Clinical Biochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

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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ARTICLE INFO

Article history:
Received 6 February 2014
Received in revised form 17 March 2014
Accepted 28 March 2014
Available online xxxx

Keywords:
Thalassemia intermedia
NGAL
Erythropoiesis
Iron metabolism
Kidney injury

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,

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http://dx.doi.org/10.1016/j.clinbiochem.2014.03.017
0009-9120/

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 glucosamidase 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]. Roudkenar 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). Hemoglobins 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 nitrilotriacetic 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 (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany). Estimation of glomerular filtration rate (eGFR) was calculated using a Cystatin C based equation: eGFR (mL/min) = $77.24 \times (\text{Cystatin-C})^{-1.2623}$ [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 5.1 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

Table 1
Hematologic and blood chemistry findings in patients with thalassemia intermedia and healthy controls.

	Thalassemia intermedia	Controls	Difference p-value
NGAL (μg/L)	139.1 ± 86.1	51.2 ± 11.8	<0.001
Erythropoiesis, iron metabolism and inflammation			
Hb (g/L)	88.0 ± 15.0	141.0 ± 10.0	<0.001
Hb F (%)	53.0 ± 30.0	<0.5	<0.001
sTfR (mg/L)	11.8 ± 3.8	1.23 ± 0.19	<0.001
Ferritin (μg/L)	627.4 ± 333.0	54.3 ± 44.6	<0.001
NTBI (μmol/L)	2.4 ± 2.1	<0.5	<0.001
hs-CRP (mg/L)	1.3 ± 0.9	0.6 ± 0.4	=0.007
Renal function			
Cystatin C (mg/L)	0.73 ± 0.12	0.75 ± 0.09	NS
β_2-Microglobulin (mg/L)	1.86 ± 0.54	1.87 ± 0.23	NS
eGFR (mL/min)	118.0 ± 23.3	122.0 ± 17.5	NS

Please cite this article as: Patsaoura A, et al, Plasma neutrophil gelatinase-associated lipocalin levels are markedly increased in patients with non-transfusion-dependent thalass..., Clin Biochem (2014), http://dx.doi.org/10.1016/j.clinbiochem.2014.03.017