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Menstrual cycle distribution of uterine natural killer cells is altered in heavy menstrual bleeding

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ABSTRACT

Heavy menstrual bleeding (HMB) affects 30% of women of reproductive age and significantly interferes with quality of life. Altered endometrial vascular maturation has been reported in HMB and recurrent miscarriage, the latter associated with increased uterine natural killer (uNK) cell numbers. This study compared endometrial leukocyte populations in controls and women with HMB. Formalin-fixed paraffin-embedded endometrial biopsies from controls (without endometrial pathology) and HMB were immunostained for CD14 (macrophages), CD56 (uNK cells), CD83 (dendritic cells), FOXP3 (regulatory T cells/Tregs), CD3 and CD8 (T cells). Leukocyte numbers were analysed as a percentage of total stromal cells in five randomly selected fields of view in the stratum functionalis of each sample. In control women across the menstrual cycle, 2–8% of total stromal cells were $CD3^+$ cells, 2–4% were $CD8^+$ T cells and 6–8% were $CD14^+$ macrophages. Compared with controls, $CD3^+$ cells were reduced during the mid-secretory phase (4%, $P < 0.01$) and increased in the late secretory phase (12%, $P = 0.01$) in HMB. $CD83^+$ dendritic cells and $FOXP3^+$ Tregs were scarce throughout the menstrual cycle in both groups. In controls, 2% of stromal cells in proliferative endometrium were $CD56^+$ uNK cells, increasing to 17% during the late secretory phase. In HMB, $CD56^+$ uNK cells were increased in the proliferative (5%, $P < 0.01$) and early secretory (4%, $P < 0.02$) phases, but reduced (10%, $P < 0.01$) in the late secretory phase. This study demonstrates dysregulation of uNK cells in HMB, the functional consequence of which may have an impact on endometrial vascular development and/or endometrial preparation for menstruation.

1. Introduction

Human menstruation is a highly regulated physiological process involving communication between the vascular and immune systems, under the control of the endocrine system. During the follicular/proliferative phase oestrogen stimulates endometrial proliferation, with angiogenesis occurring to support this endometrial growth. During the progesterone-dominant secretory phase, there is growth and coiling of the endometrial spiral arterioles. In the absence of implantation, the rapid fall in progesterone (and oestrogen) levels prompts tissue fragmentation and shedding of the superficial layer of the endometrium, the stratum functionalis. This exposes open blood vessels and glands, resulting in menstrual bleeding (Aplin et al., 2008), alongside angiogenesis to allow vascular repair in the basal region of the endometrium.

Heavy menstrual bleeding (HMB) affects approximately 10 million women annually in the USA, including 30% of women of reproductive age, with 50% of cases being idiopathic (Collins and Crosignani, 2007; NICE, 2007; Hapangama and Bulmer, 2015). The uterine vessels, especially the endometrial spiral arterioles, have been implicated in the pathogenesis of HMB by premature breakdown at the beginning of menstruation, failure to repair at the end of menstruation and increased blood flow rate through structural or functional impairment (Abberton et al., 1999a; Hurskainen et al., 1999). The importance of these vessels in the context of the normal menstrual cycle and in HMB has been further highlighted by studies that showed reduced vascular smooth muscle cell (VSMC) proliferation in spiral arterioles in the mid- and late secretory phases and in straight arterioles in the late secretory phase, and altered expression of VSMC differentiation markers in HMB (Abberton et al., 1999a,b; Biswas Shivhare et al., 2014).

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Table 1
Patient demographic information for the control and heavy menstrual bleeding (HMB) groups.

Demographic criteria		Control ($n=20$)	HMB ($n=20$)
Ethnicity	White/British	17[a]	17[b]
	European	2	1
Smoking history	Smoker	6	3[a]
	Non-smoker	14	16
Age (years)	Mean (range)	41.5 (32–50)	42.2 (30–51)
BMI	Mean (range)	30.1 (26–45.5)	28.7 (22.4–36)
Parity	Mean (range)	2.7 (1–4)	1.7 (0–4)

[a] Information available for 19/20 samples.
[b] Information available for 18/20 samples.

Menstruation, although triggered by falling levels of ovarian hormones, is considered to be an inflammatory process (Finn, 1986) maintained by proinflammatory cells such as neutrophils, macrophages and uterine natural killer (uNK) cells (Salamonsen and Woolley, 1999; Berbic and Fraser, 2013). Human endometrial immune cell populations have been widely studied (Morris et al., 1985; Kamat and Isaacson, 1987; Marshall and Jones, 1988; Bulmer et al., 1988; King et al., 1998a), with uNK cells, macrophages, mast cells and T cells being increased in the late secretory phase (Poropatich et al., 1987; Bulmer et al., 1991; Salamonsen and Woolley, 1999; Berbic and Fraser, 2013). In the menstrual phase there is increased activity of mast cells, macrophages and dendritic cells (DC), suggesting a role in tissue breakdown, clearance and regeneration (Schulke et al., 2008; Berbic et al., 2009, 2010). The endometrial immune response during the cyclic shedding, tissue remodelling and angiogenesis may help to maintain a healthy functioning endometrium. Dysregulation of leukocyte cell numbers may therefore be linked with abnormal uterine bleeding disorders, although these cell populations have not been investigated in endometrium of women with HMB.

