Effect of Oxidative Stress on Leukocyte DNA Damage in β-thalassemic Patients

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ABSTRACT

Objective: Thalassemia is a group of genetic diseases where hemoglobin synthesis is impaired. Chronic anemia leads to increased dietary iron absorption, which develops into iron overload pathology. Treatment through regular transfusions increases oxygen capacity but also provides excess iron deposited in tissues. Thalassemia patients are particularly at risk of free radical induced damage. This study has investigated that effect of oxidative stress on leukocyte DNA damage in β-thalassemic patients.
Methods: Patients with post-splenectomized and transfusion dependent β-thalassemia major; age 4-15 years were recruited. Leukocyte DNA strand breakage was performed by single cell gel electrophoresis or comet assay which was analyzed as comet tail moment.
Results: β-thalassemic patients (n=30); age 4-15 years, 25 FA and 5 EF of hemoglobin typing. Peripheral blood mononuclear cell of β-thalassemic patients showed significant higher comet tail moment than normal control (165.17 ± 2.10 vs 28.0 ± 2.50 pixels, $p < 0.05$).
Conclusion: Peripheral blood mononuclear cell of β-thalassemic patients had an increased DNA damage when compared with normal control. This evidence demonstrated that free radical and iron overload might be a cause of leukocyte DNA damage.

Keywords: Comet assay; DNA damage

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Thalassemia is a group of genetic diseases where hemoglobin synthesis is impaired. This chronic anemia leads to increase dietary iron absorption, which develops into iron overload pathology. Treatment through regular transfusions increases oxygen capacity but also provides iron through the hemoglobin of red cells. An essential treatment, in parallel with transfusions, is the use of chelating agents to remove the excess iron deposited in tissues. These deposits are found in the liver, spleen, heart, and pancreas and are associated with cardiac failure and diabetes. Thalassemic patients are particularly at risk of free radical induced damage.

In thalassemia the oxidative damage *via* free radical formation, the lipid peroxidation[1] and iron toxicity[2] has been elucidated. The mechanisms facilitating oxidative damage are multifactorial, and result from the presence of excess unpaired globin chains, the high intercellular content of non-hemoglobin iron, and the low concentration of normal hemoglobin encountered in thalassemic red blood cells (RBCs).

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As a direct result of genetic defect, thalassemic RBCs contain an excess amount of hemoglobin subunits which are β-globin chains accumulated in β-thalassemic RBCs and β-globin chains of β-thalassemic RBC.[3] Following oxidation, these unstable hemoglobins are known to generate in vitro free oxygen radicals. These free radicals form were more methemoglobins and then reversible and were irreversible hemichromes. Finally, they disintegrate to heme and globin moieties, loading the RBC membrane with denatured globin chains, heme and iron.

Free heme is capable of interacting with either lipid bilayer or with cytoskeletal membrane proteins such as protein 4.1, actin and spectrin.[4] Hemin degradation is accompanied with iron release, which plays a significant role in RBC oxidation.

Trace metals, e.g. copper and iron, have been implicated as causative agents in excessive generation of endogenous free oxygen radicals, which are capable of causing oxidative stress. In thalassemic RBCs, non-hemoglobin iron is increased. Free irons or aggregates of ferritin and deposits of hemosiderin are catalysts of lipids and protein peroxidation via the Fenton reaction[5] which is:

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$$

Data have been accumulated suggesting that increased lipid peroxidation takes place in thalassemic RBCs. The organization of membrane phospholipids in thalassemic RBCs is depicted with most of the phosphatidylcholine (PC) being present in the outer bilayer leaflet, and phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner leaflet. Not only, the distribution of membrane lipids were abnormal, but a lower percentage of PE could be detected together with a decrease in the percentage of polyunsaturated fatty acids (PUFAs), such as arachidonic acid.[6] Another indicator is the high malonyldialdehyde (MDA) contents of thalassemic RBCs detected following exogenous oxidant stress. MDA is the result of PUFA oxidation. MDA is formed from the breakdown of polyunsaturated fatty acids and serves as a convenient index for the determining the extent of the peroxidation reaction[7].

