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Chapter 6

Comparative Study of the Amount of Re-released Hemoglobin from α-Thalassemia and Hereditary Spherocytosis Erythrocytes

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Additional information is available at the end of the chapter

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Abstract

Hemoglobin release test (HRT), which is established by our lab, is a new experiment to observe the re-released hemoglobin (Hb) from erythrocytes. In this study, one-dimension HRT, double dimension HRT, and isotonic and hypotonic HRT were performed to observe the re-released Hb from the blood samples of normal adult, hereditary spherocytosis (HS), and α-thalassemia. The results showed that compared with normal adult, the re-released Hb from HS blood sample was decreased significantly; however, the re-released Hb from α-thalassemia blood sample was increased significantly. The mechanism of this phenomenon was speculated to have relation with the abnormal amount of membrane-binding Hb.

Keywords: hereditary spherocytosis, α-thalassemia, hemoglobin release test, erythrocyte, hemoglobin

1. Introduction

Erythrocytes, also called red blood cells (RBCs), are the most common type of blood cell. In humans, mature erythrocytes are flexible and oval biconcave disks. They lack cell nucleus and most organelles, in order to accommodate maximum space for hemoglobin (Hb), which has the important oxygen-transporting function. This protein makes up about 96% of the eryth-

rocytes' dry content (by weight), and around 35% of the total content (including water) [1]. Hb is an assembly of two α-globin family chains (including α and ξ chains) and two β-globin family chains (including β, γ, δ, and ε chains). Each globin subunit has an embedded heme group and each heme group contains an iron atom that can bind one oxygen molecule through iron-induced dipole forces. These subunits are bound to each other by salt bridges, hydrogen bonds, and hydrophobic interactions. Three Hb variants exist in normal adult erythrocytes, that is HbA ($\alpha_2\beta_2$, over 95%), HbF ($\alpha_2\gamma_2$, <1%), and HbA_2 ($\alpha_2\delta_2$, 1.5–3.5%) [2-3].

The erythrocyte membrane plays many roles that aid in regulating erythrocytes' surface deformability, flexibility, adhesion to other cells, and immune recognition. These functions are highly dependent on the composition of the membrane, which includes 3 layers: the glycocalyx on the exterior, which is rich in carbohydrates; the lipid bilayer, which contains many transmembrane proteins, besides its lipidic main constituents; and the membrane skeleton, a structural network of proteins located on the inner surface of the lipid bilayer. The determinant of normal membrane cohesion is the system of "vertical" linkages between the phospholipid bilayer and membrane skeleton, formed by the interactions of the cytoplasmic domains of various membrane proteins with the spectrin-based skeletal network. Band 3 and Rh-associated glycoprotein (RhAG) provide such links by interacting with ankyrin, which in turn binds to β-spectrin. Protein 4.2 binds to both band 3 and ankyrin and can regulate the avidity of the interaction between band 3 and ankyrin. Glycophorin C, band 3, XK, Rh, and Duffy all bind to protein 4.1R, the third member of the ternary junctional complex with β-spectrin and actin [4].

Thalassemia is an inherited autosomal recessive blood disorder characterized by abnormal formation of Hb, which results in improper oxygen transport and destruction of erythrocytes. Normally, the majority of adult Hb (HbA), is composed of two α- and two β-globin chains, which are arranged into a heterotetramer. The β-globin chain is encoded by a single gene on chromosome 11 [5], and α-globin chain is encoded by two closely linked genes on chromosome 16 [6]. A normal person has two loci encoding the β-chain, and four loci encoding the α-chain. Thalassemia patients have defects in either the α- or β-globin chain. According to which chain is affected, thalassemias are classified into α-thalassemias and β-thalassemia. α-thalassemias result in decreased α-globin production, which result in an excess of β-chains in adults and excess γ-chains in newborns. The excess β-globin chains form unstable tetramers (HbH, β_4), which have abnormal oxygen dissociation curves. β-thalassemias are characterized as either β^o or β-thalassemia major if formation of any β chains is prevented, the most severe form of β-thalassemia; as either β^+ or β thalassemia intermedia if some β-globin chain formation are allowed; or as β-thalassemia minor if β-globin chain production is not terribly compromised [7-8]. In contrast to the β-thalassemias, which are usually caused by point mutations of the β-globin gene, the α-thalassemia syndromes are usually caused by the deletion of one or more α-globin genes and are subclassified according to the number of α-globin genes that are deleted (or mutated): one gene deleted (α^+-thalassemia); two genes deleted on the same chromosome or in cis (α^0-thalassemia); three genes deleted (HbH disease); or four genes deleted (hydrops fetalis with Hb Bart's) [8].

