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), liperoxides 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 = 0.41; P = .007) and showed a positive trend with CD (r = 0.31; P = .07) and protein carbonyls (r = 0.35; 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 antioxidant potential, measured as trolox equivalent antioxidant capacity, appeared significantly decreased by 14%. Serum levels of vitamin E were inversely correlated with ferritin (r = −0.45; P = .003), suggesting a major consumption of this antioxidant under iron overload. Nontransferrin bound iron (NTBI) was in the range 4.5 to 54.8 μg/dL (mean, 21.8 ± 13.9). Although NTBI had a positive trend with ferritin (r = 0.37; P = .03), no clear correlation was found with either MDA or vitamin E. A mild to severe hepatic damage, as assessed by serum transaminases, was shown in 24 of 42 patients. Serum levels of vitamin E (r = −0.49, P = .015), vitamin A (r = −0.48, P = .016) and lycopene (r = −0.47, P = .029), were inversely correlated with the levels of transaminases. On the other hand, lipid-soluble antioxidants in thalassemia patients were depleted to the same extent in hepatitis C virus (HCV)-infected (31 subjects) and in HCV-uninfected (10 subjects), while in the normal range in serum from 30 nonthalassemic patients with HCV-related chronic hepatitis. These results point out that the iron-induced liver damage in thalassemia may play a major role in the depletion of lipid-soluble antioxidants. The variations of the parameters obtained 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, because ferric iron is bound to proteins, preventing it from participating in reactions that could lead to cell injury. An intermediate cellular pool of nonprotein, low molecular weight iron, has been demonstrated, as well. Under various pathological conditions associated with iron overload, including thalassemia, there is evidence of an increase in low molecular weight iron in serum and in the intracellular transit pool of iron. This promotes peroxidative damage to cell and organelle membranes in organs that accumulate excess iron, including liver, pituitary gland, pancreas, and heart. The latter, which has less developed antioxidant defenses, is particularly susceptible to the iron-induced peroxidative damage that, ultimately, can lead to congestive heart failure, which is the main cause of death in thalassemia patients.

Data concerning the antioxidant status and degree of molecular peroxidative damage in β-thalassemia are quite limited. A few studies in the past described an association between increased susceptibility of thalassemic red blood cells to oxidative stress and normal serum levels of vitamin E. This study evaluates the total antioxidant potential and several individual antioxidants, as well as parameters of peroxidative stress, including malondialdehyde, conjugated dienes, and protein carbonyls in serum of patients with β-thalassemia major, transfusion-dependent, and under regular iron chelation therapy.

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-

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tient), hypogonadism (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 hepa-
titis 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 polymer-
ase chain reaction was considered proof of active infection. Correla-
tion with anti HCV (ELISA) 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 cho-
esterol, and high-density lipoprotein (HDL) cholesterol, were evalu-
ated in serum from fasting individuals by using commercial analyti-
cal 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,28 based
on the colorimetric reaction with bathophenanthroline, as modified by
Zhang et al.29 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 nitrogen stream, resuspended in cyclohexane,
and quantitated spectrophotometrically at 234 nm, using a molar
absorption coefficient of 27,000.30 Malondialdehyde (MDA) was
evaluated in 50 µL serum samples by a colorimetric reaction with
thiobarbituric acid (TBA),31 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. Isoratic high performance liquid
chromatography (HPLC) separation of the MDA adduct was per-
formed by a Supelco Supelcosil LC-18 column (0.46 x 25 cm)
(Bellefonte, PA), eluted with 40% methanol in 50 mmol/L potassium
phosphate buffer, pH 6.8, at 1.5 mL min-1. The MDA-TBA adduct
was shown at 532 nm and quantified 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 artificial lipid peroxidation
during the assay procedure. Serum protein carbonyls were evaluated by the
2,4-dinitrophenylhydrazine method32 modified according to Gari-
baldi et al.33

The total antioxidant capacity in serum was evaluated as trolox
equivalent antioxidant capacity (TEAC) following Rice-Evans and
Miller.34 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 ex-
tracted 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:tetrahydrofu-
rane (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 al35 with minor changes, which included length of the
HPLC column (25 x 0.46 cm), and isocratic elution with 10 mmol/
L KH2PO4 buffer, pH 7.0, in 10% methanol in water, containing 10
mmol/L tetrabutylammonium bromide, at 1.2 mL min-1. Retention
time of ascorbate and urate were 5.3 and 9.0 minutes, respectively.
Thiol levels were evaluated in 200 µL serum samples by colorimetric
reaction with 5,5'-dithiobis(2-nitrobenzoic acid) as reported.36 Serum glutathione was measured after protein precipitation by a
fluorimetric assay as described.37

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 ± stan-
dard 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 hematomal 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 concentra-
tion 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-chole-
sterol, the most characteristic lipid parameter, varied in β-thalassemia,29 appeared lower than relevant controls, which
is peculiar of the disease20 (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 transami-
nases, 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.30 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 = 0.41; P = 0.007, Fig 1) and a nearly significant
trend with conjugated dienes (r = 0.31, P = 0.07) and with
protein carbonyls (r = 0.35; P = 0.054) (not shown). The
lower degree of statistical significance for the latter correla-
tions could be interpreted as the result of a lower precision
of measurements of CD and carbonyls, including 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 may be expected to increase in thalassemia patients because exception of albumin and glutathione, all the other major antioxidant species in blood contribute to a different extent to the number of radicals scavenged per mole of compound. A sharp elevation of bilirubin was measured (Table 2). Concentrations of ascorbate and vitamin E (α-tocopherol), the major lipid soluble antioxidant in human blood, decreased by 44% and 42%, respectively. Other lipid soluble antioxidants such as vitamin A, and carotenoids such as β-carotene and lycopene, which are part of the unmeasured compounds in TEAC determinations, were also markedly reduced (Table 2). A small increase of serum total thios 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 ($r = -0.45; P = .003$, 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 THALASSEMIA 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...
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 (44%), vitamin A (44%), \( \beta \)-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 each other. 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 \( \alpha \)-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, adjudicative cause of liver damage, not related to the depletion of vitamin E and the other lipid-soluble antioxidants. Levels of \( \beta \)-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 \( \beta \)-carotene, which will cause a paradoxical relative elevation of \( \beta \)-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.
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 deferoxamine. 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.

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