Expression of adhesion, attachment and invasion markers in eutopic and ectopic endometrium: a link to the aetiology of endometriosis

J. Sundqvist1, K.L. Andersson1,2, G. Scarsellì2, K. Gemzell-Danielsson1,†, and P.G.L. Lalitkumar1,*,†

1Division of Obstetrics and Gynecology, Department of Women’s and Children’s Health, Karolinska Institutet/Karolinska University Hospital, SE-171 76 Stockholm, Sweden 2Department of Woman and Child Health, Section of Gynecology and Obstetrics, Careggi University Hospital, Viale Morgagni 85, 50134 Florence, Italy

*Correspondence address. E-mail: lalit.kumar@ki.se

Submitted on October 19, 2011; resubmitted on May 3, 2012; accepted on May 22, 2012

BACKGROUND: Cell properties, such as attachment, adhesion and invasion, are important for the normal function of the endometrium. However, it is believed that the same properties may also be involved in the development of gynaecological diseases, such as endometriosis. Endometrial cells, shed by retrograde menstruation, may have an aberrant expression of molecules involved in these functions, leading to endometriosis. Therefore, the aim of this study was to investigate the expression of proteins involved in adhesion, attachment and invasion in eutopic and ectopic endometrium.

METHODS: Endometrial biopsy specimens were collected from healthy volunteers (controls: proliferative phase, n = 10; secretory phase, n = 15) and from endometriosis patients (proliferative phase: n = 9, secretory phase: n = 10). Biopsy specimens from endometriomas were also collected (proliferative phase: n = 9, secretory phase: n = 10). Expression of apolipoprotein E (ApoE), integrin β-2 (ITGB2), integrin β-7 (ITGB7), Laminin γ-1 (LAMC1), CD24 molecule (CD24) and junctional adhesion molecule-1 (JAM-1) was evaluated with real-time reverse transcriptase polymerase chain reaction and immunohistochemistry.

RESULTS: The endometrium from controls and women with endometriosis expressed ApoE, ITGB2, ITGB7, LAMC1, CD24 and JAM-1. Gene expression of ApoE and JAM-1 was decreased in both proliferative and secretory phase in the endometrium from women with endometriosis compared with control endometrium. Also, mRNA expression of LAMC1 was reduced in the endometrium from endometriosis patients compared with controls in the proliferative phase. An altered gene expression of CD24 was seen between the endometrium from endometriosis patients and endometriomas in the secretory phase. The ITGB2 protein expression was altered in epithelia cells between the endometrium from healthy volunteers and endometriosis patients in the secretory phase.

CONCLUSIONS: We have shown differential expression of adhesion, attachment and invasion proteins in proliferative and secretory endometrium from controls and endometriosis patients and in endometriomas. This study suggests that molecules with these properties may have a role in the anchoring of endometrial cells at ectopic sites, thus initiating the development of endometriosis.

Key words: endometrium / endometriosis / cell adhesion / attachment / invasion

Introduction

The human endometrium is a highly dynamic tissue, continuously undergoing cycles of growth, differentiation, shedding and self-renewal of cells during the reproductive period of a woman. These specific properties make the endometrium a perfect organ capable of attaching and implanting an embryo. A number of molecules, phenotypically relevant to adhesion, attachment, invasion, proliferation and migration, are involved in the regeneration, growth and functions of the endometrium (Horne et al., 2002; Boomsma et al., 2009). With the

† Both authors contributed equally.
Materials and Methods

Sample collection

Samples from healthy volunteers or patients with endometriosis were collected both in Stockholm, Sweden and Florence, Italy. The women in the study were all non-smokers and had not used any hormonal contraceptives or an intrauterine device or any other medication for at least 3 months before recruitment into the study. The average age of the control group was 35.6 ± 5.8 years and had a cycle length between 28 and 32 days and the patient group was 37.6 ± 6.9 years. All patients in the study were confirmed for endometriosis through laparoscopy. Endometrial biopsy specimens from healthy volunteers were collected from both the proliferative (n = 10) and secretory (n = 15) phases of the menstrual cycle using a Randall curette (Silihe, Stockholm, Sweden). The endometrium from endometriosis patients was collected with a Pipelle curette (Cooper Surgical, Trumbull, USA) from both proliferative (n = 9) and secretory (n = 10) phases of the menstrual cycle. Samples from ovarian endometriomias (proliferative phase: n = 9; secretory phase: n = 10) were collected during surgery.

