Increased erythrocyte superoxide dismutase activities in β^0 -thalassaemia/haemoglobin E and in haemoglobin H diseases

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SUMMARY Erythrocyte superoxide dismutase activities were measured in 45 subjects, 15 each of β^0 -thalassaemia/haemoglobin (Hb) E disease, Hb H disease, and normal. The erythrocyte superoxide dismutase activities were significantly higher in the patients with β^0 -thalassaemia/Hb E and Hb H diseases than in the normal subjects. The increase of erythrocyte superoxide dismutase activities is most likely due to abnormalities specific to thalassaemic red cells rather than an increased number of younger red cells for reticulocytes and nucleated red blood cells did not affect the enzyme activity. Patients with β^0 -thalassaemia/Hb E disease with lower haemoglobin concentration had significantly higher superoxide dismutase activities. In all 45 subjects haemoglobin concentrations and superoxide dismutase activities were inversely correlated ($r = -0.60$ ($p < 0.001$)). This indicates that the amounts of superoxide generated in the red cells may, at least partly, determine severity of red cell damage and thus severity of disease; the increased superoxide dismutase activity in thalassaemia is a response to superoxide generated in greater amounts because of accumulation of excessive globin chains and iron in the red cells. The superoxide dismutase activities in Hb H disease, an α -thalassaemic disease, were found to be strikingly increased, higher than in β^0 -thalassaemic disease or other conditions.

In a large number of patients with β^0 -thalassaemia/haemoglobin (Hb) E disease (in untransfused state) haemoglobin concentrations vary between 3 and 13 g/dl (mean = 7 g/dl). Why patients with the same genotype differ so much in severity of disease is interesting and important. One aspect which we have investigated concerns the relation between erythrocyte superoxide dismutase activity and severity of thalassaemia.

In the red blood cell, superoxide dismutase is an enzyme that protects the cell from the deleterious effect of the superoxide free radical (O_2^-).¹ It catalyses the breakdown of superoxide according to the reaction: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$.² The hydrogen peroxide (H_2O_2) is less dangerous than superoxide and is further reduced to water and oxygen by catalase and glutathione peroxidase.¹³ The superoxide is thought to be generated in the red blood cell by autoxidation of haemoglobin. It has been found that the autoxidation of oxyhaemoglobins

or isolated oxyhaemoglobin chains produces superoxide.⁴⁻⁶ Autoxidation of unstable haemoglobins or isolated α and β chains, however, releases superoxide at a greater rate than autoxidation of Hb A.¹⁷ Accumulation of excessive α or β -globin chains is a typical finding in thalassaemic red blood cells.⁸ Therefore, the superoxide is suspected to be generated more easily and/or in a larger amount in the thalassaemic than in the normal red blood cells. It is thus important to measure the erythrocyte superoxide dismutase activity in thalassaemia and examine its relation to severity of the disease. Any relation between erythrocyte superoxide dismutase activity and the severity of thalassaemia should reveal whether superoxide dismutase activity determines the severity of the disease or not.

Subjects

All thalassaemic subjects were patients of Division of Haematology, Department of Medicine, Faculty of

Medicine Siriraj Hospital and normal subjects were hospital personnel. Altogether 45 subjects were examined, 15 each from β^0 -thalassaemia/Hb E disease, Hb H disease and normal. In the group of β^0 -thalassaemia/Hb E disease five patients had had splenectomy. This group can be divided into two subgroups of different severity. Group 1 with haemoglobin concentrations > 8 g/dl consisted of six patients, all with spleens intact; group 2 with haemoglobin concentrations < 8 g/dl consisted of nine patients, five of whom had had splenectomies. Seven patients with Hb H disease had the α -thalassaemia 1/ α -thalassaemia 2 genotype, and eight had the α -thalassaemia 1/Hb Constant Spring (Hb CS) genotype. Diagnostic criteria for these thalassaemic diseases were according to previous reports.^{9 10}

Material and methods

Riboflavin and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co. A Beckman DB-G grating spectrophotometer was used in the study.

