The macrophage stimulating protein/RON system: a potential novel target for prevention and treatment of endometriosis

S.Matsuzaki1,2,4, M.Canis1, J.L.Pouly1, P.Dechelotte3, K.Okamura2 and G.Mage1

1Department of Gynecology, Polyclinique de l’Hôtel-Dieu, CHU, Clermont-Ferrand, France, 2Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Sendai, Japan and 3Department of Pathology, Hôtel-Dieu, CHU, Clermont-Ferrand, France

4To whom correspondence should be addressed at: Department of Gynecology, Polyclinique de l’Hôtel-Dieu, CHU Clermont-Ferrand, Bd. Léon Malfreyt, 63058, Clermont-Ferrand, Cedex 1, France. E-mail: sachikoma@aol.com

Our recent DNA microarray analysis using tissue obtained by laser capture microdissection (LCM) identified up-regulation of RON (a tyrosine kinase receptor) during the late secretory phase in eutopic endometrial epithelial cells from patients with deep endometriosis compared with control endometrium from women with macroscopically normal pelvic cavities. In the present study, we further investigated mRNA expression of RON and its ligand, macrophage stimulating protein (MSP), in deep endometriotic lesions, eutopic endometrium from patients with deep endometriosis and control endometrium by using LCM and quantitative real-time RT–PCR. MSP mRNA expression in endometrial epithelial cells was significantly up-regulated in endometriosis patients during the late secretory phase compared with expression in controls. Furthermore, we detected up-regulation of MSP mRNA in eutopic endometrial epithelial cells compared with matched eutopic endometrial epithelial cells within the same patients regardless of the menstrual phase. MSP has an intrinsically dual functional nature through its receptor RON—it is a trophic cytokine preventing apoptosis and a scatter factor promoting invasion, both of which may be necessary for the initial development and growth of endometriosis. The present findings suggest that the MSP/RON system may be involved in the pathophysiology of endometriosis.

Key words: endometriosis/endometrium/laser capture microdissection/macrophage stimulating protein/RON

Introduction

Our recent DNA microarray analysis using samples obtained by laser capture microdissection (LCM) identified up-regulation of four genes—RON, 14-3-3 protein eta, SOS and uPAR—during the late secretory phase in eutopic endometrial epithelial cells from patients with deep endometriosis compared with control endometrium from fertile women with macroscopically normal pelvic cavities (unpublished data). Endometriosis is a heterogeneous disease based on location and clinical outcome. The different theories of histogenesis proposed for the different forms of endometriosis are based on location (Nisolle and Donnez, 1997). Relation of basic science with a well-defined clinical population is the key to successful translational research that will lead to the development of new diagnostic and targeted therapeutic approaches (Murphy, 2002). Thus, we focused on patients with deep endometriosis, which is one of the severe forms of endometriosis.

RON is a receptor tyrosine kinase of the MET family and it is mainly expressed in cells of epithelial origin (Comoglio et al., 1999; Schlessinger, 2000; Danilkovitch-Miagkova, 2003). *In vitro* RON activation results in epithelial cell dissociation, migration and matrix invasion (Comoglio et al., 1999; Danilkovitch-Miagkova, 2003; Wang et al., 2003). Although *in vitro* studies have clearly demonstrated that both epithelial and stromal cells attach to and invade intact peritoneum (Witz et al., 2002, 2003), the specific factors involved in these processes are unknown. We hypothesized that the RON may be one of the factors involved in these processes.

In the present study, we further investigated mRNA expression of RON and its ligand, macrophage stimulating protein (MSP), in tissue samples from deep endometriotic lesions, eutopic endometrium from patients with deep endometriosis and control endometrium from fertile women with macroscopically normal pelvic cavities by using LCM and quantitative real-time RT–PCR techniques.