One part of each sample was taken for mRNA expression studies by real-time reverse transcriptase polymerase chain reaction (RT–PCR) and the other for immunohistochemical studies. The biopsy specimens were dated histopathologically to confirm the cycle phase of collected endometrial samples.

All women gave their oral and written informed consent prior to participating in the study. The study was approved by the Regional Ethics committees at the Karolinska Institutet, Stockholm, Sweden and the University of Florence, Italy.

RNA extraction

Tissues from endometrial samples and endometriomas were collected in RNAlater® (Ambion, USA) and kept overnight (O/N) at 4°C. RNAlater was then removed and samples were stored at −70°C until RNA extraction. Tissues were homogenized in a dismembranation apparatus (Retsch KG, Haan, Germany) and total RNA was extracted by TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA). Two micrograms of RNA were treated with RQI RNase-free DNase (Promega Biotech AB, Stockholm, Sweden) according to the manufacturer’s protocol. The DNase-treated RNA was reverse transcribed using the Superscript™ II RNase H-Reverse Transcriptase Kit (Invitrogen) with 10 mM dNTP, 250 ng pd(N)6 Random Hexamer primers (Amersham Biosciences, Buckinghamshire, UK), 40 U RNase inhibitor (Roche, Mannheim, Germany) and 200 U Superscript reverse transcriptase, according to manufacturers protocol. cDNA was stored at −70°C until use.

Real-time RT–PCR

DNA amplification was performed with real-time RT–PCR, using the Applied Biosystems 7300 Real-Time RT–PCR system (Applied Biosystems, Foster City, CA, USA). All samples were run in triplicate in Taqman Universal PCR Master Mix (Applied Biosystems) and in 96-well optical PCR plates. The housekeeping gene 18S was used as endogenous control in the same reaction. The following commercially available Taqman gene expression assays were used; ApoE: Hs00171168_m1, ITGB7: Hs00164957_m1, ITGB2: Hs01565750_m1, LAMC1: Hs00267056_m1, CD24: Hs02379687_m1, JAM-1: Hs00375889_m1 and 18S: 4319413E (Applied Biosystems). For each reaction 5 μl 1:10 diluted cDNA (20 ng total RNA), 12.5 μl Universal Master mix. 1.25 μl assay mix 18S, 1.25 μl assay mix and 5 μl sterile water was used. The real-time PCR reaction was performed according to standard manufacturer’s protocol, as
following: 50 °C x 2 min + 95 °C x 10 min, 40 (95 °C x 15 s + 60 °C x 1 min). The threshold cycles (CT), where an increase in reporter fluorescence above the baseline signal could first be detected, were determined. The mean CT value of 18S was used for normalization and was subtracted from the mean CT value of the respective gene, to obtain ΔCT values.

Immunohistochemistry
A small portion of each endometrial biopsy specimen was fixed in 4% formaldehyde and the endometrioma in 3% paraformaldehyde O/N at 4 °C, paraffin-embedded and cut into 5 μm sections for immunohistochemical analysis.

