Background

Increased adhesive events between endothelium and peripheral blood cells play a central role in the initiation of thromboembolic events that frequently complicate the outcome of β-thalassemia.

Aim

The aim of the study was to evaluate endothelial activation/dysfunction and activation of peripheral blood cells in children with β-thalassemia and to correlate endothelial and cellular activation markers with other hematological parameters in the same cohort, and to find out if these markers could be used as parameters that predict vascular complications in these patients.

Participants and methods

Endothelial adhesion molecules [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin adhesion molecule (ELAM)], and endothelin-1 (ET-1) were analyzed using immunoassays. Early activation antigen (CD69) expression on the peripheral blood leukocytes was detected using a flow cytometer in 55 β-thalassemics [30 major (β-TM); 15 intermedia (β-TI), and 10 minor] and 25 healthy age-matched and sex-matched children.

Results

The levels of sICAM-1, sVCAM-1, ELAM, and ET-1 in β-TM and β-TI were significantly higher than those in thalassemia minor and controls. The levels of sVCAM-1 were significantly increased in splenectomized patients ($P<0.001$). Serum ICAM-1, sVCAM-1, and ELAM levels were positively correlated to each other in the β-TM and β-TI groups, with a significant difference ($P<0.05$). ET-1 was positively correlated to sVCAM-1 in the β-TM group. Ferritin was positively correlated to serum ICAM-1, VCAM-1, and ELAM in β-TM and β-TI. In β-thalassemia minor, ferritin was positively correlated only with serum ICAM-1 and VCAM-1. The expression of CD69 on leukocytes was significantly greater in β-TM, followed by β-TI and then β-thalassemia minor than the control groups. In the thalassemia minor group, CD69 expression was upregulated on monocytes, but neither on neutrophils nor on lymphocytes compared with the controls. There was no significant change in CD69 levels among splenectomized and nonsplenectomized patients. ET-1 levels were significantly correlated to CD69 expression on lymphocytes of the β-TM group.

Conclusion

Endothelial activation markers and activated leukocytes are significantly increased in β-thalassemics, showing that a severe degree of endothelial activation and damage along with chronic inflammation underlie the pathophysiology of vascular complications in these patients. The parameters studied might be useful markers for the follow-up of the vascular disease and may pave the way for improvements in the therapies for this disease.

Keywords: adhesion molecules, CD69, endothelin-1, peripheral blood, thalassemia

Introduction

Despite the difficulties associated with the treatment of β-thalassemic patients, standards of care have improved in recent years, resulting in almost doubling in the average life expectancy. Consequently, undescribed complications are known to occur. In particular, marked hemostatic changes have been observed in patients with β-thalassemia major (β-TM) and β-thalassemia intermedia (β-TI) 1,2. It is suggested that β-thalassemic patients have immunomodulation 3 as during the steady state of disease, patients appear to have normal T-lymphocyte function with moderate abnormalities in T-lymphocyte and B-lymphocyte subsets 4.

Endothelial activation along with chronic inflammation underlies the pathophysiology of β-thalassemia 5. This endothelial dysfunction is characterized by reduced nitric oxide bioavailability, and pro-oxidant and proinflammatory stress 6. As a consequence, blood cells adhere and migrate through the endothelium into the tissues 7, 8, with the development of vascular intimal hyperplasia, platelet activation and neutrophil adhesiveness 9, 10, and coagulopathy, resulting in further vasomotor instability, proliferative vasculopathy, and endothelial injury 9, 10, a hallmark of the development of pulmonary hypertension in adulthood 6. This endothelial activation or injury is reflected by increased levels of endothelial adhesion proteins such as intercellular adhesion molecule-1 (ICAM-1 or CD54), vascular cell adhesion molecule-1 (VCAM-1 or CD105), and E-selectin adhesion molecule (ELAM-1) 2, 5, which, under normal conditions, are not expressed in β-thalassemics 5,11. Moreover, the levels of sICAM-1 have been shown to predict graft rejection in transplanted thalassemics 12.

