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Upregulation of Neutrophil Gelatinase-associated Lipocalin, NGAL/Lcn2, in β-Thalassemia Patients

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Background. One of the major consequences in β- thalassemia is iron overload. Oxidative statuses have been reported in β-thalassemia patients by several studies. It has been proven that iron plays a critical role in the formation of reactive oxygen species (ROS). More recently, we have found the induction of Lcn2/NGAL expression under oxidative stress condition. In this study, it was assumed that NGAL should be upregulated in β-thalassemia patients because of oxidative stress condition.

Methods. Assessment of NGAL expressions in 25 adult β-thalassemia and 9 pediatric patients was performed by semiquantitative RT-PCR, real-time RT-PCR and ELISA.

Results. Adult β-thalassemia patients upregulated NGAL expression compared with the normal samples but no upregulation was observed in pediatric patients.

Conclusions. Upregulation may play an important role in decreasing ROS or iron in β-thalassemia patients. © 2008 IMSS. Published by Elsevier Inc.

Key Words: Thalassemia, NGAL/Lcn2, Iron overload, Real-time PCR, ELISA.

Introduction

β-thalassemias are a heterogeneous group of inherited anemias resulting from reduced or absent synthesis of β-globin chains of hemoglobin A (1). Patients with β-thalassemia have partial or complete lack of synthesis of β-chains of hemoglobin (2). The remaining excess of α-chains is unstable, and they eventually precipitate and disintegrate, causing damage to the red blood cell (RBC) membrane. The affected RBCs are prematurely hemolyzed in the bone marrow and spleen, resulting in increased RBC turnover, ineffective erythropoiesis, and severe anemia (3), which can be corrected only by regular blood transfusions. One of the major consequences in this genetic disorder is iron overload due to ineffective erythropoiesis and premature hemolysis in the plasma and in major organs such as heart, liver, and endocrine glands (4,5). Oxidative statuses have been reported in β-thalassemia patients by several studies (6–9). It has been proven that iron plays a critical role in the formation of reactive oxygen species (ROS), particularly the hydroxyl radical (·OH) serving as a Fenton reagent (10,11) in β-thalassemia patients. The lipocalin superfamily, which includes the mouse and human homologues 24p3/lcn2 and neutrophil gelatinase-associated lipocalin (NGAL), comprise a group of small extracellular proteins with a common β-sheet-dominated 3-dimensional structure (12). In the past, the predominant role of lipocalins was assumed to be to act as transport proteins and, for several members, this is likely to be an important function (e.g., retinol-binding protein). 24p3/lcn2 was initially identified as a superinduced protein secreted by 3T3 cells in response to fibroblast growth factor when it was called SIP24 (13,14). Around the same time as the 24p3/lcn2 messenger RNA (mRNA) was cloned, the human homologue NGAL was identified as a 25-kDa protein associated with gelatinase/matrix metalloproteinase 9 from human neutrophils (15). 24p3/lcn2

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and NGAL share a high degree of identity at the complementary DNA (cDNA) level and 62% identical amino acid homology (16). The pathophysiological functions of 24p3/lcn2/NGAL are unclear, but it has been suggested that they may act in an immunomodulatory role by binding or inactivation of bacterial products (17) or through direct actions on the inflammatory cells (18). It has also been shown that NGAL is an iron-trafficking protein (19). Recently, upregulation of mRNA of NGAL has been shown in β-thalassemia in a mouse model (20). More recently, we have found induction of Lcn2/NGAL expression under oxidative stress conditions (21). In this study we assumed that NGAL should be upregulated in β-thalassemia patients because of oxidative stress condition. NGAL expression was studied in β-thalassemia patients compared with healthy samples. Our results revealed that NGAL was upregulated both in mRNA and protein level in adult patients and suggest that the upregulation may play an important role in decreasing ROS or iron in β-thalassemia patients.

