Cytokine 80 (2016) 18–25

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Plasma levels of TGF-β1 in homeostasis of the inflammation in sickle cell disease

Lidiane de Souza Torres [a,*], Jéssika Viviani Okumura [a], Danilo Grünig Humberto da Silva [a], Édis Belini Júnior [a], Renan Garcia de Oliveira [a], Kallyne Kioko Oliveira Mimura [b], Clarisse Lopes de Castro Lobo [c], Sonia Maria Oliani [b], Claudia Regina Bonini Domingos [a]

[a] Laboratory of Hemoglobin and Hematologic Genetic Diseases, Department of Biology, Sao Paulo State University (Unesp), Rua Cristóvão Colombo, 2265, São Jose do Rio Preto, SP 15054-000, Brazil
[b] Laboratory of Imunomorphology, Department of Biology, Sao Paulo State University (Unesp), Rua Cristovão Colombo, 2265, São Jose do Rio Preto, SP 15054-000, Brazil
[c] Institute of Hematology Arthur de Siqueira Cavalcanti (Hemorio), Rua Frei Caneca, 08, Rio de Janeiro, RJ 20211-030, Brazil

ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 20 February 2016
Accepted 23 February 2016
Available online 27 February 2016

Keywords:
Transforming growth factor
Cytokines
Platelets
Sickle cell anemia
Sickle cell disease

ABSTRACT

Sickle cell disease (SCD) represents a chronic inflammatory condition with complications triggered by the polymerization of hemoglobin S (Hb S), resulting in a series of cellular interactions mediated by inflammatory cytokines, as the transforming growth factor beta (TGF-β), which plays an important role in inflammation resolution. This study assessed the relation between SCD inflammation and the plasma concentration of TGF-β1, and also checked the influence of the presence of −509C/T polymorphism in *TGFB1* gene on TGF-β1 plasma values. The plasma levels of TGF-β1 were quantified by ELISA in 115 patients with SCD (genotypes SS, SD-Los Angeles, Sβ-thalassemia and SC) and in 58 individuals with no hemoglobinopathies (Hb AA), as the control group. The −509C/T polymorphism in *TGFB1* gene was screened by PCR-RFLP. The correlation between TGF-β1 plasma levels and the inflammation was based on its association with the count of platelets, total white blood cells (WBC) and neutrophils in the peripheral blood. Patients with SCD showed plasma levels of TGF-β1 higher than the control group, especially the Hb SS genotype, followed by the group with Hb SD. Polymorphism investigation showed no interference in the values obtained for the cytokine in the groups evaluated. All SCD groups showed TGF-β1 levels positively correlated to the platelets and WBC counts. The original data obtained in this study for SCD support the involvement of TGF-β1 in regulating of the inflammatory response and suggest that this marker possibly may become a potential therapeutic target in the treatment of the disease.

1. Introduction

Sickle cell disease (SCD) comprehends the group of hemolytic anemia caused by the presence of hemoglobin S (Hb S), either in homozygosis (sickle cell anemia), or in association with other hemoglobin variants or thalassemias [1]. Hb S originates from a point mutation in the beta globin gene (*HBB*:c.20A > T; rs334) resulting in replacement of glutamic acid for valine in the polypeptide chain, causing structural and biochemical modifications in the hemoglobin molecule [1–3]. Sickle cell anemia is the most common form of the disease, as well as the most severe. The association of Hb S with other variants such as Hb C (*HBB*:c.19G > A; rs33930165) and Hb D-Los Angeles (*HBB*:c.364G > C; rs33946267) are less frequent and less severe, while its association with beta thalassemia results in moderate to severe clinical types, depending on the beta-thalassemia mutation inherited [1,4].

Complications of SCD start with the polymerization of Hb S, erythrocyte sickling, exposure of membrane proteins, hemolytic anemia and ischemia-reperfusion cycles. These recurrent cycles result from vascular occlusion and represent the main stimulus for the inflammatory process, due to endothelial dysfunction, increased vascular inflammation, coagulation activation and oxidative stress created during the restoration of blood flow [5]. Although it plays a protective role in the infection control and promotes tissue repair, the exaggerated inflammatory response can also cause tissue damage. In SCD, inflammation may occur in acute and chronic forms, due to polymerization of Hb S, which not only results in erythrocyte sickling and intravascular hemolysis, but also a series of cellular interactions mediated by inflammatory cytokines [6].

The transforming growth factor beta (TGF-β) is a pleiotropic cytokine that affects cell proliferation, survival and migration and

* Corresponding author.
E-mail address: lidiane.unesp@gmail.com (L.S. Torres).

http://dx.doi.org/10.1016/j.cyto.2016.02.012
1043-4666/

might act as both positive and negative regulator during gene transcription, depending on the target genes and cellular context. It exists in three isoforms: TGF-β1, 2 and 3, whereas TGF-β1 is the most abundant [7]. TGF-β plays an important role in inflammation resolution, since it is associated with the inhibition of immune cells proliferation and the activity suppression of immune system precursor cells. It also acts as a potential inhibitor on T cells differentiation and apoptosis inducer in B cells, besides participating in the chemotaxis and polarization of macrophages and neutrophils at the inflammation site. On the other hand, it is involved in the releasing of proinflammatory cytokines from neutrophils and in stimulating pro-inflammatory Th17 cells lineage differentiation [8,9].