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Hereditary spherocytosis (HS) is an autosomal dominant erythrocyte membranopathy [9], but does not belong to hereditary hemoglobinopathies. This disorder is caused by mutations in genes relating to membrane proteins. These proteins include spectrin (α and β), ankyrin [10], band 3, protein 4.2 [11], and other erythrocyte membrane proteins that allow for the erythrocytes to change their shapes. The abnormal erythrocytes are sphere-shaped (spherocytosis) rather than being the normal biconcave disk shaped. This difference in shape not only interferes with the ability to be flexible to travel from the arteries to the smaller capillaries, but also makes the erythrocytes more prone to rupture.

Hemoglobin release test (HRT), also called electrophoresis release test (ERT), which is performed by electrophoresing live erythrocytes directly on a starch–agarose mixed gel with intermittent electric current, was established by our lab in 2007 [3,12]. Starch–agarose mixed gel electrophoresis is a routine method used to separate and analyze Hb in our lab since 1980. Hb within the erythrocytes can only be released once during routine starch–agarose mixed gel electrophoresis, which is performed with continuous power supply, and this phenomenon is named as "initial release" now. The difference in mobility of HbA_2 between erythrocyte and hemolysate sample (also called HbA_2 phenomenon) was found during an "initial release" experiment in 1981 [12]. In 2007, a sudden power outage was encountered during the electrophoresis of erythrocytes, however, the experiment was not abandoned and electrophoresis was continued after the power was restored. To our surprise, another new Hb band was found to be released from the origin, which was named "single-band re-release" as opposed to the "initial release". When the power outages were simulated more than once, multiple Hb bands would appear between HbA and origin, and this phenomenon was named as multiple-band re-release or ladder-band re-release [13]. Based on these experiments, isotonic and hypotonic HRT and double-dimensional HRT were developed subsequently. Then the re-released Hb was observed in many patients' erythrocytes, and its amount varied in different patients [14-15]. Some of the patients had increased Hb re-release, such as β-thalassemia, some general surgery patients, cirrhosis, and some gastro enteric tumor patients, but the specific screening experiment had not been done and the exact mechanism of this phenomenon had not been clear. The erythrocyte membrane or cytoskeleton binding Hb was speculated to have relationship with this phenomenon. To further study the mechanism of Hb re-release, the effects of blood type, blood viscosity, different membrane-destroying methods, exogenous hydrogen peroxide, and glutaraldehyde treatments on the amount of re-released Hb were observed subsequently, and the re-released Hb was speculated to have relationship with the abnormality of erythrocyte membrane and Hb. In this study, re-released Hb from two hereditary hemolytic diseases, HS (erythrocyte membrane disorder) and α-thalassemia (Hb disorder), was observed with a variety of HRT experiments.

2. The comparative study of the re-released hemoglobin from α-thalassemia and hereditary spherocytosis erythrocytes

This study had been approved by the local ethics committee, and one HS patient (coming from the first hospital of Baotou Medical College) and one α- thalassemia patient (coming from the

third Worker's Hospital of Baogang Group) were included. The HS patient, diagnosed as spectrin defect, is a 45-year-old female with hemolytic anemia, jaundice, and splenomegaly. The α- thalassemia patient, diagnosed as Southeast Asian deletion (SEA) by PCR, is a 35-year-old female with hemolytic anemia and splenomegaly. Before collecting their blood, patients were asked to sign the consent information. Venous blood samples were anticoagulated with EDTA, and then routine blood examination, osmotic fragility test and HRT were performed respectively within 24 h. The spherical erythrocytes of HS patient are more than 20% in peripheral blood smear, and the osmotic fragility is increased (max: 0.39% vs 0.40%-0.45%, min: 0.75% vs 0.55%-0.6%). As to the α-thalassemia patient, a large number of target erythrocytes exist in peripheral blood smear and the osmotic fragility is decreased.