Materials and Methods

Study Design

Patients with thalassemia and a history of receiving chronic transfusion therapy were recruited from the hematology clinics. Healthy controls were recruited from hematology clinics staff, families of thalassemia, and our colleagues at the Iranian Blood Transfusion Organization (IBTO). The study received institutional review board approval. Consent for adults and assent for children were obtained in accordance with the IBTO institutional review board guidelines. Thirty three transfused hemoglobinopathy patients were studied (eight or more transfusions per year). Case subjects included 25 adult β-thalassemia patients [12 males, ages 24.33 ± 7.09 years, ferritin (ng/mL); 2134 ± 1742 and 13 females, ages 23.46 ± 6.81 years, ferritin (ng/mL); 1888 ± 1742], 9 pediatric β-thalassemia patients (ages 8.28 ± 1.49 years, ferritin [ng/mL]; 654 ± 321) and controls [14 males, ages 28.45 ± 6.78 years, ferritin (ng/mL); 63.12 ± 70.9 and 11 females, ages 27.89 ± 9.81 years, ferritin (ng/mL); 67.6 ± 78.4]. Clinical information including demographics and history of transfusion, chelation, and therapy were obtained by interview and chart review. Subjects with systemic hypertension, kidney or heart failure, diabetes mellitus, thyroid or parathyroid dysfunction, acute or chronic infection or any inflammatory disease, as well as smokers were excluded from the study. Of 25 adult β-thalassemia patients, five sample patients had a history of liver biopsy in which liver iron was positive.

Isolation of Mononuclear Cells from Peripheral Blood

The Ficoll-Hypaque method was used to isolate mononuclear cells from cord blood. Briefly, the blood was first diluted 1:1 with phosphate-buffered saline (PBS), and 10 mL diluted blood was carefully layered onto a 3 mL Ficoll-Hypaque plus cushion (Pharmacia Biotech, Uppsala, Sweden) in a 15-mL centrifuge tube (Falcon 3033; Becton Dickinson, Franklin Lakes, NJ). The tube was centrifuged at 400 × g for 30 min at 18−20°C. The interface (containing mononuclear cells) was carefully collected and washed twice with PBS and cells were used for RNA extraction.

RNA Extraction

Total RNA from cell lines was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The quantity and quality of RNA were determined by spectrophotometry (ND-1000; Nanodrop, Wilmington, DE) and electrophoresis, respectively.

cDNA Synthesis

Reverse transcription was performed by SuperScript III reverse transcriptase (Invitrogen) with 1 μg of total RNA followed by DNaseI (Invitrogen) treatment and heat inactivation.

Assessment of NGAL Expression

Semiquantitative PCR was performed using *Taq* DNA polymerase (Cinnagene, Tehran, Iran) in a GeneAmp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA). After initial denaturation (5 min at 94°C), cDNA was subjected to 30 cycles of PCR. Primer set for the human *NGAL/Lcn2* was forward 5′-TCA CCT CCG TCC TGT TTA GG-3′ and reverse 5′-CGA AGT CAG CTC CTT GGT TC-3′. For the normalization, expression of β-actin was examined and the primer set was forward 5′-TTC TAC AAT GAG CTG CGT GTG G -3′ and reverse 5′-GTG TTG AAG GTC TCA AAC ATG AT-3′. PCR annealing temperature was 60°C for human NGAL and 59°C for β-actin. PCR products were separated in a 2% agarose gel.

Real-time PCR analysis was performed in a Rotor-Gene RG 3000 (Corbett Research, Sydney, Australia). Amplification was conducted using AB solute Syber green mix (ABgene, Surrey, UK) according to the manufacturer's instructions. PCR condition was initial denaturation at 94°C for 15 min followed by 40 amplification cycles consisting of denaturation at 94°C for 30 sec, annealing at suitable temperature for 30 sec and extension at 72°C for 30 sec. Threshold cycle values were normalized by β-actin expression.

ELISA for Human NGAL Immunoassay

The plasma fraction, after mononuclear cell separation, was used for expression of NGAL protein. Twenty-fold dilution of plasma samples was used for ELISA and was performed with Quantikine Kit in accordance with the manufacturer's protocols (R&D Systems, Minneapolis, MN). All measurements were done in triplicate and in a blinded fashion.

Statistical Analysis

Results are expressed as mean ± SD of three independent experiments. Differences between groups were compared using Student's *t* test; $p < 0.05$ was considered statistically significant.

