Oxidative Stress and Antioxidant Status in β-Thalassemia Major: Iron Overload and Depletion of Lipid-Soluble Antioxidants

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Because of continuous blood transfusions, thalassemia patients are subjected to peroxidative tissue injury by the secondary iron overload. In accordance, analysis of serum from 42 β-thalassemia patients, aged 4 to 40 years, showed that the mean concentrations of conjugated diene lipid hydroperoxides (CD), lipidperoxides evaluated as malondialdehyde/thiobarbituric acid (MDA/TBA) adducts, and protein carbonyls increased about twofold with respect to control. Ferritin levels were positively correlated with the amount of MDA (r = 41; P = .007) and showed a positive trend with CD (r = .31; P = .07) and protein carbonyls (r = .35; P = .054), as further evidence of the deleterious effects of high tissue iron levels. Marked changes in the antioxidant pattern were also observed in all patients. Evidence is presented of a net drop in the concentration of ascorbate (−44%), vitamin E (−42%), vitamin A (−44%), β-carotene (−29%), and lycopene (−67%). On the other hand, an increase of uric acid and bilirubin was observed, whereas serum albumin and glutathione were in the normal range in all patients. As a result, the total serum weight iron, has been demonstrated, as well. Under various conditions, iron overload is still a major concern in homozygous β-thalassemia. Under physiological conditions, iron ions are not available to catalyze the conversion of molecular oxygen to highly reactive radical species by Fenton chemistry, but iron overload is a major concern in homozygous β-thalassemia. Under physiological conditions, iron ions are not available to catalyze the conversion of molecular oxygen to highly reactive radical species by Fenton chemistry, but high levels of iron in the blood are associated with oxidative stress. Therefore, the iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants. The variations of the parameters evaluated in the present study were not correlated with the age of the patients. Our results suggest that the measurement of peroxidation products, matched with evaluation of antioxidants, may be a simple measure of iron toxicity in thalassemia, in addition to the conventional indices of iron status.

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EVEN THOUGH iron chelation determined considerable progress in the treatment of Cooley’s disease, secondary iron overload is still a major concern in homozygous β-thalassemia. Under physiological conditions, iron ions are not available to catalyze the conversion of molecular oxygen to highly reactive radical species by Fenton chemistry, but high levels of iron in the blood are associated with oxidative stress. Therefore, the iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants. The variations of the parameters evaluated in the present study were not correlated with the age of the patients. Our results suggest that the measurement of peroxidation products, matched with evaluation of antioxidants, may be a simple measure of iron toxicity in thalassemia, in addition to the conventional indices of iron status.

MATERIALS AND METHODS

Subjects and experimental protocol. Patients affected by homozygous β-thalassemia, 25 females and 17 males, aged 4 to 40 years (mean, 21 ± 10), were recruited, with consent, for this study, and were under observation for 1 year. All of the patients had been previously characterized for β-globin gene mutation. Patients were regularly interviewed and examined by a staff of physicians at 15 days to 1-month intervals. Serum ferritin was measured every 4 months, and cardiac, endocrinologic, and hepatologic evaluations were performed once a year. The patients received approximately 15 mL of packed red blood cells per kilogram body weight at each transfusion (2- to 4-week intervals) to maintain hemoglobin levels above 9.5 g per dL. Starting in 1980, patients were under chelation therapy with deferoxamine (DFO) at least five times a week, as a overnight subcutaneous infusion (8 to 12 hours). The average dose was about 50 mg (range, 30 to 60 mg) per kilogram body weight, per day. The therapy did not involve intake of ascorbate.

Twenty patients had undergone splenectomy. Some patients exhibited clinical complications by secondary iron overload: insulin-dependent diabetes mellitus (five patients), hypothyroidism (one pa-
Pathogenicity (17 patients), reduced left-ventricular ejection fraction (<50%; six patients). Abnormal levels of serum alanine and aspartate aminotransferases were observed in 24 patients. No patient was human immunodeficiency virus positive, 31 patients were hepatitis C virus (HCV) positive, and one was HBSAg positive.

Blood from 30 nonthalassemic patients with biopsy-proven chronic hepatitis C was taken as a control for some analysis. Fourteen of the patients were women and the mean age was 29 ± 5. HCV infection status was assessed by HCV-RNA in both nonthalassemic and thalassemic patients. A positive serum HCV-RNA by polymerase chain reaction was considered proof of active infection. Correlation with anti HCV (ELA3) was almost absolute in both groups of patients.

