Plasma neutrophil gelatinase-associated lipocalin levels are markedly increased in patients with non-transfusion-dependent thalassemia: Lack of association with markers of erythropoiesis, iron metabolism and renal function

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ABSTRACT

Background: Neutrophil Gelatinase-Associated Lipocalin (NGAL) (known as NGAL, Lipocalin 2, Siderocalin, Uterocalin, proteinase-3 and 24p3) is a mammalian small 25-kD peptide that belongs to the lipocalin superfamily, which consists of about 20 small lipoproteins. NGAL was initially discovered as an antibacterial factor of natural immunity and an acute-phase protein. NGAL is also an iron trafficking protein, a member of the non-transferrin-bound iron (NTBI) pool and an alternative to the transferrin-mediated iron-delivery pathway. Of note, NTBI, which is elevated in thalassemic patients, induces cellular toxicity. In this study we investigated the possible association of NGAL with parameters of erythropoiesis, iron metabolism and renal injury in patients with non-transfusion-dependent thalassemia (thalassemia intermedia or TI).

Patients and methods: Thirty-five patients with TI, 13 men and 22 women, aged 8–63 years, were included in the study, while, 20 healthy individuals served as controls. Plasma NGAL levels were determined using an immunoenzymatic technique. Erythroid marrow activity was estimated by measuring soluble transferrin receptors (sTfR) levels with a turbidimetric technique. NTBI levels were determined using electrothermal atomic absorption spectrometry. Cystatin C, β2-microglobulin and hs-CRP concentrations were measured by means of immunonephelometric techniques.

Results: The main results of the study showed: a) NGAL levels were significantly higher in patients with TI compared to controls (139.1 ± 86.1 vs 51.2 ± 11.8 μg/L, p < 0.0001), without significant effect of splenectomy or hydroxyurea on NGAL levels. Only 4 patients had NGAL levels within the control group range, b) no correlation was found between NGAL levels and either the parameters of erythropoiesis Hb, Hb F, reticulocytes and sTfR (p > 0.66, p > 0.67, p > 0.63 and p > 0.81 respectively), or with those of iron metabolism ferritin and NTBI (p > 0.90 and p > 0.95 respectively).

Conclusions: The increased NGAL levels reported for the first time in TI patients in this study are in agreement with the elevated expression of NGAL observed in TI mouse models. We postulate that the induction of NGAL in these patients may represent either a survival response, facilitating the survival of the less damaged thalassemic erythroid precursors, or a consequence of the abnormal iron regulation in TI.

INTRODUCTION

The term ‘thalassemia intermedia’ (TI) refers to patients with β-thalassemia major, who have a clinical phenotype that lies between the mild symptomatology of the β-thalassemia trait and the severe manifestations of transfusion-dependent β-thalassemia major. The definition of TI is based solely in clinical criteria, with the main one being the maintenance of satisfactory hemoglobin (Hb) levels of at least 6–7 g/dL without the need for regular blood transfusions [1,2].

Despite having characterized the underlying globin gene alterations in most of the patients, the severity of the clinical course remains unpredictable and shows extreme heterogeneity with frequent overlapping between the three conditions. For this reason, patients with a β-TI genotype may either be treated as patients with thalassemia major or followed as patients with thalassemia minor. Moreover, the diagnosis,
and thus the treatment, may change from TI and TM and vice versa, with time. This variability is, at least partially, explained by the role of phenotype-modifying genes and the worsening morbidity with age. In this respect, the term “non-transfusion-dependent thalassemia”, which has been recently introduced, is frequently used to better characterize the present condition of the patient [1,2].

Neutrophil gelatinase-associated lipocalin (NGAL) (known as NGAL, Lipocalin 2, Siderocalin, Uterocalin, proteinase-3 and 24p3) is a mammalian small 25-kD peptide that belongs to the lipocalin superfamily, which consists of about 20 small lipoproteins. NGAL was initially discovered as an antibacterial factor of natural immunity and an acute-phase protein [3,4]. Upon nephrotoxic and/or ischemic injury, NGAL levels are highly increased in kidney cortical tubules, blood and urine. Induction of NGAL after kidney injury precedes the elevation of classical markers for kidney damage, e.g. serum creatinine, urinary N-acetyl glucosaminidase and β2-microglobulin levels [4,5].

