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PRESENCE OF α^{CS}-GLOBIN ON MEMBRANE OF RED CELLS CONTAINING HEMOGLOBIN CONSTANT SPRING (CS)

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ABSTRACT

Hemoglobin (Hb) Constant Spring (CS) is an elongated α-globin variant present at polymorphic frequencies in Southeast Asian countries. Analysis of membrane from red blood cells of individuals with Hb H/CS and Hb CS/CS by sodium dodecyl sulfate polyacrylamide gel-electrophoresis revealed the presence of a protein band with molecular size slightly greater than globin. This band reacted with rabbit anti-human α-globin antibodies in a Western blot assay. The identity of this membrane-bound α-globin-like protein as being α^{CS}-globin was verified by comparing its ratio of biosynthetically [^{3}H]-labelled proline/[^{14}C]-labelled lysine with those of normal globin chains. The presence of α^{CS}-globin on the membrane of Hb H/CS and Hb CS/CS red cells affords an explanation for the unusually severe anemia observed in these subjects.

INTRODUCTION

Hemoglobin (Hb) Constant Spring (CS), an elongated α-globin variant, was first identified from an unusually slow electrophoretically migrating Hb obtained from three Chinese individuals living in Constant Spring, Kingston, Jamaica.[1] However, in Thailand, Wasi and co-workers had earlier observed Hb with a similar property among a few patients with Hb H disease,[2] and had given it the name Hb Thai.[3] Similar findings were reported in studies of individuals with Hb H in Greece,[4] Malaysia,[5] Hong Kong[6] and U.S.A.[7] Recent surveys have shown Hb CS reaching frequencies of 1-6% in Southeast Asia.[8]

The α^{CS}-globin gene, located at α2-globin allele, contains a point mutation in the termination codon (TAA → CAA) permitting glutamine to be incorporated and the α-globin chain is extended by another 31 amino acids before an in-frame stop codon is read by the ribosome.[9] Individuals who are heterozygous for Hb CS have only 1-2% of the variant Hb in their red cells, rather than the expected 25%.[10] This has been explained by an inherent instability of α^{CS}-mRNA rather than to that of the protein.[11,12] Thus the presence of α^{CS} gene can be considered to be equivalent to α-thalassemia 2 genotype and an individual who inherits both α^{CS} and α-thalassemia 1 gene, *i.e.* having a genotype of α-thalassemia 1/α-thalassemia 2, should be clinically less seriously affected than Hb H disease which has only one functioning α-globin gene.

Nevertheless, homozygous Hb CS subjects and heterozygotes with Hb H/CS present a more severe pathology.[13,14] The levels of Hb H and the number of red cells having inclusion bodies are higher in Hb H/CS than in Hb H individuals.[15] Hb H/CS red cells contain increased amounts of malonyldialdehyde, an indicator of lipid peroxidation,[16] and are more fragmented and rigid than Hb H cells.[17] On the other hand, Hb CS/CS and Hb H/CS red cells have near normal volumes but remain poorly hemoglobinized.[18]

It is well recognized that a change to the primary structure of a protein can often alter its normal folding and forms the molecular basis of a number of human diseases (see ref. 19 for a recent review). The extension of the C-terminus of α^{CS}-globin may interfere with its association with β-globin, and taking into consideration the above observations pointing to an unusual membrane damage in Hb CS-containing red cells, we have examined membranes of such cells for the presence of α^{CS}-globin chains.

MATERIALS AND METHODS

Subjects

Blood samples were collected into acid-citrate-dextrose solution from three subjects with Hb H/CS, two with Hb CS/CS and one normal control. Criteria for diagnosis of the disease were as previously described.[13] None of the thalassemic subjects received blood transfusion during the three months prior to the study.

Ghost membrane preparation

Blood was centrifuged at 3,000 *g* for 10 min at 4 °C. Plasma and buffy layer were removed by aspiration and the packed red cells were washed 4 times with 5 volumes of cold 10 mM Tris-HCl buffer, pH 7.4, containing 130 mM NaCl. One ml of the packed red cells was lysed by the addition of 30 ml of ice-cold 10 mM Tris-HCl buffer, pH 8.8, containing 1 mM ethylenediamine tetraacetic acid and 1 mM phenylmethyl sulfonyl fluoride. Ghost membrane was sedimented by centrifugation at 12,000 *g* for 40 min at 4 °C, and further washed 4 times with cold lysis buffer and stored at -80 °C until used. Protein content of ghost membrane was determined using the method of Bradford.[20]

Western blotting

Ghost membrane proteins (50 μg) were separated on 6-18% linear gradient sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE) according to the method of Laemmli[21] and stained with Coomassie blue R-250 dye. Portion of an unstained gel containing protein bands with molecular size less than 43 kDa (actin) were electroblotted onto nitrocellulose membrane which was then reacted with rabbit anti-human α-globin followed by peroxidase-conjugated pig anti-rabbit immunoglobulin (Dako, Denmark) using diaminobenzidine as substrate.

Hemoglobin biosynthesis

Packed blood cell preparation was enriched for reticulocytes by centrifugation at 20,000 *g* for 1 h at 4 °C and the upper fraction was removed for biosynthetic studies. A solution of 25% (v/v) cell suspension in Tris-bicarbonate buffer, pH 7.7, containing a mixture of amino acids including [^{3}H]-L-proline (4,865.5 GBq/mmol) and [^{14}C]-L-lysine (11.6 GBq/mmol) (New England Nuclear, U.S.A.), was incubated at 37 °C for 1 h. Ghost membrane proteins were separated by SDS-PAGE as described above. Gel slices at the position of globin (normal and α^{CS}) were excised from the slab using a clean razor blade and protein was then electroeluted out. Radioactivity present in the protein samples were measured using a Triton-based cocktail in a liquid scintillation counter (Beckman LS 1801, U.S.A.) equipped with a dual isotope DPM program.

