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Circulating CD133<sup>+</sup>VEGFR2<sup>+</sup> and CD34<sup>+</sup>VEGFR2<sup>+</sup> cells and arterial function in patients with beta-thalassaemia major

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Abstract Arterial dysfunction has been documented in patients with beta-thalassaemia major. This study aimed to determine the quantity and proliferative capacity of circulating CD133<sup>+</sup>VEGFR2<sup>+</sup> and CD34<sup>+</sup>VEGFR2<sup>+</sup> cells in patients with beta-thalassaemia major and those after haematopoietic stem cell transplantation (HSCT), and their relationships with arterial function. Brachial arterial flow-mediated dilation (FMD), carotid arterial stiffness, the quantity of these circulating cells and their number of colony-forming units (CFUs) were determined in 17 transfusion-dependent thalassaemia patients, 14 patients after HSCT and 11 controls. Compared with controls, both patient groups had significantly lower FMD and greater arterial stiffness. Despite having increased CD133<sup>+</sup>VEGFR2<sup>+</sup> and CD34<sup>+</sup>VEGFR2<sup>+</sup> cells, transfusion-dependent patients had significantly reduced CFUs compared with controls (<i>p</i>=0.002). There was a trend of increasing CFUs across the three groups with decreasing iron load (<i>p</i>=0.011). The CFUs correlated with brachial FMD (<i>p</i>=0.029) and arterial stiffness (<i>p</i>=0.02), but not with serum ferritin level. Multiple linear regression showed that CFU was a significant determinant of FMD (<i>p</i>=0.043) and arterial stiffness (<i>p</i>=0.02) after adjustment of age, sex, body mass index, blood pressure and serum ferritin level. In conclusion, arterial dysfunction found in patients with beta-thalassaemia major before and after HSCT may be related to impaired proliferation of CD133<sup>+</sup>VEGFR2<sup>+</sup> and CD34<sup>+</sup>VEGFR2<sup>+</sup> cells.

Keywords Arterial function · Beta-thalassaemia major

Introduction

The importance of arterial dysfunction in patients with beta-thalassaemia major is increasingly recognized. In these patients, we and others have demonstrated endothelial dysfunction as evidenced by reduced brachial flow-mediated dilation [1, 2] and increased stiffness of the carotid artery [1], ascending aorta [2] and abdominal aorta [3]. Endothelial dysfunction in these patients has important clinical implications on the stiffening of the arterial tree, with the consequences of suboptimal ventriculo-arterial interaction [4] and the development of premature atherosclerosis [5, 6].

Understanding of the mechanisms of arterial dysfunction in patients with beta-thalassaemia major is hence of paramount importance. In patients with β-thalassemia/haemoglobin E, an increased level of circulating endothelial cells reflecting endothelial damage and denudation has been demonstrated [7]. Repair of the denuded endothelium might be instrumental for the restoration of endothelial function. Endothelial progenitor cells (EPCs) have been regarded as restorative cells that home to sites of endothelial damage and denudation [8]. Furthermore, the activities of EPCs have been shown to correlate with the functional integrity of the endothelium [9].

In patients with beta-thalassaemia, markers of oxidative stress have been reported to be increased [10], whilst in a rodent model of iron overload, increased generation of reactive oxygen species within the arterial wall has been demonstrated [11]. Importantly, reactive oxygen species may accelerate the onset of EPC senescence [12]. Taken together, iron overload in patients with beta-thalassaemia
major may potentially result in arterial dysfunction via its effects on the quantity and function of EPCs.

The definitions of EPC are nonetheless varied and controversial [13–15], which result in the generation of a complicated list of putative EPC immunophenotypes. Whilst recent data suggest that CD133+/CD34+ VEGF receptor 2 (VEGFR2) cells do not appear to be true EPCs [13–15], CD133+, CD34+, or VEGFR2+, or any combination of these immunophenotypes, have been commonly used to enumerate putative EPCs [15]. Importantly, circulating concentration of these cells have been correlated with cardiovascular disease and events [16–18] and occurrence of cardiovascular disease and events [19, 20].

