

Adrenomedullin and its receptor, calcitonin receptor-like receptor, are aberrantly expressed in women with idiopathic menorrhagia

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Abstract. The human endometrium undergoes a unique process of benign angiogenesis under the control of ovarian steroids during reproductive life. Aberrant angiogenesis has been implicated in idiopathic menorrhagia, a common gynaecological complaint. One of the key factors involved in endometrial angiogenesis is adrenomedullin (AM), a multifunctional 52-amino acid peptide. AM mediates the activities of endometrial angiogenesis via calcitonin receptor-like receptor (CLR). The objective of the present study was to compare the endometrial expression of AM and CRL in women with and without idiopathic menorrhagia. Endometrial biopsies were obtained from 9 women with menorrhagia (≥ 80 ml per menstruation) and 12 women with normal blood loss (< 80 ml per menstruation). Protein and mRNA expression levels of AM and CLR were determined using immunohistochemistry and real-time PCR. Compared to the controls, patients with menorrhagia exhibited low immunostaining intensity of AM, while high CLR staining was observed in the epithelium ($p < 0.05$). No difference in mRNA expression was observed between the groups. These data suggest that an imbalance in the AM/CLR system might alter endometrial angiogenesis in menorrhagia.

Introduction

The human endometrium undergoes a unique process of benign angiogenesis under the control of ovarian steroids during reproductive life. One of the key factors involved in endometrial angiogenesis is adrenomedullin (AM), a multifunctional

52-amino acid peptide originally isolated from a human pheochromocytoma (1,2). AM belongs to the calcitonin family, which comprises six known members: calcitonin, amylin, two calcitonin-gene related peptides (CGRP- α and CGRP- β), AM and intermedin (3,4). Originally identified as a hypotensive peptide, AM was subsequently shown to be an angiogenic factor (1,5). Since then, it has become apparent that it has a wide range of other biological actions, including the regulation of cell growth and differentiation.

AM mediates its activities via heterodimeric receptors composed of calcitonin receptor-like receptor (CLR) and one of the three receptor activity-modifying proteins (RAMPs) (3,6). RAMPs alter the affinity of the CLR receptor to the related peptides. Under normal conditions, the CLR/RAMP1 heterodimer shows greater affinity for CGRP. However, when associated with RAMP2 or RAMP3 it shows greater affinity for AM (3,6).

Menorrhagia is a common gynaecological condition – the main presenting complaint in women referred to gynecologists – and accounts for two-thirds of all hysterectomies. Between 1994 and 1999, one in every nine women aged 35-45 years had a hysterectomy in the US (7). Thus, menorrhagia is an important healthcare issue. In approximately 50% of cases, no pathology is found at hysterectomy. Aberrant angiogenesis has therefore been implicated (8,9).

The aim of this study was to examine AM and CLR expression as well as vascular density in the endometrium of women with menorrhagia compared to healthy controls.

Materials and methods

Study population. Endometrial biopsies were obtained from healthy volunteers ($n=12$, mean age 34 years) and from women with menorrhagia (menstrual blood loss ≥ 80 ml per menstruation, $n=9$, mean age 42 years). All the women had regular menstrual cycles of 25-32 days. None had used hormonal or intrauterine contraception for at least three months prior to the study.

Menstrual blood loss was assessed with pictorial blood loss assessment charts (PBAC) (10). Clinical examination, transvaginal ultrasound and hysteroscopy revealed no pathology. Platelet count, activated prothrombin thromboplastin

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Table I. Adrenomedullin staining intensity and number of stained vessels in the endometrium of healthy women (n=12) and of women with menorrhagia (n=9).

Group	Luminal epithelium	Glandular epithelium	Stoma	Vessels
Control	2.0 (0.0-3.0)	2.0 (1.0-2.5)	2.0 (1.0-2.5)	1.0 (0.5-1.5)
Menorrhagia	2.0 (0.5-2.5)	1.5 (1.0-2.5)	0.5 (0.0-1.0) ^a	1.0 (0.5-2.0)

Median value and range is shown; p<0.05 considered statistically different.

time, INR, bleeding time and von Willebrand factor were all normal.

