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Association of homocysteine and inflammatory-related molecules in sickle cell anemia

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Objective: Investigate the role of homocysteine (Hcy), Th17-related cytokines, and adhesion molecules in the inflammatory state seen in the sickle cell anemia (SCA).

Methods: We studied the Hcy, interleukin (IL)-17, and transforming growth factor β (TGF- β) cytokine levels of 62 SCA patients, as well as the expression levels of inflammatory and endothelial activation markers.

Results: We found significant associations between Hcy levels and increased expression of IL-17 and TGF- β among SCA patients, and a positive significant correlation between Hcy and soluble vascular cellular adhesion molecules (sVCAM). SCA individuals had raised IL-17 levels when compared with controls.

Discussion: These results suggest a possible role of Hcy in the induction of TGF- β and IL-17. Other authors proposed that Hcy may contribute to the initiation and progression of vascular disease by monocyte activation, resulting in the secretion of cytokines that amplify the inflammatory response. The role of Hcy in cytokine production and oxidative stress in the endothelium may explain the increase of sVCAM expression and, the vascular activation currently described among the SCA individuals with the highest Hcy serum levels. The chronic inflammation was observed in hyperhomocysteinemic mice, with an increased expression of VCAM-1 and plasma levels of tumor necrosis factor- α , showing an association of this inflammatory molecule and vascular changes.

Conclusion: Our findings suggest that the increased levels of IL-17, Hcy and sVCAM contributes to the vascular inflammation and activation presented by SCA patients, which probably have an important role in vaso-occlusion. On the basis of the presented data, IL-17 and Hcy might be considered as important components in the pathogenesis of SCA.

Keywords: Sickle cell anemia, Cytokines, Soluble adhesion molecules, Homocysteine, IL-17

Introduction

Sickle cell anemia (SCA) is associated with a pro-inflammatory state, characterized by an elevated baseline leukocyte count, known to be correlated with several specific complications and mortality of the disease, and by increased plasma levels of pro-inflammatory cytokines, such as interleukin (IL)-6 and IL-8.¹⁻⁴ Inflammation, white blood cell adhesion to vascular endothelium, and consequent endothelial damage contribute to SCA pathogenesis.^{5,6} The chronic inflammatory state, which is different from

one patient to another, may partly account for the variability of the disease expression.

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are cytokine-inducible, single-chain glycoproteins belonging to the immunoglobulin superfamily. The ICAM-1 is constitutively expressed on vascular endothelium and cells of the immune system and is upregulated in response to various stimuli, including cytokines synthesized during inflammation.^{7,8}

The VCAM-1 is present in activated endothelial cells after stimulation with cytokines, such as IL-1 β , tumor necrosis factor- α (TNF- α), and IL-4.^{9,10} The VCAM-1 serves as a counter receptor for integrin very late activation antigen-4 ($\alpha 4\beta 1$), and is important

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in the recruitment of leukocytes to inflammatory sites.¹¹ The VCAM-1 also participates in the cellular interactions involved in lymphocyte activation, and both ICAM-1 and VCAM-1 also can be found as soluble forms circulating in plasma although the physiological roles of these soluble cell adhesion molecules are not yet clearly known.^{12–15}

The IL-17 is a pro-inflammatory T cell-derived cytokine with numerous biological actions. It has been reported that IL-17 plays an essential role in microbial host protection by connecting lymphoid and myeloid host defenses.¹⁶ Although considered to be dependent on IL-23 for differentiation from naive T cells into Th17 cells, current work has shown that initial differentiation is dependent on transforming growth factor beta (TGF- β), with subsequent Th17 lineage expansion dependent on IL-23.¹⁷ The role of the Th17 response in vascular cell alterations is not well known.

The homocysteine (Hcy), a sulfur-containing amino acid, is found at low concentration in blood and cells and is an important intermediate molecule involved in the biosynthesis of methionine and cysteine.¹⁸ The high plasma concentration of Hcy is a well-established risk factor for several disorders including cardiovascular disease, stroke, venous thrombosis, and arteriosclerosis.^{19,20}