This study aimed to determine the quantity and proliferative capacity of circulating CD133+/VEGFR2+ and CD34+/VEGFR2+ cells in patients with beta-thalassaemia major and their relationships with endothelial function and arterial stiffness. To further address the effect of varying iron loads on these cell number and function, we compared the findings in transfusion-dependent thalassaemia patients with those in patients who had undergone haematopoietic stem cell transplantation (HSCT) and in healthy control subjects.

Materials and methods

Subjects

Seventeen patients with beta-thalassaemia major receiving regular blood transfusion and 14 patients who had undergone HSCT were recruited from the haematology clinic. Eleven healthy subjects were recruited as controls. The latter included adult volunteers, healthy siblings of thalassaemia patients and individuals with functional heart murmur or nonspecific chest pain or palpitation attending a cardiac outpatient clinic for which no underlying organic causes were identified.

All subjects rested for at least 15 min before cardiovascular assessment as described below. The body weight and height were measured and the body mass index was calculated accordingly. Blood pressure in the right arm was measured twice using an automatic oscillometric device (Dinamap, Critikon, Inc.), and the average of two readings was taken. The serum ferritin levels of all subjects were determined.

Assessment of arterial function

Endothelial function of the brachial artery was assessed noninvasively by ultrasound examination of the vasodilation response to endothelium-dependent stimulus, as previously reported [1]. The right brachial artery, proximal to the antecubital fossa, was imaged longitudinally using the linear array transducer of the Vivid 7 ultrasound machine (General Electric, Horten, Norway). Flow-mediated endothelium-dependent vasodilation was assessed by measuring the brachial artery diameter at baseline and during reactive hyperaemia. Reactive hyperaemia was induced by deflating a cuff inflated to 300 mmHg for 4 min in the forearm. The percentage of diameter change during reactive hyperaemia was calculated. Low intra- and inter-observer coefficients of variation for flow-mediated dilation have previously been reported by our group [1].

The cross-sectional stiffness of the right carotid artery was measured by calculating the stiffness index [5]. The carotid artery segment about 1 cm proximal to the carotid bifurcation on the right side was imaged using the linear transducer, and the systolic and diastolic diameters were measured. The stiffness index was calculated according to the formula: \( \frac{\ln(\text{systolic BP/diastolic BP})}{(\delta D/D)} \), where \( \delta D \) is the difference between systolic and diastolic diameters and \( D \) is the mean diameter.

Quantification and proliferative assay of CD133+ VEGF2+ and CD34+ VEGFR2+ cells

The circulating cells with the aforementioned predefined immunophenotypes were quantified by flow cytometry as previously reported [16]. Briefly, 100 \( \mu \)l of peripheral blood was incubated with fluorescein isothiocyanate-conjugated anti-CD34 (Becton Dickinson, NJ, USA), phycoerythrin-conjugated anti-kinase insert domain receptor (R&D Systems) and allophyococyanin-conjugated anti-CD133 in the dark at room temperature for 30 min. Control isotype antibodies were obtained from Becton Dickinson. The red blood cells were lysed by the addition of 2 ml of BD FACSTM lysing solution (Becton Dickinson). Fluorescence-activated cell sorting analysis was performed using BD FACSAria analyzer, and the data were analysed using FlowJo software. Each analysis included all the events in the lymphocyte region. The frequency of peripheral bloods positive for the aforementioned reagents was determined by a two-dimensional side scatter fluorescence dot-plot analysis (Fig. 1). The percentages of positive cells were converted to cells per millilitre of blood using the complete blood count.

Cell isolation and colony-forming assay were performed as previously reported [17]. Mononuclear cells were isolated using Ficoll-PaqueTM PLUS density-gradient centrifugation from the peripheral blood of patients and controls. Five million cells were cultured in a six-well fibronectin-coated plate (Becton Dickinson) with CFU-Hill Liquid Medium (STEMCELL Technologies, BC, Canada) for 2 days, followed by harvesting of non-adherent cells. One million cells were then transferred into a 24-well fibronectin-coated plate (Becton Dickinson) for further
culturing with fresh medium for 3 days. After 5 days of plating, the cells were fixed and stained with Giemsa staining solution. The number of colony-forming units (CFUs) was enumerated in duplicate.

