Plasma levels of stromal cell-derived factor-1 (CXCL12) and circulating endothelial progenitor cells in women with idiopathic heavy menstrual bleeding

E. Elsheikh1, E. Andersson2, C. Sylvén3, B.-G. Ericzon1, J. Palmblad4, and M. Mints5,*

1Division of Transplantation Surgery, Department of Clinical Science, Intervention and Technology, Karolinska University Hospital Huddinge, Stockholm 141 86, Sweden 2Uppsala University and Department of Women’s and Children’s Health, Karolinska Institutet at Karolinska University Hospital Solna, Stockholm 171 76, Sweden 3Division of Cardiology, Department of Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm 141 86, Sweden 4Center for Inflammation and Hematology Research, Departments of Medicine and Dermatology, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm 141 86, Sweden 5Department of Women’s and Children’s Health, Karolinska Institutet, Karolinska University Hospital Solna, Stockholm 171 76, Sweden

*Correspondence address. Department of Obstetrics and Gynecology, Karolinska University Hospital Solna, S-171 76 Stockholm, Sweden; Tel: +46-8-51770000; Fax: +46-8-517-717-74; E-mail: miriam.mints@ki.se

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Study Question: Do plasma levels of stromal cell-derived factor-1 (CXCL12, sometimes termed SDF-1) and the numbers of circulating endothelial progenitor cells (EPCs), EPC colony-forming units (EPC-CFU) and mature endothelial cells (ECs) differ between women with idiopathic heavy menstrual bleeding of endometrial origin (HMB-E) and controls and are they related to plasma levels of other angiogenic growth factors?

Summary Answer: Angiogenesis is altered in women with HMB-E, characterized by a reduction in mean plasma levels of CXCL12, a low number of EPC-CFUs and a high level of circulating ECs.

What is Known Already: Plasma levels of CXCL12 are significantly higher during the proliferative than the secretory phase of the menstrual cycle in healthy women and exhibit a negative correlation with blood EPC-CFUs.

Study Design, Size, Duration: A prospective cohort study in a university hospital setting. Between 2008 and 2009 10 HMB-E patients were recruited from Karolinska University Hospital. Ten healthy women were also included in the analysis.

Participants/Materials, Setting, Methods: Ten healthy control women and 10 HMB-E patients, all with regular menstrual cycles, provided 4 blood samples during a single menstrual cycle: 2 in the proliferative phase, 1 at ovulation and 1 in the secretory phase. We assessed plasma levels of CXCL12, vascular endothelial growth factor A165 (VEGFA), basic fibroblast growth factor (bFGF) and granulocyte and granulocyte–macrophage colony-stimulating factors by ELISA. We counted circulating EPC-CFUs by culture, and ECs and EPCs by flow cytometry and immunostaining for cell surface markers.

Main Results and the Role of Chance: Plasma levels of CXCL12 were significantly lower in HMB-E patients compared with control women ($P < 0.0001$), with a significant decrease ($P = 0.013$) between the proliferative phase and ovulation. VEGFA showed a trend towards the same decreasing pattern as CXCL12, although not statistically significant ($P = 0.086$), whereas systemic VEGFA levels in control women remained unchanged across the different phases of the menstrual cycle ($P = 0.473$). HMB-E patients had a lower number of EPC-CFUs compared with control women ($P = 0.014$), with a positive correlation between the level of CXCL12 and EPC-CFUs ($r = 0.428; P = 0.047$). Whilst the level of circulating endothelial cells in HMB-E patients was higher than in control women, this did not reach statistical significance. In contrast, the levels of the hematopoietic/EPC marker CD34 were significantly lower in HMB-E patients than control women ($P < 0.020$).

