ORIGINAL RESEARCH REPORT

Clinical events and their relation to the tumor necrosis factor-alpha and interleukin-10 genotypes in Sickle-Cell-Anemia patients

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Abstract
Objective/background: Sickle-cell anemia (SCA) is a genetic blood disease characterized by chronic inflammation and a heterogeneous clinical picture. Serum tumor necrosis factor (TNF-alpha) and interleukin 10 (IL-10) levels are associated with the clinical course of SCA. This study aimed to evaluate the association between the frequency of the polymorphisms TNF-alpha-308 G → A, IL-10-1082 G → A, IL-10-819 C → T, and IL-10-592 A → C; serum TNF-alpha; and IL-10 levels, and the incidence of clinical events in SCA patients.

Methods: Polymerase chain reaction–restriction fragment length polymorphism and enzyme-linked immunosorbent assay were performed on 25 adults with SCA at the steady state; their data were compared with those for 26 healthy individuals.

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Introduction

Sickle-cell anemia (SCA) results from a point mutation, in which an adenine nucleobase in the sixth codon of the β-globin gene is replaced by a thymine (GAG → GTG). The molecular translation replaces glutamic acid with valine, thereby producing an abnormal form of hemoglobin called hemoglobin S [1]. Although the molecular lesion is limited to a single nucleotide, the SCA gene is pleiotropic and leads to multiple phenotypic expressions. SCA patients may present with various complications, such as recurrent episodes of vaso-occlusion, acute chest syndrome (ACS), stroke, infections, and priapism. These complications vary considerably among patients and over time [2–4].

The repeated polymerization of hemoglobin S can cause definitive damage to the structure of red blood cells, producing mainly intravascular hemolysis and vaso-occlusion, and triggering a cyclical cascade of reactions that culminates with the generation of reactive oxygen species and increased oxidative stress, reduced bioavailability of nitric oxide, endothelial injury, hypercoagulability, increased expression of adhesion molecules in blood and endothelial cells, ischemia/reperfusion injury, and chronic inflammation. Alone or in combination, these reactions are associated with inflammatory responses in various organs and can produce an array of other secondary pathological conditions [5].

For reasons not yet fully understood, the clinical outcome of SCA is highly variable. Several factors have been identified as modulators of clinical severity in SCA, including fetal hemoglobin (HbF) levels, association with α-thalassemia, β-globin haplotypes, and the presence of single-nucleotide polymorphisms (SNPs) [6–8]. SNPs are sites in which the genomic DNA sequence of a percentage of individuals in the population differs by a single base. It is the most common type of variation in the human genome [9].

Some authors have hypothesized that the phenotype of SCA patients is modulated by polymorphisms in genes involved in inflammation, cell interaction (vascular cell adhesion molecule 1, complement receptor 1, P-selectin, and alpha V integrin), modulation of oxidant injury, and nitric-oxide biology (nitric oxide synthase 2, nitric oxide synthase 3, and arginase, type II) [10–12].

The polymorphism tumor necrosis factor (TNF-alpha)-308 G → A has been shown to be associated with an increased risk of stroke in patients with SCA [13]. The polymorphism in the interleukin 10 (IL-10) gene is expressed in three genotypes: AA, AG, and GG, associated with low, intermediate, and high IL-10 production, respectively.

Results: The most frequent genotype of the TNF-alpha polymorphism was GG (low producer), and the most frequent genotype of the IL-10 polymorphisms was “low producer” (ACC ACC, ACC ATA, ATA ATA). The TNF-alpha levels were significantly higher in SCA in patients with acute chest syndrome (ACS). The IL-10 levels were reduced in polytransfusion and in patients with ACS.

Conclusion: The patients presented prevalence of TNF-alpha and IL-10 low-profile producer. The cytokine serum levels presented an association with the presence of polytransfusion and ACS in SCA patients.

