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Prolidase Activity and Oxidative Status in Patients with Thalassemia Major

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Aim: Prolidase is a specific imidodipeptidase involved in collagen degradation. The increase in the enzyme activity is believed to be correlated with the increased intensity of collagen degradation. The study aimed to evaluate the relationship between prolidase activity and oxidative status in patients with thalassemia major. *Methods*: Comparison was made between 87 patients diagnosed with thalassemia major and 33 healthy children of similar age and gender. Mean age of the subjects was 7.5 ± 4.3 years in the group of patients with thalassemia major and 8.9 ± 3.1 years in the control group. Serum prolidase activity was measured spectrophotometrically. Oxidative status was determined using total oxidant status (TOS), total antioxidant capacity (TAC), and oxidative stress index (OSI) measurement. *Results*: Prolidase activity was significantly increased in patients with thalassemia major (53.7 ± 8.7 U/l) compared to the control group (49.2 ± 7.2 U/l, $P < 0.001$). TOS was significantly increased in the patient group (5.31 ± 3.14 mmol H_2O_2 equiv./l) compared to the control group (3.49 ± 2.98 μmol H_2O_2 equiv./l) and the OSI was also significantly increased in the patient group (3.86 ± 3.28 arbitrary unit) compared to the control group (2.53 ± 2.70 arbitrary unit) ($P < 0.0001$ and $P < 0.001$, respectively), while there were no significant differences between the patient (1.61 ± 0.30 μmol Trolox equiv./l) and control (1.64 ± 0.33 μmol Trolox equiv./l) groups with respect to TAC. *Conclusion*: Significant increases in prolidase activity in patients with thalassemia major may constitute a key parameter in demonstrating a disorder of the collagen metabolism. J. Clin. Lab. Anal. 24:6–11, 2010. © 2010 Wiley-Liss, Inc.

Key words: thalassemia major; prolidase activity; collagen; oxidative status

INTRODUCTION

Connective tissue protein collagen constitutes one-third of total body protein. Prolidase [EC 3.4.13.9] is a cytosolic enzyme that catalyses the hydrolysis of imidodipeptides with C-terminal proline or hydroxyproline (1,2). The enzyme has an important role in the recycling of proline from imidodipeptides derived from degradation products of collagen and other proline-containing proteins for collagen synthesis (3) and cell growth (4). The efficiency of recycling of proline was found to be about 90% (5). Because of the high proportion of the iminoacids in collagen (25% proline and hydroxyproline together), this enzyme has an important role in its degradation and its activity might be correlated with the rate of collagen degradation (6). The presence of this enzyme in various tissues has been demonstrated (7). Thalassemia is an inherited disorder with an autosomal recessive mode of inheritance and constitutes one of the most serious health problems worldwide (8). It is well known that increased erythropoiesis in bone marrow in thalassemic patients results in the expansion of marrow cavity and reduced bone mass (9). Regular blood transfusions from infancy until adulthood in thalassemia major patients have facilitated transformation of severe bone deformities into less marked skeletal lesions, such as osteoporosis (10).

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Osteoporosis is a common disease characterized by reduced bone mass, microarchitectural deterioration of bone tissue, and increased risk of fragility fractures (11,12). Genetic factors have an important role in the pathogenesis of osteoporosis, involving variation in several genes such as *collagen type I A1* (*COLIA1*), vitamin D, estrogen receptors, and interleukin (IL)-6 that regulate bone mineral density (BMD) and bone geometry and quality (13). One of the most important candidate genes for predisposition to osteoporosis is the *COLIA1* gene, which encodes the α-1 (I) protein chain of type I collagen, the major protein of bone. The G-T substitution at base 1 of intron 1 at the binding site of the Sp1 transcription factor of the *COLIA1* gene is a putative marker for low BMD and osteoporotic fractures. Several studies demonstrated that Sp1 polymorphism has been associated with low BMD and an increased risk of osteoporotic fracture in thalassemic patients in different populations (12,14).