Materials and methods

Patients

Patients undergoing laparoscopy and/or laparotomy for deep endometriosis were recruited for this study that began in May, 2001 in the Polyclinique de l’Hôtel Dieu, CHU Clermont-Ferrand, Clermont-Ferrand, France. As control samples, endometrial tissues were obtained from fertile women with macroscopically normal pelvic cavities who underwent laparoscopic tubal ligation or reversal of tubal sterilization. None of the patients received hormonal treatments such as gonadotrophin-releasing hormone agonist or sex steroids, and none used intrauterine contraception for at least 6 months prior to surgery. Any patients with anti-inflammatory/antibiotics treatment were excluded in the present study. Recruited patients had regular menstrual cycles (between 26 and 32 days) with confirmed menstrual history, and serum 17β estradiol and progesterone levels were measured. The endometrial
dating criteria, as described by Noyes et al. (1950), and menstrual history were utilized to assess menstrual cycle phase. Endometriotic biopsies were classified into four groups: late proliferative (LP) (days 11 ± 14), early secretory (ES) (days 15 ± 18), mid-secretory (MS) (days 19 ± 23), and late secretory (LS) (24 ± 28). Endometrial samples from 26 patients with deep endometriosis (LP, n = 7; ES, n = 6; MS, n = 6; LS, n = 7) and 23 control women (LP, n = 6; ES, n = 5; MS, n = 6; LS, n = 6) were used for real-time RT–PCR analysis. Among 26 patients with deep endometriosis, 14 paired samples (LP, n = 6; ES, n = 3; MS, n = 2; LS, n = 3) of tissue representing deep endometriosis and eutopic endometrium were also analysed. Clinical characteristics are summarized in Table I. Endometrial tissue biopsies were performed just before surgery by using an endometrial suction catheter (Pipelle, Laboratoire CCD, Paris, France). Samples of endometriosis and endometrium were divided into two portions. The first tissue portion was fixed in 10% formalin–acetic acid and embedded in paraffin for histological examination. The second portion was immediately collected in RNA-later (Ambion, Cambridgeshire, UK) and stored at −20°C until further analysis was performed. All tissue samples were obtained with full and informed patient consent. The research protocol was approved by the Consultative Committee for Protection of Persons in Biomedical Research (CCPPRB) of the Auvergne region.

**LCM**

From each fresh frozen tissue sample, 8 μm thick frozen sections were prepared for endometriotic tissues and 10 μm thick frozen sections were prepared for the matched eutopic endometrium. During our preliminary studies we found that it was more difficult to microdissect endometriotic tissues than endometrial tissues. Therefore, 8 μm thick frozen sections were prepared for endometriotic tissues. Sections were mounted on positively charged slides (Super frost Plus, Menzel GmbH, Braunschweig, Germany). Haematoxylin and eosin (H&E) staining on frozen sections was performed by using the National Cancer Institute (NCI) protocol (http://cgap.msf.nih.gov/Protocols/index.html) with some minor modifications as previously described (Matsuzaki et al., 2004).

Briefly, slides were fixed in 70% ethanol for 15 s and stained with H&E, followed by dehydration in two 15 s washes in 95% ethanol, two 60 s washes in 100% ethanol and two final washes in xylene for 3 min each. Slides were air-dried for 5 min and stored in a desiccator for no more than 1 h. Glandular epithelial cells and stromal cells from endometrial or endometriotic tissues were isolated from the slides by using the PdxCell II LCM System (Arcturus, Plaisir, France) according to the manufacturer’s instructions. A 7.5 and 15 μm beam diameter was utilized for epithelial cells and for stromal cells, respectively. Microdissected cells were collected on optically transparent LCM Macro caps for endometrium and LCM HS caps for endometriosis (Arcturus).

**RNA extraction and quantification**

After LCM, RNA extraction was performed by using the Picopure RNA extraction kit (Arcturus). The caps were placed in microcentrifuge tubes (Eppendorff, le Pecq, France) containing lysis buffer and incubated at 42°C for 30 min. After centrifugation, the caps were removed and RNA was isolated by using the Picopure RNA extraction protocol. To eliminate potential genomic DNA contamination, RNA samples were treated with DNaseI (15U; DNaseI, Courtaboef, Qiagen, France) at room temperature for 15 min. Finally, total RNA was resuspended in 11 μl RNAse-free water and was kept at −80°C until needed. RNA quantities were measured with the Ribo-Green RNA Quantitation Kit (Molecular Probes Europe BV, Leiden, The Netherlands). All procedures were performed according to the manufacturer’s instructions.

**Quantitative real-time RT–PCR with a Light cycler**

Quantitative real-time RT–PCR with a Light cycler was performed on non-amplified total RNA from microdissected tissues. Total RNA (10 ng) was subjected to an RT reaction by using Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed in a Light Cycler System by using the Fast Start DNA master SYBR green I kit as recommended by the manufacturer (Roche, Mannheim, Germany). In a total volume of 20 μl, each reaction contained 2 μl SYBR green I reaction mix (consisting of Taq DNA polymerase reaction buffer, dNTP mix, SYBR green I, MgCl2 and Taq DNA polymerase), 0.3–0.5 μM of each primer, 3–4 mM MgCl2 and 2 μl cDNA, standard or nuclease free water as a negative control. Primer sets are shown in Table II. Quantification of the targets in the unknown samples was performed by using a relative quantification method with external standards. The target concentration is presented relative to the concentration of a reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After each run, melting curve analysis was performed to verify the specificity of the PCR reaction.