Results

Expression of NGAL in Normal Samples

RT-PCR was carried out to determine whether there is any difference among normal samples in terms of NGAL expression. RNA was extracted from 25 healthy samples and the quality of the RNA was checked on agarose by electrophoresis. As expected, there was no difference among healthy samples in terms of NGAL expression, which is necessary for normal physiology of the cells. NGAL expression was further analyzed by real-time RT-PCR and ELISA. Again no significant difference was observed (data not shown). Value of ELISA in normal samples was 32.85 ± 12 (mean ± SD) and median was 33.5.

Expression of NGAL in β-thalassemia Patients

Next we studied NGAL expression in β-thalassemia patients. First we performed semiquantitative RT-PCR. Because normal samples expressed the same level of NGAL, we used only two of them to compare with β-thalassemia patients. As shown in Figure 1 in all cases (Lanes 3–12), NGAL was upregulated compared to normal samples (Lanes 1 and 2). Then we quantified NGAL expression in β-thalassemia patients by real-time RT-PCR. NGAL expression increased from 1.8- to 20-fold compared with healthy samples (Table 1). ELISA was performed to evaluate NGAL expression in protein level. To calculate fold changes in ELISA, the obtained value of thalassemia samples was divided by the value obtained from the pool plasma of normal samples. The ELISA again verified the RT-PCR and real-time RT-PCR results. In all cases, except one sample, NGAL protein was expressed more compared to the controls (healthy samples) (Table 1). There was a positive correlation between NGAL concentration and fold changes in real-time PCR (Figure 2; $r = 0.943$, $p = 0.003$). Further analysis of NGAL (values obtained from ELISA), comparing it with ferritin, also showed a positive correlation between NGAL and ferritin (Figure 3; $r = 0.707$, $p = 0.003$). NGAL expression was not sex dependent, i.e., in both male and female β-thalassemia patients it was upregulated. NGAL upregulation was not found in pediatric β-thalassemia patients (Table 1, Nos. 27–35). But in our study groups of adult patients, there was no relationship between age of β-thalassemia and NGAL expression. In other words, NGAL expression in older patients

Table 1. Fold changes in real-time PCR, concentration of NGAL obtained in ELISA and concentration of ferritin

No.	Fold changes (real-time PCR)	NGAL concentration (ng/mL) ELISA	*p* value[a]	Ferritin (ng/mL)
1[b]		30 ± 4.04[c]		70 ± 72
2	14.8 ± 1.01	131 ± 21	$p < 0.001$	1890
3	1.81 ± 0.32	51.5 ± 8	$p = 0.016$	1010
4	12.9 ± 0.44	114 ± 25	$p < 0.001$	2198
5	2.97 ± 0.87	50 ± 8.5	$p = 0.01$	825
6	11.98 ± 1.06	115 ± 23.5	$p < 0.001$	1981
7	2.47 ± 0.482	52 ± 9.9	$p = 0.007$	1904
8	7.89 ± 0.10	83.6 ± 15	$p < 0.001$	1820
9	13.8 ± 1.01	108 ± 16.5	$p < 0.001$	2108
10	9.04 ± 0.91	87 ± 24	$p < 0.001$	2300
11	13.97 ± 0.46	128 ± 29	$p < 0.001$	2001
12	15.42 ± 0.42	131 ± 17	$p < 0.001$	1950
13	8.09 ± 0.32	99.5 ± 14.6	$p < 0.001$	1201
14	7.98 ± 1.02	77.5 ± 4.5	$p < 0.001$	1870
15	11.09 ± 0.79	101 ± 12	$p < 0.001$	2009
16	17.98 ± 1.05	153 ± 19.9	$p < 0.001$	2450
17	1.24 ± 0.82	38 ± 3.5	$p = 0.06$	1056
18	6.17 ± 0.89	84 ± 9.9	$p < 0.001$	1560
19	10.23 ± 0.98	98 ± 18	$p < 0.001$	1841
20	15.96 ± 1.03	131 ± 19	$p < 0.001$	2156
21	20.12 ± 1.04	152 ± 18.8	$p < 0.001$	2292
22	12 ± 1.09	113 ± 21	$p < 0.001$	1860
23	9 ± 0.68	72 ± 6.8	$p < 0.001$	1950
24	8.91 ± 0.79	105 ± 21.54	$p = 0.004$	1808
25	8 ± 0.83	70 ± 5.03	$p < 0.001$	1760
26	5.24 ± 0.47	100 ± 12.45	$p = 0.007$	1806
27[d]	1.12 ± 1.01	33.2 ± 5.68	$p = 0.3$	560
28	1.81 ± 0.32	28 ± 6.8	$p = 0.48$	320
29	0.90 ± 0.44	27.30 ± 10.21	$p = 0.53$	290
30	1.07 ± 0.32	27.90 ± 7.8	$p = 0.33$	180
31	0.92 ± 0.32	38 ± 10.8	$p = 0.37$	220
32	0.97 ± 9.96	20.5 ± 5.19	$p = 0.9$	260
33	0.89 ± 0.96	22.9 ± 9	$p = 0.58$	398
34	1.80 ± 1.02	33.30 ± 8.6	$p = 0.53$	350
35	1.02 ± 0.45	27.30 ± 8	$p = 0.43$	423