The TGF-β production can be controlled by single nucleotide polymorphisms (SNP) in its gene, such as the −509C/T on *TGFB1* (rs1800469), wherein the mutant allele T is associated with high circulating levels of TGF-β1 [10]. The elevation in the TGF-β1 levels has been described in sickle cell anemia [11,12] and genetic polymorphisms in *TGFB*, its receptors and members of its activation pathway were related to subphenotypes of the disease, including clinical complications such as myocardial infarction, osteonecrosis, priapism, leg ulcer and pulmonary hypertension [13].

In the present study we built on the observation of the inflammatory condition in SCD patients – genotypes SS, SD-Los Angeles (SD), S/beta-thalassemia (Sβ-thal) and SC – based on the association of TGF-β1 plasma levels to the total amount of white blood cells (WBC), neutrophils and platelets in the peripheral blood. In addition, we evaluated the relation between the −509C/T polymorphism in the *TGFB1* gene and the TGF-β1 plasma values.

2. Methods

2.1. Subjects

The study consisted of 115 SCD patients from the *Arthur de Siqueira Cavalcanti* Institute of Hematology (Hemorio) in Rio de Janeiro, RJ, Brazil. In order to minimize biases in the analysis, all patients were selected according to exclusion criteria, namely: anti-inflammatory prescription for three weeks prior to sample collection, use of hydroxyurea for up to six months preceding the collection date and blood transfusions carried out in <60 days (or Hb A > 10.0%) [14]. In addition, only individuals over 10 years old were included in the study, since at this age the hemoglobin profile is usually stable [15]. Fifty-eight volunteers, adults of both genders and with normal hemoglobins, without the use of anti-inflammatory drugs for three weeks, were part of the control group. The work has the approval by the Research Ethics Committee from *Sao Paulo State University* (*UNESP*) under Certificated of Presentation for Ethics Consideration (CAAE) number 08813112.7.0000.5466.

2.2. Samples, hemoglobin profile and genotyping for SCD

Peripheral blood samples (5 ml) were collected into tubes containing 5% ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The hemoglobin migration pattern was evaluated by electrophoresis on cellulose acetate at pH 8.6 [16] and agar-agar gel electrophoresis at pH 6.2 [17]. The red cell morphology was analyzed in light microscope with 40× objective lens. The quantification of the hemoglobin fractions was performed by high performance liquid chromatography (HPLC) by VARIANT™ automated equipment (Bio-Rad Laboratories, CA, USA).

To confirm SCD genotype by molecular biology, DNA was extracted from leukocytes by phenol-chloroform method [18] and then subjected to polymerase chain reaction followed by restriction fragment analysis (PCR-RFLP) for identification of mutations that result in hemoglobins S, C, and D-Los Angeles (Table 1) [19].

After genotyping, individuals were separated into four SCD study groups: Hb SS, Hb SD, Hb Sβ-thal and Hb SC; and the control group: Hb AA.

2.3. TGF-β1, platelets and WBC

The −509C/T polymorphism in the *TGFB1* gene was screened by PCR-RFLP using primers sense 5′- CCGCTTCTGTCCTTTCTAGG - 3′ and antisense 5′- AAAGCGGGTGATCCAGATG - 3′. The reaction mix consisted of 1× reaction buffer, $MgCl_2$ (5 mM), dNTPs (0.4 mM), 0.8 mM of each primer, 1 unit of Taq polymerase and 12 ng/μL of DNA. The cycling conditions applied were: 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension of 7 min at 72 °C. The 406 bp fragment was treated with *Bsu36* I restriction enzyme (5′-TNAG G↓CC-3′), which recognizes the cleavage site in the presence of the normal allele C and generates two fragments: 223 bp and 183 bp. The fragments were visualized on 2.5% agarose gel stained with ethidium bromide.

The plasma levels of TGF-β1 cytokine were evaluated by multiplex instrument LUMINEX xMAP MAGPIX (Millipore Corporation, Billerica, MA, USA). The platelets, WBC and neutrophils count was carried out by flow cytometry and spectrophotometry methods.

2.4. Statistical analysis

The *chi-square* and *Fisher's exact* tests were applied to the genotypic and allelic frequencies analysis of the −509C/T (*TGFB1*) polymorphism in the study groups. The relationship between plasma levels of TGF-β1 and the −509C/T polymorphism, as well as TGF-β1 levels between the SCD and control groups, were evaluated by *Student's t-test* or *Mann-Whitney* test, depending on nature of the data (parametric or non-parametric distribution). The TGF-β1, platelets, WBC and neutrophils comparisons among the four SCD genotypes were performed using the *one-way Anova* followed by *post hoc Tukey-Kramer*. Relations between circulating levels of the markers were evaluated by *Pearson* correlation test. Simple linear regression analysis was applied to verify the dependence of TGF-β1, platelets and WBC variables. In all cases, non-parametric data were transformed into square root or base-10 logarithm in an attempt to prioritize the use of parametric tests. The adopted confidence interval was 95%, with a significance level of $p < 0.05$.

3. Results

3.1. Characterization of the study groups

After characterization of the 115 samples obtained from patients with SCD, 67 (58.3%) were from patients with Hb SS, 30 (26.1%) with Hb SC, 10 (8.7%) with Hb Sβ-thal and eight (6.9%) with Hb SD. All 58 control subjects were confirmed with Hb AA profile. The hemoglobin profile presented by the five study groups are detailed in Table 2.

3.2. −509C/T polymorphism frequency and its influence on circulating levels of TGF-β1

The −509C/T polymorphism was investigated in all samples. In the case group we found 39.1% of the CT genotype and 9.6% of TT