Limitations, Reasons for Caution: Small sample, unknown source of CXCL12, unknown balance between influx and efflux of EPCs from bone marrow and to the endometrium.
Introduction

Heavy menstrual bleeding (HMB), i.e. >80 ml per menstruation, is common in women of reproductive age, accounting for >20% of visits to gynecology outpatient clinics. An etiologic explanation for this bleeding, such as fibroids, carcinoma or coagulation disturbances, can be assigned to approximately half of patients with an HMB diagnosis. However, the remaining 50% of HMB occurs in the absence of any recognized uterine pathology, and is therefore referred to as idiopathic HMB or HMB of endometrial origin (HMB-E) (Mints et al., 2007). HMB leads to iron deficiency and anemia due to continuous blood loss, and may ultimately require hysterectomy. The psychological, social and employment consequences of this condition in combination with the growing number of women it affects have led to its designation as a public health problem (Mints et al., 2010).

It has been suggested that disturbances in the complex mechanisms of endometrial angiogenesis are the probable cause of HMB-E. The four known principal mechanisms of angiogenesis are vessel sprouting, elongation of pre-existing vessels, intussusception and incorporation of endothelial progenitor cells (EPCs) from the blood into the endothelial wall (Holash et al., 1999). Recently, attention has been focused on this last mechanism, namely EPCs.

In a previous study we suggested that some blood vessels in the endometrium develop from EPCs derived from bone marrow; 14% of the endothelial cells in endometrial blood vessels in human leucocyte antigen-mismatched women who received bone marrow transplants (Mints et al., 2008) were donor derived. Recently, we reported that the chemokine stromal cell-derived factor-1 (CXCL12, sometimes termed SDF-1) may play a role in homing EPCs to the endometrium, as significantly higher plasma levels of CXCL12 were found during the proliferative phase and showed a negative correlation with blood EPC-colony forming units (EPC-CFU) (Elsheikh et al., 2011).

CXCL12 belongs to the chemokine family, a superfamily of chemoattractant cytokines (Vindrieux et al., 2009; Sun et al., 2010), and is an important player in angiogenesis. CXCL12 is mostly expressed in highly vascularized organs such as the liver, spleen and kidneys, and binds to its receptor CXCR4, which is expressed on various target cells including EPCs (Janowski, 2009). CXCL12 secretion by the stromal cells is thought to create a chemogradient in the microenvironment around blood vessels, attracting circulating EPCs in the blood to integrate into the vessel walls. This mechanism may be one way for the tissue to repair small constitutional damage in the vessel walls, but it could also be active during greater events that trigger angiogenesis (Kucia et al., 2006).

Studies of different granulation tissues have shown that the number of EPCs ranges between 5 and 25% of luminal vessel cells, and that the level of CXCL12 correlates positively with the ability for wounds to heal among diabetic individuals (Bermudez et al., 2011). This further indicates that the CXCL12 pathway could play a significant role in the neovascularization of human tissues, including the endothelium (Kawamoto and Asahara, 2007).

The CXCL12/CXCR4 axis is also involved in the release of various hematopoietic cells from the bone marrow, including CD34+ cells and EPCs. When the axis is active, those progenitors remain tethered to stroma cells in the bone marrow. But when the ligation is interrupted, for instance by Plerixafo (used for mobilization of CD34+ progenitor cells for hematopoietic stem cell transplantation), an efflux of EPCs to the blood also occurs.

The aim of the present study was to expand our previous studies on EPCs and the plasma level of CXCL12s in healthy women and apply these results to patients with HMB-E. Here, we investigated plasma levels of stromal cell-derived factor-1 (CXCL12), and the numbers of EPCs, EPC colony forming units (CFU) and mature endothelial cells (ECs) in the circulation of women with HMB-E and examined the relationship between these factors and variations in plasma levels of other angiogenic growth factors. These include vascular endothelial growth factor A165 (VEGFA) and basic fibroblast growth factor (bFGF). In addition, we studied granulocyte and granulocyte macrophage colony-stimulating factor (G-CSF and GM-CSF), which are involved in the release of myeloid progenitor cells from the bone marrow and can accelerate neovascularization (Minamino et al., 2005; Capoccia et al., 2006). Finally, we assessed the number of circulating mature ECs (and/or inflammatory ECs) that detached from the vessel walls, which may be found in the blood as a result of vascular injury (Woywodt et al., 2002; Blann et al., 2005).

