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Serum IL-6, IL-10, and TNFα levels in pediatric sickle cell disease patients during vasoocclusive crisis and steady state condition

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ABSTRACT

Vaso-occlusive crisis (VOC) is a significant complication of sickle cell disease (SCD), and altered production of pro-inflammatory and anti-inflammatory molecules contributed to its pathogenesis. In view of the association of chronic inflammation with VOC onset, and given the capacity of interleukin (IL)-10 as anti-inflammatory, and IL-6, and TNFα as pro-inflammatory cytokines, we tested the association of altered IL-10, IL-6, and TNFα secretion with VOC pathogenesis and its severity. Study subjects comprised 147 SCD patients with active VOC (VOC Group), and 63 pain-free SCD patients for at least 9 months before blood collection (*Steady-state Group*). Serum cytokine concentrations were determined by ELISA. IL-10 levels were significantly reduced, while IL-6 levels were increased in *VOC* compared to *Steady-state* groups; serum TNFα levels were comparable between both groups. There was enrichment of low IL-10, but high IL-6 and TNFα quartiles in VOC Group, which translated into increased VOC risk. In contrast, high IL-10, but low IL-6 and TNFα quartiles were seen in *Steady-state* Group. Correlation analysis demonstrated significant association between reduced IL-10 levels and the frequency, type, severity, and duration of VOC and requirement for hydroxyurea treatment, while IL-6 correlated with duration of VOC episodes. Our data support strong association of reduced IL-10 and increased IL-6 levels with VOC, and their modulation of VOC-related parameters.

1. Introduction

Sickle cell disease (SCD) is an autosomal recessive hematological disorder caused by a single nucleotide substitution in the 6th codon of the β-globin gene, resulting in replacing the hydrophilic glutamate by the hydrophobic valine [1,2]. This amino acid change results in the polymerization of deoxygenated hemoglobin, and the typical distortion, decreased flexibility, and fragility of red blood cells, leading to microvascular occlusion, and hypoxia [2,3]. SCD is characterized by hemolytic anemia, occlusions of the microcirculation, frequent infections and fever, joint pain, lethargy, and weakness, resulting in painful vaso-occlusive crisis (VOC) and chronic organ injury [4–7]. Several lines of evidence implicated a state of chronic inflammation with VOC pathogenesis, evidenced by the elevation in the levels of pro-inflammatory cytokines and acute phase proteins in the sera of SCD patients during VOC episodes [8–10].

Cytokines were implicated in several VOC processes, such as vascular endothelial activation, adhesion of erythrocytes and leukocytes to vascular endothelium, platelet activation, and deregulation of the apoptosis of endothelial cells [11,12]. Several studies reported on altered balance of inflammatory and anti-inflammatory cytokines in SCD patients during VOC, highlighted by elevation in pro-inflammatory cytokines [5,13,14], and reduction in anti-inflammatory cytokines levels [13,15] in SCD patients compared to healthy individuals. Among the anti-inflammatory cytokines, interleukin-10 (IL-10), a pleiotropic cytokine produced by T helper type 2 (Th2) and regulatory T cells (Treg), monocytes/macrophages, B cells, NK cells, and dendritic cells, and was described to be associated with SCD complications [16]. Several mechanisms were proposed for IL-10 suppression of inflammation, which included suppression of the production of inflammatory

Abbreviations: CI, confidence interval; IL-10, interleukin-10; NSAID, non-steroidal anti-inflammatory drugs; OR, odds ratios; SCD, sickle cell disease; VOC, Vaso-occlusive crisis.

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cytokines [17,18] and attenuation of the toxic effects of reactive oxygen species (ROS) and free radicals, such as nitrites [18,19]. However, the effects of IL-10 on vascular cells vary according to the cell origin [19], and the specific signaling pathways elicited by pro-inflammatory stimuli [17,19].

Cytokine imbalance was suggested to contribute to the pathogenesis of sickle cell pain crisis [20,21]. Reduced expression of anti-inflammatory cytokines, including IL-10, were reported in unselected SCD patients compared to healthy subjects [20–22], and in SCD patients with VOC compared to pain-free SCD patients [23]. However, contradictory findings were reported by other studies [8,24,25], principally due to the low number of subjects and selection criteria employed in those studies. We recently reported on the utility of analyzing changes in IL-10 serum levels in predicting VOC in SCD patients [26]. Here we extend these findings by testing the association of reduced IL-10 secretion with VOC and its associated features, by comparing its serum in levels in VOC cases and pain-free SCD control patients.

2. Patients and methods

2.1. Study subjects

This was a retrospective case-control study, conducted between October 2011 and April 2013. Study subjects comprised 211 consecutively-recruited SCD patients, who were diagnosed with SCD according to hemoglobin profile (HbA, HbS, HbA2, and HbF), and were assigned to 1 of 2 groups: SCD patients who had any VOC event (*VOC Group*; $n = 148$), or clinically asymptomatic SCD control patients who reported no VOC events for the past 9 months (*Steady-state Group*; $n = 63$) (Table 1). VOC was defined as acute events characterized by diffuse pain occurring in the upper or lower extremities, back, chest, and abdomen, that was related to SCD, but not to SCD-unrelated cause such as trauma or cancer. Treatment of VOC consisted of oral (24.8%) or intravenous (42.9%) non-steroidal anti-inflammatory drugs (NSAID), narcotics alone (2.2%), and narcotics plus NSAID (20.7%). Comparable frequencies of hydroxyurea- ($P = 0.80$) and folic acid- ($P = 0.32$) treated patients were seen in both groups.

