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Status of Red Cell Membrane Protein Phosphorylation in Thalassemia

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ABSTRACT The steady-state levels of β-spectrin phosphorylation in HbH (α-thalassemia 1/α-thalassemia 2), HbH/HbConstant Spring (α-thalassemia 1/HbCS, hereafter called HbH/HbCS) and nonsplenectomized β-thalassemia/HbE (hereafter called β-thal/HbE) red cells were quantitated using Western hybridization. Phosphorylation of β-spectrin serine and threonine residues from thalassemic samples was not significantly different from normal control. However, tyrosine phosphorylation was higher than normal control in HbH ($p<0.01$), HbH/HbCS ($p<0.05$) and β-thal/HbE ($p<0.05$) samples. Tyrosine phosphorylation of β-spectrin was observed only in the presence of vanadate, a phenomenon not hitherto reported. As tyrosine kinase activity has been linked to oxidative stress, loss of membrane lipid asymmetry and procoagulant activity of the red cell membrane, the observed increase in β-spectrin tyrosine phosphorylation of the thalassemic red cells is likely, at least in part, to account for these parameters.

KEYWORDS: Thalassemia, tyrosine phosphorylation, spectrin.

INTRODUCTION

The thalassemic red cell 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. The rheological properties of red cells are principally controlled by the protein cytoskeleton underlying the membrane. Although both α- and β- thalassemic red cells are less deformable than normal red cells, the two cell types exhibit differences in membrane stability, namely, the maximum extent of deformation that a membrane can undergo beyond which it fragments. The α-thalassemic membrane exhibits slightly increased stability whereas β-thalassemic membrane shows markedly decreased stability.[1, 2] It is believed that differences in the properties inherent to the α- and β- globin chains are the basis for the pathophysiological differences observed between α- and β- thalassemic red cells.[1, 2]

The morphology and mechanical properties of the red cell membrane are controlled by the cytoskeletal network underlying the lipid bilayer. Spectrin is the principal structural element of the red cell cytoskeleton, regulating membrane cytoskeletal functions.[3] Indeed, individuals expressing a reduced level or a mutant form of spectrin, as in hereditary spherocytosis and hereditary elliptocytosis, exhibit abnormal shaped red cells with altered mechanical properties.[3]

Spectrin has been shown to be a substrate for a number of kinases *in vitro*.[4] Phosphorylation sites have been mapped to 1 threonine and 3 serine residues located near the carboxy terminal of the β-chain.[5] Nevertheless, the role of spectrin phosphorylation *in vivo* is not yet fully understood. Manno *et al* (1995) have shown, by *in vitro* ^{32}P-incorporation and mechanical assay, that the extent of spectrin phosphorylation is inversely correlated with deformability of normal ghost cells, implying a role of spectrin phosphorylation in the mechanical control of the red cell cytoskeleton.[6] Based on these data, one should predict that membrane with marked reduction in deformability, such as that seen with thalassemic red cells, should exhibit a higher level of spectrin phosphorylation. In agreement with Manno's group, Erusalimsky et al (1985) reported that intact thalassemic red cells incorporate less [^{32}P]- orthophosphate compared to normal control, suggesting a higher basal level of phosphorylation in thalassemic cells.[7] However, when isolated ghost membranes, rather than intact red cells, were used in the assay, no significant difference in the extent of ^{32}P incorporation was observed between thalassemic and normal red cells.[7]

Instead of quantitating ^{32}P-incorporation in membrane as in previous work, in this study we

assessed the steady-state level of β-spectrin phosphorylation in thalassemic membranes using an immunological approach. Our data showed that β-spectrin phosphorylation at tyrosine residues of HbH, HbH/HbCS and β-thal/HbE samples, was significantly greater than that of normal control.

Materials and Methods

Patients

Normal control subjects were healthy volunteers with normal hematological profile and hemoglobin typing. Blood samples were obtained from thalassemic subjects of the Out-Patient Division, Department of Hematology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand. These patients have been identified through standard hematological criteria and had not received blood transfusion for at least 3 months prior to blood sample collection. The hematological profiles of the patients (HbH, HbH/HbCS and β-thal/HbE) are shown in Table 1.

Materials

Chemicals for red cell ghost preparation, sodium dodecyl sulfate acrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Sigma (St Louis, MO,USA). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH, USA). Monoclonal anti-phosphoserine, -phosphothreonine, and –phosphotyrosine antibodies were obtained from Sigma. Horse-radish peroxidase (HRP)-conjugated goat-anti-mouse IgG was from Dako (Glostrup, Denmark). ECL ™ reagent and X-ray film were from Amersham Life Science. GS 700 imaging densitometer, SDS-PAGE and Immunoblot apparatus were from Biorad (Hercules, CA, USA).