The hyperhomocysteinemia has an important role in vascular disorders and may act through increasing cytotoxic activity, especially for endothelial cells; elevating hydrogen peroxide (H₂O₂) levels and decreasing nitric oxide (NO) synthesis, inducing cytokine production to stimulate the inflammatory state, activation of procoagulant factors, and dysregulation of lipid metabolism via oxidative modification of low-density lipoprotein cholesterol with enhanced atherogenesis. Higher levels of Hcy have also been implicated in causing changes in the rheological properties of blood, such as decreasing antithrombin III and tissue plasminogen activator, and increasing factor VII and C-protein.^{21,22} Additionally, Hcy has been associated with enhanced interaction between endothelial cells and leukocytes.²³

The possibility that Hcy may contribute to the ischemic phenomena present in SCA has attracted some interest in total plasma Hcy. Lowenthal *et al.*²⁴ showed that the median plasma concentration of Hcy among SCA subjects was 1.5-fold higher than that of controls. Additionally, sickle cell disease (SCD) patients have higher plasma concentrations of Hcy in spite of higher plasma folate levels and vitamin B₁₂ concentrations compared with those observed in healthy individuals.

Since SCA patients are prone to ischemic complications, the search for mechanism is particularly challenging because the pathogenesis of vaso-occlusion involves several molecules and processes across

multiple length and timescales, including polymerization and melting of hemoglobin, vascular inflammation, adhesion molecules, cytokines, and still unclear factors such as the contribution of Hcy. We hypothesized that the plasma Hcy may contribute to the increased endothelial activation and inflammatory states seen in SCA individuals, and we performed a study to assess serum concentrations of Hcy, inflammatory markers, such as IL-18, IL-23, IL-17, TGF- β , and soluble adhesion molecules (sICAM and sVCAM) in SCA patients.

Materials and methods

Subjects

We studied 62 patients (28 men and 34 women; mean age of 21 ± 14) from Northeast, Bahia, Brazil who were diagnosed with SCA and were followed at the hematology ambulatory from the Fundação de Hematologia e Hemoterapia do estado da Bahia (HEMOBA). All patients maintained a steady-state, transfusion-free treatment course with the use of 1 mg of oral folate supplementation. Patients had no other systemic diseases that could have potentially altered their inflammatory functions. Blood samples were obtained during regular clinical visits. The study was approved by the Fundação Oswaldo Cruz's Human Research Board and is in accordance with the Declaration of Helsinki of 1975, as revised in 2000. All subjects signed informed consent forms. Forty age-matched control individuals were included for the IL-17 analysis.

Biochemical analyses of Hcy

Serum concentrations of Hcy were measured by a chemiluminescence immunoassay using the automatic device IMMULITE 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA), according to the manufacturer's instructions.

Cytokine and soluble adhesion molecule measurements

The IL-18, IL-23, IL-7, and TGF- β serum levels were measured using Cytokine ELISA OptEIA kits (BD Pharmingen, San Diego, CA, USA) and soluble adhesion molecules (sICAM and sVCAM) were characterized using ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's recommendations.

Statistical analysis

Baseline characteristics were summarized as proportions and means of selected variables. The Kolmogorov–Smirnov test was performed to determine the distribution of quantitative variables. Bivariate correlation analyses were carried out to verify correlations between pairs of variables using Pearson's or Spearman's rank correlations (r) and two-tailed test was used. The non-parametric

Table 1 Steady-state hematologic markers, soluble adhesion molecules, and cytokine values of the SCA patient group

	Mean	SD
Hematologic markers		
RBC ($\times 10^6/\mu\text{l}$)	2.69	0.67
Hemoglobin (g/dl)	8.07	1.60
Hematocrit (%)	24.42	5.26
Platelets ($\times 10^3/\mu\text{l}$)	368	178
Leukocytes ($10^6/\text{ml}$)	10.9	3.7
Reticulocytes count (%)	6.9	3.1
Cytokines		
IL-18 (pg/ml)	3556.81	1279
TGF- β (pg/ml)	1588.78	430.88
IL-23 (pg/ml)	101.52	180.47
IL-17 (pg/ml)	3.9	7.8
Soluble adhesion molecules		
sICAM (ng/ml)	393.37	95.74
sVCAM (ng/ml)	516.06	226.94

SD, standard deviation.

Kruskal–Wallis test was used to compare two or more groups as measured by interval variables. Test analyses were considered significant if *P*-values obtained were less than 0.05. Data analyses were conducted using the software programs STATA 10 (StataCorp, TX, USA) and GraphPad Prism 5 (Graphpad Software, San Diego, CA, USA).

Results

Data of hematological markers, soluble adhesion molecules, and cytokine of SCA patients in steady-state are represented in Table 1.