**Statistical analysis**

The Statview 4.5 program (Abacus concepts, Inc., Berkeley, CA, USA) was used for statistical analysis. The Mann–Whitney U-test or Kruskal–Wallis test was applied to compare the results from different groups. The Wilcoxon sign rank test was performed to compare the differences in paired eutopic and ectopic endometrial samples. Statistical significance was defined as a P value of less than 0.05.

**Results**

**Eutopic endometrium from patients with endometriosis versus control endometrium**

Results are shown in Figure 1. During the late secretory phase, MSP mRNA expression was significantly higher in endometrial glandular epithelial cells from patients with deep endometriosis than in those from control endometrium (P < 0.02). There was no significant

<table>
<thead>
<tr>
<th>Table II. Clinical characteristics of patients</th>
<th>Patients with endometriosis</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eutopic endometrium (n = 26)</td>
<td>34.7 ± 3.1</td>
<td>32.8 ± 4.1</td>
</tr>
<tr>
<td>Endometriosis (n = 14)</td>
<td>37.9 ± 1.7</td>
<td>37.9 ± 1.7</td>
</tr>
<tr>
<td>Agea</td>
<td>34.7 ± 3.1</td>
<td>32.8 ± 4.1</td>
</tr>
<tr>
<td>Parityb</td>
<td>0 (0–3)</td>
<td>0 (0–3)</td>
</tr>
<tr>
<td>rAFSc</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Stage I</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Stage II</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

a Mean ± SD.

b Median (range).

c Revised American Society for Reproductive Medicine classification of endometriosis (American Society for Reproductive Medicine, 1997).
Figure 1. Results of quantitative real-time RT–PCR in endometrium from patients with endometriosis and endometrium from controls. Expression levels of MSP and RON genes are given relative to the expression levels of the reference gene, GAPDH. Results are presented as the mean ± SD. Bars indicate SD. Gray bars indicate patients with endometriosis. White bars indicate controls. LP = late proliferative phase (eutopic endometrium, \(n = 7\); controls, \(n = 6\)); ES = early secretory phase (eutopic endometrium, \(n = 6\); controls, \(n = 5\)); MS = mid-secretory phase (eutopic endometrium, \(n = 6\); controls, \(n = 6\)); LS = late secretory phase (eutopic endometrium, \(n = 7\); controls, \(n = 6\)). (A) \(P < 0.02\) versus controls during the late secretory phase.

Figure 2. Results of quantitative real-time RT–PCR in endometriosis and matched eutopic endometrium from patients with deep endometriosis. The values indicate the fold change of each gene in endometriosis relative to the matched eutopic endometrium within same patients. The horizontal lines represent median value. The menstrual cycle phase classification of endometriosis is based on the phase of matched eutopic endometrium. \(P = \) proliferative phase (\(n = 6\)); \(S = \) secretory phase (\(n = 8\)). (A) \(P < 0.03\) versus eutopic endometrium during the proliferative phase. (B) \(P < 0.02\) versus eutopic endometrium during the secretory phase.
difference in RON and MSP mRNA expression in stromal cells between patients with and without endometriosis at different points of the menstrual cycle. There was no significant cyclical difference in MSP and RON expression levels both in epithelial (RON, unpublished data) and stromal cells from the two different populations.

**Ectopic versus matched eutopic endometrium**

Results are shown in Figure 2. Expression of MSP mRNA in glandular epithelial cells of deep endometriotic tissue was significantly higher than that in matched eutopic endometrium during the proliferative ($P < 0.03$) and secretory phases ($P < 0.02$), whereas no significant difference was detected in stromal cells. There was no significant difference in RON mRNA expression in glandular epithelial and stromal cells between deep endometriotic tissue and matched eutopic endometrium during the proliferative and secretory phases. There was no significant cyclical difference in RON and MSP mRNA expression in epithelial and stromal cells within deep endometriotic tissue.