Preparation of ghost membrane

Ghost cell preparation was adapted from the method of Dodge et al[8], and was performed within 6 hours of blood collection. Five ml of EDTA blood were centrifuged at 1,000 g for 10 min, after which the plasma and buffy coat were removed. The packed red cells were washed 3 times with and finally resuspended in an equal volume of isotonic buffer (140 mM NaCl, 5 mM Tris-HCl, pH 7.4), followed by lysis in 14 volumes of hypotonic buffer (7 mM NaCl, 5 mM Tris-HCl, pH 7.4, 100 mM phenylmethylsulfonyl fluoride). For the detection of tyrosine phosphorylation, packed red cells were pre-incubated in an equal volume of isotonic buffer containing 1 mM sodium meta-vanadate at 37 °C for 30 min, followed by washing in isotonic buffer and lysis. The lysate was then centrifuged at 10,000 g, 4 °C, for 1 hour to sediment the ghost preparation, followed by 3 more washes in hypotonic buffer. The washed ghost pellets were then resuspended in sample buffer (0.125 M Tris-HCl, pH 6.8, 6% SDS, 20% glycerol, 10% β-mercaptoethanol) and boiled for 2 min prior to analysis by SDS-PAGE.

Quantitation of β-spectrin

Ghost membrane proteins were separated by SDS-PAGE at a constant voltage of 150 V for 1.5 hours. Gels were stained with 0.2% (w/v) Coomassie Blue R 250 in 50% (v/v) 95% ethanol and 7% (v/v) glacial acetic acid at room temperature for 30 min, and destained in 25% (v/v) 95% ethanol and 12.5% (v/v) glacial acetic. After drying, protein bands corresponding to the position of β-spectrin were quantitated using the GS 700 Imaging densitometer (Biorad) (Hercules, CA, USA).

Quantitation of phosphoserine, -threonine and -tyrosine in β-spectrin

A duplicate gel was run as described above, but instead of staining with Coomassie Blue, the gel was transferred by electroblotting onto nitrocellulose membrane at 100 V for 2 hours. The blotted membrane was incubated in blocking buffer (5% skim milk, 150 mM NaCl, 5 mM Tris-HCl, pH 8.8, 0.2% Tween 20) at 4 °C overnight, followed by a 1:2000 dilution of monoclonal antibodies against

Table 1. Hematologic data of the normal and thalassemic patients used for the determination of β-spectrin tyrosine phosphorylation.

Subjects	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Normal (n=14)	12.46 ± 2.12*	37.41 ± 5.45	87.94 ± 4.51	28.36 ± 2.68	33.35 ± 1.52
HbH (n=12)	8.18 ± 2.17	29.28 ± 7.69	71.55 ± 7.44	20.03 ± 2.08	27.99 ± 1.79
HbH/HbCS (n=5)	7.82 ± 1.47	29.78 ± 4.48	79.2 ± 3.42	20.84 ± 0.76	26.2 ± 1.25
β-thal/HbE (n=12)	6.39 ± 1.91	20.42 ± 5.92	59.63 ± 4.62	18.63 ± 1.96	31.23 ± 1.74

* Mean ± SD; Hb = hemoglobin; Hct = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration

phosphoserine, -threonine and –tyrosine in blocking buffer at room temperature for 2 hours. The membrane was washed twice with TBST buffer (150 mM NaCl, 5 mM Tris-HCl, pH 8.8, 0.2% Tween 20) for 10 min each time, before incubating with 1:1000 dilution of HRP-conjugated goat anti-mouse IgG in blocking buffer for 2 hours. After washing with TBST buffer twice, 10 min each time, the antigen-antibody complex was visualized using the ECL™ method and exposure to X-ray film. The signal corresponding to the position of β-spectrin was quantitated by scanning the X-ray film in a GS 700 Imaging densitometer. The amounts of phosphoserine, -threonine and –tyrosine in β-spectrin in each sample were normalized with respect to the amounts of β-spectrin.