[a] *p* value of ELISA.
[b] Normal samples.
[c] From pool plasma.
[d] Nos. 27–35 are pediatric samples.

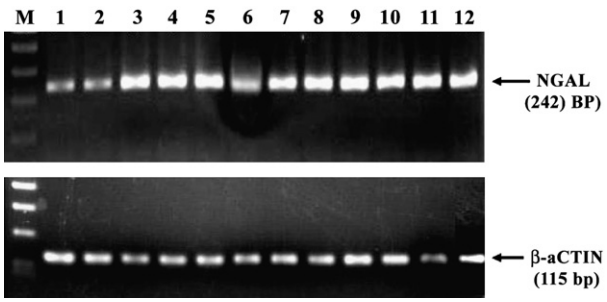

Figure 1. Upregulation of NGAL in β-thalassemia patients. Gene expressions were measured by semiquantitative RT-PCR. NGAL was upregulated in β-thalassemia patients (Lanes 3–12), Only 10 samples have been shown, compared to two normal samples (Lanes 1 and 2). M, 100-bp marker.

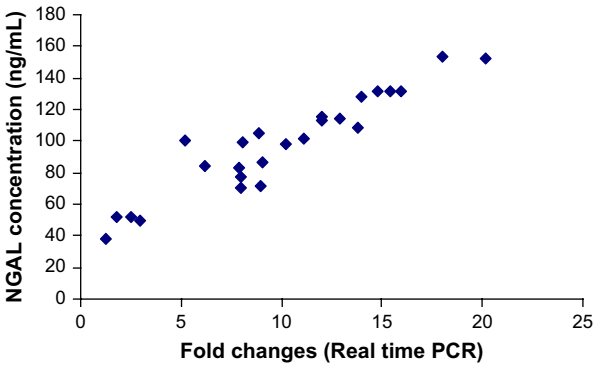

Figure 2. Correlation between real-time PCR and ELISA results. There was a positive correlation between NGAL concentration and fold changes in real-time PCR ($r = 0.943$, $p = 0.003$). Color version of this figure available online at www.arcmedres.com

was not more upregulated compared to that of the younger patients.

Discussion

Several reports indicated that expression of *NGAL Lcn2* was induced during harmful conditions such as cancer, intoxication, infection, inflammation, kidney injury, heart and burn injuries where production of free radicals has been reported (22–31). Our previous study revealed expression of NGAL/Lcn2 by ROS and the radiation (21). Several studies also reported the oxidative statuses in β-thalassemia (6–9). This study was conducted to investigate the expression of NGAL in β-thalassemia patients. Healthy samples express the same level of NGAL expression necessary for normal physiology of the cells. In β-thalassemia samples, NGAL upregulated both in mRNA and protein level. There was a positive correlation between Q (real-time)-PCR and ELISA. However, RNA values of isolated cells from peripheral blood do not exactly reflect ELISA levels in plasma. NGAL can be secreted by other organs such as liver and kidney. It is more likely that the upregulation is due to oxidative