Hydroxyl radicals ($OH^{\bullet}$) are the most reactive oxygen free radical species capable of direct oxidative damage to macromolecules including DNA, protein, and lipid membranes. Of note, the effect of excess results of oxygen free radicals, such as DNA strand breaks and membrane blebbing, match the hallmark feature of apoptosis. The production of $OH^{\bullet}$ mediates lipid peroxidation, oxidative damage to DNA and sulfhydryl modification of proteins.[8]

Recently, an effect of iron overload on cells oxidatively stressed with hydrogen peroxide (H_2O_2) has been determined. Iron overload was stimulated with ferric chloride ($FeCL_3$) and ferrous sulphate ($FeSO_4$). Human lymphocytes of male and female donors and human adenocarcinoma colonic cells showed an increase in DNA damage in the Comet assay after treatment with H_2O_2. Ferric chloride produced an increase in DNA damage in human colonic cells, but little or no damage in human lymphocytes, but ferrous sulphate produced a dose-related response. When H_2O_2 was combined with $FeCl_3$ or $FeSO_4$, the DNA damage produced was slightly greater than with H_2O_2 alone.[9]

In this study, peripheral blood mononuclear cells (PBMC) of normal cord blood and β-thalassemic patients were measured for DNA strand break by single cell gel electrophoresis or comet assay. The effect of oxidative stress on leukocyte DNA damage has been determined.

MATERIALS AND METHODS

Subjects

Thirty normal control samples include normal cord blood from the Delivery Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University. All have normal hematological indices. The drain blood before blood transfusion of post splenectomy thalassemic patients (n=30) were recruited from Department of Pediatrics, Faculty of Medicine, Chiang Mai University. About 20 mL of normal cord blood and whole blood of β-thalassemic patients were collected in acid citrated dextrose (ACD).

Complete blood cell count and peripheral blood mononuclear cell (PBMC) preparation

Complete blood cell count was analyzed by automated cell counter (Hycell Groope Lisabio) and hemoglobin typing was analyzed by a Primus Variant HPLC Analyzer (Primus Corporation). Peripheral blood mononuclear cells (PBMC) were isolated by centrifuging in Ficoll-Angiografin density gradient (1.077) at 400 g for 10 minutes. The PBMC was resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and about 100,000 units of penicillin and 0.1 g of streptomycin. Later, cells were counted and stained with Wright's Giemsa.

Examination of single cell gel electrophoresis (SCG) or comet assay

The cell suspension was prepared at 1.0 x 10^6 cells/mL in the RPMI-1640. Comet assay was modified from Henderson L, et al.[10] Visualization of DNA damage, after fluorochrome-staining, the DNA damage was observed under a 100 x objective on a fluorescent microscope. The Comet analysis system used in this study was developed by kinetic imaging, Zeiss Microscope model axioskop 2 with both fluorescent and light function linked to a charge-coupled device camera (CCD camera) to quantify the length of DNA migration and percentage of migrated DNA. To distinguish between populations of cells differing in size (e.g. PBMC *versus* NRBC), we also measured the nuclear diameter. Finally, the ISIS3 comet image program calculates comet tail moment (*mt*). The parameters were calculated according to the formula:

$$mt = Xm^{\bullet}Ft$$
$$Xm = [\sum_t (Ii^{\bullet}Xi)] / (\sum_t Ii)$$
$$Ft = (\sum_t Ii) / \sum cIi)$$

Comet tail moment (*mt*) is the product of the DNA portion in the comet tail (*Ft*) and its median (*Xm*). Where *Ii* was fluorescence intensity at point *i*, and *Xi* was the distance from the comet head median to point *i*. Indices under the sum symbols imply the summation region : *t*, the summation is over the comet tail only; *c*, the summation is over the whole comet. Generally, 30 randomly selected cells were analyzed and reported in the value of comet tail moment (*mt*) per sample.

Statistical analysis for in *vitro* data was independent-sample t test. Data were presented as mean ± standard deviation (SD). All statistical data analysis was performed using SPSS (version 11.5). A 2 sided p-value of 0.05 was considered statistically significant.

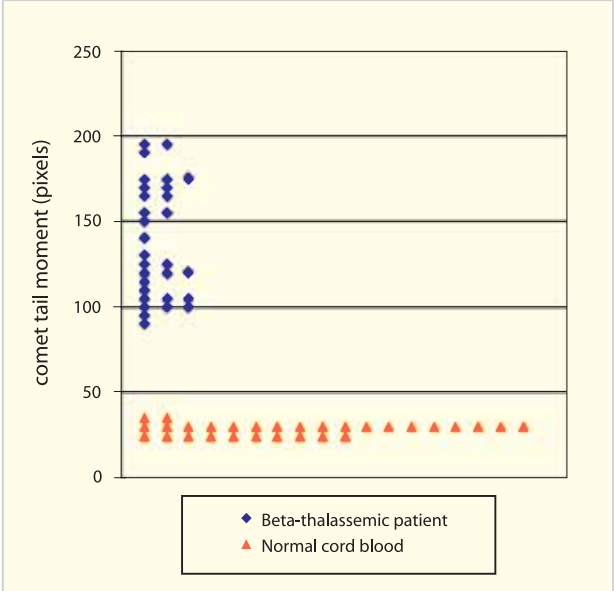

Fig 1. Comparison the comet tail moment of PBMC between normal cord blood and β-thlassemic patient. Mean ± SD comet tail moment of PBMC were 28.0 ± 2.5 pixels in normal cord blood (n=30) and 165.17 ± 2.10 pixels in β-thalassemic patient (n=30) which were significant (p<0.05).