Materials and Methods

Study population

Blood samples were collected from 10 HMB-E patients (mean age 37.0 years) and 10 healthy volunteers recruited from hospital staff (control women, mean age 32.8 years). The age difference between HMB-E patients and control women was not significant. All women had a regular menstrual cycle of 25–32 days. None of the women had used hormonal or intrauterine contraception in the 3 months preceding the study. Blood samples of HMB-E patients showed normal values for platelets, activated prothrombin thromboplastin time, international normalized ratio (INR), bleeding time and von Willebrand factor. The uterine cavity of HMB-E patients appeared normal as evaluated by hysteroscopy.

The phases of the menstrual cycle were determined based on the first day of menstruation, and estradiol and progesterone levels. Participants identified their ovulation day (luteal hormone surge) by testing their morning
urine. Four blood samples were collected in EDTA tubes: one in Days 1 – 2 of menstrual cycle, one in the Day 5 of menstrual cycle one at ovulation (Day 14) and one in the secretory phase (Days 22 – 24).

The diagnosis of HMB was based on an accurate history of menstruation and pictorial blood loss assessment charts. A pictorial chart score higher than 100 was diagnostic for HMB (Higham et al., 1990). Patients for this study were recruited from out patient’s clinic for women with HMB. In order to avoid any bias in patient selection, we enrolled patients consecutive-ly. As controls we considered only women with a regular normal menstrual cycle according to pictorial chart score (<80). Moreover, all women had undergone examination and transvaginal ultrasound that revealed no pathology in the uterine cavity.

Blood samples were used to isolate peripheral blood mononuclear cells (PBMCs) and to culture EPCs. Remaining blood was centrifuged at (1000 g) for 15 min and the plasma was frozen at – 70 °C until analysis.

Plasma angiogenic growth factor levels

Plasma from all four samples was tested for the following soluble angiogenic growth factors: CXCL12, VEGFA, G-CSF, GM-CSF and bFGF by means of an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. For each growth factor all determinations were made in a single plate and read by kinetic microplate reader (v max). The intra-assay coefficient of variation range was 3 – 9%.

Flow cytometry

To find out which cell types in the circulation may correlate with the formation of the endometrium, PBMCs were phenotyped with an array of antibodies to specific markers expressed on EPCs (VEGFR-2, VEGFR-1, Tie-2, CD133, CD146 and CD34) (Timmermans et al., 2009) and mature EC markers (CD141, CD144 and CD31), as well as various homeostasis-regulating molecules [i.e. vascular adhesion protein-1 (VAP-1 ), CD142, heparin sulfate proteoglycan (HSP) and plasminogen activator inhibitor-1 (PAI-1)].

The following antibodies were used: anti-VEGFR-2 (Reliatech, Maschendorf, Germany), anti-VEGFR-1 (R&D Systems), anti-CD133 (Miltenyi Biotec, GmbH, Bergisch, Germany), anti-VAP-1 (Serotec, Düsseldorf, Germany), anti-CD141, anti-PAI-1, HSP (Biogenesis Ltd, Poole, UK), Tie-2, CD34, CD144, CD31 and CD142 (Becton Dickinson, San Jose, CA, USA). Corresponding control isotypes were used for evaluation of non-specific-binding of monoclonal antibodies. The cells were analyzed on a fluorescence-activated flow cytometer (FACSorter, Becton Dickinson).

EPC-CFU assay

To study the functional capacity of EPCs during menstrual angiogenesis, we performed a two-step CFU assay to detect the number of EPC-CFUs, as described by Hill et al. (2003). Briefly, PBMCs were cultured on fibronectin (20 μg/ml)-coated tissue culture plates in an endothelial-selective medium (EndoCult, Stem Cell Technologies, Vancouver, BC, Canada) in an incubator (37°C, 95% humidity, 78.09% nitrogen, 20.95% oxygen, 0.93% argon, 0.039% CO2). On Day 2, the non-adherent cells were collected and plated in a second set of fibronectin-coated plates. The number of CFUs that formed in the different wells was counted on Day 5 after culturing.