Unexpectedly, NGAL is abundantly expressed in erythroid progenitor cells. In vitro culture experiments demonstrated that NGAL induces apoptosis and inhibits differentiation of erythroid progenitor cells. During acute anemia, the expression of NGAL was reduced in erythroid cells by a feedback system. Furthermore, NGAL represents a key factor in the regulation of erythrocyte growth owing to its ability to inhibit the maturation and differentiation of bone marrow erythroid precursors and is also involved in an iron delivery pathway [6]. NGAL is also an iron trafficking protein, a member of the non-transferrin-bound iron (NTBI) pool and an alternative to the transferrin-mediated iron-delivery pathway [7]. Of note, NTBI, which is elevated in thalassemic patients, induces cellular toxicity [8]. Several systemic diseases associated with the presence of secondary anemia, such as chronic renal failure, chronic inflammation and cancer, are known to induce a dramatic increase in circulating NGAL levels [9–12]. Roudknar et al. showed that NGAL mRNA and protein levels are increased in patients with transfusion-dependent thalassemia major as a result of iron overload, while other studies suggested that elevated NGAL levels in these patients are mainly due to renal injury. To our knowledge there are no data so far concerning NGAL levels in patients with non-transfusion-dependent thalassemia [13–15]. In this study we investigated whether NGAL levels in patients with non-transfusion-dependent thalassemia are associated with renal injury, iron overload, erythropoiesis and/or inflammation.

**Patients and methods**

Thirty-five patients with TI, 13 men and 22 women, aged 8–63 years, were included in the study. The blood samples were collected in an outpatient basis, as the patients’ clinically steady state did not require hospitalization. Seven (7/35) patients were smokers, while one (1/35) presented with cardiac insufficiency, two (2/35) presented with diabetes mellitus and one (1/35) suffered from rheumatoid arthritis. Eight patients (8/35) received hydroxyurea (HU) and only 4 (4/35) had been transfused occasionally but none of them had received any transfusion at least 6 months before entering the study. Twenty-five (25/35) patients had been splenectomized. Twenty healthy age and sex-matched individuals were included in the control group. The study was approved by the Ethics Committee of the “Aghia Sophia” Children’s Hospital and was performed according to the Helsinki Declaration. Written informed consent was obtained from the parents of the patients and the apparently healthy controls.

Hematologic parameters and red blood cell indices were measured using a Siemens-ADVIA 120 whole blood auto-analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Hemoglobin levels were characterized and quantitated using weak cation-exchange high-pressure liquid-chromatography (CE-HPLC) with the Bio-Rad Variant Hemoglobin Testing system and the β-Thalassemia Short Program (Bio-Rad Laboratories, Hercules, CA, USA). Ferritin was quantitatively determined using the Roche E411 Cobas immunoassay analyzer (Roche Diagnostics, Mannheim, Germany), using an electrochemiluminescence technique. Intra- and inter-assay CVs were <3.5% and 4.4% respectively. Soluble transferrin receptors (sTfR) levels were measured using the Siemens Advia 1800 Clinical Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

Determination of serum non-transferrin-bound iron (NTBI) was performed using electrothermal atomic absorption spectrometry (GFAAS) (A-Analyst 800, Perkin Elmer AAS). Briefly, NTBI was chelated using nitriilotriacetic acid (NTA) and then ultrafiltrated. Serum ultrafiltrates were diluted six-fold with distilled water. NTBI from the Fe–NTA complex present in the serum ultrafiltrate was measured by GFAAS at 2100 °C element atomization. Serum NGAL concentration was determined using a solid phase ELISA technique (R&D Systems, Minneapolis, MN, USA). The intra-assay and inter-assay CVs ranged between 3.1% and 4.1% and between 5.6% and 7.9%, respectively, according to the manufacturer.