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Materials and methods

Participants

This analytical study was based on a cross-sectional sample of 25 adults diagnosed with SCA attending the hematology outpatient service of the Walter Cantidio University Hospital in Fortaleza, Brazil, in 2013 and 2014. All patients were at the steady state in accordance with the criteria proposed by Ballas (2012) [15]: absence of painful episodes and/or intercurrent illnesses, such as infections and inflammation in the 4 weeks preceding the study; no hospital admissions in the 3 days preceding the study; and no blood transfusions in the 4 months preceding the study. Patients with infectious diseases, hemoglobin profiles incompatible with SCA, history of blood transfusion within the preceding 4 months, and/or inflammatory episodes during the study were excluded. Clinical data, complete blood count, and HbF values were collected from the patients’ medical records. Demographic data were obtained through interviews with the patients. The study protocol was approved by the Walter Cantidio University Hospital Research Ethics Committee. All patients and controls gave their informed written consent.
consent, and all study procedures followed the guidelines set forth in Resolution 466/12 of the National Health Council, which regulates research involving human participants.

The control group consisted of 26 healthy individuals (HbAA), adults, volunteers, and both sexes, with aleatory selection and enrolled in the Pharmacy School of the Federal University of Ceará. They had normal hemoglobin profiles, and no recent history of anemia, inflammatory conditions, or hematological disease.

**Serum levels of TNF-alpha and IL-10**

The serum TNF-alpha and IL-10 levels were measured by enzyme-linked immunosorbent assay (BD Biosciences, Pharmingen, California, USA), following the manufacturer’s instructions.

**Typing of SNPs**

Genomic DNA was extracted from leukocytes and stored at −80°C until the moment of typing. The TNF-alpha-308 G → A, IL-10-1082 G → A, IL-10-819 C → T, and IL-10-592 A → C polymorphisms were evaluated by polymerase chain reaction and restriction fragment length polymorphism, as described elsewhere [16,17]. The polymorphisms were stratified according to the TNF-alpha profile: AA (high producer), GA (intermediate producer), and GG (low producer), and IL-10 profile: GCC GCC (high producer), GCC ATA GCC ACC (intermediate producer), and ACC ACC ATA ATA ATA (low producer). The association between the genotype and the cytokine profile (high/intermediate/low producer) was determined using the reference values specified by the manufacturer of the typing kit One Lambda (Canoga Park, CA, USA) and data published in the literature [17].

**Statistical analysis**

The associations between polymorphisms and clinical events were analyzed with the two-tailed Fisher’s exact test, while the Kruskal–Wallis test, the unpaired t test, and the Mann–Whitney test were used to assess the relationship between the genotypes of the polymorphisms and the respective serum cytokine levels, and to compare patients and controls. The p values were calculated for each comparison. The level of statistical significance was set at 5% (p < .05), with a 95% confidence interval.

**Results**

The hematologic details of the SCA patients can be observed in Table 1.

The study included 25 SCA patients (11 women and 14 men) aged 35.72 years on the average (range: 19–63 years) and 26 healthy controls (19 women and 7 men) with an average age of 32.2 years (18–59 years). The most frequently observed clinical complication was pain crisis (75%), followed by polytransfusion and hospitalization (both 46.4%), leg ulcers (42.9%), gallstones (32.1%), and ACS and avascular necrosis of the femoral head (both 14.3%) (Table 1).

The average TNF-alpha levels were significantly higher in SCA patients (27.01 pg/mL) than in controls (4.34 pg/mL) (p = .0089) (Figure 1).

The IL-10 serum levels were not significantly different between the SCA patients (10.93 pg/mL) and the control group (9.98 pg/mL) (p = .5088) (Figure 2).

The most frequent genotype of patients with the TNF-alpha-308 G → A polymorphism was GG (low producer/ n = 20; 80%), followed by GA (intermediate producer/n = 5; 20%). None of the patients were AA (high producer/ n = 0; 0%).