The life expectancy of patients with β-thalassemia has greatly improved over recent years as a result of regular transfusions and increased compliance with iron chelation therapy. However, this increase is often accompanied by a series of serious complications including osteopenia and osteoporosis (15,16). As a rule, untreated thalassemia major patients present with severe bone deformities very early in life (17). The etiology of bone disease in thalassemia is multifactorial and is still under investigation. Factors such as hormonal deficiency, especially gonadal failure, bone marrow expansion, increased iron stores, desferrioxamine toxicity, and calcium/vitamin D deficiency all seem to have a serious impact on the impaired bone metabolism of the disease (12,18).

Given these circumstances regarding the life expectancy, further studies on the causes and management of long-term morbidities in this patient population should be carried out. However, there is no report concerning prolidase activity in patients with thalassemia major and healthy control group. A scan of the literature for research investigating prolidase enzyme activity, a significant factor in the collagen "destruction-production turnover", did not yield any studies.

The primary aim of this study is to investigate relationships among prolidase activity and oxidative status and blood parameters in patients with thalassemia major.

MATERIALS AND METHODS

Subjects

A total of 87 (53 boys and 34 girls) subjects with thalassemia major (7.5 ± 4.3 years old) and 33 (25 boys and 8 girls) healthy controls (8.6 ± 3.3 years old) were included in this study. 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 of their body weight at each transfusion to maintain hemoglobin (Hb) levels above 9.5 g/dl. This value is that which we tried to keep above but as the patients' socio-cultural and socio-economic levels are low in our region, during follow-ups, various unwanted problems arose from the patients themselves. Some patients did not attend for check-ups or came late so this is the reason for the low level of mean Hb in our patients. Deferoxamine was administered as chelation therapy. The therapy did not involve intake of ascorbate.

Their diagnoses were confirmed through Hb electrophoresis, blood count results, and physical examination. Since most thalassemic patients require intermittent blood transfusions, blood was collected as late as possible, at least 3–4 weeks after the last transfusion. Hb electrophoresis was performed on all patients with healthy controls to exclude the β-thalassemia trait. They all had normal blood counts and Hb electrophoresis results. Hb electrophoresis was performed using alkaline cellulose acetate electrophoresis densitometry. Complete blood count was performed using Celdyne 3700 Haematology Analyser (Abbott®, Abbott Park, IL). Serum ferritin was measured using an automated chemiluminescence autoanalyzer (Roche®, Basel, Switzerland). All the thalassemia major patients who had undergone a splenectomy or not were included in the study group and this group was compared with the control group.

The study protocol was carried out in accordance with the Helsinki Declaration as revised in 1989. Written consent was obtained from all parents prior to their children's participation in the study. The study was approved by the local Ethics Committee.

Exclusion Criteria

Exclusion criteria included usage of supplemental vitamins, smoking, presence of diabetes mellitus, coronary artery disease, rheumatoid arthritis, malignancy, hypertension, hyperlipidemia, systemic or local infection, acute-chronic liver diseases, renal dysfunction, and iron deficiency anemia.

Blood Sample Collection

Blood from thalassemia major patients was collected just before the transfusion. Control blood was obtained from healthy individuals, who were not taking any medication. Blood samples were obtained following an

overnight fasting state and collected into empty tubes and immediately stored on ice at 4°C. The serum samples were then separated from the cells by centrifugation at 3000 rpm for 10 min, lipid parameters and the enzyme activities were measured freshly. The remaining serum portions were stored at −80°C for a time no longer than 6 months and used to analyze total oxidant status (TOS), total antioxidant capacity (TAC), and total peroxide concentration levels.