Blood from thalassemia patients was collected just before the transfusion. Control blood was from healthy individuals, aged 4 to 40 years who were not taking any medication. After clotting, serum was separated by centrifugation and divided in several aliquots. The analytical determinations described below were either performed immediately, or serum was stored at -80°C and used within 72 hours.

**Clinical chemistry analyses.** Iron, total iron binding capacity (TIBC), total bilirubin, aspartate and alanine transaminase, total cholesterol, and high-density lipoprotein (HDL) cholesterol, were evaluated in serum from fasting individuals by using commercial analytical kits from Sigma (St Louis, MO). Ferritin was determined by an enzyme-immunoassay (Abbott Labs, North Chicago, IL). Albumin was measured electrophoretically.

**Biochemical analyses.** Nontransferrin bound iron (NTBI) was measured in 500 μL serum samples by the Singh’s method, based on the colorimetric reaction with bathothenaldehyde, as modified by Zhang et al.

Conjugated diene lipid hydroperoxides (CD) were extracted from 500 μL of serum by CHCl3:MeOH (2:1, vol:vol). The organic extract was dried under a nitrogen stream, resuspended in cyclohexane, and quantitated spectrophotometrically at 234 nm, using a molar absorption coefficient of 27,000. Malondialdehyde (MDA) was evaluated in 50 μL serum samples by a colorimetric reaction with thiobarbituric acid (TBA), followed by neutralization of samples by equivalent volumes of a mixture consisting of 4.5 mL 1.0 mol/L NaOH and 45.5 mL methanol. Isotonic high performance liquid chromatography (HPLC) separation of the MDA adduct was performed by a Supelco Supelcosil LC-18 column (0.46 × 25 cm) (Bellefonte, PA), eluted with 40% methanol in water, containing 10 mM KH2PO4, buffer, pH 6.8, at 1.5 mL min⁻¹. The MDA-TBA adduct was shown at 532 nm and quantitated by reference to a calibration curve of tetrahydroxypropane, submitted to the TBA colorimetric procedure. Butylated hydroxytoluene (0.03%) was added to the thiobarbituric acid reagent to prevent artifactual lipid peroxidation during the assay procedure. Serum protein carbonyls were evaluated by the 2,4-dinitrophenylhydrazine method modified according to Gari-baldi et al.

The total antioxidant capacity in serum was evaluated as trolox equivalent antioxidant capacity (TEAC) following Rice-Evans and Miller. All-trans retinol and α-tocopherol were extracted from 200 μL of serum samples diluted to 1.0 mL with 0.15 mol/L NaCl, with 2 volumes of absolute ethanol and 8 volumes of petroleum ether. The organic extracts were gathered, dried under nitrogen, resuspended in suitable solvent, and analyzed by a LC-18 HPLC column as above, with 2% water in methanol at 1.5 mL min⁻¹. All-trans retinol and α-tocopherol were detected at a wavelength of 320 nm and 290 nm, respectively. Under the conditions described, all-trans retinol eluted after 4 minutes and α-tocopherol after 12 minutes. An automatic wavelength change after 9 minutes allowed the detection of both compounds in the same sample. β-carotene and lycopene were extracted from 500 μL serum samples, diluted 1:2 with 0.15 mol/L NaCl, with 1 volume of methanol and 3 volumes of hexane:diethyl ether (1:1, vol:vol). The extracts were then dried under nitrogen, resuspended with a mixture of acetonitrile:methanol:tetrahydrofuran (58.5:35:6.5, vol:vol:vol), and analyzed with the same solvent by a HPLC Supelco column as above, at a flow rate of 2.5 mL min⁻¹. Under these conditions, lycopene eluted at 8.2 minutes and β-carotene at 13.8 minutes. Revelation was at 450 nm. Ascorbic acid and uric acid were determined in 500 μL serum from blood collected in 1.0 mmol/L dithiothreitol. Extraction, HPLC separation, and spectrophotometric revelation at 266 nm were as reported by Lazzarino et al. with minor changes, which included length of the HPLC column (25 × 0.46 cm), and isotonic elution with 10 mmol/L KH2PO4 buffer, pH 7.0, in 10% methanol in water, containing 10 mmol/L tetrahydroxypropane, at 1.2 mL min⁻¹. Retention time of ascorbate and urate were 5.3 and 9.0 minutes, respectively. Thiols were evaluated in 200 μL serum samples by colorimetric reaction with 5.5′-dithiothriols(2-nitrobenzoic acid) as reported. Glutathione was measured after protein precipitation by a fluorimetric assay as described.