SCD controls comprised patients presenting for routine follow-up in outpatient clinics. Inclusion criteria included afebrile state, no VOC episode, hospitalization or transfusion for at least 9 months, and were excluded if they were on pain medication, or presented with other coexisting condition. Steady-state SCD control patients were matched with VOC patients according to gender, hemoglobin profile (HbS, HbF), and other hematological indices (Table 1). All subjects were Bahraini Arabs and non-Arabs or recently naturalized Bahrainis were excluded. The Arabian Gulf University Research and Ethics Committee approved the study protocol, which was in agreement with the Helsinki declaration of 2000. All participants (or guardians in the pediatric cases) gave written informed consent, after the purpose of the study was explained to them.

Table 1
Characteristics of study participants.

	VOC Group[a]	Steady-state[a]	P[b]
Age	11.4 ± 6.4	13.5 ± 11.8	0.208
Gender (male:female)	89:58	36:27	0.649
HbS (%)	70.7 ± 9.0	70.8 ± 7.5	0.956
HbF (%)	19.2 ± 7.7	20.1 ± 6.5	0.464
Total hemoglobin (g/dL)	9.7 ± 1.4	10.7 ± 1.5	0.277
WBC ($\times 10^9$/L)	9.6 ± 4.8	9.4 ± 2.0	0.516
Platelets ($\times$109/L)	313.6 ± 177.5	332.7 ± 215.6	0.556
Hematocrit (%)	26.6 ± 5.4	27.4 ± 4.9	0.257
Mean corpuscular volume (fL)	75.7 ± 10.0	77.1 ± 10.6	0.419
Mean corpuscular Hb (pg)	25.8 ± 5.6	26.5 ± 4.8	0.465
Mean corpuscular Hb Conc (g/dL)	36.4 ± 2.7	35.3 ± 1.8	0.403
Reticulocytes (%)	5.0 ± 3.5	5.7 ± 3.9	0.392
Blood transfusion [number (%)]	72 (56.3)	20 (31.7)	0.002
Hydroxyurea Rx [number (%)]	42 (28.7)	24 (38.1)	0.250

[a] Study subjects comprised 147 SCA patients who had any VOC event during study (VOC Group) and 63 SCA patients who had no VOC events (Steady-state Group).
[b] Pearson's chi square test (*categorical variables*), 2-tailed *t*-test (*continuous variables*).

2.2. Serum IL-10 cytokine measurement

Peripheral venous blood was collected into plain tubes (no anticoagulants). Serum was prepared by centrifugation of coagulated blood at 2000*g* for 10 min at 4 °C, and was stored in aliquots at or below −30 °C. Samples were tested for IL-10 (Cat. D1000B), IL-6 (Cat. D6050), and TNFα (Cat. DTA00C) using human sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Assay sensitivity for the cytokine ELISA kits ranged from 3.9 to 5.5 pg/ml, and inter-assay and intra-assay precision (CV%) ranged from 4.2–7.5% and 1.7–5.4%, respectively.

2.3. Statistical analyses

Statistical analyses were performed on SPSS version 21 (Statistical Package for the Social Sciences). Categorical variables were expressed as percentages of total, while continuous variables were presented as mean ± SD. Student's *t*-test was used to determine differences in means, and Pearson $\chi 2$ or Fisher's exact test was used to assess inter–group significance. For continuous variables that did not follow a normal distribution, we used nonparametric analysis: Mann–Whitney U-test for two group comparisons, or Kruskal–Wallis test for multiple group comparisons; quantitative data described as medians and range values. Scatter plot analyses were initially used to present the distribution of IL-10, IL-6, and TNFα among groups of individuals, with values out of percentiles 5–95 interval shown as individual points. Correlations among continuous variables were determined by Spearman correlation coefficient (r). The VOC risk was estimated in VOC patients relative to steady-state control patients by calculating the odds ratios (OR) and 95% confidence interval (CI), according to the method of Woolf. Cytokine serum levels were compared using comparison of quartiles technique to detect systematic switch of values toward one of the two groups. OR and 95% CI were also calculated for different cutoff points, based on the distribution in control subjects; IL-10, IL-6, and TNFα levels were used as continuous and then as categorized variables.

3. Results

3.1. Study subjects

Table 1 summarizes the demographics and clinical characteristics of study participants. Both gender distribution ($P = 0.649$) and age at examination ($P = 0.208$) were comparable between VOC and steady-state SCD patients. HbS ($P = 0.956$), HbF ($P = 0.464$), along with other hematological and inflammatory indices, including total hemoglobin, hematocrit, WBC, platelet count, MCV, MCH, MCHC, and reticulocyte counts, were comparable between both SCD patients groups. VOC patients reported a mean 4.6 ± 2.2 VOC episodes/year, of mean duration of 4.6 ± 2.5 days/episode. The majority of VOC cases required blood transfusion (56.3%) and hospital admission (84.0%) principally to manage the painful crisis, with the pain score (on a scale of 0–10) being 7.1 ± 1.8.

3.2. Cytokine serum levels

Mean serum IL-10 levels were reduced in VOC cases than in control SCD patients ($P < 0.001$) (Fig. 1). In contrast, serum IL-6 levels