Determination of Prolidase Activity

Serum was diluted 40-fold with 2.5 mmol/L Mn^{2+}, 40 mmol/L trizma HCl buffer (pH 8.0), and preincubated at 37°C for 2 hr. The reaction mixture containing 30 mmol/l gly-pro, 40 mmol/l trizma HCl buffer (pH 8.0), and 100 µl of preincubation serum in 1 ml was incubated at 37°C for 30 min. A total of 0.5 ml of 20% trichloroacetic acid solution was added and then the incubation reaction was stopped. The supernatant was used for the measurement of proline by the method proposed by Myara (6,19), which is a modification of Chinard's method (20). Intraassay CV of the assay was 3.8%.

Measurement of the TAC

TAC of the serum was determined using a novel automated measurement method developed by Erel (21). In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequentially produced radicals such as brown colored dianisidinyl radical cation produced by the hydroxyl radical are also potent radicals. Using this method, antioxidative effect of the sample against the potent free radical reactions initiated by the produced hydroxyl radical is measured. The assay has got excellent precision values lower than 3%. The results are expressed as µmol Trolox equiv./l.

Measurement of TOS

TOS of serum was determined using a novel automated measurement method developed by Erel (22). Oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 equiv./l).

Oxidative Stress Index (OSI)

Percentage ratio of TOS level to TAC level was accepted as OSI. For calculation, the resulting unit of TAC was changed to mmol/l, and the OSI value was calculated according to the following formula (23): OSI (Arbitrary Unit) = TOS (µmol H_2O_2 equiv./l)/TAC (µmol Trolox equiv./l).

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Qualitative variables were assessed by χ^2 test. Correlation analyses were performed using Pearson's correlation test or Spearman's correlation test. The differences between the different groups of controls and patients were analyzed by unpaired *t*-test or Mann–Whitney *U* test. A *P*-value <0.05 was accepted as significant. Data were analyzed using the SPSS® for Windows computing program (Version 11.5).

RESULTS

Demographic and clinical data in thalassemia major patients and controls are summarized in Table 1. There were no significant differences between thalassemia major subjects and controls with respect to age and gender (all $P>0.05$) (Table 1). The correlation between prolidase activity and the given variables in thalassemia major patients are summarized in Table 2. There were significant differences between thalassemia major subjects and controls with respect to height, weight, and body mass index (BMI) (all $P<0.05$) (Table 1). There were significant differences between thalassemia major subjects and controls with respect to serum iron, Hb, hematocrit, mean corpuscular volume, and ferritin ($P<0.002$, $P<0.0001$, $P<0.0001$, $P<0.0001$ and $P<0.0001$; respectively) (Table 1). Prolidase activity was significantly increased in patients with Thalassemia major (53.7 ± 8.7 U/l) compared to the control group (49.2 ± 7.5 U/l, $P<0.001$) (Table 1). Eighteen of the thalassemia major patients had undergone splenectomy. Prolidase activity in patients who had and had not undergone splenectomy was 61.4 ± 3.4 U/l and 52.5 ± 8.8 U/l, respectively. Comparison of increases in prolidase activity between patients who had and had not undergone splenectomy yielded a significant difference ($P<0.001$) (Table 1).

There were no correlations between prolidase and TAC, TOS, OSI, ferritin, phosphor, alkaline phosphatase, liver size (cm), spleen size (cm), BMI, and the age of onset for transfusion treatments.

TABLE 1. Demographic Data, Blood Parameters, Prolidase Activity Levels, and Oxidative Status of Patient and Control Groups