Statistical analysis

The Friedman non-parametric test was used to test for significant differences in repeated estimates. The non-parametric Wilcoxon matched pairs test was used to compare growth factor levels and the number of CFUs at the different time points. Statistical analysis was performed with the SPSS software version 18.0, and P < 0.05 was considered significant. Data are presented as median and interquartile range (IQR).

The non-parametric Kruskal–Wallis test was used to compare the growth factor levels between HMB-E patients and control women at the different time points. Correction for multiple tests was not performed. For each woman the median value of the four measurements was used. For women with one or more missing values the median of the observed measurements was used. For the statistical correlations between the growth factors and the number of EPC-CFUs, the Pearson correlation test was used. The software R version 2.15.2 was used for these calculations (R Core Team, 2012).

Ethical approval

The study was approved by the Ethics Committee of Karolinska University Hospital. All participating women gave written informed consent.

Results

Levels of circulating angiogenic growth factors

Level of CXCL12s varied significantly over the menstrual cycle, but differently in HMB-E patients and control women (P = 0.003). First, the average level of CXCL12 across the menstrual cycle was 16% lower in HMB-E patients than in control women (P < 0.0001). Secondly, the pattern of changes in the levels of CXCL12s during the cycle was different. HMB-E patients showed the highest levels on Days 1 – 2 of the menstrual cycle, whereas control women did so on Day 5. In HMB-E patients the lowest level of CXCL12s was seen at ovulation (Day 14), but in control women during the secretory phase (Days 22 – 24). At Day 5 of the menstrual cycle the median level of CXCL12 was significantly lower (P = 0.005) in HMB-E patients than control women (Fig. 1). Hence, levels of CXCL12s in HMB-E patients showed a dysregulated cyclicity, with appearances of the peak and the nadir earlier in the menstrual cycle compared with control women.

Figure 1 Boxplots showing plasma stromal cell-derived factor-1 (CXCL12) levels during different time points of the menstrual cycle. Level of CXCL12 in the mid-proliferative phase (Day 5) were significantly lower in HMB-E patients (n = 10) than the control group (n = 6). Values are given as medians and IQRs; error bars represent minimum and maximum values.
We also analyzed two angiogenic growth factors, VEGFA and bFGF. Whilst systemic VEGFA in HMB-E women showed a qualitatively similar pattern to CXCL12 neither its level nor that of bFGF was significantly different from the control group. Neither did levels of these factors vary during the menstrual cycle in a statistically significant way in either of the two groups (Fig. 2A and B).

In a manner similar to CXCL12, bFGF showed a statistically insignificant decreasing pattern from the Days 1–2 of menstrual cycle to ovulation day (Day 14) in HMB-E patients, and from the Day 5 to the secretory phase (Days 22–24) of the menstrual cycle among control women (Fig. 2b). In both groups, we found no detectable levels of the myeloid growth factors GM-CSF or G-CSF, showing that the response we were interested in was not a result of general bone marrow stimulation, which is relevant since G-CSF can interrupt the CXCL12/CXCR4 axis in the bone marrow, causing an influx of EPCSs to the blood.

**Figure 2** (A) VEGFA and (B) FGF-b levels during different time points of the menstrual cycle (HMB-E patients, $n = 10$; control women $n = 6$). Values are given as medians and IQRs; error bars represent minimum and maximum values.

**Figure 3** (A) Boxplots showing the numbers of EPC-CFUs in HMB-E patients and control women. Number of EPC-CFUs significantly decreased in HMB-E patients ($n = 6$) when compared with control women ($n = 10$). Values are given as medians and IQRs; error bars represent minimum and maximum values. (B) A scatter plot showing positive correlation between all CXCL12 concentrations (pg/ml, $x$-axis) and number of circulating EPCs (EPC-CFUs, $y$-axis) in the HMB-E patients. $r$ = Pearson correlation coefficient. The inserted figure shows the negative correlation between levels of CXCL12 and the numbers of circulating EPCs in the control group (adopted from Crosby et al., 2000; Elsheikh et al., 2011) (EPC, endothelial progenitor cell; EPC-CFU, EPC-colony forming unit.).
EPC-CFUs and CXCL12