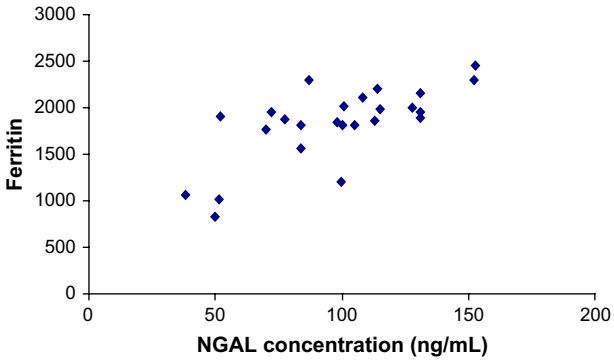

Figure 3. Correlation between ferritin concentration and ELISA results. There was a positive correlation between NGAL (values obtained from ELISA) and ferritin ($r = 0.707$, $p = 0.003$). Color version of this figure available online at www.arcmedres.com

status condition in β-thalassemia. The main source of ROS in this genetic disorder is iron overload (10,11). Patients with β-thalassemia, like those with genetic hemochromatosis, develop iron overload due to increased iron absorption, and their iron burden is further exacerbated by transfusion therapy. Circulating forms of iron that are not tightly bound to plasma transferrin have been termed as non-transferrin-bound iron (NTBI) (7,31). NTBI is detected whenever the capacity of transferrin to incorporate iron derived either from the gastrointestinal tract (GIT) or from reticuloendothelial cells becomes a limiting factor (32). Walter et al. reported a significant elevation of NTBI in all thalassemia patients compared with healthy samples and even sickle cell disease (8). Both NTBI and a labile plasma iron (LPI) pool appear primarily in heavily transfused patients where the transferrin iron-binding capacity has been surpassed (32), although this is not always the case (33). LPI refers to cell-penetrating forms of iron that are redox active and susceptible to chelation (34). Cell damage associated with iron overload has been attributed to the emergence of excessive levels of cell LPI that promote production of ROS exceeding cellular defense capacities (35). In other words, in thalassemia major, there is as an outpouring of catabolic iron, a Fenton reagent that overwhelms the iron-carrying capacity of plasma transferrin and generates redox-active forms. Oxidative stress statuses have been proven in β-thalassemia patients (6–9). In our previous study, we showed that ROS induced NGAL expression (21) and, according to the results of the present study, we think that ROS plays an important role in the upregulation of NGAL in this disorder. However, further and complementary studies are required in this aspect. Investigating the correlation between ROS and NGAL expression could be one of the possible studies in the future. Induction of NGAL, in addition to ROS production, could be due to NTBI. NGAL is an iron-trafficking protein, a member of the NTBI pool and an alternative to the transferrin-mediated iron-delivery pathway (36). Elevated expression of NGAL in thalassemia patients supports the role of NTBI proteins in the abnormal iron regulation in thalassemia. Overall, upregulation of NGAL may have two reasons: first it acts as an antioxidant, and second as an iron chelating agent. Administration of vitamin E, which is a lipid antioxidant, normalized the increased levels of ROS and exhibited improvement in oxidant-antioxidant balance in the plasma (37). Therefore, NGAL may have a similar function; however, further studies are required to prove this hypothesis. Recently, in accordance with our study, Weizer-Stern et al. reported upregulation of Lcn2/NGAL mRNA in β-thalassemia intermedia and β-thalassemia major mouse models (20). They also found downregulation of hepcidin, a key iron regulatory gene, regulates systemic iron homeostasis by inhibiting the absorption of iron from the diet and the recycling of iron by macrophages. However, Kattamise et al. (38) showed that hepcidin expression is regulated

mainly by increased erythropoietic activity rather than by iron load. More recently, Origa et al. (39) reported that hepcidin levels were elevated in thalassemia major due to transfusion that reduces erythropoetic drive and delivers large iron load and led to higher ferritin. In our study, the ferritin concentration in β-thalassemia was also higher compared to normal samples. Upregulation of NGAL was not observed in pediatric β-thalassemia samples, which indicates that the inducing factor, iron overload, was not sufficient, probably due to fewer blood transfusion therapies, to induce NGAL expression compared to adult.

In conclusion, iron overload and oxidative status in β-thalassemia patients induce NGAL/Lcn2 expression. Upregulation of NGAL in this disorder may play a beneficial role in decreasing ROS or chelating iron. Obviously, chelating of iron is one of the major therapeutic goals in β-thalassemia. It may be possible in the future to use exogenous NGAL for β-thalassemia patients. The beneficial role of NGAL has been reported previously (40). Moreover, NGAL measurement may be useful as part of the diagnostic and prognostic evaluation of thalassemia syndromes. However, further studies are required to clarify the precise function of NGAL in β-thalassemia patients.

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