Parameters	TM (*n*: 87)	Control (*n*: 33)	*P* value
Age (years)	7.5±4.3	8.9±3.1	>0.05
Sex (M/F)	53/34	25/8	>0.05
Height (cm)	112.7±21.8	127.7±16.2	<0.0001
Weight (kg)	22.2±8.9	26.±6.7	<0.02
BMI (kg/m^2)	16.8±1.9	15.8±0.9	<0.002
Hb (g/dl)	8.3±1.4	12.0±0.7	<0.0001
Hct (%)	23.9±4.3	34.2±2.8	<0.0001
Serum Iron (mg/dl)	164.1±63.2	79.7±18.6	<0.0001
Ferritin (μg/l)	3245.4±1936	37.5±20.0	<0.0001
Age of onset for transfusions (years)	0.86±0.73	–	–
Total number of transfusions (Units)	91.9±74.3	–	–
Number of patient who had undergone splenectomy	18	–	–
TAC (mmol Trolox equiv./l)	1.61±0.30	1.64±0.33	>0.05
TOS (μmol H_2O_2 equiv./l)	5.31±3.14	3.49±2.98	<0.0001
OSI (arbitrary unit)	3.86±3.28	2.53±2.70	<0.001
Prolidase (U/l)	53.7±8.7	49.2±7.5	<0.001
Undergone splenectomy Prolidase (U/l)	61.4±3.4	–	
Not undergone splenectomy Prolidase (U/l)	52.5±8.8	–	<0.001

Values are expressed as mean±SD. Mann–Whitney *U* test. A *P* value <0.05 was accepted as significant. TM, thalassemia major; TAC, total antioxidant capacity; TOS, total oxidant status; OSI, oxidative stress index.

TABLE 2. Correlations between Prolidase Activity and Clinical and Blood Parameters in Patients with Thalassemia Major

Parameters		*P* value	*r*
Prolidase	Age (years)	0.044	0.276
Prolidase	Height (cm)	0.003	0.399
Prolidase	Body weight (kg)	0.003	0.402
Prolidase	Duration of transfusion treatment (years)	0.026	0.303
Prolidase	Duration of chelation treatment (years)	0.022	0.311
Prolidase	Total number of transfusions (Units)	0.008	0.248
Prolidase	Total protein (g/dl)	0.0001	0.559
Prolidase	Calcium (mg(dl)	0.003	0.397
Prolidase	Iron (mg/dl)	0.019	0.322

Total oxidant capacity and OSI were significantly increased in patients with thalassemia major ($P<0.0001$ and $P<0.001$, respectively), whereas there were no differences between the groups with respect to TAC (Table 1). In the thalassemia major patient group, a positive correlation was determined between prolidase activity and age (years), height (cm), body weight (kg), duration of transfusion treatment (years), duration of chelation treatment (years), total number of transfusions (Units), total protein (g/dl), calcium (mg/dl), and iron (mg/dl) (Table 2).

DISCUSSION

In this study, we observed that thalassemia major patients had increased prolidase activity, TOS levels, and OSI levels and the TAC level remained unchanged. Prolidase enzyme is present in several tissues and plasma, and it is believed that the presence of extensive tissue distribution, as well as alterations in prolidase enzyme activities may have significant involvement in the development and outcome of several diseases (24). A lack of studies in literature assessing prolidase activity in thalassemia major patients makes it difficult to interpret the data on the activity of this enzyme found during our study.

As a rule, untreated thalassemia major patients present with severe bone deformities very early in life (17). The etiology of bone disease in thalassemia is multifactorial and is still under investigation. Factors such as hormonal deficiency, especially gonadal failure, bone marrow expansion, increased iron stores, desferrioxamine toxicity, and calcium/vitamin D deficiency, all seem to have a serious impact on the impaired bone metabolism of the disease (18,25). Osteoporosis is a common disease that is characterized by a decrease in mineral density and increased fracture risk. Ongoing laboratory studies are contributing to the growing accumulation of knowledge about osteoporosis. Recent studies have indicated that osteoporosis is caused by complex interactions among local and systemic regulators of bone cell function (26). The rate of occurrence of osteoporosis in patients with thalassemia major has been reported as 90% in the literature (27–29). In two studies evaluating osteoporosis and prolidase activity in postmenopausal women, no correlations were determined between serum prolidase activity and BMD or indicators of bone turnover (30,31). A study comparing prolidase activity in patients with end-stage renal disease