Whole blood was divided into two parts, one part was used to prepare blood samples, and the other part was used to prepare RBC samples. Blood samples were prepared by adding the same volume of CCl_4 into the anticoagulated blood. After turbulent mixing and centrifuging (12000 rpm for 10 minutes), the upper layer was whole blood hemolysate, the middle layer between hemolysate and CCl_4 was whole blood stroma. The other part of whole blood was firstly made into packed RBCs by washing the RBCs with saline for 4-5 times until the supernatant was colorless [3,12,13]. Then the same volume of CCl_4 was added into the packed RBCs, and after mixing and centrifuging (3000 rpm for 10 minutes), the upper red solution was RBC hemolysate, and the middle layer between hemolysate and CCl_4 was RBC stroma.

The starch–agarose mixed gel was prepared by dissolving 0.24 g of agarose and 1.72 g of starch in 90 mL of TEB buffer (42.1 mmol/L Tris, 1.71 mmol/L EDTA, 6.47 mmol/L boric acid, pH 8.6) [3,12,13]. The solution was heated until the agarose melts, and then the gel was laid on a 17×17 cm glass while hot. After solidification, about 8 μL of samples were applied on the cathodic side of the gel by using 3 MM filter paper. After adding blood samples on the starch–agarose mixed gel, electrophoresis was carried out in borate buffer (0.3 mol/L boracic acid, 0.06 mol/L NaOH, pH9.0) at 5 V/cm for 2 hours, then paused for 15 minutes and ran for 15 minutes by turns. It took about 6 hours for the entire electrophoresis. After electrophoresis, the red bands on the gel were firstly observed directly with eyes, and then the gel was sequentially stained with Ponceau Red (0.1% Ponceau S, 5% glacial acetic acid and 2% glycerol) and Benzidine (0.6 g of benzidine, 25 mL of glacial acetic acid, 10 mL of glycerol, add deionized water to 500 mL, then keep the solution in 75℃ water bath for 1 hour until the benzidine is dissolved completely. Sodium nitroprusside and 30% H_2O_2 should be added to this solution before use) for 4 hours, respectively. Finally, the gel was rinsed with rinsing solution (5% glacial acetic acid, 2% glycerin) until the background was clear.

Routine one-directional HRT was performed to compare the re-released Hb from normal, α-thalassemia, and HS patients' blood samples, which were prepared from whole blood, whole blood hemolysate, whole blood stroma, RBCs, RBCs hemolysate, RBCs stroma and plasma respectively. Comparing with the normal control, the re-released Hb from HS and α-thalassemia erythrocytes had opposite changes (Figure 1). In normal control, there was nearly no HbA in the sample of whole blood stroma, but a small amount of HbA in the RBCs stroma; both whole blood and RBCs sample of normal control had re-released Hb; however re-released Hb did not appear in whole blood and RBCs hemolysate samples. As to HS, there was more

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HbA appearing in RBCs stroma sample, and no re-released Hb appeared in any of the blood samples. On the contrary, the HbA of α-thalassemia whole blood stroma increased significantly, but that of α-thalassemia RBCs stroma was hard to see; in addition, the re-released Hb from whole blood and RBCs sample of α-thalassemia were increased significantly and Hb ladder was formed obviously.