All immunohistochemical stainings were performed with the MACH 3™ Mouse-Probe HRP-polymer kit or MACH 3™ Rabbit-Probe HRP-polymer kit (Biocare Medical, CA, USA). Slides were preheated at 60 °C for 60 min before removal of paraffin in DNA decloaker (Biocare Medical). Slides were then washed in Tris-buffered saline (Biocare Medical) and endogenous peroxidase activity was eliminated by Peroxidase dazed (Biocare Medical). Non-specific bindings were blocked with Background Sniper (Biocare Medical). Slides were incubated with the primary antibody for 30 min for LAMC1 and CD24, and 1 h for ApoE, ITGB2, ITGB7 and JAM-1. Primary antibodies were diluted in Da Vinci Green Diluent (Biocare Medical) and used at following dilutions; mouse monoclonal anti-ApoE 1:250 (Ab1906; Abcam, Cambridge, UK), monoclonal mouse anti-ITGB2 1:2000 (LS-B1849; LifeSpan Biosciences, Seattle, WA, USA), rabbit polyclonal anti-ITGB7 1:200 (11328-1-AP; Proteintech Group, Inc., Chicago, IL, USA), mouse monoclonal anti-Laminin B2 gamma 1 (LAMC1) 1:4 (ab54174; Abcam), mouse monoclonal anti-CD24 (Ab-2, Clone SN3b) 1:100 (MS-1279-P1; NeoMarkers, Fremont, CA, USA) and rabbit monoclonal anti-JAM-1 1:500 (ab52647; Abcam). Slides were incubated with first MACH 3 mouse-probe or MACH 3 rabbit-probe (Biocare Medical) and then M-polymer HRP or R-polymer HRP (Biocare Medical), followed by staining with Betazoid DAB (Biocare Medical). They were counterstained with Mayer’s haematoxylin and dehydrated in increasing concentrations of ethanol and finally xylene and mounted with Pertex® (Histolab, Gothenburg, Sweden). Following negative isotype controls were used: Dako Universal Negative Control Mouse (N1698; Dako, Carpinteria, CA, USA) and ChromPure Rabbit IgG (011-000-003; Jackson ImmunoResearch, West Grove, PA, USA). Negative controls, omitting the primary antibody, were also performed. Stainings were observed in a Zeiss Axiosvert 200 M microscope (Zeiss, Göttingen, Germany) and images were captured with QCapture, version 3.1.1. (QImagin, Surrey, BC, Canada).

Scoring of protein expression was performed according to intensity of staining and percentage of positive cells. The intensity of staining was graded as 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. The percentage of stained cells was graded as following; 0 = no staining, 1 ≤ 10%, 2 = 11–50%, 3 = 51–80% and 4 ≥ 81%.

The final score was calculated by multiplying the two scores. Scoring was done blindly and by two independent observers.

Statistical analysis
Comparisons between the independent groups were done with the Kruskal–Wallis test, followed by multiple comparisons with Dunn’s correlation. A P-value of <0.05 was considered to be statistically significant. All calculations were performed using GraphPad Prism 5, version 5.01 (GraphPad Software, Inc., CA, USA).

Results
Expression of ApoE in the endometrium and endometrioma
mRNA expression of ApoE was seen in the endometrium from controls (HE) and endometriosis patients (EE), but also in endometriomas (EL) (Fig. 1A). The ApoE expression within the menstrual cycle was similar; however, a significant difference was seen between HE and EE in both proliferative and secretory phase (P < 0.001, <0.01).

ApoE protein expression in HE and EE was observed as cytoplasmic staining in stromal cells, in both proliferative (Fig. 2A and B) and secretory phase (Fig. 2D and E). Weak staining was also observed in isolated areas of stromal matrix in the endometrium. Vessels were positive for ApoE, as seen in EL (Fig. 2C). Also, ApoE was expressed by some cells, localized close to the epithelial cells of the capsule in EL, independent of cycle phase (Fig. 2C and F). Immunohistochemical scoring showed no significant difference in either stromal or epithelial cells (Fig. 3A) between HE and EE in both cycle phases.

Expression of ITGB2 in the endometrium and endometrioma
Gene expression of ITGB2 (Fig. 1B) was found in HE and EE in both cycle phases. EL also expressed ITGB2 (Fig. 1B). No significant difference was observed between the groups.

Some stromal cells showed membrane staining of ITGB2 in HE and EE in both proliferative and secretory phase, as shown in Fig. 2G, H, J and K. Almost no positive staining of ITGB2 was seen in the luminal and glandular epithelium. Expression of ITGB2 was found in a few stromal cells in the sub epithelial region of the EL, independent of menstrual cycle phase (Fig. 2I and L). No statistical difference was observed in ITGB2 protein expression in stroma during the menstrual cycle, however, the HE epithelial cells (Fig 3B) had increased expression of ITGB2 in the secretory phase (P < 0.01).

Expression of ITGB7 in the endometrium and endometrioma
ITGB7 mRNA expression was seen in HE, EE and EL (Fig. 1C). No significant differences were observed between cycle phases or groups.