Results

The steady-state levels of β-spectrin phosphorylation at serine, threonine and tyrosine residues were determined using an immunoblot technique. Phosphorylation of β-spectrin at serine and threonine residues were not significantly different between normal control and all the thalassemic cell types examined (HbH, HbH/HbCS, β-thal/HbE). Figure 1 shows a typical set of results for normal, HbH and β-thal/HbE red cell membranes. The presence of okadaic acid did not help to increase the signal intensity, indicating that serine/threonine phosphatases were not a problem in the ghost preparation (data not shown). In addition to β-spectrin, α-spectrin reacted with the anti-phosphoserine and anti-phosphothreonine antibodies. Phosphorylation of α-spectrin has previously been shown in low-salt extract of red cell membrane stimulated with cAMP.[4]

Tyrosine phosphorylation of β-spectrin became evident only in the presence of vanadate, and its level was significantly higher in HbH ($p<0.01$), HbH/HbCS ($p<0.05$) and β-thal/HbE ($p<0.05$) compared to normal control (Table 2). Figure 2 shows the results for a set of samples from normal and β-thal/HbE red cell membranes. In addition to β-spectrin, two other membrane proteins (215 Kd and 90-100 Kd) reacted weakly with the anti-phosphotyrosine antibody; presumably these were ankyrin and band 3 respectively, based on their migration in SDS-PAGE. These two proteins have also been shown to be phosphorylated at tyrosine residues.[4]

Discussion

The thalassemic red cell membrane offers a useful system with which to study the role of spectrin phosphorylation in regulating the mechanical

Table 2. β-spectrin tyrosine phosphorylation in normal and thalassemic ghosts.

Subjects	Normalized values of tyrosine phosphorylation
Normal (n=14)	0.69 ± 0.47 *
HbH (n=12)	2.14 ± 1.74 **
HbH/HbCS (n=5)	1.48 ± 0.92 ***
β-thal/HbE (n=12)	1.35 ± 0.95 ***

* Mean ± SD; ** $p < 0.01$ versus normal control ghost; *** $p < 0.05$ versus normal control ghost.

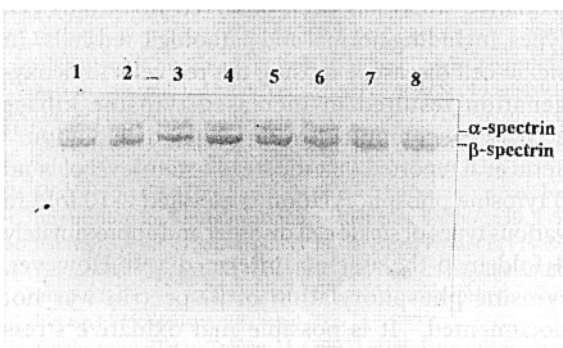

Fig 1. Immunoblot of ghost proteins probed with anti-phosphoserine monoclonal antibody. Lane 1, normal control; lanes 2-3, HbH; Lanes 4-8, β-thal/HbE. One hundered μg of red cell membrane protein were loaded in each lane of the SDS-PAGE gel. After electrotransfer, the antibody-antigen complex was visualized by ECL method. The two bands reacted with the antibody correspond to α- and β- spectrin, as determined by SDS-PAGE and Coomassie staining of a duplicate gel.

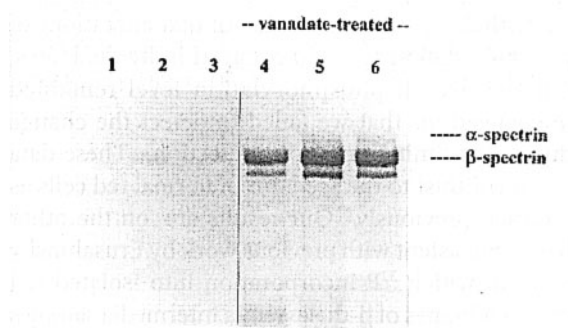

Fig 2. Immunoblot of ghost proteins probed with anti-phosphotyrosine monoclonal antibody. Lanes 1 & 4, normal control; lanes 2-3 & 5-6, β-thal/HbE. One hundred μg of red cell membrane protein were loaded in each lane of the SDS-PAGE gel. After electrotransfer, the antibody-antigen reaction was visualized using ECL method. Strong immunoreaction was evident in the presence (lanes 4-6), but not in the absence (lanes 1-3), of meta-vanadate. Positions corresponding to α-spectrin and β-spectrin are indicated.

property of the red cell membrane. Unlike normal red cell membranes, which are highly deformable, thalassemic membranes are more rigid, due to oxidative damage induced by membrane-bound unmatched globin chains.[1,2] The effects of the α- and β- globin chains on membrane stability are different at high shear stress. α- Thalassemic membranes exhibit mechanical stability, whereas β-thalassemic membranes are fragile.[1, 2]

Normal red cell membrane's deformability gradually decreases as the degree of β-spectrin phosphorylation increases, suggesting that this phenomenon is regulated by phosphorylation of β-spectrin.[6] If that is the case, one should expect that thalassemic red cells, whose membrane deformability is markedly reduced, should exhibit a higher level of β-spectrin phosphorylation compared to that of the normal red cells. With that in mind, we have quantitated the steady-state level of β-spectrin phosphorylation in various thalassemic red cell membranes by immunoblot, as a first step towards understanding if, and how, β-spectrin phosphorylation regulates red cell membrane mechanical properties.