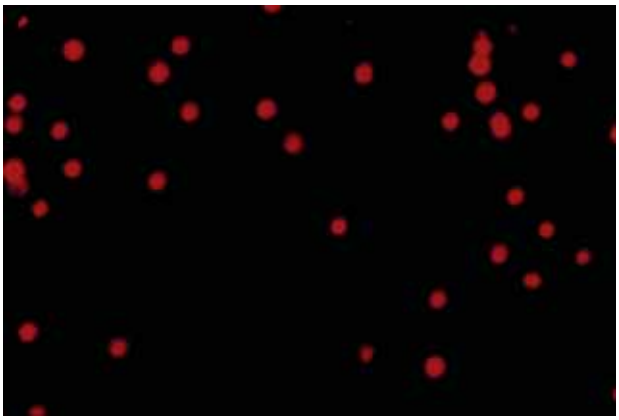

Fig 2. PBMC of normal cord blood showed undetectable of DNA fragmentation, resulting in the cellular DNA remaining in the nucleus. Digital image of PBMC was captured by Zeiss Microscope model axioskop 2 both fluorescent and light function linked to a charge-coupled device camera (CCD camera).

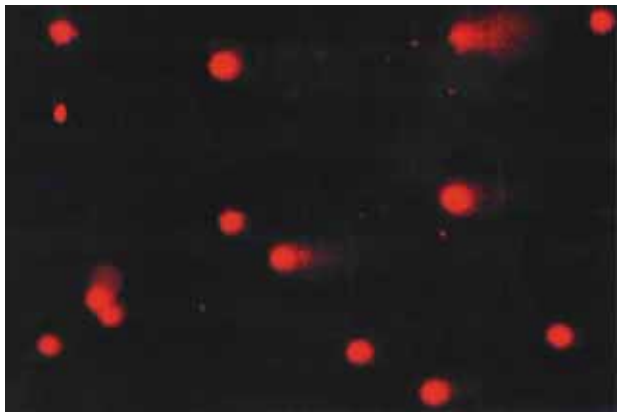

Fig 3. PBMC of β-thalasssemic patient showed a migration of the DNA out of the cell body. Digital image of PBMC was captured by Zeiss Microscope model axioskop 2 both fluorescent and light function linked to a charge-coupled device camera (CCD camera).

RESULTS

Eleven male and 19 female patients with post-splenectomized and transfusion dependent β-thalassemia major; age 4-15 years, 25 of FA and 5 of EF hemoglobin typing were studied. Normal control samples include normal cord blood and all had normal hematological indices (data not showed). When compared the values of DNA strand break of PBMC between normal cord blood and β-thalassemic patients it was found that the mean ± SD comet tail moment were 28.0±2.5 pixels in normal cord blood (n=30) and 165.17±2.10 pixels in β-thalassemic patients (n=30) which were significant ($p<0.05$). This revealed that there is DNA damage in PBMC of β-thalassemic patients (Fig 1-3).

DISCUSSION

In this study the hematological indices of 30 β-thalassemic patients showed severe anemia when compared with normal cord blood (data not shown). This result contributed ineffective erythropoiesis in the bone marrow and peripheral hemolysis of red blood cells.

Hydroxyl radicals ($OH^{\bullet}$) were the most reactive oxygen free radical species capable of direct oxidative damage to macromolecules including DNA, protein, and lipid membranes. The effect of excess results of oxygen free radicals, such as DNA strand breaks and membrane blebbing, match the hallmark feature of apoptosis. The effect of iron salt on cells oxidatively stressed measured in the comet assay was studied by Anderson D, et al. They found that the human lymphocytes from a male and a female donor and human adenocarcinoma colonic cells showed an increased in DNA damage in comet assay after treatment with H_2O_2.

In this study we found that peripheral blood mononuclear cells of β-thalassemia major had an increased DNA damage when compared with normal controls. This evidence demonstrated that free radical and iron overload might be a cause of leukocyte DNA damage.

Clearly, further studies are required to understand the effect of free radicals on basic cellular functions. At the same time, endeavors into identifying new and more efficacious antioxidants as a therapeutic strategy should continue. Indeed, elucidating the mechanism of action for some of the naturally occurring antioxidants, such as the potent enzymes mimetics and polyphenols, may lead to new therapeutic targets that can be modulated through more conventional pharmacological approaches.

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