Informed consent was obtained from all participants. The Ethics Committee of the Karolinska Institute approved the study.

Endometrial biopsies. Biopsies were obtained using a Pipelle sampler (Pipelle Laboratoire C.C.D., Paris, France), and were performed during the luteal phase on the anterior wall of the uterine cavity on day following the surge of luteinizing hormone (LH)+6 to LH+8. Participants identified the day of the LH surge by testing their morning urine. All biopsies were fixed in 4% formaldehyde for a maximum of 24 h, then stored in 70% ethanol until embedding. An additional three biopsies from each group were frozen in liquid nitrogen for RT-PCR. All biopsies used in the study had a normal histology compatible with the phase of the cycle.

Immunohistochemistry. Paraffin-embedded biopsies were sectioned, dewaxed in Bioclear (Bio-Optica, Milan, Italy) and rehydrated by decreasing ethanol concentrations and washing in phosphate-buffered saline (PBS). Endogenous peroxidases were blocked using 3% hydrogen peroxide in methanol for 10 min, and slides were then washed in PBS. Thereafter, the samples were blocked using 1.5% normal goat serum for AM and CLR and horse serum for CD31, then incubated for 30 min. Rabbit monoclonal IgG against human AM (Peninsula Laboratories Inc., San Carlos, CA, USA) and rabbit immune serum against CLR were used as the primary antibodies (11), respectively diluted to 1:500 and 1:1500. Human myometrium was used as the positive control for ADM. Normal rabbit serum was used as the negative control for CLR and AM. The slides were incubated at 4°C in a humidified chamber overnight.

The sections were subsequently washed in PBS with 0.01% Tween-20. Biotinylated goat anti-rabbit IgG was used as a secondary antibody. The slides were incubated for 45 min with the secondary antibody, followed by incubation for 45 min in a freshly-prepared solution of peroxidase avidin biotin complex (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA, USA). Thereafter, the slides were washed and stained using 3,3-diaminobenzidine in H₂O₂ (DAB-kit, Vector Laboratories for AM and AP kit for CLR), then counterstained with haematoxylin and dehydrated before mounting with Pertex (Histolab, Gothenburg, Sweden).

Evaluation of immunohistochemistry. Two independent viewers, blinded to the identity of the slides, assessed each

sample. Staining intensity was evaluated semi-quantitatively using the following grading system: 0, no staining; +, faint staining; ++, moderate staining; +++, strong staining.

To quantify the number of vessels in the endometrium, the vessels in six randomly selected fields of views were counted in each slide at a magnification of x400. The average number of vessels in each view was then calculated.

Data were expressed as the mean ± range and statistically evaluated using the Mann-Whitney rank sum test. A difference of p<0.05 was considered significant.

RNA and cDNA preparation. Frozen endometrial tissue was homogenized in lysis buffer then extracted using the Qiagen RNA mini kit (Invitrogen, Stockholm, Sweden) according to the manufacturer's instructions. The concentration and purity of the RNA were measured using Quantity-One (Bio-Rad, Clinical Diagnostic, Sundbyberg, Sweden).

Total RNA (600 µg) was reverse transcribed using Invitrogen's SuperScript according to the manufacturer's instructions. The samples were then stored at -20°C until PCR.

Quantitative-PCR. Real-time PCR was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA USA). cDNA (8 µg) combined with 5 µl SYBR Green mixture, 1 µl each of the forward and reverse primers and 2 µl of water was subjected to quantitative-PCR using a SYBR green assay. The expression of each gene was normalized against GAPDH using a standard curve constructed from the cDNA dilution. The cDNA was then heated to 50°C for 2 min and denatured at 95°C for 10 min. The template was amplified over 45 cycles of 60 sec melted at 95°C and 60 sec at 62°C for annealing and extension. Primer sequences were as follows: AM, sense 5'-AAG AAG TGG AAT AAG TGG GCT-3', antisense 5'-TGG CTT AGA AGA CAC CAG AGT-3'; CLR, sense 5'-CTC CTC TAC ATT ATC CAT GG-3', antisense 5'-CCT CCT CTG CAA TCT TTC C-3'; GAPDH, sense 5'-GAA GGT GAA GGT CGG AGT CAA C-3', antisense 5'-CAG AGT TAA AAG CAG CCC TGG-3'. Fluorescence data was acquired by measurement every 7 sec and presented as a plot of fluorescence intensity versus cycle number.