In HE and EE, ITGB7 protein expression was observed in the cytoplasm in some of the stromal cells, but also in the luminal and glandular epithelium, showing a mainly apical staining pattern in both phases of the menstrual cycle (Fig. 4A, B, D and E). Vessels also showed positive staining for ITGB7 (data not shown). In EL, ITGB7 expression was found in some sub epithelial cells and in vessels (Fig. 4C and F).

Some old glands also expressed ITGB7 (data not shown). Both stromal and epithelial cells showed similar pattern of protein expression in HE and EE (Fig. 3C).

Expression of LAMC1 in the endometrium and endometrioma
mRNA expression of LAMC1 was observed in HE and EE from both cycle phases and in EL, as shown in Fig. 1D. The expression of LAMC1 was significantly different between HE and EE in the proliferative phase of the menstrual cycle.
Staining with the antibody against LAMC1 in the endometrium gave a cytoplasmic expression pattern in the luminal and glandular epithelium, with a stronger staining in the apical part of the epithelium, in both HE (Fig. 4G and J) and EE (Fig. 4H and K). Stromal cells also showed weak staining in the cytoplasm and vessels were positive for LAMC1 (Fig. 4J). EL showed staining of LAMC1 in sub epithelial cells (Fig. 4I and L), independent of cycle phase, and a weak staining in old glands (data not shown). Scoring of protein expression in stromal and epithelial cells gave no statistical difference during the menstrual cycle in the groups (Fig. 3D).

Expression of CD24 in the endometrium and endometrioma

Gene expression of CD24 was seen in HE and EE in both phases of the menstrual cycle, and in EL (Fig. 1E). No cyclical pattern was observed in the endometrium; however, a statistically significant difference was seen between EE and EL in the secretory phase ($P < 0.001$).

Immunostaining of CD24 in the endometrium, both from HE and EE was observed in the membranes of luminal and glandular epithelium, with a stronger staining in the apical borders of the epithelium.
Stromal cells were mostly negative for CD24. In EL, expression of CD24 was found at the apical borders of the epithelium in both cycle phases (Fig. 5C and F). Scoring of protein expression was similar in stromal cells, but in epithelial cells a statistical difference ($P < 0.001$) was found between proliferative and secretory phase in HE (Fig. 3E).

Expression of JAM-1 in the endometrium and endometrioma

Expression of JAM-1 mRNA was found in HE, EE and EL (Fig. 1F). Statistical difference was observed between HE and EE in both cycle phases ($P < 0.05$).

JAM-1 showed membrane expression in the luminal and glandular epithelium in HE and EE (Fig. 5G, H, J and K). Vessels were also positive for JAM-1 (Fig. 5J). Expression was found in the epithelium of EL in the proliferative phase (Fig. 5I). No staining was observed in the interior part of the EL (Fig. 5L). Some vessels were also positive for JAM-1 (data not shown). Furthermore, no differences were seen in protein expression in stromal or epithelial (Fig. 5F) cells between HE and EE.

Discussion

The role of the analysed proteins in the pathogenesis of endometriosis is not clear from the literature; however, it could be hypothesized that they play a role in the adhesion, attachment and invasion of endometrial cells. ApoE may have an important role in the initiation of endometriosis, as well as in the progression of the disease: it has been shown that ApoE contributes to the formation of foam cells and
increased ApoE production creates a self-propagating loop, exacerbating lesion progression (Neyen et al., 2009). It has been reported that women with endometriosis have an unfavourable lipid profile with increased levels of low-density lipoproteins and ApoE plays an important role in lipoprotein metabolism and cellular lipid transport by interacting with specific cell surface receptors. In addition, ApoE has been identified as a marker of cell survival and proliferation (Chen et al., 2005), which are important events during the establishment of endometriosis.