**Discussion**

The present study, together with our previous study (unpublished data), demonstrates that both RON and its ligand MSP are up-regulated in endometrial epithelial cells from patients with endometriosis compared with those of controls during the premenstrual phase. MSP has an intrinsically dual functional nature through its receptor RON—it is a trophic cytokine preventing apoptosis and a scatter factor promoting invasion (Iwama et al., 1996; Medico et al., 1996; Tamagone and Comoglio, 1997; Trusolino et al., 1998; Comoglio et al., 1999; Danilkovitch et al., 2000; Danilkovitch-Miagkova, 2003), both of which may be necessary for initial development and growth of endometriosis. The studies establishing MSP function clearly demonstrated that MSP prevents apoptosis of epithelial cells and that it could be considered a survival factor for epithelial cells (Iwama et al., 1996; Tamagone and Comoglio, 1997; Trusolino et al., 1998; Danilkovitch et al., 2000). A recent study demonstrated that apoptosis was decreased in glandular epithelial cells from patients with endometriosis compared with cells from women without endometriosis during the late secretory phase (Dmonowski et al., 2001). Endometrial epithelial cells undergo MSP autocrine stimulation, and up-regulation of MSP in glandular epithelial cells from patients with endometriosis during the premenstrual phase may prevent apoptosis through RON during retrograde menstruation. Thus, the MSP/RON system may facilitate survival of the endometrial epithelial cells in the pelvic cavity via autocrine pathways.

Furthermore, RON can be activated both in an MSP-dependent and MSP-independent manner (Danilkovitch-Miagkova et al., 2000). Adhesion of RON-expressing epithelial cells to extracellular matrix (ECM) via integrins causes ligand-independent phosphorylation and activation of RON (Danilkovitch-Miagkova et al., 2000). In the presence of nanomolar concentrations of MSP, RON induces cell ‘scattering’ and ECM invasion (Medico et al., 1996). Addition of MSP to collagen-adherent cells causes higher levels of RON phosphorylation and kinase activity than either MSP or collagen alone (Danilkovitch-Miagkova et al., 2000). We hypothesized that surviving RON-expressing endometrial epithelial cells in the pelvic cavity may initially invade peritoneal ECM in an MSP-dependent manner. After adhesion to ECM, MSP-dependent and/or MSP-independent phosphorylation and activation of RON may occur, leading to further invasive growth of endometrial epithelial cells into peritoneum. An *in vitro* study demonstrated that the initial adhesion of endometrial cells to mesothelium is not mediated by beta 1 integrins, whereas beta 1 integrins may be involved in transmesothelial invasion of endometrial cells (Witz et al., 2002a). These findings may partly support our hypothesis. *In vitro* studies demonstrated that proliferative, secretory and menstrual-phase endometrium both from patients with and without endometriosis can equally adhere to intact peritoneal mesothelium (Debrock et al., 2002; Witz et al., 2002b). Although these *in vitro* models are lacking in quantitative evaluations, one of the reasons why endometrium has a similar potential to implant into peritoneum may be that MSP and RON mRNA expressed both in eutopic endometrium from patients with and without endometriosis (regardless of menstrual phases) results in the invasive phenotype of endometrial cells throughout the menstrual cycle. However, in the present study, we did not include endometrial tissues during menstrual, early and mid-proliferative phases. Further studies, especially on menstrual-phase endometrium, should be necessary.

The present study also detected a significant up-regulation of MSP in ectopic endometrial epithelial cells compared with matched eutopic endometrial epithelial cells within the same patients without regard to menstrual cycle phase. Although endometriosis is a benign disease, clinical observations and *in vitro* experiments imply that the endometriotic cells are invasive and able to metastasize (Gaetje et al., 1995; Zeitvogel et al., 2001). Apoptosis levels of ectopic endometrium are significantly lower than those of matched eutopic endometrium (Gebel et al., 1998). After development of endometriotic lesions, both anti-apoptotic/survival and invasive/growth activities of MSP in ectopic endometrial epithelial cells may be required to sustain endometriotic lesions.

Although further studies to clarify molecular regulatory mechanisms for MSP/RON and the functional roles of MSP and RON genes both in eutopic and ectopic endometrium from patients with endometriosis are necessary, the present study suggests that the MSP/RON system may be involved in the pathophysiology of endometriosis. However, because we selected only patients with deep endometriosis in the present study, it is necessary to investigate if the present findings are constant for any type of endometriotic lesion (Nisolle and Donnez, 1997) or are specific to deep endometriosis.

**Acknowledgements**

We are grateful to all the staff at the Polyclinique, l’HÔtel Dieu, CHU Clermont-Ferrand, particularly to all the residents and staff in the operating room. We are indebted to all the staff in the Department of Pathology, l’HÔtel Dieu, CHU Clermont-Ferrand. This study was supported in part by grants PHRC 2002 and PHRC 2003 of CHU Clermont-Ferrand.

**References**


Iwama A, Yamaguchi N and Suda T (1996) STK/RON receptor tyrosine kinase mediates both apoptotic and growth signals via the multifunctional docking site conserved among the HGF receptor family. EMBO J 15,5866–5875.


Submitted on September 27, 2004; accepted on November 9, 2004