The presence of chronic antigenic stimuli, extrinsic (infectious or noninfectious) or intrinsic (tissue inflammation, cytokines, hemolysis, vascular endothelial damage), might be involved in the mechanisms of cell activation and immunomodulation 13. Cellular activation is reflected by expression of several activation markers on peripheral blood cells such as CD69. CD69 is a widely expressed type II transmembrane glycoprotein with a C-type lectin-binding domain in the extracellular part of the molecule 14. It is one of the earliest cell surface antigens expressed by T cells following activation; hence, it is known as an activation inducer molecule or very early activation antigen 15. It is detected within 1 h of ligation of the T-cell receptor/CD3 complex. In addition to mature T cells, it shows regulated expression on a variety of cells of the

hematopoietic lineage, including neutrophils, monocytes, B cells, natural killer cells, and platelets 14 and also epidermal Langerhans cells 15, eosinophils, and thymocytes 16. Once expressed, CD69 acts as a costimulatory molecule for T-cell activation and proliferation. It is proposed to be involved in the pathogenesis of many diseases such as rheumatoid arthritis, chronic inflammatory liver diseases, mild asthma, and acquired immunodeficiency syndrome 15.

Endothelin-1 (ET-1) is an endothelial cell-derived peptide of the 21 amino acid residue, synthesized through the proteolytic processing of the 203-residue peptide termed preproendothelin 17. It is produced by a variety of tissues, especially vascular endothelium 18. ET-1 is a potent vasoconstrictor, proinflammatory factor, sensitive to cell injury 17. It plays a significant role in the pathogenesis of many diseases such as hypertension and in chronic anemia such as thalassemia and sickle cell anemia 19. This study aims to evaluate endothelial activation in children with β-thalassemia by detecting the levels of soluble endothelial adhesion molecules, ET-1, and peripheral blood cell activation by identifying CD69 expression on different blood cell and also to correlate the levels of those parameters with each other and other hematological parameters. To find out if these markers could be used as parameters that predict or reflect vascular complications in these patients.

Patients and methods

The study was carried out on 55 β-thalassemic patients (31 males and 24 females, M/F ratio 1.2 : 1) regularly attending the Hematology Specialized Clinic, Pediatric Hospital, Ain Shams University. They were compared with 25 age-matched and sex-matched healthy patients as a control group. Parents of the patients signed informed consents before enrollment. The study was approved by the local institutional review board. Patients were subdivided into three groups, on the basis of the clinical, hematological, and biosynthetic characteristics: 30 with β-TM; 15 with β-TI; and 10 with β-thalassemia minor; β-thalassemics with intercurrent infection, HIV, a history of hypertension, and concomitant intensive exercise were excluded.

All patients were subjected to the following: full history taking (with a focus on the onset and frequency of blood transfusion and a history of viral infection) and a thorough clinical examination. Laboratory investigations for both patients and controls included complete blood count (CBC), reticulocytic count, estimation of fetal hemoglobin (HbF), liver function tests; quantification of serum adhesion molecules (VCAM-1, ICAM-1, ELAM), and ET-1. Flow cytometric analysis of early activation antigen (CD69) on white blood cells was carried out. Patients' files were reviewed for assessment of serum ferritin levels and its mean.

Sampling

Seven milliliters of venous blood was collected from each individual (patients and controls) into sterile vacuum tubes; 3 ml was EDTA anticoagulated: 2 ml for CBC, reticulocytic count, and HbF determination and 1 ml for CD69 detection. The remaining 4 ml was allowed to clot for VCAM-1, ICAM-1, ELAM, and ET-1 analysis. The serum was separated by immediate centrifugation at 1500g for 15 min at 4°C to avoid cytokine synthesis or breakdown in vitro and was stored at 20°C until analysis.

Methods

CBC and reticulocytic count were carried out using Coulter Gen S (Beckman Coulter, Inc., Fullerton, California) and blood film. Serum ferritin by Ferr Gen 2 kits (Roche Diagnostics GmbH) using Cobas Integra 800 (Roche, Mannheim, Germany).