Data analysis for quantitative-PCR. All samples were run in duplicate. Data were imported and analysed using ABI PRISM 7000 Software version (Applied Biosystems, Foster City, CA, USA) and normalised against GAPDH.

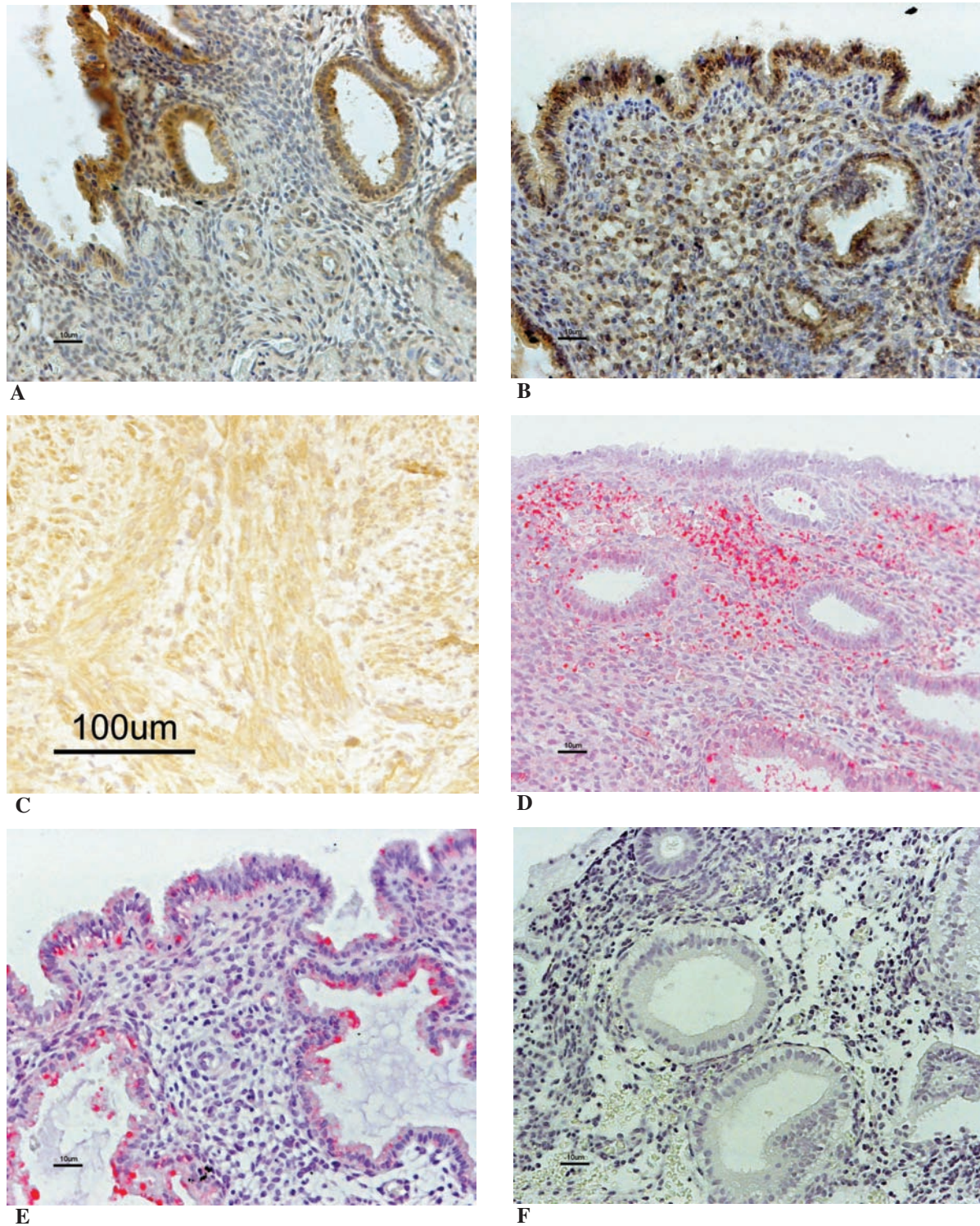


Figure 1. Distribution of adenomedullin (AM) and calcitonin receptor-like receptor (CLR) in the endometrium. (A) Immunostaining for AM in a control sample; (B) immunostaining for AM in a menorrhagia sample; (C) positive control for AM; (D) immunostaining for CLR in a control sample; (E) immunostaining for CLR in a menorrhagia sample; (F) negative control for AM and CLR.

Results

Immunohistochemical expression of AM in the endometrium. Immunostaining of AM was seen in all endometrial cell types (Fig. 1). There was a lower staining intensity in the stroma in tissue obtained from women with menorrhagia compared to the controls (Table I).

Immunohistochemical expression of calcitonin receptor-like receptor in the endometrium. Immunostaining of CLR was seen in all endometrial cell types (Fig. 1). Immunostaining in the endometrium of the menorrhagia group was more intense in the luminal epithelium ($p=0.002$) and glandular epithelium ($p=0.0059$) compared to that observed in the controls (Table II).

Table II. Calcitonin receptor-like receptor staining intensity and number of stained vessels in the endometrium of healthy fertile women (n=12) and of women with menorrhagia (n=9).

Group	Luminal epithelium	Glandular epithelium	Stroma	Vessels	No. of stained vessels
Control	0.5 (0.0-2.0)	1.0 (0.0-2.5)	0.5 (0.0-2.0)	1.6 (0.6-2.4)	7.5 (1.0-16.0)
Menorrhagia	1.5 (0.5-2.5) ^a	2.0 (1.5-3.0) ^a	1.0 (0.0-1.5)	1.9 (1.6-2.2)	7.5 (3.4-12.0)

Median value and range is shown; p<0.05 considered statistically different.

Table III. Relative expression of adrenomedullin and calcitonin receptor-like receptor mRNA in the endometrium of healthy fertile women (n=3) and of women with menorrhagia (n=3).

Group	AM	CLR
Control	1.04 (3.61-0.41)	1.07 (0.20-1.08)
Menorrhagia	3.34 (0.21-1.33)	0-66 (0.60-0.66)

Median value and range is shown.