The most frequent genotype of patients with the IL-10-1082 G → A, IL-10-819 C → T, and IL-10-592 A → C gene polymorphisms was ACC ACC, ACC ATA, ATA ATA (low producer/n = 13; 52%), followed by ATA GCC, GCC ACC (intermediate producer/n = 10; 40%) and GCC GCC (high producer/n = 2; 8%) (Table 2).

The patients showed no significant difference in serum levels of cytokines when compared in relation to the IL-10 and TNF-alpha producer cytokine profile.

The SCA patients with ACS showed a significant increase in TNF-alpha serum levels (p = .0417) (Figure 3), and IL-10 serum levels presented reduced significantly in polytransfused patients (p = .0485) (Figure 4). The patients with IL-10 low-profile producer showed a significant association with the presence of ACS (p = .0463) (Figure 5).

**Discussion**

Our sample of patients presented a hematological profile typical of SCA: normochromic and normocytic anemia, leukocytosis, and increased HbF levels. The finding of moderate anemia, mild leukocytosis, and high HbF levels even in steady-state patients is supported by the literature. An excellent marker of severity of SCA, increased HbF levels are associated with a reduction in episodes of vaso-occlusion, transfusions, and hospitalizations [18,19]. The high HbF levels observed in this study may also be attributed to the use of hydroxyurea, which has been documented to increase the HbF concentrations in SCA patients.

In this study, the most frequent clinical complication was pain crisis, followed by hospitalization, blood transfusion, and recurrent infection. This matches the findings of Boas, Cerqueira, Zanette, Reis, and Gonçalves (2010) [20] for adult patients with active SCA treated at a referral center in Salvador (Bahia, Brazil).

Produced by macrophages and T lymphocytes, TNF-alpha is known for its pro-inflammatory activities, including the activation of endothelial cells, stimulation of inflammation, induction of the coagulation cascade, synthesis of acute-phase proteins, activation of neutrophils, and stimulation of neutrophil adhesion. Thus, increased serum TNF levels in SCA patients indicate a persisting inflammatory process.

In the present study, the TNF-alpha levels were significantly higher among patients than controls. This finding agrees with other studies that have shown this cytokine to be elevated in children and adults with SCA both in the steady state and during crisis [21–23]. The increase in TNF-alpha levels is due to chronic inflammatory processes, even in steady-state SCA [24,25].
Produced primarily by monocytes, IL-10 is an anti-inflammatory cytokine that contributes to regulating the production of pro-inflammatory cytokines. In this study, the patients and controls did not differ significantly with regard to IL-10 levels. Likewise, Graido-Gonzalez et al. (1998) [26] found the IL-10 levels in adults with SCA to increase during crisis, but not in the steady state.

**Table 1** Hematologic details of Sickle-Cell-Anemia patients (n = 25).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>37.28 (range: 19–63)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14 (50%)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (50%)</td>
</tr>
<tr>
<td>Hb (g/DL)</td>
<td>8.147 ± 1.129</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>25.35 ± 3.753</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>92.20 ± 12.84</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>33.12 ± 1.919</td>
</tr>
<tr>
<td>Leukocytes (/mm³)</td>
<td>11,091 ± 4,403</td>
</tr>
<tr>
<td>Platelets (/mm³)</td>
<td>432,854 ± 112,640</td>
</tr>
<tr>
<td>HbF (%)</td>
<td>7.475 ± 4.175</td>
</tr>
</tbody>
</table>

Note. Data values are expressed as mean ± standard error of the mean (SEM). Hb = hemoglobin; HbF = fetal hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = Mean Corpuscular Volume.