RESULTS AND DISCUSSION

When ghost membrane proteins of red cells from Hb H/CS and Hb CS/CS subjects were analyzed by SDS-PAGE, it was apparent that they contained an additional protein component which migrated slightly slower than globin (Fig. 1), with the band being more prominent in samples from Hb CS/CS (Fig. 1, upper portion, lanes 1 and 2) than in Hb H/CS samples (lanes 3-5). The apparent molecular weight of this band was 18 kDa, consistent with that of α^{CS}-globin (18.5 kDa). When the lower portion of the gel was electroblotted onto a nitrocellulose sheet, and the protein bands treated with antibody against human α-globin, only the material corresponding to the position of the putative α^{CS}-globin was reactive (Fig. 1, lower portion, lanes 1-5). It is worth noting that the band at the normal globin position in membrane samples from Hb CS-containing cells did not react with the antibody indicating that normal globin species associated with membranes of Hb H/CS and Hb CS/CS cells were not α-globin but β-globin (presumably from binding of Hb H, β_4).

In order to verify that this membrane-bound globin of higher molecular weight was indeed α^{CS}-chain, its amino acid composition was compared with that of α- and β-globin by measuring the ratio of proline:lysine (RPL) incorporated by *in vitro* biosynthesis employing reticulocyte-enriched cells. Normal globin (both α and β) contains 7 proline residues and 11 lysine whereas α^{CS}-globin has 11 proline and 11 lysine. Table 1 shows the results of experiments using blood samples from three Hb H/CS individuals. Since each experiment

Table 1 Ratio of [^{3}H]-L-proline to [^{14}C]-L-lysine incorporated into membrane-bound normal and α^{CS}-globin in reticulocytes of three Hb H/CS individuals

Subject	[^{3}H]/[^{14}C] normal globin	[^{3}H]/[^{14}C] α^{CS}-globin	Column 2/Column 3
1	0.46	0.74	0.62
2	0.64	0.97	0.66
3	4.16	6.83	0.61

employed different specific activities of the two radiolabelled amino acids, the ratios of RPL value for normal globin to that of the putative α^{CS}-globin were measured and compared with the expected ratio of 0.64. There was good agreement between the experimental and calculated ratios in all three experiments confirming that the membrane-bound α-globin band observed in SDS-PAGE was indeed α^{CS}.

Dissimilarity in the properties of thalassemic red cells of different genotypes has been attributed to the species of globin chains bound to the membrane: α-globin in the case of β-thalassemia and Hb H (β_4) in α-thalassemia.[22] Membrane association of α-globin has a more deleterious effect on red cell membrane than does β-globin chain.[23] The presence of both α^{CS}-globin and Hb H on the membrane of Hb H/CS and

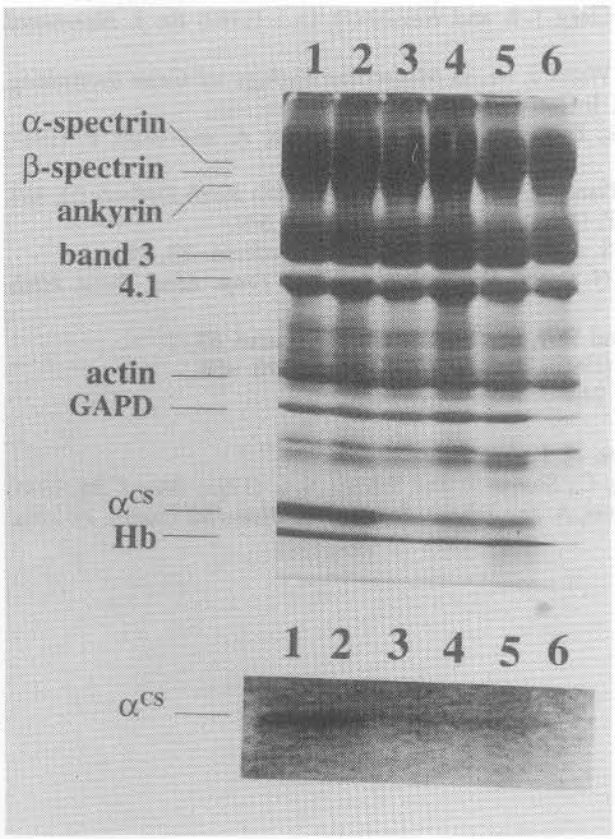

Fig. 1 SDS-PAGE of ghost membranes from Hb H/CS, Hb CS/CS and normal cells. Fifty microgram of membrane proteins were separated on 6-18% linear gradient polyacrylamide gel. Lanes 1 and 2 were from Hb CS/CS cells, lanes 3-5 from Hb H/CS and lane 6 was normal control. In the upper panel the gel slab was stained with Coomassie blue. In the lower panel the α^{CS} band was Western blotted onto nitrocellulose membrane sheet which was then treated with rabbit anti-human α-globin antibodies followed with peroxidase-conjugated pig anti-rabbit immunoglobulin using diaminobenzidine as substrate. GAPD, glyceraldehyde 3-phosphate dehydrogenase; Hb, globin.

Hb CS/CS red cells would explain the unusual membrane properties of these cells and account for the severity of the disease among such individuals who, in the light of this study, have now to be considered as having both α- and β-thalassemia phenotypes.

While this study was in progress, Aljurf *et al.*[24] reported a similar observation of the presence of α^{CS}-globin on membranes of Hb H/CS and Hb CS/CS red cells.

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