As shown in Fig. 3A, HMB-E patients had a lower number of EPC-CFUs (median 2; IQR 0–7) compared with control women (median 11; IQR 6–17) \((P = 0.0142)\). Interestingly, there was a positive correlation between the level of CXCL12 and EPC-CFUs \((r = 0.428; P = 0.047)\) (Fig. 3B). We did not find any correlation between VEGFA or bFGF levels and EPC-CFUs. Thus, the higher the blood levels of CXCL12, the higher the number of EPC-CFUs. The insert shows the previously published values for the control women in our study, among whom a negative correlation was observed, i.e. the lower the CXCL12 values, the higher the number of EPC-CFUs (Elsheikh et al., 2011).

Levels of circulating mature ECs in HMB-E patients and control women (Figs. 4 and 5)

In general, we found that the level of circulating mature ECs in HMB-E patients was higher than in control women (Fig. 4A and B). The percentages of mature ECs positive for VEGFR-2, VEGFR-1, Tie-2, CD144, CD141 and CD31 in HMB-E patients were 5.0, 3.4, 0.2, 0.9, 25.0 and 30.7%, respectively, and in control women they were 2.6, 2.4, 0.4, 0.4, 16.9 and 12.9%, respectively. These differences were however not statistically significant. In contrast, the levels of the hematopoietic/EPC marker CD34\(^+\) cells were significantly lower in HMB-E patients than control women (median, 0.0 and 0.1%, respectively, \(P < 0.020\)) (Fig. 4C). However, the number of CD133-positive cells did not vary between HMB-E patients and control women in a statistically significant way (median 0.2 for patients and 0.3 for controls).

HSP expression was significantly lower in HMB-E patients than control women (median, 0.02 and 0.3%, respectively, \(P = 0.045\), Fig. 4D). These results might be a sign of an increased vascular inflammation in the endometrium and also indicate that the healing process in the endometrium in women with HMB-E is altered.

Representative picture of flow cytometric analysis of single staining for specific markers expressed on EPCs and mature EC markers as well as various homeostasis-regulating molecules are shown in Fig. 5.

**Figure 4** (A, B and C) Characterization of ECs markers in the peripheral blood of HMB-E patients and normal women. (D) Expression of various molecules regulating homeostasis. Values are given as medians and IQRs. For the patients, \(n = 4\). For the healthy control women, \(n = 10\) except for VEGFR-1 \((n = 6)\), CD34 \((n = 8)\), CD141 \((n = 7)\) and PAI-1 \((n = 7)\).
Discussion

The present study provides three important findings. First, we have demonstrated that levels of CXCL12, an important factor in angiogenesis, were significantly lower in HMB-E patients compared with control women ($P < 0.0001$), with a significant change of the menstrual cyclicity of CXCL12s so that the peak and nadir for the HMB-E patients occurred prior to those of the healthy women. Indeed, HMB-E patients exhibited a decrease ($P = 0.013$) between the early proliferative phase and ovulation, whereas control women showed a decrease a week later. In addition, VEGFA showed a trend, albeit not statistically significant, towards the same decreasing pattern as CXCL12, whereas the systemic VEGFA levels in control women were not significantly different in the different phases of the menstrual cycle we studied. Secondly, HMB-E patients had a lower number of EPC-CFUs compared with healthy control women, with a positive correlation between the level of CXCL12 and EPC-CFUs. And thirdly, the level of circulating mature ECs in HMB-E patients was generally higher than in control women. In contrast, the levels of the hematopoietic/EPC marker CD34 were significantly lower in HMB-E patients than control women.