On 6% SDS-PAGE, α- and β- spectrin appear to be the only two major proteins that reacted with anti-phosphoserine and anti-phosphothreonine antibodies. Our data show that the steady-state level of β-spectrin phosphorylation at serine and threonine residues was not significantly different between thalassemic samples and normal controls, indicating that the reduced deformability of the thalassemic red cells was not associated with β-spectrin phosphorylation at these amino acids. Nevertheless, we cannot rule out that alterations of phosphorylation level do occur at individual sites, but the overall phosphorylation level remained unchanged, or that we failed to detect the change due to the limitation of our detection. These data are in contrast to the scenario in normal red cells as reported previously.[6] Our results are, on the other hand, consistent with previous work by Erusalimsky et al, in which ^{32}P incorporation into isolated red cell membranes of β-thalassemia intermedia samples was shown to be similar to that of the normal membranes, although ^{32}P incorporation into intact thalassemic red cells was somewhat less than that of the normal cells.[7]

The steady-state level of β-spectrin tyrosine phosphorylation could not be detected by the immunoblot method, unless meta-vanadate was present during ghost preparation, indicating a relatively higher rate of dephosphorylation over phosphorylation of β-spectrin. Although vanadate has been shown to act primarily by inhibiting protein tyrosine phosphatase[9, 10], vanadium compounds have been suggested to exert multiple effects on diverse cellular processes including direct activation of tyrosine kinase[11], which could probably account for the increased tyrosine phosphorylation observed. It is also possible that vanadate indirectly affects the level of β-spectrin tyrosine phosphorylation by controlling Ca^{2+}-ATPase and hence a number of Ca^{2+}-dependent kinases which phosphorylate β-spectrin, such as casein kinase and cAMP-dependent protein kinase. Although Mg^{2+}, Na^{+} and K^{+}, but not Ca^{2+} ions have been shown to enhance inhibition of Ca^{2+}-ATPase by vanadate[12-14], it is not known how this reaction is modified in the thalassemic red cells, where intracellular concentrations of many ions are altered.

This is the first report of tyrosine phosphorylation of β-spectrin, although tyrosine phosphorylation of the non-erythroid homolog of spectrin, foldrin, has been reported.[15] The inability of Manno et al[6] to detect β-spectrin reactivity with anti-phosphotyrosine antibody is probably due to the lower concentration of vanadate present in their ghost preparation (10 μM) compared to this study (1 mM).

Tyrosine phosphorylation was significantly higher than that of normal control in HbH (n=12, $p<0.01$), HbH/HbCS (n=5, $p<0.05$) and β-thal/HbE (n=12, $p<0.05$) samples. Tyrosine phosphorylation of membrane proteins has been implicated in regulating various functions of the red cell, including glycolysis, morphology and ion transport activity.[4, 16-17] Increased tyrosine kinase activity induced by oxidative stress has been observed in various cell types, including normal and pathologic red cells. In sickle-cell disease, exposing the red cells to deoxygenation resulted in increased tyrosine kinase activity, concommittant with cellular dehydration.[18] Terra et al reported that the steady-state level of band 3 tyrosine phosphorylation is elevated 2-10 fold in various types of sickle cell disorder and approximately 4 fold in β-thalassemia intermedia.[19] However, tyrosine phosphorylation of β-spectrin was not documented. It is possible that oxidative stress affects β-spectrin phosphorylation by modification of spectrin kinases/phosphatases, or by alterating the spatial arrangement of membrane proteins.[18-22]

Reduced tyrosine kinase activity of membrane proteins is observed in Scott syndrome, a hereditary bleeding disorder characterized by a deficiency in prothrombinase activity caused by defective phospholipid scrambling activity of the blood cell

membrane.[23] The decrease in membrane phospholipid scrambling results in reduction in phosphatidyl serine (PS) exposure and in prothrombinase activity on the cell surface. On the other hand, thalassemic red cells exhibit an increase in PS exposure and prothrombinase activity[24,25], correlating with the elevation of tyrosine kinase activity. Thus, under oxidative stress, high PS exposure and prothrombinase activity of thalassemic red cells are accompanied by increased β-spectrin tyrosine phosphorylation.

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