We also observed significant changes of the protein expression in epithelial cells for ITGB2 in the secretory phase between the endometrium from healthy controls and endometriosis patients. The attachment of the menstrual endometrium to the peritoneum may be mediated through integrins present in the menstrual endometrium (van der Linden et al., 1994; Koks et al., 2000). It is also interesting to note that integrin-mediated endometrial adhesion is enhanced by TNF-α (Sillem et al., 1999), an important cytokine involved in endometriosis (Halis and Arici, 2004). In addition, ITGB7 mediates migration of lymphocytes.
towards the inflammation site, thus contributing to an increase in inflammatory cytokines, such as interleukins and TNF-α (Wagner et al., 1998). This could be one of the mechanisms involved in the increase in inflammatory molecules seen in the peritoneal fluid in endometriosis (Halis and Arici, 2004). Furthermore, it is known that in some specific cell types, steroid hormones influence the intracellular signals leading to cellular attachment, motility and differentiation by integrins (Juliano and Haskill, 1993; Giancotti and Ruoslahti, 1999). There are reports that peritoneal fluid consists of various estrogen derivatives and this may mediate integrin signalling, leading to extracellular cell functions (Xu et al., 2008). Since endometriosis is an estrogen-dependent disease, this correlation would be interesting to investigate further.

Moreover, we found altered gene expression of LAMC1 in the endometrium from endometriosis patients compared with the healthy endometrium in the proliferative phase. Laminin promotes adhesion and migration of monocytes (Pedraza et al., 2000), which may contribute to the altered functions of the immune system in endometriosis patients. This may also explain the role of LAMC1 in endometriosis as it may promote the adhesion of the menstrual endometrium to the peritoneum during proliferative phase. Inagaki et al. (2003) have reported the association of anti-Laminin antibodies with infertility, especially when caused by endometriosis. The same group has also shown that there is an expression of Laminin mRNA in endometriotic lesions. Expression of Laminin has been found in the endometrium and endometriosis in glands and stroma (Beliard et al., 1997; Harrington et al., 1999), however, the type of Laminin investigated was unclear. Our result support previous results of LAMC1 expression in the endometrium and endometrioma. Protein expression of CD24 and JAM-1

---

**Figure 4** Immunolocalization of ITGB7 (A–F) and LAMC1 (G–L). During proliferative phase ITGB7 was present in HE (A), EE (B) and EL (C). Also, protein expression of ITGB7 was seen in HE (D), EE (E) and EL (F) during secretory phase. Staining of LAMC1 was shown in proliferative HE (G), EE (H) and EL (I) and in secretory HE (J), EE (K) and EL (L). HE, endometrium from healthy women; EE, endometrium from endometriosis patients; EL, endometrioma. Scale bar, 10 µm.
in the endometrium has also been seen in previous studies. Our findings support earlier reports of membrane as well as cytoplasmic expression of CD24 in endometrial glands (Kim et al., 2009). Also, our results regarding JAM-1, which is localized at tight junctions at intracellular borders (Aurrand-Lions et al., 2001; Ebnet et al., 2004), corroborates with the finding of Koshiba et al. (2009), where they showed that the expression of JAM-1 is cytoplasmic as well as membrane bound in endometrial glandular epithelium, independently of the stage of the menstrual cycle (Koshiba et al., 2009). Aberrant expression of JAM-1 and CD24 may lead to changed adhesive properties of endometrial cells, allowing them to adhere to ectopic places. In addition, CD24 expression was reported to be significantly enhanced in the hyperplastic endometrium with an inverse correlation to the expression of estrogen and progesterone receptor (Kim et al., 2009), suggesting a hormonal regulation of the protein. This is in line with our observations of a cyclic regulation of CD24 in the control endometrium. It is interesting to note that the level of CD24 in endometriosis patients is not changed during different phases of the menstrual cycle when compared with the control. This phenomenon may contribute to the derailment of endometrial receptivity observed among endometriosis patients (Brosens et al., 2012). CD24 showed a cyclic regulation in a healthy endometrium and its expression in endometriosis patients indicating a trend towards a cyclical pattern, however, this was not statistically significant. The differences between gene and protein expression could be explained by post-transcriptional modifications between mRNA, mature protein and protein degradation, a phenomenon which has been discussed previously (Greenbaum et al., 2003). Another reason for this observation could be that the mRNA analysis was performed on the whole endometrium, while protein expression was analysed on either stromal or epithelial cells alone from a couple of sections by immunohistochemistry. Tissue distribution of molecules may vary and biopsy specimens from individuals may have different ratios of stromal/epithelial cells.