HbF quantification was performed routinely by ion-exchange high-performance liquid chromatography (D10; BioRad, Marnes La Coquette, France). Serum levels of VCAM-1, ICAM-1, and ELAM were measured using specific immunoenzymatic assays (R&D Systems, Minneapolis, Minnesota, USA). Serum ET-1 level was assessed using an ELISA kit supplied from Peninsula Laboratories Inc. (Bechmen, California, USA). Monoclonal antibodies (moAbs) and flow cytometric analysis of CD69. Cell surface antigens were detected by direct immunofluorescence evaluated by a flow cytometer (Coulter Epics XL; Coulter Electronics). The leukocytes were stained in whole blood to avoid any in-vitro manipulation that could affect activation. The anti-CD69 mouse fluorescein isothiocyanate-conjugated moAbs were tested (R&D systems) according to the manufacturers' instructions 18. MoAbs were used with 50 µl whole blood, and were incubated on ice for 30 min. After washing with 3 ml of an ice-cold PBS, the red cells were lysed before fixation on the system. Nonspecific immunofluorescence was determined using anti-mouse isotype-matched control moAbs. Subtypes were identified according to side scatter and forward scatter criteria. The florescence mean channel value, as an indicator of antigen expression density, was recorded by subtracting the control florescence mean channel value from that of the sample. In each case, 105 positively gated events were analyzed. The % and the mean fluorescence index (MFI) were used to determine the antigen density [Figure 1].

Figure 1: Flow cytometric detection of CD69 on lymphocytes (a), monocytes (b), and neutrophils (c) in the case of thalassemia major. LOG, logarithmic scale.

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Statistical analysis

Statistical analysis was carried out on a personal computer using the Statistical Package of Social Sciences (SPSS Inc., Chicago, Illinois, USA), version 8. Normally distributed numerical data were presented as mean±SD. The means between two groups were compared using the

Student t-test. Correlation was assessed using Pearson's test (r). Differences were considered significant when the P value was less than 0.05.

Results

This study was carried out on 55 β-thalassemic patients (31 males and 24 females, M/F ratio 1 : 1.2) and were compared with 25 age-matched and sex-matched healthy individuals. Patients were subdivided into 30 with β-TM (mean age 8.86±2.5 years); 15 with β-TI (mean age 12.3±3.1 years); and 10 with thalassemia minor (mean age 18.1±3.2 years). β-TM patients were receiving regular packed cell transfusion therapy, 10–15 ml/kg every 2–3 weeks, and β-TI at varying frequency every 3–6 weeks. In terms of iron chelation therapy, 17 patients in the β-TM group showed were on subcutaneous desferrioxamine 30–40 mg/kg/day (desferal; Ciba-Geigy, Basle, Switzerland) and 12 patients were on oral L1 (deferiprone; Ciba-Geigy) combined with subcutaneous desferrioxamine every other day. The mean β-TM ferritin concentration of patients was 1050±530 ng/ml, ranging between 300 and 2100 ng/ml, and serum iron levels were high, indicating inadequate iron depletion through patients' incompliance or inadequate dosing. Twenty-one patients (38.2%) were splenectomized (17 TM; four TI). Liver function tests in transfused patients, namely, alanine transaminase had a mean value of 53.2±8.8 IU/l, which is significantly higher than that of the control group (t=4.56; P=0.001). Nine patients had a previous history of hepatitis B virus infection, whereas eight patients had been infected previously with the hepatitis C virus.

The hematological parameters of our patients showed reduced levels of hemoglobin and increased levels of corrected total leukocytic counts, platelet counts, ferritin, and HbF [Table 1].