About 5–6 ml of venous blood were drawn both into EDTA and into heparinised tubes. EDTA blood was used for routine complete blood count and haemoglobin typing and the heparinised blood was used for superoxide dismutase study.

HAEMATOLOGICAL STUDIES AND HAEMOGLOBIN TYPING

Red blood cell count (RBC), mean corpuscular volume (MCV), haematocrit (HCT) and white blood cell count (WBC) were determined in a Coulter Counter model ZF 6. Haemoglobin concentrations were measured on a Coulter Haemoglobinometer. Haemoglobin types were characterised by starch gel electrophoresis in Tris-EDTA-Borate buffer, pH 8.6.^{11 12}

ERYTHROCYTE SUPEROXIDE DISMUTASE ASSAY

The heparinised blood was centrifuged; plasma and buffy coat were removed by aspiration. Packed red cells were washed three times with normal saline. Erythrocyte superoxide dismutase activities in chloroform-ethanol extract were measured by

following the method of Winterbourn *et al.*¹³ This method depends on the ability of the enzyme to inhibit the reduction of NBT by superoxide, which is generated by the reaction of photoreduced riboflavin and oxygen.

Results

Results are summarised in Table 1 and illustrated in Figs. 1 and 2. Thalassaemic erythrocytes had higher superoxide dismutase activities than the normal erythrocytes, both on the basis of U/g Hb and U/cell. In Hb H disease superoxide dismutase activities were strikingly higher than in β^0 -thalassaemia/Hb E disease. Differences between normal and β^0 -thalassaemia/Hb E, normal and Hb H disease, and

Fig. 1 Erythrocyte superoxide dismutase activities as U/g Hb of normal subjects and patients with β^0 -thalassaemia/Hb E and with Hb H diseases.

Table 1 Mean $\pm$ SD of haemoglobin concentrations and erythrocyte superoxide dismutase activities

	Normal (n = 15)	β^0 -thalassaemia/ Hb E (n = 15)	Hb H disease (n = 15)
Haemoglobin (g/dl)	13.4 $\pm$ 1.20	8.1 $\pm$ 1.83	9.3 $\pm$ 1.38
Superoxide dismutase U/g Hb	2563 $\pm$ 376	4392 $\pm$ 888	6154 $\pm$ 1169
U/cell ($\times 10^{-4}$)	8.30 $\pm$ 1.33	11.05 $\pm$ 3.55	13.98 $\pm$ 2.34

Table 2 Haematological data and erythrocyte superoxide dismutase activities of mild and more severe β^0 -thalassaemia/Hb E disease

		Hb (g/dl)	HCT (%)	RBC ($\times 10^{12}/l$)	MCV (fl)	Superoxide dismutase	
						U/g Hb	$\times 10^{-8}$ U/cell
<i>Group 1</i>							
Mild β^0 -thalassaemia/Hb E	Mean	9.9	28.6	3.94	72	3901	9.77
(n = 6)	SD	1.37	4.68	0.56	4	481	1.15
<i>Group 2</i>							
More severe β^0 -thalassaemia/Hb E	Mean	7.0	20.6	2.74	75	4718	11.90
(n = 9)	SD	0.95	3.17	0.37	5	967	1.83
						p < 0.001	p < 0.05

Fig. 2 Erythrocyte superoxide dismutase activities as U/cell of the same three groups of subjects as shown in Fig. 1.

β^0 -thalassaemia/Hb E and Hb H disease were all statistically significant ($p < 0.001$). Haematological data and erythrocyte superoxide dismutase activity of the mild (group 1) and the more severe (group 2) β^0 -thalassaemia/Hb E are shown in Table 2. The group 2 patients (more severe) had significantly higher superoxide dismutase activities than the patients in group 1 (mild) ($p < 0.001$ for U/g Hb and $p < 0.05$ for U/cell).