We hypothesised that the endometrial immunological response reflected by the various endometrial immune cells might be altered in women with HMB and that this may contribute to abnormal vascular development. To test this hypothesis we compared endometrial leukocytes across the different phases of the menstrual cycle in healthy controls with those in women with HMB.

2. Materials and methods

2.1. Samples

Endometrial biopsies were obtained with informed consent from women undergoing hysterectomy at the Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne or at the Liverpool Women's Hospital, Liverpool. The experimental group were women with a history of HMB, defined as "excessive menstrual blood loss which interferes with the woman's physical, emotional, social and material quality of life, and which can occur alone or in combination with other symptoms" (NICE, 2007). The control group were fertile women undergoing hysterectomy for prolapse, cystocele, rectocele, or urinary or stress incontinence. For both HMB and control groups, women with pathology potentially associated with endometrial abnormality (e.g. endometriosis, adenomyosis, leiomyomata) were excluded, as were women who had received hormone treatment within three months of the operation. Menstrual cycle phase was determined using histological assessment according to standard morphological criteria (Noyes et al., 1975) by a specialist gynaecologist histopathologist (JNB); cycle day was not used to define the different menstrual cycle phases. Patient details are shown in Table 1. The study was approved by the Newcastle and North Tyneside 1 Research Ethics Committee (Ref: 10/H0906/71) and Liverpool Adult Ethics committee (Ref: 09/H1005/55). Endometrial biopsies from both control and menorrhagic women ($n=5$ for each of the proliferative, early secretory, mid-secretory and late secretory phases) were fixed in 10% neutral buffered formalin for 24–48 h, routinely processed into paraffin wax, and 3-μm serial sections cut for immunohistochemistry.

2.2. Immunohistochemistry

Sections were dewaxed in xylene, rehydrated in alcohols and incubated in 1% H_2O_2 in water for 10 min to block endogenous peroxidase activity. All washes were performed in 0.15-M Tris-buffered 0.05-M saline (TBS) pH 7.6. Details of source, dilution, pretreatment and incubation times for all antibodies are provided in Table 2. Unless otherwise stated, sections were immunostained using an avidin–biotin–peroxidase technique (mouse Vectastain Elite ABC kit; Vector Laboratories, Peterborough, UK), which has been described in detail previously (Schiessl et al., 2009). The reaction was developed for 1–2 min with 3,3′-diaminobenzidine (DAB; Sigma Chemical Co., Poole, Dorset, UK) containing 0.01% H_2O_2 to give a brown reaction product. Sections were lightly counterstained with Mayer's haematoxylin, dehydrated, cleared in xylene and mounted with DPX synthetic resin (Raymond A. Lamb Ltd., London, UK). Positive and negative (replacement of the primary antibody by appropriate non-immune serum) controls were performed for all antibodies and samples, respectively.

2.3. Double immunohistochemical labelling

Detection of $CD3^+$ cells in paraffin-embedded sections relied on an antibody that detects the epsilon (ϵ) chain of human CD3. However, $CD56^+$ uNK cells may also express cytoplasmic CD3ϵ (King et al., 1998b). Therefore, double immunohistochemical labelling was used to identify the proportion of $CD3^+$ cells that were $CD56^+$ $CD3^+$, representing only uNK cells rather than $CD3^+$ T cells. The first immunostain was visualised using a Vector Silver substrate kit for peroxidase (Vector Laboratories Inc., Burlingame, CA, USA) and the second with a Vector NovaRED substrate kit for peroxidase (Vector Laboratories Inc.). Appropriate positive and negative controls were performed for each antibody run, including single immunohistochemical labelling of serial sections with each antibody. Negative controls showed no immunostaining for any of the protocols used.

2.4. Quantitative image analysis

Sections were examined and images captured using a Nikon Eclipse 80i microscope and NIS Elements software (Nikon Instruments Inc., Kingston upon Thames, Surrey, UK). Leukocyte numbers were counted manually as the percentage of positive cells compared with the total number of stromal cells in five randomly selected fields of view (×200 magnification) within the stratum functionalis in each sample (Fig. 1).

The proportion of $CD3^+CD56^+$ double positive was assessed in double immunostained mid- and late secretory phase samples from both control and HMB groups ($n=3$ each group). Single and double positive cells were counted in five randomly selected fields (×400 magnification) in the stratum functionalis in each sample using image analysis software ImageJ (Version 1.46; NIH, Bethesda, MD, USA). The number of $CD3^+CD56^+$ double positive cells was quantified both as a proportion of the total number of $CD3^+$ cells and as a proportion of the total number of $CD56^+$ cells.