Table 1: Descriptive data of hematological parameters in the groups studied

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The serum levels of immunoreactive ICAM-1, VCAM-1, and ELAM as well as ET-1 were high in the patient groups than the healthy controls (P<0.05; [Table 1], being the highest in β-TM, followed by β-TI and then β-thalassemia minor patients, with a statistical difference, when compared with the control group (P<0.05; [Table 2]. No significant difference was found between serum ICAM-1, ELAM, and ET-1 levels among splenectomized and nonsplenectomized patients (t=0.412, 1.19, and 0.418, respectively; P>0.05). However, sVCAM-1 showed a statistically significant increase among splenectomized patients (t=2.12; P=0.04). Serum ICAM-1, VCAM-1, and ELAM levels were positively correlated to each other in the β-TM and β-TI groups, with a significant difference (P<0.05), whereas ET-1 serum levels were positively correlated to sVCAM-1 only in the β-TM group (r=0.42, P=0.02). In the β-thalassemia minor group, a positive correlation was found only between ICAM-1 and sVCAM-1 (r=0.77, P=0.001; [Table 3].

Table 2: Comparative study of the different parameters studied in different groups

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Table 3: Correlation between different hematological parameters in the groups studied

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Serum ferritin level was positively correlated to serum ICAM-1, VCAM-1, and ELAM in β-TM and β-TI groups (P<0.05), whereas in β-thalassemia minor, ferritin was only correlated to sICAM-1 and sVCAM-1 (P<0.05). No significant correlation was found between ET-1 and ferritin levels in all the groups studied (P>0.05; [Table 3]. Also, no significant correlation was found between the molecules studied and other hematological parameters (total leukocytic counts, hemoglobin, and platelet counts; P>0.05).

In terms of the immunophenotype of peripheral blood cells, the levels of CD69 were found to be upregulated on the lymphocytes, monocytes, and neutrophils in β-TM and β-TI patients compared with β-thalassemia minor and control groups (P<0.05). The increase was significantly greater in the β-TM group than in the other three groups (P<0.001). Compared with the controls, the β-thalassemia minor group had significantly higher levels in the % and MFI of CD69 on monocytes (P<0.05) but not lymphocytes or neutrophils (P>0.05; [Table 1]. Moreover, CD69 levels in different blood cells did not differ between splenectomized and nonsplenectomized patients (P>0.05).

ET-1 levels were significantly correlated to CD69 expression (% and MFI) in lymphocytes of β-TM patients (r=0.38 and 0.41; P=0.04 and 0.02, respectively). The % and MFI of CD69 expression showed no other significant correlations compared with the other parameters studied (P>0.05; [Table 1].

Discussion

The presence of a high incidence of thromboembolic events has led to the identification of a hypercoagulable state in β-thalassemia 1. It is evident that endothelial cells participate actively in inflammatory reactions and thrombosis by undergoing profound changes in function 16.

Fifty-five β-thalassemics were evaluated for the presence of endothelial and peripheral blood cell activation. The study found significantly higher serum levels of ICAM-1, VCAM-1, and ELAM in all β-thalassemics compared with the control group, confirming their overexpression and indicating activated endothelial cells 2,20. These results are in agreement with previous studies 4, 10, 11, 13, which have reported that cellular activation present in β-thalassemics is related to exposure to pathogens and chronic immune stimulation through repeated blood transfusion and iron overload, whereas the possibility of the involvement of drugs (e.g. desferrioxamine,

ferriprone) remains to be investigated. Serum VCAM-1 levels were found to be higher in splenectomized than in nonsplenectomized β-thalassemics, indicating higher endothelial activation as reported by other researchers 10, 12, suggesting the greater likelihood of development of thromboembolic manifestations as a long-term complication in splenectomized patients. However, another previous study 21 reported no difference between the levels of adhesion molecules in splenectomized and nonsplenectomized patients; meanwhile, decreased levels of these parameters among splenectomized patients have been reported previously 5. This discrepancy may be because of the heterogeneity of β-thalassemia patients in their genotype, age, number of transfusions, and iron overload.

ET-1 serum levels, in this study, were increased in β-thalassemic patients than the healthy controls as reported previously 17 that, thalassemia, chronic anemia, and tissue hypoxia may contribute toward transduction of ET-1 and that its levels increase by 15–20% in β-thalassemic than in normal controls. Again, according to our results, the highest ET-1 levels were found in the β-TM group, followed by those of the β-TI and β-thalassemia minor groups ($P<0.05$), indicating the ongoing process of inflammation, in agreement with previous studies 13,19.