developing uremic bone disease during follow-up and the control group reported decreased prolidase activity in the patient group compared to the control group. That study reported that prolidase activity was not a reliable factor in diagnosing uremic bone disease (32). Another study (33) reported that serum prolidase activity could be a good marker during osteoporosis in type 2 diabetes mellitus. However, Myara et al. (6) reported that plasma prolidase activity was not elevated in bone metastasis and primary bone cancers. They also emphasized that its plasma level was also unrelated to age and sex. In our study, there was a positive correlation between prolidase activity and age, height, and body weight in patients with thalassemia major. There were also positive correlations in the patient group between prolidase activity and iron, total protein, duration of transfusion, duration of chelation treatment, and total number of transfusions. In our study, increases in the prolidase activity in thalassemia major patients was significantly different compared to the control group and there was a positive correlation between calcium levels and prolidase activity in the patient group. There were no correlations between prolidase activity and TAC, TOS, OSI, ferritin, phosphor, alkaline phosphatase, liver size (cm), spleen size (cm), BMI, or the age at which transfusion treatment was started.

Although increases in the collagen turnover rate are correlated with increases in the prolidase enzyme activity, how the prolidase is regulated, its involvement in pathology, and metabolism in different tissues are issues not yet fully understood (34,35).

In our study, we observed that thalassemia major subjects had increased TOS and OSI levels and the TAC level remained unchanged.

In the patients with thalassemia major, the level of iron associated with blood transfusion and ferritin was significantly higher than that of the control group. In concurrence with previous studies (36,37), we found a marked increase in serum ferritin and serum iron concentrations in thalassemia major patients. The increment in serum iron and in the intracellular transit pool of iron due to continuous blood transfusions promotes peroxidative damage in thalassemia major patients (36). Under conditions of iron overload, increased free radical production, peroxidative damage to tissues, and depletion of endogenous antioxidants may be expected (38). Accordingly, a study reported peroxidative damage to lipids and proteins in thalassemia major patients, i.e. indicated by about a 2-fold increase of the serum malondialdehyde/thiobarbituric acid adducts, conjugated diene lipid hydroperoxides, and protein carbonyls, together with a significant decrement in the total serum antioxidant capacity due to depletion of lipid soluble antioxidants (36). In comparison with the control group, no statistically significant decrease was observed in the TAC level of the patients with thalassemia major and a significant increase was observed in TOS levels and OSI. In experimental animal models, high liver iron levels were shown to induce elevation of lipid peroxides and oxidants presumably through iron initiated Fenton chemistry (39,40). A similar mechanism is likely to have contributed in our present study. As reported in a previous study (41), antioxidant supplementation, which can be administered to these patients, can be useful for decreasing oxidative stress by strengthening their antioxidant system.

In several studies (38,42,43), increased oxidative stress has been reported in thalassemia major, and increased oxidative damage in thalassemia has been related to the generation of free radicals by an excess of denaturated α or β-globin chains, intracellular iron overload, and low concentration of normal Hb. A study investigated oxidative status in thalassemia major subjects, where a significantly increased oxidative stress compared to other forms of microcytic anemias including δ thalassemia major and iron deficiency anemia was detected (44). In respect of increased TOS and OSI, our findings were consistent with that study (44). In addition, the TAC level was similar to that of the control group.

The primary limitation of this study was that BMD, gene polymorphism, and hormones involved in bone turnover (parathormone, vitamin D) were not investigated.

The increase in prolidase enzyme activity is believed to be correlated with increased intensity of collagen degradation and may be a useful tool in diagnosis and/or monitoring osteoporosis.

In conclusion, significant increases in prolidase activity in patients with thalassemia major may constitute a key parameter in demonstrating a disorder of the collagen metabolism. More comprehensive biochemical and biophysical analyses are required to elucidate pathologic mechanisms underlying these alterations observed in the prolidase activity.

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