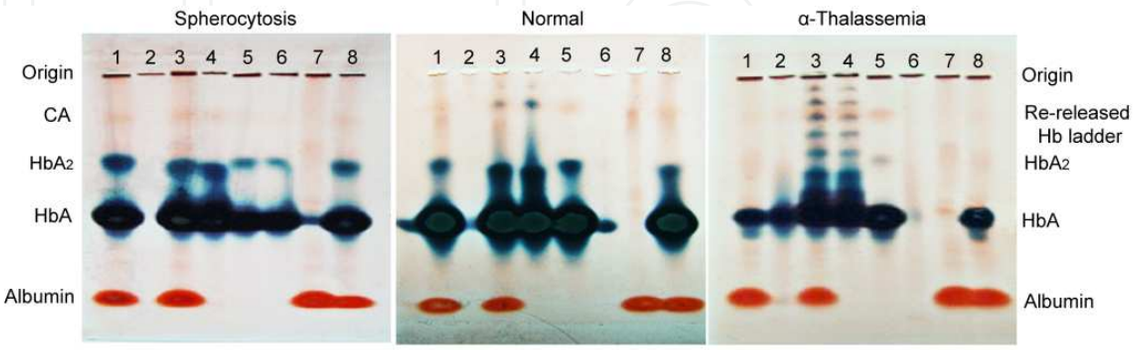

Figure 1. One dimension HRT of spherocytosis and α-thalassemia blood samples. Samples 1–8 were whole blood hemolysate, whole blood stroma, whole blood, RBCs, RBCs hemolysate, RBCs stroma, plasma, whole blood hemolysate.

To observe the effect of hypotonic treatment on the re-released Hb, isotonic and hypotonic HRT was performed with normal adult, HS patient, and α-thalassemia patient at room temperature or 37℃ for 1 hour. During the experiment, the whole blood or packed RBCs were firstly diluted with H_2O in the proportion from 10: 0 to 1: 9 (named as tube 1 to 10, respectively), and then kept at room temperature or 37℃ for 1 hour. Then one-direction HRT was performed as described above. The result of room temperature isotonic and hypotonic HRT showed that the whole blood sample (tube 1) of normal control had slight ladder of re-released Hb, when diluted with H_2O (tube 2 to tube 5), the re-released Hb was decreased, but increased from tube 6 to tube 8, and then decreased again from tube 9 (Figure 2). Compared with the normal control, the re-released Hb ladder decreased obviously in the HS patient, but increased significantly in the α-thalassemia patient. (Figure 2).

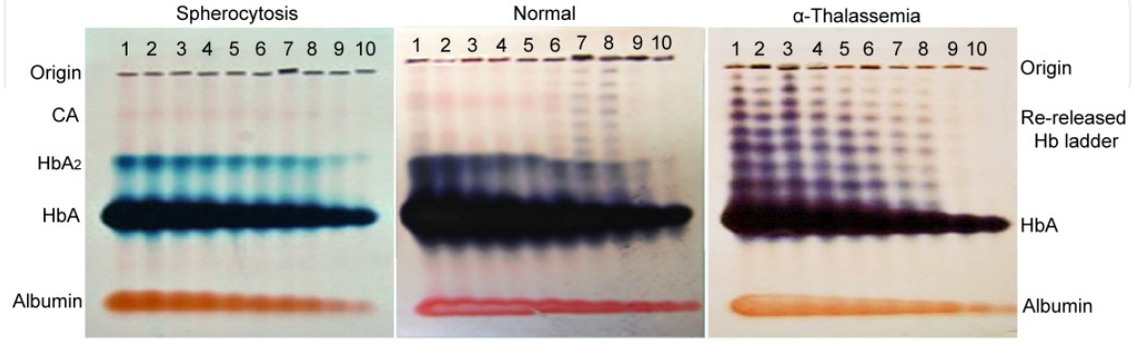

Figure 2. Whole blood isotonic and hypotonic HRT results of normal adult, spherocytosis, and α-thalassemia at room temperature. Whole blood was diluted with H_2O in the proportion from 10: 0 to 1: 9, respectively (tube 1 to 10), and kept at room temperature for 1 hour.

The result (Figure 3) of 37℃ isotonic and hypotonic HRT was similar with that at room temperature except for the disappearance of re-released Hb ladder from tube 1 of the normal control.