Statistical analysis

Data are presented as the mean ± SD or median and range, where appropriate. Demographic characteristics, arterial indices and the number of CFUs among the three groups were compared by simple analysis of variance (ANOVA) with post hoc comparisons by multiple \( t \) tests. The numbers of CD133\(^+\)VEGFR2\(^+\) and CD34\(^+\)VEGFR2\(^+\) cells were compared among the three groups using Kruskal–Wallis test with post hoc comparisons by Mann–Whitney \( U \) test. Relationships between arterial functional indices and CFUs were determined by Pearson correlation analysis. Stepwise multiple linear regression was used to identify significant determinants of brachial flow-mediated dilation and carotid arterial stiffness. A \( p \) value < 0.05 was regarded as statistically significant. All of the analyses were performed using SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA).

Results

Subjects

The demographic data and clinical parameters of patients and controls are summarized in Table 1. For the 17 transfusion-dependent thalassaemia patients, the types of chelation therapy were deferoxamine in eight, deferoxamine in four, deferasirox in three and deferoxamine in two patients. The 14 patients who had undergone HSCT at a median age of 6.4 (range, 1.6–20.1) years were significantly younger than transfusion-dependent patients and controls (\( p < 0.001 \)). Regular phlebotomy was performed in these transplanted patients until serum ferritin level was reduced to <2,000 pmol/l. At the time of study, 2 of the 14 patients were still receiving regular phlebotomy. All of the patients did not have heart failure clinically. The systolic blood pressure was lower in patients with and without HSCT (\( p = 0.009 \)), whilst serum ferritin level was expectedly the highest in transfusion-dependent patients (\( p < 0.001 \)) compared with patients after HSCT and controls.

Fig. 1 Two-dimensional side scatter fluorescence dot-plot analysis of the frequency of peripheral blood cells positive for CD133, CD34 and VEGFR2
Arterial function

Compared with controls, transfusion-dependent patients \((p<0.001)\) and post-BMT patients \((p=0.002)\) had significantly lower brachial flow-mediated dilation (Fig. 2a). On the other hand, carotid arterial stiffness was significantly greater in transfusion-dependent patients \((p=0.009)\) and post-BMT patients \((p=0.028)\) than controls (Fig. 2b). There was a significant positive correlation between flow-mediated dilation and carotid arterial stiffness \((r=0.57, p<0.001)\).

Quantity and function of CD133+VEGFR2+ and CD34+VEGFR2+ cells

The number of these circulating cells had a skewed distribution in the two patient groups (Fig. 3). There were significant differences in the quantity of CD133+VEGFR2+ \((p=0.049)\) and CD34+VEGFR2+ cells \((p=0.01)\) among the three groups as analysed by the Kruskal–Wallis test. Post hoc comparisons revealed that the number of these cell types was significantly higher in transfusion-dependent patients compared with post-HSCT patients and control subjects (Fig. 3). The proliferative capacity of these circulating cells was assessed by its ability to form CFUs. A CFU is defined by a cluster of central round cells surrounded by radiating spindle cells (Fig. 4a). Significant differences in CFUs were found among the three groups (ANOVA, \(p=0.038\); Fig. 4b). Post hoc comparisons showed that the number of CFUs was significantly smaller in transfusion-dependent patients compared with controls \((p=0.002)\). For post-transplant patients, their number of CFUs was similar to those of controls \((p=0.16)\). There was, however, a trend of increasing CFUs across the three groups \((p=0.011)\).

The number CFUs correlated positively with the magnitude of flow-mediated dilation \((r=0.42, p=0.029)\) and negatively with carotid arterial stiffness \((r=-0.45, p=0.02)\). There was, however, no correlation between serum ferritin level and CFU counts \((p=0.39)\). Multiple linear regression analysis of the entire cohort was further performed to identify significant determinants of flow-mediated dilation and arterial stiffness. The dependent variables included were age, sex, body mass index, systolic and diastolic blood pressures, and serum ferritin level. The only significant determinant identified was CFU counts for both flow-mediated dilation \((\beta=0.4, p=0.043)\) and carotid arterial stiffness \((\beta=-0.45, p=0.02)\).