To test whether the higher superoxide dismutase activity in the patients with more severe disease was attributable to greater reticulocytosis superoxide dismutase activity was also measured in the

Table 3 Superoxide dismutase activities in top and bottom layers of red blood cells from three patients with autoimmune haemolytic anaemia

Subject	Reticulocyte (%)	Superoxide dismutase (U/g Hb)
Case I (Hb = 10.0 g/dl)		
Whole blood	1.8	
Top layer	5.7	3175
Bottom layer	0.4	2604
Case II (Hb = 10.0 g/dl)		
Whole blood	6.6	
Top layer	13.6	2665
Bottom layer	1.0	2732
Case III (Hb = 5.4 g/dl)		
Whole blood	19.0	
Top layer	38.6	3019
Bottom layer	9.0	2807

reticulocyte-rich and reticulocyte-poor fractions of red blood cells from three patients with autoimmune haemolytic anaemia. The results are shown in Table 3.

Discussion

The erythrocyte superoxide dismutase activities in our thalassaemic patients were significantly higher than normal. With the exception of one value the distribution of superoxide dismutase activities in β^0 -thalassaemia/Hb E disease did not overlap with that of normal, when calculated as U/g Hb. When calculated as U/cell two values of β^0 -thalassaemia/Hb E patients came within the normal range. One unusually high value in the control red cells was probably due to error in red cell counting. The erythrocyte superoxide dismutase activities in Hb H disease were in general higher than in β^0 -thalassaemia/Hb E disease.

There have been only three reports on erythrocyte superoxide dismutase activity in thalassaemia and their results conflict. Concetti *et al*¹⁴ and Gerli *et al*¹⁵ reported no significant difference in erythrocyte superoxide dismutase values between homozygous β^0 -

Table 4 *Superoxide dismutase activities in splenectomised and non-splenectomised patients (all means $\pm$ SD)*

	Hb (g/dl)	WBC ($\times 10^9$ /l)	NRC* (10^9 /l)	Superoxide dismutase activity	
				U/g Hb	$\times 10^{-8}$ U/cell
Splenectomised (n = 5)	7.4 $\pm$ 0.20	13.67 $\pm$ 7.42	51.03 $\pm$ 35.60	4466 $\pm$ 861	11.54 $\pm$ 1.45
Non-splenectomised (n = 10)	8.5 $\pm$ 2.18	7.58 $\pm$ 1.94	0.55 $\pm$ 0.59	4354 $\pm$ 945	10.80 $\pm$ 2.09
				p >> 0.50	p > 0.40

*Nucleated red cells (NRC) were calculated from total nucleated cell counts (WBC + NRC) and NRC/WBC ratios in the blood smear.

thalassaemia subjects and normal controls. Vanella *et al*¹⁶, however, observed significant differences in erythrocyte superoxide dismutase activities between similar groups of subjects. It should be pointed out that the patients in these three series had received blood transfusion shortly before the studies. Most of our patients received no blood transfusion although a few had been transfused several months before superoxide dismutase measurement. Thus the superoxide dismutase activities found in our study represented a truer picture of this enzyme activity in thalassaemic red cells.

There is a question whether raised erythrocyte superoxide dismutase activities are due to abnormalities specific to thalassaemia or due to an increase in the proportion of younger red cells. In general erythrocyte enzyme activities are greater in younger red cell populations.¹⁷ Thalassaemic diseases are usually associated with reticulocytosis and also often with the presence of nucleated red cells. We checked the effect of reticulocytosis on the erythrocyte superoxide dismutase activities in three patients with autoimmune haemolytic anaemia as shown in Table 3. The red cells were separated into a reticulocyte-rich top layer and a reticulocyte-poor layer and superoxide dismutase activities were determined. In spite of reticulocyte counts between the two layers differing by as much as 14 times, the superoxide dismutase activities were not significantly different. Furthermore the erythrocyte superoxide dismutase activity in splenectomised patients with β^0 -thalassaemia/Hb E did not differ from that of the non-splenectomised ones, although the former had numerous NRC (Table 4). Thus the increased erythrocyte superoxide dismutase activities in thalassaemia found in our study were unlikely to be due to reticulocytosis or reticulocytosis alone. Most likely it was caused by increased production of