Here, for the first time to our knowledge, we show that plasma levels of CXCL12 are altered in HMB-E patients compared with healthy women. The impact of the reduction of the mean plasma levels and the altered cyclicity of CXCL12 on the angiogenic process in the endometrium is still unclear, mainly because we do not know the source of CXCL12. However, since there was a cyclical pattern to the levels, which coincided with the menstrual cycle, it is reasonable to consider that the endometrium might be one significant source. Our prior demonstration of cyclical CXCL12 protein expression in the endometrial stroma with a tendency of increased levels in the secretory phase compared with the proliferative phase ($P = 0.051$) (Elsheikh et al., 2011) supports that hypothesis. In contrast, Laird et al. (2011) showed that mRNA expression of CXCL12 in the endometrium does not change during the menstrual cycle. There are methodological differences between that study and ours. We investigated the expression of the protein by immunohistochemistry, whereas Laird et al. studied the mRNA expression. Since the human endometrium is a complex tissue, comprising different types of cells, including epithelial, stromal, inflammatory, perivascular and blood vessel cells, we suggest that immunohistochemistry may be more specific in terms of CXCL12 protein expression and localization. Thus, it is conceivable that lower local amounts of CXCL12 in the endometrium, reflected as low blood levels, together with poor timing of CXCL12 peaks might disturb the recruitment of EPCs to the endometrium.

In the present study we also showed that HMB-E patients had a lower number of EPC-CFUs compared with control women. Because EPCs have been shown to contribute between 5 and 25% of new ECs in other granulation tissues (Crosby et al., 2000), it would be no surprise if they constituted a relatively big fraction of new ECs in the endometrium. If there is a lack of CXCL12, lesser amounts of EPCs will be incorporated, which could lead to a lack of cells to form vessel walls.

Figure 5 Representative picture of flow cytometric analysis showing single staining for specific markers expressed on EPCs and mature EC markers as well as various homeostasis-regulating molecules on PBMCs from healthy controls (upper panel) and HMB-E patients (lower panel). SSC, side scattered.
together with local VEGFA over-expression, would make the new vessels fragile and possibly leakier than normal. The lower numbers of CD34+ cells together with low levels of G-CSF and GM-CSF point to a reduction of outflow of EPCs from the bone marrow.

Furthermore, we found generally higher numbers of circulating mature ECs, which could be a sign that the body is compensating for the loss of EPCs. Papers that have focused on the possible role of EPCs in atherosclerosis have shown that a vessel wall that uses EPCs instead of proliferating local ECs to repair endothelial damage has a significantly lower risk of developing neointimal hyperplasia, and that people with higher levels of circulating EPCs have a lower lifetime risk of developing cardiovascular disease (Hill et al., 2003; Werner et al., 2003; Moreno et al., 2004).

Previously, we reported a strong association between HMB-E and a family history of HMB (Kuzmina et al., 2011). These results suggested that a family history of HMB has the highest predictive value for the development of HMB-E, and thus that the condition might be hereditary. The difference in blood levels of CXCL12, as well as the number of circulating EPCs described in the present paper, might be part of such a trait. Xiao et al. (2008) reported that certain CXCL12 gene variations were associated with blood levels of CXCL12 and number of EPCs. These researchers also found that the number of EPCs was inversely correlated with level of CXCL12 and that one AA genotype in the CXCL12 gene was associated with higher blood levels of this cytokine. Further research is warranted to see if our HMB-E patients segregate into a specific CXCL12 genotype.

Accumulated evidence from a variety of systems suggests a model where EPCs are released from the bone marrow by means of processes that disrupts the tethering system of, inter alia, the CXCL12/CXCR4 axis between bone marrow stroma cells and EPCs. Subsequently, blood EPCs are recruited to regions of active angiogenesis by means of locally expressed adhesion and chemotactic factors. In this respect the CXCL12/CXCR4 axis has received considerable interest, since agents blocking this interaction reduce the capacity for wound healing and other aspects of angiogenesis (Cheng and Qin, 2012). Hence, the net number of blood ECPs depends on the balance between influx and efflux from bone marrow and tissues. A high level of CXCL12 might be interpreted as a signal from the tissues to attract EPCs, but the source of CXCL12 remains elusive in most cases. It can be anticipated that during different phases of the menstrual cycle, the endometrium sends signals of varying magnitude depending on the angiogenic activity; that during different phases of the menstrual cycle, the endometrium is recognized as a source of CXCL12, but the mechanism by which the signal is generated is left to be elucidated (Cheng and Qin, 2012).

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Authors’ roles


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Conflict of interest

The authors have no conflict of interest to declare.

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