Hcy concentration, cytokine profile, and adhesion molecules

The median of Hcy concentration in the 62 SCA patients was 7.3 $\mu\text{mol/l}$ (± 3.3 $\mu\text{mol/l}$). Considering

the classification commonly used for the degree of homocysteinemia as normal (5–14 $\mu\text{mol/l}$) and hyperhomocysteinemia as mild (15–30 $\mu\text{mol/l}$), moderate (31–100 $\mu\text{mol/l}$), and severe (>100 $\mu\text{mol/l}$), hyperhomocysteinemia was not found among the studied patient group.¹³ The association between cytokines and Hcy serum levels revealed a significant positive association between IL-17, quantified in 32/62 SCA patients, and TGF- β quantified in 13/62 SCA patients ($P = 0.019$, $r = 0.38$ and $P = 0.027$, $r = 0.610$, respectively). However, the IL-18 (13/62) and IL-23 (13/62) levels were not associated with Hcy concentration in this group (Fig. 1).

To assess the hypothesis that Hcy is related to vascular activation, analysis was performed with soluble adhesion molecules. The association between soluble vascular and intercellular adhesion molecules (sVCAM and sICAM) with Hcy showed a significant positive correlation with sVCAM ($P = 0.014$, $r = 0.396$), but not with sICAM (Fig. 2). When we considered the mean concentrations of Hcy in the 50th and 75th percentiles as a cutoff point, we can also report a statistically significant association between the sVCAM concentration and Hcy levels in these patients ($P = 0.021$, $\chi^2 = 5.28$ and $P = 0.031$, $\chi^2 = 4.64$, respectively).

Analysis of IL-17 levels in the 25th and 75th percentiles of Hcy, in the SCA patients, confirms the positive correlation between them ($P = 0.047$) (Fig. 3).

IL-17 levels are increased in SCA patient group

SCA patients presented raised levels of IL-17 cytokine compared with control group ($P = 0.011$) (Fig. 4).

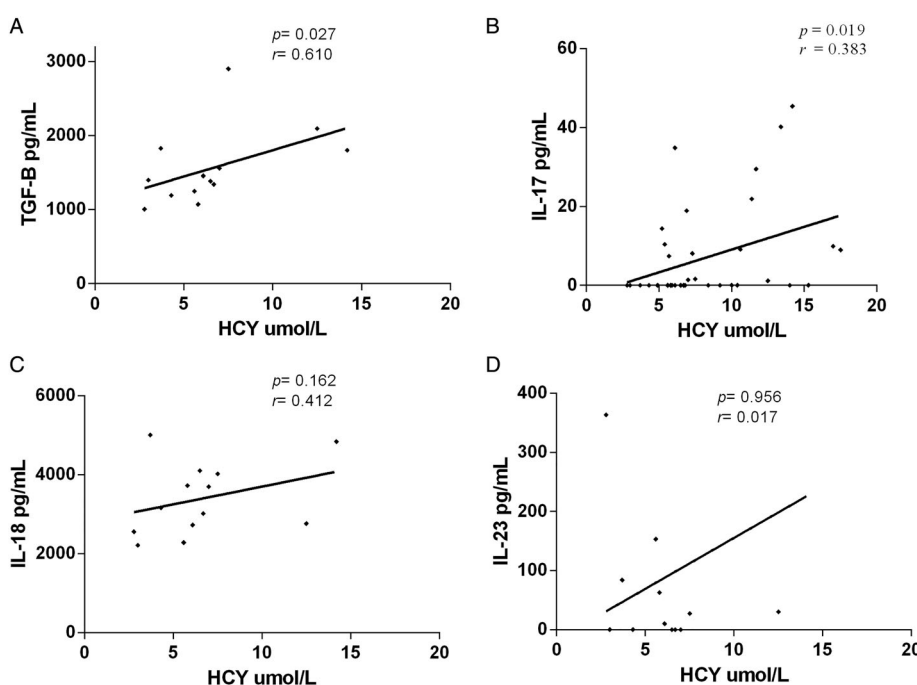


Figure 1 Hcy levels and correlation with TGF- β , IL-17, IL-18, and IL-23 cytokines in SCA patients.