superoxide in thalassaemic red cells. In thalassaemic red cells there are always excessive globin chains. Isolated haemoglobin subunits have been shown to generate superoxide.^{6,7} Also lipid membrane peroxidation of thalassaemic red cells is increased.¹⁸ These indicate that superoxide and other free radicals are generated in increased amounts in thalassaemic red cells and are probably important factors causing red cell membrane damage in this disease. The increased superoxide dismutase activity in thalassaemic red blood cells may be a response to the increase of the free radicals.

Defective globin chain synthesis in thalassaemia leads to decreased haem synthesis.¹⁹ This results in accumulation of non-haem iron in the red cells as indicated by the presence of sideroblast and increased erythrocyte ferritin and haemosiderin.²⁰⁻²² Iron is a strong oxidant; together with haemoglobin it leads to superoxide generation by stimulating haemoglobin autoxidation.¹ Excessive amount of iron in the thalassaemic red cells may be another contributory factor in increased superoxide production.

Erythrocyte superoxide dismutase activities in the splenectomised and non-splenectomised patients did not significantly differ (Table 4). Splenectomised patients had numerous nucleated red cells. The presence of nucleated red cells did not seem significantly to affect the erythrocyte superoxide dismutase activities. Splenectomy in thalassaemia is usually associated with increased proportion of red cells with excessive haemoglobin chains which appear as inclusion bodies.²³ These inclusion bodies may be expected to generate more superoxide but perhaps not enough to result in significantly increased superoxide dismutase activities.

As shown in Table 2 the group of patients with haemoglobin <8 g/dl had significantly higher erythrocyte superoxide dismutase activities (p < 0.001 and p < 0.05) than the group with haemoglobin >8 g/dl. The erythrocyte superoxide dismutase activities showed inverse correlation with haemoglobin concentrations with a correlation coefficient of -0.60 (Table 5).

Whether a raised erythrocyte superoxide dismutase activity determines the severity of the thalassaemic disease or not is interesting. If the

Table 5 *Correlation between superoxide dismutase activities and haemoglobin concentrations of all 45 subjects*

	Correlation (γ)	p
Hb v superoxide dismutase (U/g Hb)	-0.609	<0.001
Hb v superoxide dismutase (U/cell)	-0.604	<0.001

erythrocyte superoxide dismutase activity is independent to severity of the disease one would expect that thalassaemic patients with higher erythrocyte superoxide dismutase activities would run a milder course, for superoxide would be neutralised to a larger extent and would therefore be less deleterious to red cells. However, the opposite was observed in this study—that is, higher erythrocyte superoxide dismutase activities were associated with more severe disease. This indicates that increased erythrocyte superoxide dismutase activities in thalassaemia is a reaction to or compensates for the increase in superoxide.

In Hb H disease the erythrocyte superoxide dismutase activities were strikingly high, probably highest among conditions so far reported. This has not been known before. This is probably due to the presence of excessive β -chains generating increased amounts of superoxide in red blood cells. The red blood cells of patients with Hb H disease have also been found to show a remarkable increase in glutathione peroxidase activity.²⁴ The most likely explanation is that erythrocytes with Hb H, which is an unstable haemoglobin, generate superoxide in increased amounts leading to increased superoxide dismutase activities which catalyse the breakdown of superoxide into hydrogen peroxide resulting in high activities of glutathione peroxidase, an enzyme participating in conversion of hydrogen peroxide to water and oxygen.

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