Discussion

The present study demonstrates significant vascular dysfunction as characterized by impaired flow-mediated dilation and arterial stiffness in not only transfusion-dependent patients with beta-thalassaemia major but also in patients after HSCT. In transfusion-dependent patients who have the greatest iron load, impaired proliferation despite an increase in the number of circulating CD133+VEGFR2+ and CD34+VEGFR2+ cells is noted. Our data further suggest a dose-dependent effect of iron load on the proliferation of these cells. Importantly, we have shown that impairment of the proliferative capacity of these cells in thalassaemia patients is associated with vascular dysfunction.

Evidence of vasculopathy in patients with beta-thalassaemia major is accumulating. The findings of endothelial dysfunction and carotid arterial stiffening in iron-overloaded transfusion-dependent patients concur with those

| Table 1 Comparison of demographic and clinical parameters among transfusion-dependent thalassaemia patients, patients after HSCT, and controls |
|-----------------|-----------------|-----------------|---------------|
|                 | Transfusion-dependent patients \((n=17)\) | Patients post-HSCT \((n=14)\) | Controls \((n=11)\) | \(p\) |
| Age (years)     | 26.9±6.0        | 17.8±7.0\(^b\)  | 26.2±4.0      | <0.001\(^a\) |
| Sex (M/F)       | 7:10            | 5:9             | 5:6           | 0.88          |
| Weight (kg)     | 48±8            | 42±10           | 61±14\(^b\)  | <0.001\(^a\) |
| Height (cm)     | 158±6           | 151±15          | 164±9\(^b\)  | 0.018\(^a\)  |
| Body mass index (kg/m\(^2\)) | 19±2           | 18±2            | 22±3\(^b\)   | 0.001\(^a\)  |
| Systolic blood pressure (mmHg) | 105±11         | 105±7           | 117±12\(^b\) | 0.009\(^a\)  |
| Diastolic blood pressure (mmHg) | 67±13          | 64±7            | 66±6          | 0.64          |
| Ferritin (pmol/l) | Median: 4,383\(^b\) | Median: 572    | Median: 168  | <0.001\(^a\) |
|                 | (range, 2150–11866) | (range, 96–4672) | (range, 16–998) |               |

\(^a\)Statistically significant

\(^b\)\(p<0.05\) by post hoc \(t\) test or Mann–Whitney \(U\) test (for ferritin level) compared with the other two groups
reported previously by our group [1, 4, 5] and others [2, 3, 21]. In patients after HSCT, however, data on vascular function are scarce. In the only study published to date, Limsuwan et al. [22] have reported significant arterial intima–media thickening despite normal carotid arterial stiffness in transplanted thalassaemia patients when studied at a mean age of 10 years. In the present study, however, we found that despite HSCT, our patients studied at a mean age of 18 years had persistent endothelial dysfunction and carotid arterial stiffening.

Despite regular phlebotomy, thalassaemia patients after HSCT remain in a state of iron overload. In fact, iron overload is a recognized common long-term issue in patients with autologous and allogeneic HSCT [23, 24]. In vivo studies have provided a link between iron load and vascular dysfunction. Iron loading may reduce endothelium-derived nitric oxide directly by decreasing endothelial and inducible nitric oxide activity [25], and indirectly by stimulating membrane lipid peroxidation to generate lipid peroxyl radicals [26]. In mice overloaded with iron dextran,
increased generation of reactive oxygen species within the arterial wall and impaired acetylcholine-induced endothelial-dependent vasorelaxation have been shown [11].

Whilst previous studies have focused on assessing the arm of endothelial damage in the generation of vascular dysfunction in thalassaemia [1, 7, 15, 27, 28], repair of the endothelial damage by EPCs may also be instrumental in restoring normal endothelial function [29]. To our knowledge, this is the first study to explore the associations between cells with putative immunophenotypes of EPCs and vascular function in transfusion-dependent thalassaemia patients and in patients after HSCT.