All procedures were performed under red light to avoid artifactual photooxidation of lipids by low energy quanta of visible light and to preserve light sensitive vitamins.

**Statistical analysis.** All results are expressed as means ± standard deviation (SD). Comparison between controls and thalassemia patients was performed by the unpaired Student’s t-test. Pearson’s correlations were used to determine relationships between covariates.

**RESULTS**

Some hematological and clinical characteristics of our thalassemia patients are listed in Table 1 and compared with those of healthy controls. With the exception of TIBC, iron indices were markedly increased, and the mean concentration of serum ferritin was more than twenty times higher than normal (Table 1). Nontransferrin bound iron, absent in serum from healthy controls, was detected in all thalassemia patients (Table 1). Total cholesterol, as well as HDL-cholesterol, the most characteristic lipid parameter, varied in β-thalassemia, appeared lower than relevant controls, which is peculiar of the disease (Table 1).

No correlation existed between the age of the patients and hematological parameters, as well as any other parameter examined throughout this study. However, 24 of 42 patients exhibited abnormal values of aspartate and alanine transaminases, showing hepatic necroinflammation, more severe in the older patients (16 to 40 years) than in the younger ones (4 to 15 years) (Table 1).

Under conditions of iron overload, increase of free radical production, peroxidative damages to tissues, and depletion of endogenous antioxidants may be expected. Peroxidative damage to lipids and proteins is indicated by the increase of about twofold of the serum MDA, conjugated diene lipid hydroperoxides, and protein carbonyls (Table 2). Ferritin levels showed a significant positive correlation with MDA values (r = .41; P = .007, Fig 1) and a nearly significant trend with conjugated dienes (r = .31, P = .07) and with protein carbonyls (r = .35; P = .054) (not shown). The lower degree of statistical significance for the latter correlations could be interpreted as the result of a lower precision of measurements of CD and carbonyls, involving extractions and a number of analytical steps. Changes of the serum antioxidant pattern were then investigated. A preliminary
Moreover, blood antioxidants, such as uric acid and bilirubin, total antioxidant activity due to their relative concentration known to contribute significantly to the plasma TEAC value, of hemolysis and liver damage. A detailed quantitative analysis of individual antioxidants was then imperative. With the determination, were also markedly reduced (Table 2). A small increase of serum total thiols was observed (Table 2). Albumin and uric acid have been reported to account for most of the blood total antioxidant capacity. Since these antioxidant species are unmodified, or even slightly higher than control, the decreased TEAC value appears to be the result of the marked decrease of vitamin C and lipid soluble antioxidants.

Serum levels of vitamin E were inversely correlated with ferritin values (r = –.45; P = .003, Fig 2), which suggests a consumption of the vitamin as a radical scavenger. No other clear correlation was found between ferritin values and the levels of other antioxidants.

Nontransferrin bound iron, ranging from 4.5 to 54.8 µg/
OXIDATIVE STRESS IN THALASSEMA MAJOR

Fig 2. Correlation between ferritin and vitamin E in serum from patients with β-thalassemia major. Each serum sample was simultaneously analyzed for ferritin and vitamin E (n = 42; r = −.45; P = .003).

dL, had a positive trend with ferritin (r = .37; P = .03, not shown). However, no clear correlation was found with either MDA or vitamin E.

Serum levels of aspartate transaminase were inversely correlated with vitamin E (r = −.49; P = .015), vitamin A (r = −.48; P = .016), and lycopene (r = −.47; P = .02) (Fig 3A-C), suggesting that liver damage may play a major role in the extent of depletion of these lipid soluble antioxidants. Levels of alanine aminotransferase were similarly inversely correlated with vitamin E, vitamin A, and lycopene (not reported). By contrast, no correlation was found between serum levels of β-carotene and either aspartate (Fig 3D) or alanine aminotransferase. Notably, levels of β-carotene of patients with the highest serum transaminase activities (>100 U/L) were only 10% to 15% lower than control. Depletion of lipid-soluble antioxidant vitamins was observed both in HCV-infected and in HCV-uninfected thalassemia patients, while these compounds were in the normal range in serum from nonthalassemic patients with HCV-related chronic hepatitis (Table 3). This suggests that in thalassemia patients the observed depletion of lipid-soluble antioxidants may be ascribed to chronic iron-related liver oxidative damage and is independent of the HCV-related cytopathic damage.