Cystatin C, β2-microglobulin and hs-CRP concentrations were measured by means of immunonephelometric techniques using the BN Prospec nephelometer (Siemens Healthcare Diagnostics, Liederbach, Germany). Estimation of glomerular filtration rate (eGFR) was calculated using a Cystatin C based equation: eGFR (ml/min) = 77.24 × (Cystatin-C–0.1327) [14].

**Statistical analyses**

Data are presented as mean ± SD, and the level of statistical significance was considered at p < 0.05. All the statistical procedures were performed using the STATGRAFICS PLUS version 3.1 for Windows program (Graphic Software System). We used the standardized skewness and standardized kurtosis, to determine whether the sample comes from a normal distribution. Values of these statistics outside the range of −2 to +2 indicate significant departures from normality, which would tend to invalidate many of the statistical procedures normally applied to this data. These values integrated automatically from the program indicated the parameters needed to transform in either log or reciprocal or square root, where needed. These transformations were then used for correlations between parameters.

**Results**

We initially analyzed and compared the levels of NGAL’s expression in patients with TI and in the normal control group. We found that NGAL levels were significantly higher in patients with TI compared to controls (139.1 ± 86.1 vs 51.2 ± 11.8 μg/L, p < 0.001, Table 1 and Fig. 1). Only 4/40 or 10% of the patients with TI had NGAL levels comparable to the control group’s range. No correlation was found between patients’ age

<p>| Table 1 Hematologic and blood chemistry findings in patients with thalassemia intermedia and healthy controls. |
|----------------------------------------------------------|------------------|-------------------|</p>
<table>
<thead>
<tr>
<th><strong>Thalassemia intermedia</strong></th>
<th><strong>Controls</strong></th>
<th><strong>Difference p-value</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NGAL (μg/L)</strong></td>
<td>139.1 ± 86.1</td>
<td>51.2 ± 11.8</td>
</tr>
<tr>
<td><strong>Erythropoiesis, iron metabolism and inflammation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB (g/dL)</td>
<td>88.0 ± 15.0</td>
<td>141.0 ± 10.0</td>
</tr>
<tr>
<td>Hb (g%)</td>
<td>53.0 ± 30.0</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>sTfR (mg/L)</td>
<td>11.8 ± 3.8</td>
<td>1.23 ± 0.19</td>
</tr>
<tr>
<td>Ferritin (μg/L)</td>
<td>627.4 ± 333.0</td>
<td>543 ± 44.6</td>
</tr>
<tr>
<td>NTBI (μmol/L)</td>
<td>24 ± 2.1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>1.3 ± 0.9</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td><strong>Renal function</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cystatin C (mg/L)</td>
<td>0.73 ± 0.12</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>β2-Microglobulin (mg/L)</td>
<td>1.86 ± 0.54</td>
<td>1.87 ± 0.23</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>118.0 ± 23.3</td>
<td>122.0 ± 17.5</td>
</tr>
</tbody>
</table>
and NGAL levels \( r = 0.211, p = 0.270 \). When patients with TI were divided into two groups according to splenectomy (Spl) status, we observed that NGAL levels of Spl(+) and Spl(−) did not differ significantly \( 162.1 \pm 61.7 \) vs. \( 128.3 \pm 47.9 \mu g/L \) respectively, \( p = 0.348 \) (Fig. 2a). Similarly, when patients with TI were divided into two groups according to hydroxyurea treatment (HU), we also observed that NGAL levels of HU(+) and HU(−) did not differ significantly \( 149.7 \pm 140.5 \) vs. \( 136.8 \pm 34.8 \mu g/L \) respectively, \( p = 0.767 \) (Fig. 2b).

**Correlation between NGAL levels with markers of erythropoiesis and iron metabolism**

Patients with TI had significantly lower Hb levels compared to controls \( p < 0.001 \), while they had significantly higher ferritin and NTBI levels \( p < 0.001 \) indicative of iron overload and iron-mediated toxicity compared to controls \( p < 0.001 \), as well as significantly higher sTfR and hs-CRP levels compared to controls \( p < 0.001 \) and \( p = 0.007 \), respectively), indicative of ineffective erythropoiesis and low grade inflammation (Table 1).