**Table 2** Tumor Necrosis Factor-Alpha and Interleukin-10 Genotype Frequency in 25 Sickle-Cell-Anemia Patients in Steady State.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency</th>
<th>Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>5 (20%)</td>
<td>Intermediate producer</td>
</tr>
<tr>
<td>GG</td>
<td>20 (80%)</td>
<td>Low producer</td>
</tr>
<tr>
<td>AA</td>
<td>0 (0%)</td>
<td>High producer</td>
</tr>
<tr>
<td>ACC</td>
<td>1 (4%)</td>
<td>Low producer</td>
</tr>
<tr>
<td>ACC ATA</td>
<td>7 (28%)</td>
<td>Low producer</td>
</tr>
<tr>
<td>ATA ATA</td>
<td>5 (20%)</td>
<td>Low producer</td>
</tr>
<tr>
<td>GCC ATA</td>
<td>5 (20%)</td>
<td>Intermediate producer</td>
</tr>
<tr>
<td>GCC ACC</td>
<td>5 (20%)</td>
<td>Intermediate producer</td>
</tr>
<tr>
<td>GCC GCC</td>
<td>2 (8%)</td>
<td>High producer</td>
</tr>
</tbody>
</table>

Note. IL = interleukin; TNF = tumor necrosis factor.
In our sample, the predominant genotype of the TNF-alpha-308 G → A polymorphism was GG (80%), followed by GA (20%) and AA (0%). From the study by Cajado et al. (2011) [22] based on a sample of SCA patients from Brazil northeast, Bahia, the frequency follows the same profile: 80.2%, 18.2%, and 1.6%. In SCA patients from the state of São Paulo, Brazil, the frequency was 83.7% for GG and 16.3% for GA [27]. According to Hoppe et al. (2007) [13], in a sample of African–American children classified as HbSS, GG was observed with a frequency of 75%.

In our study, polymorphisms were typed in the IL-10 gene regions -1082 G → A, -819 C → T, and -592 A → C. The genotype ACC/ATA was the most frequent among the patients (25%).

As expected, in the patient group, the serum TNF-alpha levels were higher for GA (intermediate producer) than for GG (low producer), although the difference was not statistically significant. This indicates that the GG genotype of the TNF-alpha-308 G → A polymorphism in the population studied is associated with a good prognosis with regard to low serum TNF-alpha production. However, when the genotypes were correlated with serum concentrations, an increase in TNF-alpha levels was observed, supporting the hypothesis of a multifactorial etiology. In addition, Cajado et al. (2011) [22] found an association between the A allele of the TNF-alpha-308 G → A polymorphism and the increased serum TNF-alpha levels in adults with SCA in the steady state.

Clinical events, such as leg ulcers, ACS, femoral necrosis, and recurrent infection, were more frequent in patients with increased serum levels of TNF-alpha, but the difference was only significant for ACS. Defined as the appearance of new pulmonary infiltrate along with respiratory symptoms or chest pain, ACS is the most common cause of death and the second most common cause of hospitalization in adult SCA patients. All these events are associated with chronic inflammation, and TNF-alpha is a pro-inflammatory cytokine [27]. Serum IL-10 levels were reduced in patients with ACS and blood transfusion. Polytransfusion in response to severe anemia often leads to iron overload. Interestingly, another study based on the same patients showed serum IL-10 levels to be lower in participants with than without iron overload secondary to polytransfusion [28].

The main effect of IL-10 is to inhibit the synthesis of other cytokines, such as interferon alpha, IL-2, IL-12, and TNF-β. Sarray, Mahdi, Saleh, and Almaoui (2014) [29] demonstrated that decreased serum IL-10 levels is a predictor of vaso-occlusive crisis in SCA. Little research has been published on the association between cytokine-gene polymorphisms, serum cytokine concentrations, and clinical events in SCA. Much remains to be clarified regarding the dynamics of TNF-alpha and IL-10 in SCA, and their use as markers of vaso-occlusive crisis.

Conclusions

Our results showed that SCA patients in the Ceará, Brazil showed an increase in TNF-alpha production and a predominance of low-profile producer of the cytokines IL-10 and TNF-alpha. IL-10 low levels were associated with the presence of multiple transfusions. Patients with ACS were associated with increased TNF-alpha production and low IL-10. Additional studies are needed to clarify the mechanisms involved in infectious processes in such patients in order to establish the prognostic value of genetic polymorphisms in the development of SCA-related clinical complications.

Conflicts of interest

The authors declare no conflicts of interest.

References


