over a dish containing 1.0 ml of tissue culture mixture. After 2 days of labeling, cells were harvested by diluting the culture mixture with PBS and aspirating the content of

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entire plates with a Pasteur pipet. Samples thus collected were analyzed for overall biosynthetic rates of Hb as described previously. \(^1\) Briefly, cell pellets were washed with a medium, stored at \(-70^\circ\text{C}\) overnight, thawed at room temperature, and lysed by the addition of a mixture of KCl and Nonidet P-40 solutions. Hemolysates were then subjected to isoelectric focusing on an LKB Multiphor apparatus under the conditions described by Drysdale et al. \(^12\) and Allen et al. \(^13\) After completion of the run, the gels were fixed in trichloroacetic acid, stained with Coomassie blue R-250, destained, and prepared for autoradiography on Kodak NS-2T x-ray film. The densities of autoradiogram bands were determined from densitometric tracings that were carried out on a Joyce/Loebl 3CS microdensitometer.

Hemoglobin biosynthesis in individual erythropoietic bursts was analyzed by use of fluorography. \(^14\) Details of this method have been described in a previous communication. \(^15\) Briefly, individual bursts were lifted from culture dishes by using a 10-\(\mu\)l Eppendorf pipet and placed in microcentrifuge tubes containing 200 \(\mu\)l PBS and 0.1 \(\mu\)l of packed red blood cells prepared from the original blood samples. Hemolysates of individual bursts were prepared as described above and subjected to isoelectric focusing. The gels were fixed in 15% trichloroacetic acid, immersed in distilled water, equilibrated with dimethylsulfoxide (DMSO), impregnated with 2,5-diphenyloxazole in the presence of DMSO, and soaked in water. Dried gels were then placed for 1 wk in contact with Kodak RP “X-Omat” film that had been preflashed using an electronic photographic flash unit (Vivitar 283). After densitometric scanning of the fluorogram bands, empirical probits of each sample differing in percent HbF biosynthesis were calculated as described previously. \(^15\) The absence of significant subunit exchange during these procedures had been proven by a mixing experiment and by the demonstration of a constant ratio of HbS:HbC in individual bursts derived from a patient with HbSC disease. \(^15\)

RESULTS

Identification of HbF Malta-I in Cord Blood

Hemolysate of the cord blood of the second child (E.W.) was subjected to four types of electrophoretic analyses. \(^16\) In cellulose acetate electrophoresis in Tris EDTA borate (TEB) buffer, pH 8.5, a distinct band constituting about 20% of total Hb moved cathodically to HbF (between HbS and HbC). In citrate agar electrophoresis, pH 6.0, this fraction moved with HbF. In alkaline buffer (TEB, pH 8.9) containing mercaptoethanol and 6 \(M\) urea, the mutant globin chain moved cathodically to \(\gamma\)-chain. However, in acidic TEB citrate buffer (pH 6.0) containing mercaptoethanol and urea the mutant chain did not separate from \(\gamma\)-chain.

This highly distinctive electrophoretic behavior strongly suggested that the variant was HbF Malta-I. Dr. Fred Garver, Department of Molecular and Cellular Biology, Medical College of Georgia, confirmed the identification by an immunologic method. \(^17\)

Biosynthesis of HbF Malta-I in Culture

Cultures from the father (L.W.) who had hematocrit (HCT) of 48% and less than 1% HbF in his peripheral blood erythrocytes, revealed the presence of
to a normal distribution of the 57 samples is presented in Fig. 3. Analysis with D’Agostino’s test revealed the value of $D = 0.27743$ and also supported the normality of the distribution. The mean and SD of the relative HbF biosynthesis in the 57 bursts was 39.8% ± 15%, which is in basic agreement with the overall HbF biosynthetic rate of 35.5%.

The relative ratio of HbF Malta-I to total HbF in individual bursts also revealed significant variation. The frequency distribution of this ratio, however, did not show a normal distribution. The mean and SD of the relative biosynthesis of HbF Malta-I in 57 bursts was 22.4% ± 10.0% and was in agreement with the overall biosynthetic rate (24.2%) of the mutant HbF measured with autoradiography. There was a positive correlation ($0.01 < p < 0.02$) between the ratios of HbF Malta-I/HbF and HbF/Hb of individual bursts (Fig. 4).

**DISCUSSION**

In this article we demonstrated significant augmentation of synthesis of HbF Malta-I in culture of peripheral blood erythropoietic precursors from an adult carrier. Clonal erythropoietic cell culture of adult blood may provide a novel and unique means for identification of carriers of HbF variants. In addition, the availability of radiolabeled $\gamma$-chains synthesized in culture may allow determination of molecular properties of hitherto uncharacterized $\gamma$-chain mutants.

HbF Malta-I, a $G\gamma$ variant, comprised about 24% of total HbF synthesized in culture. We recently observed that the ratio of $G\gamma$ to $A\gamma$ chains of HbF synthesized in culture of adult erythropoietic precursors assumes the "adult" ratio. Thus, the value of the biosynthetic rate of HbF Malta-I in culture appears to be in accord with the predicated value of expression of one of the two $G\gamma$ genes in adults. Recently, Farquhar et al. presented evidence that HbF biosynthesis in culture is regulated at transcriptional levels. Biosynthetic studies of HbF variants, such as those presented in this report, will provide more direct methods for determining the control mechanisms for synthesis of HbF variants than analysis of proteins in the erythrocytes of umbilical cord blood.

The ratio of HbF Malta-I to total HbF in individual bursts revealed wide variations. Earlier, we have reported the presence of a relatively constant ratio of HbC/(HbS + C) in individual bursts derived from cultures of cells from a HbSC double heterozygote. The latter information may be interpreted as showing a balanced allelic gene expression in individual erythropoietic bursts. If so, the variation of HbF Malta-I/total HbF ratios in individual bursts may suggest variations in the expression of $G\gamma$ and $A\gamma$ chain synthesis in each burst. A correlation existed between the ratio of HbF Malta-I/total HbF and the ratio of HbF/Hb. This result suggests that, in culture of adult erythropoietic precursors, there is a correlation between $\gamma/(\gamma + \beta)$ and $G\gamma/(G\gamma + A\gamma)$ ratios in individual bursts. Furthermore, the values represented by the least-square regression line corresponded to the
relationship between HbF levels and $G\gamma/(G\gamma + A\gamma)$ ratios in human erythrocytes. Cell culture of peripheral blood BFU-E may be an appropriate model for studies of human Hb synthesis.

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