We found no correlation between NGAL levels and the concentration of the parameters of erythropoiesis (Hb, Hb F, reticulocyte number and sTfR) or iron metabolism and toxicity (ferritin and NTBI) \( p > 0.55, p > 0.70, p > 0.63, p > 0.85, \) and \( p > 0.69, \) respectively), while a weak but significant non-linear correlation was found between NGAL and hs-CRP levels \( r = 0.392, p = 0.03 \) (Table 2).

**Correlation between NGAL levels and renal function parameters**

Patients with TI had serum Cystatin C levels comparable to controls and within normal limits \( 0.73 \pm 0.12 \) vs. \( 0.75 \pm 0.09 \) mg/L, \( p > 0.564 \), and normal eGFR ranged from 80.0 to 168.0 mL/min. Similarly, no difference was found between serum \( 2\)-microglobulin levels in patients with TI and normal controls, \( 1.86 \pm 0.54 \) vs. \( 1.87 \pm 0.23 \) mg/L, \( p > 0.92 \).

No correlation was found between NGAL levels and markers of renal function, namely Cystatin C, eGFR and \( 2\)-microglobulin \( p > 0.73, p > 0.73 \) and \( p > 0.64 \), respectively) (Table 2).

**Discussion**

The decreased synthesis of the \( \beta \)-globin chains in the developing \( \beta \)-TI erythroid precursors results in accumulation and precipitation of the \( \alpha \)-globin chains, leading to their premature death and thus, to ineffective erythropoiesis. Iron overload, which may cause serious tissue damage, is due to increased iron absorption from the gastrointestinal tract and from blood transfusions [1,2] Circulating forms of iron, not tightly bound to plasma transferrin, have been termed as NTBI. NTBI usually appears when the iron-carrying capacity of plasma transferrin is overwhelmed and they may generate redox-active forms (superoxide \( O_2^- \) and hydrogen peroxide \( H_2O_2 \)). The fact that high levels of NTBI are detected even in TI patients with low iron load is probably due to the considerable increase of bone marrow erythroid activity, which increases plasma iron turnover and, thus, releasing substantial amounts of NTBI. This is also supported by previous reports, which showed that

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**Table 2**

Correlation matrix of NGAL with parameters of erythropoiesis, iron metabolism, inflammation and renal function.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NGAL Correlation coefficient</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Hb</td>
<td>−0.113</td>
<td>0.55</td>
</tr>
<tr>
<td>Hb F</td>
<td>0.074</td>
<td>0.70</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>0.09</td>
<td>0.63</td>
</tr>
<tr>
<td>sTfR</td>
<td>0.033</td>
<td>0.85</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.052</td>
<td>0.78</td>
</tr>
<tr>
<td>NTBI</td>
<td>0.075</td>
<td>0.69</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>0.392</td>
<td>0.03</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>−0.065</td>
<td>0.73</td>
</tr>
<tr>
<td>( \beta )-Microglobulin</td>
<td>−0.089</td>
<td>0.64</td>
</tr>
<tr>
<td>eGFR</td>
<td>0.065</td>
<td>0.73</td>
</tr>
</tbody>
</table>
the mean sTfR was higher in severe compared to mild TI and positively correlated to plasma NTBI levels [8].

The main finding of this study is that patients with TI have markedly elevated NGAL levels compared to normal controls independent of age, splenectomy and/or hydroxyurea therapy. Furthermore, these levels are higher from those reported for patients with transfusion-dependent thalassemia major who had normal renal function. As we used the same assay for the determination of plasma NGAL levels, comparison with previously published data was feasible, although the authors acknowledge the limitation of this approach [13–15].