Although an unequivocal definition of circulating EPCs by flow cytometry remains to be agreed upon, widely used markers include CD34, VEGFR2 and CD133 [29]. Nonetheless, recent studies suggest an important phenotypic and functional overlap between cells with these immunopheno-

Whereas inverse associations between the quantity of circulating CD133+VEGFR2+ and CD34+VEGFR2+ cells and the occurrence of cardiovascular events [20], cardiovascular risk score [17], severity of coronary artery disease [19], risk of peripheral vascular complications in diabetic patients [16] and risk of development subclinical atherosclerosis [18] have been widely reported, our finding of an increase in these circulating cells in our transfusion-dependent patients with yet significant vascular dysfunction is intriguing. Several explanations can perhaps be offered. Increased serum erythropoietin, documented in thalassaemia patients despite repeated blood transfusion [30], has been shown to increase the mobilization of circulating EPCs in both experimental models [31] and human subjects [32]. Additionally, induction of heme oxygenase-1 secondary to increased oxidative stress [10] and iron overload [33] in thalassaemia may also play a role. In a rabbit model of aortic balloon injury, heme oxygenase-1 has been shown to contribute to vascular repair by increasing bone marrow-derived circulating EPCs [34]. In our patients after HSCT, the reduction of serum erythropoietin and heme oxygenase-1 might have accounted for the normalization of number of circulating CD133+VEGFR2+ and CD34+VEGFR2+ cells.

Although the number of these circulating cells has been once regarded as a surrogate of function, discordance between their quantity and CFUs is increasingly recognized [35, 36]. Notwithstanding the suggestion of increased mobilization of these cells in our transfusion-dependent patients, which might be considered an intrinsic homeostatic mechanism to restore endothelial function, their proliferative capacity is limited and appears to worsen progressively across the three groups (Fig. 4b). Although there was a lack of an association between ferritin and CFUs in this study, we did not measure non-transferrin-bound iron that may be a better reflection of the free iron potentially acting on these cells. It is noteworthy that whilst cells of the CFUs express proteins similar to primary endothelial cells, they also express myeloid progenitor cell markers and mature into macrophages [13].

The exact mechanism of impaired cellular proliferative capacity in our patients remains speculative. Increased oxidative stress secondary to iron loading in patients with beta-thalassaemia major [10] and in those after HSCT [24] has been documented. A recent study showed an inverse relationship between the level of oxidized low-density lipoprotein and arterial endothelial function [37]. Nonetheless, the effect of oxidative stress on EPCs remains controversial [38]. Compared with mature endothelial cells, EPCs have been shown to be more resistant to oxidative stress in relation to the high intrinsic expression of...

![Morphologic characteristics of a colony-forming unit (CFU).](image)

**Fig. 4** a Morphologic characteristics of a colony-forming unit (CFU). b Number per high power field (HPF) in patients and controls.
manganese superoxide dismutase [39]. This aforementioned study did not, however, examine specifically the effect of oxidative stress on proliferative capacity of EPCs. On the other hand, other studies have shown that endothelial colony-forming cells are highly sensitive to oxidant stress [38]. It remains to be proven that increased oxidative stress secondary to iron load in thalassaemia patients both before and after HSCT is the culprit for impaired cellular proliferation found in this study. Nonetheless, the present study has shed some light on the physiological significance of reduced cellular proliferation on the arterial function in these patients. Our findings are also consistent with the reported relationship between colony number and endothelial function in healthy and diseased population with varying cardiovascular risks [17, 19, 40].

Several limitations of this study warrant comments. Firstly, the transfusion-dependent patients were put on different regimens of iron chelation. It would have been ideal to tease further the effect of various regimens on the quantity and function of CD133+/VEGFR2+ and CD34+/VEGFR2+ cells. Secondly, the number of subjects studied is relatively small, and these findings can only be regarded as preliminary. Thirdly, whereas the present study is a cross-sectional one, a longitudinal study to monitor the changes in the quantity and function of these cells and corresponding changes in arterial function in patients before and after HSCT would provide more insight on the impact of iron load on the mobilization and proliferation of these cells and the physiological implications on vascular function. Fourthly, the age of transplanted patients was younger. Nonetheless, this does not abrogate the findings of the study as younger age should be associated with better vascular [41] and EPC function. We have further performed multivariate analysis to adjust for the possible confounding influence of age on the vascular indices. Finally, we did not measure erythropoetin in our patients as the hypothesis set priori did not mandate its determination.

In conclusion, our findings suggest arterial dysfunction in patients with beta-thalassaemia major even after HSCT, which may perhaps be related in part to impaired proliferation of the CD133+/VEGFR2+ and CD34+/VEGFR2+ cells in the milieu of iron overload.

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