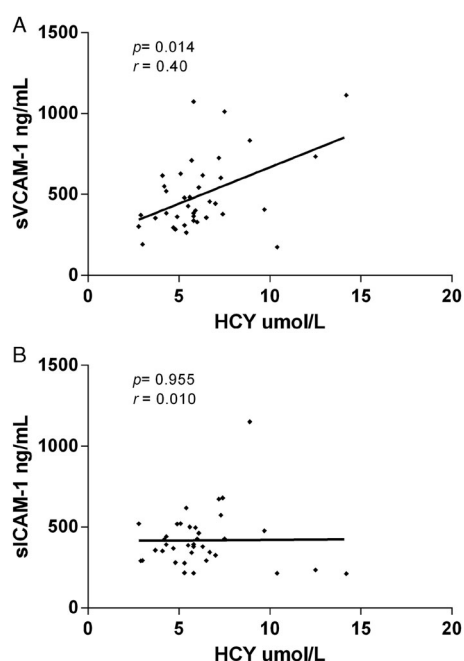


Figure 2 Hcy levels and correlation with soluble adhesion molecules in SCA patients.

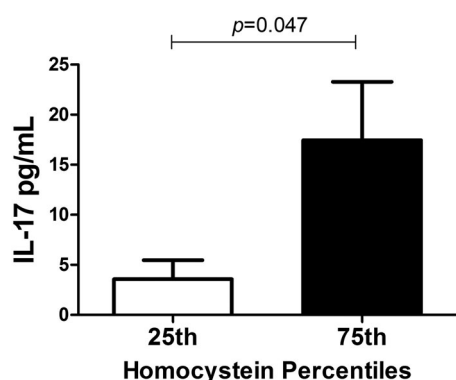


Figure 3 Hcy percentiles and levels of IL-17 in SCA individuals.

Hcy concentration, cytokines, or adhesion molecules with any clinical manifestations

We performed the analyses of Hcy, adhesion molecules, and cytokine data associated with patient’s clinical profile and there were not significant results. Interestingly, we noted that one patient who had acute chest syndrome (ACS) also had high-Hcy level (14 $\mu\text{mol/l}$) compared with mean Hcy in the group (7.3 $\mu\text{mol/l}$).

Discussion

A chronic inflammatory state in vascular tissue is recognized to contribute to thrombotic and vaso-occlusive events in SCA. Since Hcy has been associated with vascular complications in the pathology of other diseases, it may contribute to the vascular complications presented by patients with SCD.²⁴

In our study, we investigated Hcy levels and markers of vascular activation, including cytokines and soluble

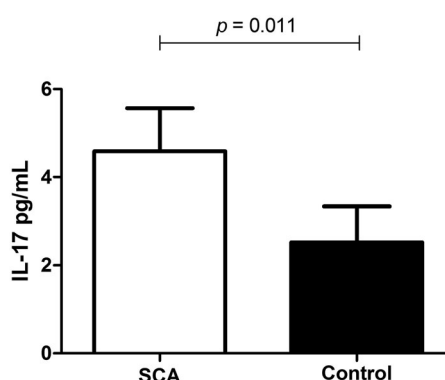


Figure 4 Levels of IL-17 in SCA individuals and controls.

adhesion molecules in SCA patients. Our results related to Hcy analyses showed that the studied group did not present increased levels of Hcy (7.3 $\mu\text{mol/l}$) when compared with previous results from Lowenthal *et al.*²⁴ that found a mean concentration of 12.6 $\mu\text{mol/l}$ among SCD patients, or when compared with the common classification criterion for hyperhomocysteinemia (>14 $\mu\text{mol/l}$).²⁵ However, Balasa *et al.*²⁶ found Hcy levels even lower (5.8 $\mu\text{mol/l}$) in SCA patients and there was no difference between controls. Despite the use of folic acid by our patients, this supplementation did not influence Hcy levels, confirm previous report about no association of hyperhomocysteinemia and folic acid supplementation among SCA patients.²⁷ In this work, We did not realize Hcy dosage in the control group to compare with Hcy levels in patients with SCA, but previous work by our team carried out a cross-sectional study comprising 143 healthy neonates, from the same population, that showed Hcy mean levels about 7.4 $\mu\text{mol/l}$ in this group, showing that in the SCA group investigates, the Hcy levels were not elevated when compared with individuals from the same population.²⁸

The effect of Hcy on cytokine production is not well understood. We found a positive association among Hcy and IL-17 and TGF- β . The IL-17 promotes inflammation by induction of various pro-inflammatory cytokines and chemokines, recruitment of neutrophils, enhancement of antibody production, and activation of T cells.²⁹ The TGF- β is a pleiotropic cytokine involved in several human diseases, including cardiovascular disease, and also can induce IL-17 production.³⁰ Our results suggest a possible role of Hcy in the induction of TGF- β and IL-17. This hypothesis is supported by Su *et al.*,³¹ who proposed that Hcy may contribute to the initiation and progression of vascular disease by monocyte activation, resulting in the secretion of cytokines that amplify the inflammatory response. We also found an elevated basal level of IL-17 in SCA patients when compared with controls, representing the chronic inflammatory state in these individuals.