DISCUSSION

Iron toxicity is involved in various human pathologies, including idiopathic (primary) hemochromatosis, acute iron poisoning, congenital atransferrinemia, as well as secondary iron overload in β-thalassemia. Nevertheless, data concerning products of peroxidative processes in iron-overload diseases are quite limited. TBA-reactivity, as a marker of lipid peroxidation, was found in the plasma of six iron-loaded patients in whom measurable amounts of “free iron” were evaluated. Increase of serum TBA reactive substances has also been described in hereditary hemochromatosis. We evaluated peroxidative stress in transfusion-dependent β-thalassemia patients, under iron-chelation therapy. Early studies had investigated the generation of MDA in thalassemic red blood cells, but failed to demonstrate an increase of MDA, unless exogenous peroxidative stress was provided. Then, Giardini et al were able to demonstrate that in thalassemia patients red blood cell MDA was significantly higher as compared with controls. Our work gives evidence that oxidative alterations to cell components can be shown in serum as a marked increase of conjugated diene lipid hydroperoxides, malondialdehyde, and protein carbonyls.

The introduction of iron chelation as an essential component of β-thalassemia therapy considerably delayed tissue injuries from iron overload, thus improving life expectancy. The significant positive correlation found in our study between ferritin levels and an index of peroxidative stress, such as MDA, and the positive trend with CD and protein carbonyls, highlights the importance of a compliant, continued iron chelation therapy to prevent hoarding of damages from high tissue iron levels.

A direct evaluation of the radical aggression by iron burden to cells is difficult. Therefore, potential serum markers
Table 3. Serum Ferritin and Lipid-Soluble Antioxidants in HCV-infected (HCV+) and -Uninfected (HCV−) Thalassemia Patients and in HCV-Related Chronic Hepatitis

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Ferritin μg/L</th>
<th>VA μmol/L</th>
<th>VE μmol/L</th>
<th>β-Carotene μmol/L</th>
<th>Lycopene μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalassemia major HCV+</td>
<td>31</td>
<td>1,894 ± 1,150</td>
<td>1.14 ± 0.35*</td>
<td>11.64 ± 5.6*</td>
<td>0.29 ± 0.08*</td>
<td>0.18 ± 0.08*</td>
</tr>
<tr>
<td>Thalassemia major HCV−</td>
<td>10</td>
<td>1,579 ± 783</td>
<td>0.97 ± 0.28*</td>
<td>9.82 ± 2.2*</td>
<td>0.29 ± 0.07*</td>
<td>0.18 ± 0.05*</td>
</tr>
<tr>
<td>HCV-related chronic hepatitis</td>
<td>24</td>
<td>230 ± 185</td>
<td>1.71 ± 0.4</td>
<td>19.0 ± 0.5</td>
<td>0.41 ± 0.06</td>
<td>0.56 ± 0.18</td>
</tr>
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</table>

Values are the mean ± SD of (n) determinations performed in duplicate on blood samples from different subjects. Each thalassemia patient contributed the mean of two values determined during 1 year of observation.

Abbreviations: VE, vitamin E; VA, vitamin A.

* With respect to the HCV-related chronic hepatitis, P < .001 (Student’s t-test).

should be attained. The measurement of NTBI as a test for potential toxicity of iron overload did not appear reliable in our hands and gave discrepant results in studies with thalassemia patients without chelation therapy or treated with DFO, according to the compliance. The measurement of peroxidation products, matched with evaluation of antioxidants, may be a simple measure of iron toxicity, in addition to the conventional indices of iron status.