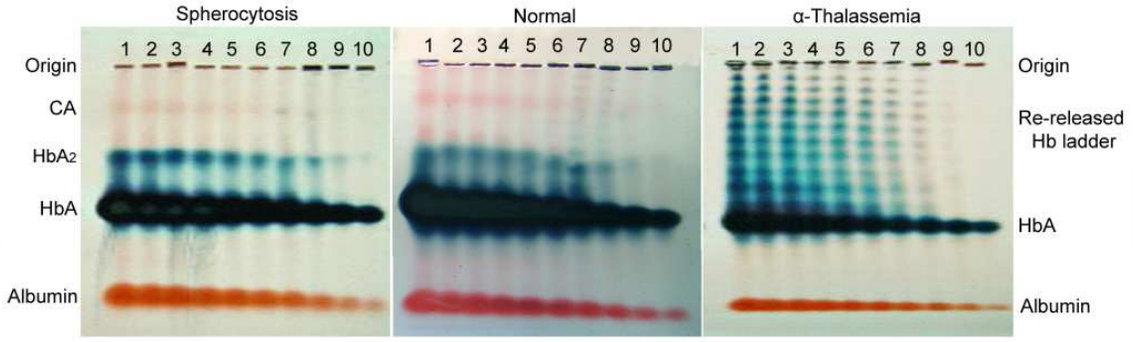

Figure 3. Whole blood isotonic and hypotonic HRT results of normal adult, spherocytosis, and α-thalassemias at 37℃. Whole blood was diluted with H2O in the proportion from 10: 0 to 1: 9, respectively (tube 1 to 10), and kept at 37℃ for 1 hour.

Double-direction HRT (or diagonal HRT) was performed to observe not only the re-released HbA but also the re-released HbA_2. Firstly, one-direction HRT was performed as described above, then the direction of electric field was changed vertical to the original one, and another cycle of HRT was performed. The result showed that there was few re-released HbA in normal whole blood, but the re-released HbA_2 was difficult to observe (Figure 4A). Compared with normal, the re-released HbA from HS whole blood was decreased, but that from α-thalassemia whole blood was increased significantly. The amount of HbA_2 in α-thalassemia whole blood was less than the normal control obviously, and the re-released HbA_2 could not be detected in our experiment (Figure 4B).

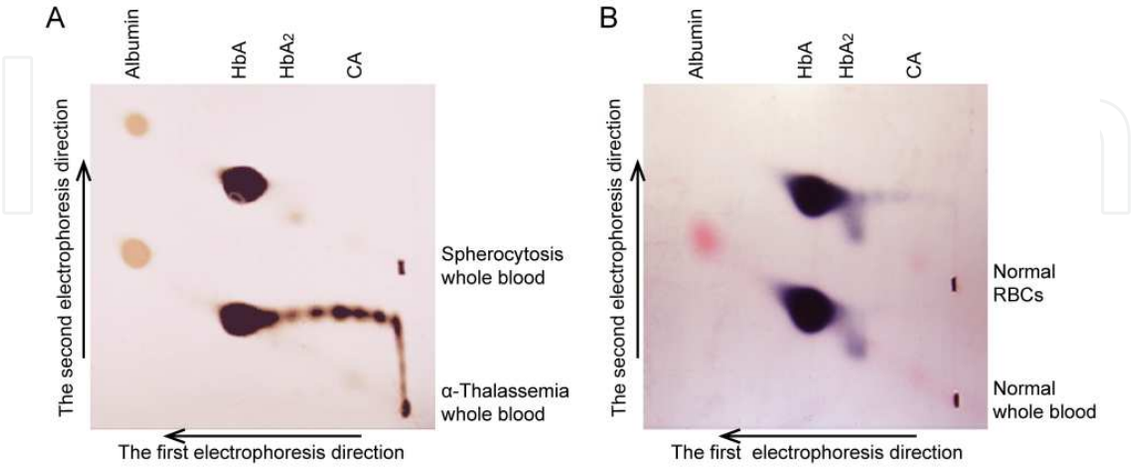

Figure 4. Double-direction HRT of normal adult, spherocytosis, and α-thalassemias blood sample. A was the double-direction HRT of spherocytosis and α-thalassemia whole blood sample; B was the double-direction HRT of the normal whole blood and RBCs sample.