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A new field of research in SCA is rising on Th17 cells. These IL-17 producing cells are involved in the inflammatory response and a little information is available regarding studies in SCA. Thus, it becomes important to investigate the role of cytokines in endothelial dysfunction and activation, once the role of other molecules, such as IL-1 and TNF- α , is yet not very established. Our team has previously demonstrated a significant and positive association of TGF- β and circulating arginase levels in SCA patients, showing a vascular relevance of this cytokine and the need to clarify the role of these molecules in SCA.³²

We found lower levels of IL-17 and TGF- β in this study when compared with previous reports.^{21,22} These discrepancies can be due to genetic differences in the studied populations. More importantly, the IL-17 control group in this work is age-matched and from the same population as the SCA patients. Many patients and controls had undetectable IL-17, so levels of this cytokine appear to be usually very low.

We believe that the role of Hcy in cytokine production and oxidative stress in the endothelium may explain our findings related to the increase of sVCAM expression and, consequently, with the vascular activation currently described among the SCA individuals with the highest Hcy serum levels. In agreement with our results, Hofmann *et al.*³³ observed evidence of chronic inflammation in hyperhomocysteinemic mice, including an increased expression of VCAM-1 and elevated plasma levels of TNF- α , showing an association of this inflammatory molecule and vascular changes. Hcy could be considered an emerging potential target of inflammation in SCA and it may be significant in the investigation of vaso-occlusion. The ACS is a combination of radiographic evidence of new pulmonary infiltrates and respiratory symptoms, and is a frequent cause of hospitalization in SCA, also, it likely involves alterations of normal homeostatic functions of vascular endothelium in the lungs.³⁴ We did not find other studies that related to our finding of increased Hcy in ACS, indicating the importance of further investigation of this potential link.

We conclude that the combination of IL-17, Hcy, and sVCAM may be involved in vascular damage in SCA and this might be implicated in the disease pathogenesis. Additional studies including SCA patients under vaso-occlusive crises are warranted in order to confirm our findings and show the behavior of these molecules in crisis condition. More importantly, we suggest the development of a longitudinal study in order to answer more accurately the influence of these molecules on vascular damage in SCA.

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Disclaimer statements

Contributors W.V.-B. is graduated in Biological Sciences from the Universidade Federal da Bahia (UFBA) and PhD in Experimental Human Pathology of the Centro de Pesquisas Gonçalo Moniz (CPqGM). B.C. is graduated in Biochemistry Pharmacy from UFBA and PhD in Experimental Human Pathology of the CPqGM. A.Z. is graduated in Medicine from the Universidade Federal do Rio Grande do Sul, and she is currently the Medical Hematologist of HEMOBA. C.F. is Pharmacy undergraduate student at the UFBA. R.S. is graduated in Biological Sciences from UFBA and currently is Master's student in Experimental Human Pathology of the CPqGM. C.G. is graduated in Biomedicine from Universidade Estadual de Santa Cruz (UESC) and currently is Master's student in Experimental Human Pathology of the CPqGM. T.P. is graduated in Pharmacy from UFBA and PhD student in Experimental Human Pathology of the CPqGM. S.S. is graduated in Biomedicine from UESC and PhD student in Experimental Human Pathology of the CPqGM. M.G. is graduated in Biochemistry Pharmacy from UFBA and PhD at Genetics from Universidade Estadual de Campinas, and she is currently titular researcher of CPqGM and professor in the Faculdade de Farmácia da Universidade Federal da Bahia.

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Conflicts of interest The authors stated that there are no conflicts of interest regarding the publication of this article.

Ethics approval The study was approved by the Fundação Oswaldo Cruz's Human Research Board and is in accordance with the Declaration of Helsinki of 1975, as revised in 2000. All subjects signed informed consent forms.

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