Recent epidemiological studies show that women with endometriosis have an increased risk of ovarian cancer (Melin et al., 2006). Some of the genes investigated in this study are also associated with malignancy. It is possible that abnormal expression of adhesion molecules, such as cadherins and integrins, could have a central role in the invasion and spread of endometriotic cells (Starzinski-Powitz et al., 1999). Studies have shown the differential expression of ApoE, CD24 and JAM-1 in both ovarian and endometrial carcinomas Chen et al., 2005; Kim et al., 2009; Koshiba et al., 2009). Furthermore, C-16, a peptide derived from LAMC1, had been shown to have metastasis-promoting activities, such as enhancement of migration and metastasis formation of melanoma (Kuratomi et al., 2002).

Thus, it would be interesting to further investigate the role of the
molecules studied here in the development of gynaecological cancers.

This study is limited to investigate the expression of cell adhesion, attachment and invasion markers, as the aim of the study was to see the presence of relevant molecular markers in the healthy endometrium, and compare it with that in women with endometriosis. It has been shown earlier that the interaction between endometrial tissue, present in the menstrual flow and the peritoneum, has an important role in the aetiology of endometriosis (Witz et al., 1999). Even though we do not have a clear picture of the peritoneal fluid estrogen level and its influence on the molecules studied, there are reports of a large number of estrogen metabolites which are present in the peritoneal cavity of women (Xu et al., 2008). Estrogen receptors are present in the endometrioma and endometriotic lesions (Nisolle et al., 1994). The effect of estrogen on these molecular factors and their properties of adhesion, attachment and invasion need to be investigated in further studies. In this study, out of six molecules studied, having involved in attachment, adhesion or invasion namely, ApoE, ITGB2, ITGB7, LAMC1, CD24 and JAM-1, only the expression of LAMC1 was altered in the eutopic endometrium of endometriosis patients compared with healthy women. Thus, it also stresses the importance to explore the theory of invagination or metaplasia.

In summary, this study reports for the first time the expression of ApoE, ITGB2, ITGB7, LAMC1, CD24 and JAM-1 in endometriomas. Furthermore, to our knowledge, the expression of ApoE, ITGB2, ITGB7 and LAMC1 has not previously been reported in the endometrium. We found significant differences in the mRNA expression of ApoE and JAM-1 between the healthy endometrium and endometriosis patients in both the proliferative and secretory phases. The mRNA expression of LAMC1 was significant different in the healthy proliferative endometrium compared with proliferative endometrium from endometriosis patients. Also, the mRNA expression was altered between the endometrium from endometriosis patients and endometriomas in secretory phase. Furthermore, we found a cyclic regulation of CD24 protein expression in epithelial cells in the healthy endometrium and a difference in protein expression of ITGB2 in epithelial cells between the endometrium from healthy controls and endometriosis patients in the secretory phase. This study provides new information about molecules involved in adhesion, attachment and invasion in the healthy endometrium, and in the endometrium from endometriosis patients and endometriomas. Thus the results support the possibility that these factors could be involved in the aetiology of endometriosis.

Acknowledgements

We are grateful to Lena Elfors-Söderlund and Eva Broberg at the World Health Organisation (WHO) collaboration centre in Human Reproduction at the Karolinska University Hospital, Stockholm, Sweden, Cecilia Berger and Rama Svensson at Södersjukhuset, Stockholm, Sweden and Helena Koppen-Kalner at Danderyds Hospital, Stockholm, Sweden for their assistance in contacting volunteers and endometriosis patients before biopsy specimen collection. We would also like to thank Eva Andersson, Birgitta Byström and Mohamed Pourian at the FRH-lab, Karolinska Institutet, Stockholm, Sweden for their assistance in scoring immunohistochemistry.

Authors’ roles

J.S. participated in study design, execution, analysis, manuscript writing and critical discussion. K.L.A collected samples and participated in mRNA purifications. G.S was involved in study design. K.D.G. participated in study design, sample collection, manuscript writing and critical discussion. P.G.L.L participated in study design, analysis, manuscript writing and critical discussion.

Funding

This study was supported by grants from the Swedish Medical Research Council (K2010:54X-14212:09-3), Stockholm, Sweden and the RGD network, Karolinska Institutet, Stockholm, Sweden.

Conflict of interest

None declared.

References


