Blood Cells, Molecules and Diseases xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Aberrant bone marrow vascularization patterns in untreated patients with Gaucher disease type 1

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ARTICLE INFO

Article history:
Submitted 25 September 2016
Accepted 19 October 2016
Available online xxxx

Keywords:
Angiogenesis
Bone marrow
Gaucher disease
VEGF
Angiopoietins
Pericytes

ABSTRACT

Bone marrow (BM) in subjects with Gaucher disease (GD) displays accumulation of Gaucher cells (GC), i.e. glucocerebroside-laden macrophages. Following the assumption that macrophage proliferation and perturbation in GD modulates local inflammation-associated phenomena including angiogenesis, BM biopsies from 11 untreated GD patients and 36 controls were investigated for morphology and angiogenesis-associated features. These included microvascular density, (MVD), vessel structure and pericyte coverage, expression of VEGF-A and angiopoietins (ANGPT1 and 2). In GD BM, cellularity was higher, and GC clustered in cohesive but poorly demarcated areas, leaving irregular islands with normal hematopoiesis. MVD was 2.6-fold higher in GD marrows than in controls ($p < 0.001$). In GC-rich areas, MVD was 1.4-fold higher ($p = 0.026$), and vessel architecture was abnormal compared with GC-poor areas. MVD correlated with BM cellularity, particularly in GC-rich areas. Moreover, 30 ± 17% of GD BM vessels were pericyte-coated, significantly fewer than in controls (48 ± 16%; $p < 0.001$). Expression of ANGPT1 and 2 was significantly higher in GD BM vessel walls than in controls (7.2- and 13.2-fold higher), whereas VEGF expression was 20-fold lower ($p < 0.05$ for all). Thus, human GD BM shows increased angiogenesis with defective pericyte coating and skewed VEGF/ANGPT1 and 2 balances, presumably related to local accumulation of GC.

1. Introduction

Gaucher disease (GD) is a progressive, multisystem lysosomal storage disorder, related to deficient activity of the enzyme glucocerebrosidase due to mutations in the *GBA1* gene [1,2]. Three clinical types of GD are distinguished according to the absence (type 1) or presence (types 2 and 3) of neurological symptoms and the dynamics of developing clinical signs. The most prevalent form of GD is non-neuronopathic GD type 1 (GD1) presenting mostly with thrombocytopenia, anemia, hepatosplenomegaly, and bone manifestations [3]. Tissue infiltration by glucocerebroside-laden macrophages (Gaucher cells, GC) is a hallmark of GD, leading to impaired organ function and eliciting clinical signs and symptoms [4,5]. Since monocytes/macrophages play a major role in chronic inflammation, it can be assumed that macrophage/GC proliferation in GD can locally induce or modify inflammation-associated phenomena.

Increased angiogenesis is part of tissue remodeling in response to processes such as inflammation, malignant transformation and hypoxia [6]. In the bone marrow (BM), intense angiogenesis and deranged 3-dimensional vessel architecture is found in leukemias, lymphomas, myelomas and other hematological malignancies [6]. Mechanisms for the enhanced blood vessel formation include release of various growth factors from malignant and stromal cells, e.g. vascular endothelial growth factors (VEGFs) or angiopoietins (ANGPTs). The balance between these and other factors determines outgrowth, networking and functionality of the new vessels, as well as the final step of vessel maturity, *viz.* recruitment and coverage of the endothelial tube by pericytes (PC) [7,8]. Assessment of angiogenesis, for instance by microvessel density (MVD) and vessel lumen perimeters, has also prognostic value for the outcomes of underlying disease. Thus, in myelomas, MVD increases gradually from MGUS to myeloma grade I though III [9]. Previously in a case study, skin biopsies from GD patients have shown endothelial and pericyte (PC) abnormalities in blood vessels [10], suggesting aberrant angiogenesis related to GD.

Here, we assessed if GD is associated with signs of increased angiogenesis in the BM (measured as MVD and vessel perimeters), and evaluated expression and localization of some endothelial growth factors (VEGF-A_{165}, ANGPT1 and 2). We also assessed the PC coverage since this step in angiogenesis is believed to reflect vessel maturity and is often reduced in malignancies [8]. Finally, we related these assessments to disease activity/staging.

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http://dx.doi.org/10.1016/j.bcmd.2016.10.009
1079-9796/

2. Methods

2.1. Patients and controls

The study population consisted of 11 adults (3 women and 8 men) aged 21–86 years with untreated GD1, followed between 2002 and 2015 at the Hematology Center Karolinska, Karolinska University Hospital Huddinge, who initially underwent BM biopsy as part of the diagnostic work-up for cytopenia and splenomegaly.

In all studied patients, the diagnosis of GD was confirmed by a low activity of glucocerebrosidase in peripheral blood (PB) leukocytes and increased activity of plasma chitotriosidase. Furthermore, direct DNA sequencing revealed mutations in the *GBA1* gene in all cases. All but one patient (91%) carried at least one allele with c.1226A>G (N370S) mutation in the *GBA1* gene. The patients' medical records were reviewed to collect relevant clinical data. Patient demographical and clinical characteristics are shown in Table 1; information concerning *GBA1* mutations is provided in Supplementary data Table 1.