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3. Discussion

Both HS and thalassemia belong to hereditary hemolytic disorders, which include hemoglobinopathies, erythrocyte membranopathy, and erythrocyte enzymopathy [16]. HS is the representative erythrocyte membranopathy [17], and thalassemia is the classic hemoglobinopathy [18-20]. In this study, Clinical tests showed that anemia, splenomegaly, and jaundice were the common clinical signs and symptoms of these two patients [16]. Some of the erythrocytes of the HS patient were spherical, but that of α-thalassemia were target-shaped. The osmotic fragility of erythrocytes increased in HS, but decreased in α-thalassemia. The morphology and osmotic fragility changes were caused by the defects of these two disorders. The abnormalities in HS erythrocyte membrane proteins, particularly ankyrin, α- and β-spectrin, band 3 and protein 4.2, result in the loss of membrane surface area relative to intracellular volume, which leads to spherically shaped erythrocytes with decreased deformability and increased fragility. Increased erythrocyte fragility leads to vesiculation and further membrane loss [21], so HS erythrocytes are unable to withstand the introduction of small amounts of free water that occurs when they are placed in increasingly hypotonic saline solutions. As a consequence, HS erythrocytes hemolyze more readily than normal erythrocytes at any saline concentration [17].

The thalassemic erythrocyte membranes exhibit morphological, biochemical, and mechanical abnormalities due to oxidative damage induced by binding of unmatched globin chains to the cytoplasmic surface of the membrane. So both α- and β- thalassemic erythrocytes become renitent and are less deformable than normal erythrocytes. The morphology and mechanical properties of the erythrocytes membrane are controlled by the cytoskeletal network underlying the lipid bilayer. Spectrin is the principal structural element of the erythrocyte cytoskeleton, regulating membrane cytoskeletal functions [22].

The re-released Hb was compared between these two kinds of hereditary hemolytic disorders by HRT. The results showed that comparing with the normal control, the re-released Hb from HS whole blood or erythrocytes was decreased, but increased distinctively from that of α-thalassemic erythrocytes during routine, two-directional, and isotonic and hypotonic HRT. The re-released Hb is speculated to have relationship with membrane-binding Hb, and the abnormal membrane-binding Hb will lead to abnormal Hb re-release. As known, most of the Hb exists in cytoplasm; only small amount of Hb binds with the membrane through interaction with the cytoskeletal proteins or membrane lipids. The abnormality of both membrane and Hb will change the amount of membrane-binding Hb, and will further lead to the variation of re-released Hb during HRT. HRT was established by our lab in 2007, and in the previous studies, the re-released Hb usually increased from some patients' erythrocytes during HRT, such as β-thalassemia patients, some general surgery patients, cirrhosis, and some gastroenteric tumor patients. In our study, the re-released Hb from α-thalassemic erythrocytes was increased significantly like before [12], but the re-released Hb from HS erythrocytes was decreased a lot. The abnormal membrane-binding Hb was speculated to be the reason.

It is well known that in vivo and under normal physiological conditions, intraerythrocytic hemoglobin may exist in three different forms represented by oxygenated, deoxygenated and partially oxidized Hb. Apart from the first two derivatives whose relative proportions are

continuously changing during the oxygenation deoxygenation cycle, met-hemoglobin (MetHb) is normally present at a steady-state level of about 1% [23]. MetHb usually binds with membrane, and the re-released Hb from normal erythrocytes is speculated to be the membrane-binding MetHb. Oxidative damage can lead to the oxidative membrane damage and increased proportion of MetHb. The oxidization of band 3 leads to dissociation of ankyrin from band 3, and then tetrameric MetHb cross-link with the cytoplasmic domain of oxidized band 3 dimer [24]. In addition to MetHb, the abnormal Hb in all kinds of hemoglobinopathies is speculated to be the other main source of re-released Hb. α-thalassemia has the defect in α-globin syntheses, the relative excess of β-globin increases and the abnormal HbH (β_4) forms, which can bind with the membrane and lead to the increased Hb re-release.

Hb usually has interaction with spectrin, and the spectrin defect in HS patient interfere the binding of Hb with membrane, so the membrane-binding Hb and re-released Hb decreased obviously. There are five main kinds of erythrocyte skeleton proteins; defect of different cytoskeletal protein might leads to different results.

In conclusion, the change of re-released Hb is only an experimental phenomenon of HRT, and the mechanism of HRT has not been clear very much. In the future, more and more studies are needed to clarify these.

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