Many reasons can be contemplated for elevated NGAL levels in patients with TI, including anemia/hypoxia, renal damage, and iron homeostatic disturbances. The fact that no correlations with indices of iron overload and anemia could be demonstrated in this study may be due to the small number of patients and their significant heterogeneity.

Elevated systemic NGAL levels may contribute to anemia as a direct inhibitor of erythrocyte maturation. Local synthesis of NGAL by immature medullary erythroid stem cell progenitors constitutes an autocrine regulatory pathway promoted by interleukin-1 that induces inhibition of erythropoiesis through induction of apoptosis and arrest of differentiation [6]. The increased systemic NGAL levels would have a negative impact on the bone marrow red cell homeostasis, thus the bone marrow counteracts this potential negative effect by reducing the production of NGAL by the same erythroid precursor and by enhancing the survival mechanisms of these cells. Another fact that should also be mentioned is that the peripheral levels (of the factors) that are being measured may not consistently reflect the levels measured in the bone marrow [6,12].

Upregulation of LCN2 gene expression and elevation of plasma NGAL protein levels have been reported in mouse models with beta-thalassemia major and/or intermedia or anemia induced by phlebotomy, iron deprivation, or phynylhydrazine treatment [16,17]. NGAL is also induced during hypoxia, indicating that tissue oxygenation levels may be a factor in the regulation of NGAL expression by anemia. Induction of NGAL expression by anemia/hypoxia was not found to require increased erythropoiesis and is not directly mediated by erythropoiesis [17]. NGAL is an iron-trafficking protein, a member of the NTBI pool and an alternative to the transferrin mediated iron-delivery pathway. NGAL circulates in iron-associated siderophore and iron-free forms siderophore. The iron-associated siderophore NGAL delivers iron into cells following 24p3R or megalin receptor-mediated uptake and trafficking into acidic endosomes, where iron is released and subsequently accumulates in the cytoplasm. In parallel, siderophore iron-free NGAL is proposed to scavenge excess-excess intracellular and extracellular iron, limiting labile iron-mediated cytotoxicity. Elevated expression of NGAL in TI patients supports the role of NTBI proteins in the abnormal iron regulation in thalassemia. However, it must be noticed that previous studies have shown that serum and tissue iron levels are unlikely to account for the regulation of hepatic NGAL in situations of disrupted iron homeostasis [13].

NGAL is rapidly released by a variety of cell types, including renal tubular cells, liver hepatocytes, endothelial and smooth muscle cells, in response to diverse cellular stresses, including inflammation and ischemia. The storage of considerable amounts of iron in the kidney causes significant metabolic and functional damages, leading to renal dysfunction. NGAL was expressed in response to typical stimuli which trigger renal disease such as depletion of ATP, exposure to H2O2 or exposure to bacteria. Microscopic analysis provided additional confirmation that damaged nephrons generate NGAL. Hence, NGAL is a direct response to infectious damage and consequently might also serve as a prophylactic peptide in aseptic injury. The greater the dysfunction of the kidney, the greater is the expression of NGAL, implying that NGAL derived from the damaged nephrons by an autonomous response [18, 19].

Interestingly, we found that NGAL levels correlated with hs-CRP levels. We have recently reported that patients with TI as well as patients with sickle cell-beta thalassemia have a chronic low-grade inflammation [20,21]. Low-grade inflammation has been implicated in the etiology of clusters of so-called metabolic syndromes, such as insulin resistance, visceral adiposity and atherosclerosis. All these are known risk factors for chronic kidney and chronic heart disease where a correlation between NGAL and hs-CRP has been reported [11]. In conclusion, the increased NGAL levels reported for first time in patients with non-transfusion-dependent thalassemia in this study are in agreement with the elevated expression of NGAL observed in non-transfusion-dependent thalassemia mouse models. We postulate that the induction of NGAL in these patients may represent either a survival response, facilitating the survival of the less damaged thalassemic erythroid precursors, or a consequence of abnormal iron regulation. Further research is required to validate these hypotheses.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

Funding was received from Athens University to Dr Ioannis Papassotiriou (ELKE 70/3/5924-7303). The funding source played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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