Eight patients had thrombocytopenia (i.e. platelets $< 150 \times 10^9$/L), and 2 patients had blood hemoglobin concentration < 120 g/L. Four patients were splenectomized. Two patients had a monoclonal gammopathy of unknown significance and one a monoclonal IgG of 24–33 g/L. Majority of patients were middle-aged at the time of the study, had evidence of bone disease, mild to moderate disease according to the Zimran Severity Score Index (SSI) (Supplementary data Table 1) [11,12] and a rather stable disease as judged from SSI during the past 8 years. Only one patient had a mildly raised C-reactive protein serum level (14 mg/L, reference value < 3), indicating absence of acute generalized inflammation in nearly all subjects.

The study was performed according to ethical guidelines of the Declaration of Helsinki and approved by the local ethics committee in Stockholm. All patients provided their informed consent.

Control group consisted of 36 patients (18 men and 18 women, aged 27–89 years; mean age 63.6) who underwent BM sampling as part of hematological diagnostics or before hematopoietic stem cell donation. These patients were then followed up for the next 4–5 years without showing evidence of a hematological disease or malignancy; the majority has been described previously [13,14].

Table 1
Patient characteristics.

Characteristic (n = 11)	Result
Sex	
Male	8 (73%)
Female	3 (27%)
Age at the time of BM sampling (years)	
Range	21–86
Mean	60
Median	57
Splenectomy	4 (36%)
Bone disease	
Symptomatic	8 (73%)
Radiological	10 (91%)
Neurological symptoms	
Parkinson's disease and neuropathy	1 (9%)
Parkinson's disease and epilepsy	1 (9%)
Neuropathy	1 (9%)
Zimran Severity Score Index (SSI)	
SSI scores: mean/median/range	8/7/3–14
Mild Gaucher disease (SSI scores 0–10)	7 (64%)
Moderate Gaucher disease (SSI scores 11–25)	4 (36%)
GBA1 gene mutations	
c.1226A>G/c.1226A>G	3 (27%)
c.1226A>G/c.1448T>C	2 (18%)
c.1226A>G/other[a]	5 (45%)
Other/other[b]	1 (9%)

[a] c.115+1G>A (1 patient); c.330delA (1 patient); c.437C>T (1 patient); c.721G>A (1 patient); RecNci I: c.1448T>C, c.1483G>C, c.1497G>C (1 patient).
[b] c.798C>G/c.1040T>G.

2.2. Bone marrow samples

Trephine BM biopsies from GD patients and controls were processed and analyzed at the Department of Clinical Pathology and Cytology, Karolinska University Hospital (Supplementary data). Biopsies were formalin fixed, formic acid decalcified, and paraffin embedded. Morphological evaluation was performed on hematoxylin-eosin (HE) stained slides.

Bone marrow cellularity was assessed by light microscopy as part of routine procedure, with semiquantitative evaluation of proportion of hematopoietic cells to adipocytes in BM spaces [15]. Degree of infiltration by GC was assessed likewise, and expressed as percentage of non-fatty tissue in BM spaces. For GC burden evaluation, cohesive infiltrates and dispersed cell groups were considered.

Aspirated BM samples from some controls were also formalin fixed, paraffin embedded (see Supplementary data for details).

2.3. Immunohistochemical assays

Antibodies. The antibodies are notated in the Supplementary data Table 2.

Microvascular density (MVD) in BM samples was determined by light microscopy (Olympus BX40 microscope) in CD34-stained slides. Firstly, MVD values in BM of GD patients were compared with those in healthy controls. Results are given as mean number of vessels in 10 randomly chosen 40x high power fields (HPF). Secondly, in BM biopsies from GD patients, areas with prominent GC infiltration (≥50%, GC-rich areas) as well as areas with apparently normal hematopoiesis or less prominent infiltration (<50% GC infiltration, GC-poor areas) were identified. For each patient, assessment of MVD was performed separately in 5 GC-rich and in 5 GC-poor HPF areas.

Vessel perimeters were assessed in BM biopsies from GD patients by imaging techniques. (see Supplementary data for details). For that purpose, digital scans of CD34-stained slides were obtained. For each sample, five HPF pictures (40x) were obtained from GC-rich and five from GC-poor areas respectively, with adequate metric scales provided. All microvessels were identified in each image, and the metric scale bar was used for reference. Perimeters were highlighted by manual tracing the inner (luminal) CD34 staining, and then measured by the software.

Expression of VEGF, ANGPT1 and 2 in BM was evaluated as described previously [13,14,16–18] Briefly, >20 megakaryocytes and >20 blood vessels per sample were assessed by light microscopy with a 40x or a 100x oil immersion objective, respectively. Results are given as mean values for the percentages of positively stained vessels.

Quantification of pericyte (PC) coverage. To visualize PC coverage of BM vessels by immunofluorescence, cells were labeled with the CD34 and SMA-α antibodies by standard and previously reported techniques [13,14,16–18]. Vessels that were covered with >50% of their perimeters with PC were categorized as PC-positive. *Analysis of vessel and PC morphology* was done by immunofluorescent- and double laser confocal microscopy (see Supplementary data for further details).

2.4. Statistical analyses

Analyses were performed with the Statistica™ software package using Student's *t*-test and Spearman non-parametric correlation. Results are given as mean and SD values. P values < 0.05 were considered as significant.

3. Results

3.1. Bone marrow cellularity, GC infiltration and general features

The BM was hypercellular for age in most biopsies from GD patients (mean value 60% ± 19%), with markedly varied proportion of GC infiltrates (mean value 27% ± 16%, Supplementary data Table 3). Those were mostly cohesive but poorly demarcated, inter- or paratrabecular,

Please cite this article as: M. Klimkowska, et al., Blood Cells Mol. Diseases (2016), http://dx.doi.org/10.1016/j.bcmd.2016.10.009