We observed that thalassemia patients, continuously exposed to iron-induced oxidative injury, possess an extremely altered pattern of all serum antioxidants. Various methods, including TEAC, developed to measure the total antioxidant capacity of serum, have been indicated as useful tools to predict the risk of free radical-induced tissue damage. Nevertheless, with thalassemia patients, as also recently reported with patients subjected to hemodialysis, such an approach appears unsuitable. Changes in contributors such as urate and bilirubin, the levels of which increase in thalassemia because of hemolysis, may mask, as they do, marked changes in other essential antioxidants. Then, despite a mean decrease of 14% in the serum total antioxidant potential, a dramatic fall in the amount of ascorbate (44%) and lipid soluble antioxidants, vitamin E (42%), vitamin A (44%), β-carotene (29%), and lycopene (67%) is observed in all patients. A considerable ascorbic acid deficiency has also been described in patients with idiopathic hemochromatosis or in conditions of secondary iron overload and is suggested to be the result of irreversible oxidation by iron.

The moderate increase of serum total thiols is puzzling and unexplained. Protein thiols are expected to decrease under conditions of oxidative stress. On the other hand, no change of serum albumin was observed. The possibility that undefined components in serum from thalassemia patients may interfere with the assay cannot be ruled out.

The body’s antioxidant system is an integrated one, in which some components may interact to spare or replace other each. However, the deficiency of individual antioxidants observed in thalassemia is such that no effective compensation could be brought about. Dehydroascorbate cannot be regenerated to its reduced form, as its regenerating system involves erythrocyte glutathione, most of which in thalassemia patients, can be oxidized. Moreover, as vitamin C is essential to maintain vitamin E status and function, depletion of vitamin C, in turn, contributes to further exacerbate the depletion of vitamin E. Although efficient antioxidants such as uric acid and bilirubin are high, they cannot compensate for lipid-soluble antioxidants, so that tissue lipid compartments are not suitably preserved.

The observed depletion of serum levels of vitamin E and vitamin A can be explained by impairment of liver function and peroxidative processes. Chronic hepatic iron overload, while causing a substantial reduction of serum lipids, can lead to a concurrent reduction of serum vitamin E and vitamin A. In accordance, although the absolute amount is markedly decreased, the level of vitamin A and vitamin E, corrected for serum cholesterol, is very similar to control (Table 2). In vivo studies with iron-loaded animal models showed that dietary excess of iron did cause liver damage and hepatic vitamin E depletion in mice and rats and that progressive liver iron loading induced a progressive lipid peroxidation and a hepatic decrease of α-tocopherol. This may support the idea that in thalassemic chronic hepatic iron overload, a significant consumption of vitamin E, and possibly of other lipid-soluble antioxidants, may occur for neutralizing oxidative processes at the liver level, well before these essential compounds can reach other sites of action.

Serum levels of vitamin E showed a significant inverse correlation with serum ferritin and with serum aminotransferase activities. High levels of serum ferritin have been reported in chronic HCV-hepatitis. Although 31 of 42 thalassemia patients were HCV-infected, data reported in Table 3 suggest that HCV is to be considered only an independent, additive cause of liver damage, not related to the depletion of vitamin E and the other lipid-soluble antioxidants. Levels of β-carotene, although substantially lower than normal, did not appear correlated with serum transaminases. A similar lack of correlation, reported in subjects with different stages of liver disease, has been explained by considering that very severe liver damage may in some way interfere with the hepatic uptake or metabolism of β-carotene, which will cause a paradoxical relative elevation of β-carotene in serum of patients with the most diseased liver.

It should be stressed that thalassemia patients, whose liver damage was not so severe as to affect serum transaminases, also showed very low levels of vitamin A, vitamin E, carotene, and lycopene. Similarly, patients with hemochromatosis, showing very high levels of serum iron and ferritin, exhibited a substantial decrease of serum vitamin E, even in the absence of clinical signs of liver cell damage. Presence in the membranes of vitamin E and other lipid soluble antioxidants in suitable amounts and their synergistic interactions guarantee membrane structural integrity. Tis-
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Sue concentrations of lipid soluble antioxidants is determined by their plasma concentrations. The dramatic depletion observed in thalassemia may be crucial for the erythrocyte membrane and heart tissue, where iron overload can start chain reactions leading to peroxidative destruction of myofibrils. Administration of antioxidant compounds could be advisable. Vitamin C should be carefully administered, as it can have prooxidant activity in the presence of iron overload and defereroxamine. Beneficial effects by supplementation of vitamins E and A may be expected. Although therapeutic trials with orally or parenterally administered canthaxantin and tocopherols have been attempted in the past with various results, intravenous administration of vitamins E and A in liposomes, enhancing organ targeting and distribution, should be studied.

Acknowledgment

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