Adrenomedullin and calcitonin receptor-like receptor mRNA expression in the endometrium. There were no differences in mRNA expression between the two groups (Table III).

Discussion

AM is a multifunctional peptide hormone that plays a significant role in vasodilatation and angiogenesis (12,13). The human endometrium is a mucosa served by a microvascular blood supply that involves benign angiogenesis, which is under the control of ovarian steroids throughout reproductive life (14,15). In agreement with previous studies, we found AM and CLR to be expressed in all endometrial cell types (2).

Aberrant angiogenesis has been implicated in menorrhagia (5,8). As has been previously reported, we found vessel density to be similar in the endometrium of patients with idiopathic menorrhagia compared to that of the controls (16). The expression of AM was weaker in the stroma of the endometrium of menorrhagia patients than in that of the controls, although staining in the vessels was similar. These results suggest that paracrine interaction between endothelial and stromal cells may be critical to normal endometrial endothelial function. While AM is a vasodilator, most models indicate that it also stabilises the endothelial barrier (17-20). Thus, decreased stromal expression of AM could result in increased vascular permeability, resulting in increased blood and fluid loss (21).

CLR expression was increased in the epithelial cells of the endometrium of women with menorrhagia. This increased expression may lead to heightened sensitivity to AM and to the production of prostaglandins, agents known to be involved in menstrual bleeding (22,23) and endometrial growth (24).

Immunostaining revealed some differences in protein expression compared to mRNA levels in tissue homogenates. This is most likely due to the fact that fewer samples were used for real-time PCR than for immunohistochemistry. Individual variation between samples could also contribute to this occurrence.

In conclusion, our data indicate that the AM/CLR system is critical to normal endometrial function, and that imbalances in this system might be involved in the pathogenesis of idiopathic menorrhagia.

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