On studying the correlations between soluble adhesion molecules, this study found a positive correlation between sICAM-1, VCAM-1, and ELAM levels in the β-TM group; also, a positive correlation was found between serum ET-1 and VCAM-1 levels, in agreement with Wu et al. 22, who suggested the use of the correlation between the VCAM-1 and ET-1 as a predictive marker of an increased risk for coronary heart disease. Again, in the β-TI group, a positive correlation was found between sICAM-1, VCAM-1, and ELAM levels; this interaction may indicate the extent of vascular damage found in β-thalassemics, who are otherwise characterized by a subtle clinical phenotype as reported previously 23. Recently, this endothelial dysfunction has been attributed to severe complications such as pulmonary hypertension and thromboembolic phenomena in both β-TI and β-TM; thus, reduction of this procoagulant activity by blood transfusion was proposed to be useful in preventing the occurrence of thromboembolic manifestations, especially in β-TI 5,12. In the β-thalassemia minor group, only the ICAM-1 and VCAM-1 levels were correlated positively, as reported previously 8.

In the current study, serum ferritin was significantly higher in the patient groups, especially β-TM and β-TI. This iron overload was established previously 12, in response to repeated transfusions, ineffective erythropoiesis, and repeated infections. Again, in this study, ferritin levels were found to be positively correlated to serum ICAM-1, VCAM-1, and ELAM in β-TM and β-TI, whereas in β-thalassemia minor, ferritin was found to be correlated positively to sICAM-1 and sVCAM-1 levels only, in agreement with other studies 10,24. The correlation of ferritin and activation molecules in β-thalassemics indicates the possible causative role of iron overload in endothelial damage and atherogenesis, with a higher risk of thrombosis 5. The activation status

of peripheral blood cells, in the current study, showed that the expression of CD69 was the highest in β-TM patients who showed increased activated peripheral blood cells compared with other groups as reported previously 25 to be because of repeated transfusions and chronic antigenic stimulation resulting in prolonged activation of endothelial cells, causing increased leukocyte activation, adhesion, and migration into the tissues promoting thrombosis. Moreover, CD69 expression was found to be correlated significantly to ET-1 levels in lymphocytes of patients with β-TM. This correlation was proposed previously 26 as a predictive marker of the risk of atherogenesis in these patients. Meanwhile, in the β-TI group, CD69 expression on blood cells showed a significant increase compared with thalassemia minor and the control groups, in agreement with Cappellini et al. 1, indicating that the presence of a large number of circulating activated cells in β-TI patients together with high levels of serum ICAM-1 and VCAM-1 molecules might explain the hypercoagulable status.

However, the thalassemia minor group showed statistically higher levels of CD69 (% and MFI) on monocytes but not neutrophils or lymphocytes compared with the controls, in agreement with Kanavaki et al. 5, confirming the activation state in β-thalassemia carriers despite the lack of transfusion-transmitted stimuli and the significantly lower iron burden, thus raising the hypothesis that the hemolytic process and red blood cells (RBCs) abnormalities are not the only factors contributing to the peripheral cells and endothelial activation but also other antigenic stimuli may be involved in the activation process. In addition, different body iron status, degree of anemia, and episodes of infection may contribute toward the variable expression of CD69 found in thalassemia patients. Thus, it was proposed that the presence of peripheral blood elements in thalassemics such as ICAM-1, VCAM-1, ELAM, and ET-1 indicates that endothelial injury or activation may be an aspect of the disease, aiding in the recruitment and activation of white blood cells and RBCs, promoting thrombosis. Consequently, these parameters might be used as useful markers for the follow-up of vascular disease. An individualized approach is recommended to establish an optimal strategy for preventing the occurrence of this complication in β-thalassemia. Such an understanding may pave the way for the development of improvements in the therapies available for the treatment of this disease. More studies are required to identify further implications of these parameters with possible therapeutic protocols to improve transfusion regimens (i.e. use of prestorage leukocyte-depleted RBCs), to further reduce iron load, to improve RBC rheological properties, and suppress ineffective erythropoiesis (i.e. by overtransfusion of normal RBCs).[26]