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Increased endometrial expression of CC-chemokine receptor-1 in women with adenomyosis

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Summary. Abnormal endometrial expression of CC-chemokine receptor-1 (CCR1) may play a role in the pathogenesis of endometriosis. Adenomyosis, also called endometriosis interna, occurs when the endometrium invades the myometrium. The objective of this study was to determine CCR1 expression in endometrium in women with adenomyosis as compared to women without adenomyosis. We evaluated endometrial mRNA and protein expression in women with and without adenomyosis using quantitative polymerase chain reaction (PCR), immunohistochemical staining and western blot analysis, respectively. We detected CCR1-immunoreactive expression in endometrium in all women with and without adenomyosis. CCR1-immunoreactive staining in endometrial cells was significantly higher in women with adenomyosis (4.89±1.06) compared to those without adenomyosis (2.21±1.16, P<0.001). Women with adenomyosis had higher levels of CCR1 mRNA in endometrium compared to women without adenomyosis (P<0.05). CCR1 protein levels in endometrium were significantly higher in women with adenomyosis (1.66±0.79) compared to women without adenomyosis (0.56±0.13, P<0.001), and positively correlated with the severity of dysmenorrhea (r=0.87, P<0.001). These results suggest that increased CC-chemokine receptor expression may play a role in the pathogenesis of adenomyosis.

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Introduction

Adenomyosis is a disease involving myometrial invasion by endometrial glands and stroma presenting as localized or diffuse lesions in the myometrium (Zhang et al., 2010). Women with adenomyosis may present with dysmenorrhea, menorrhagia, chronic pelvic pain, and subfertility, though dysmenorrhea is often the key clinical symptom (Zhang et al., 2010). Although the causes of adenomyosis are poorly understood, recent studies have shown that adenomyosis is a chronic inflammatory disease, and that eutopic endometrium in women with adenomyosis is different from normal endometrium (Sotnikova et al., 2002; Yang et al., 2009; Xiao et al., 2010; Benagiano and Brosens, 2012). Increased expression of interleukin (IL)-18, 8, 6 and monocyte chemotactic protein (MCP)-1 in eutopic endometrium is a common feature in women with adenomyosis (Ulukus et al., 2005; Yang et al., 2006; Huang et al., 2010), suggesting a role of pro-inflammatory factors in the pathogenesis of adenomyosis (Inagaki et al., 2003).

CC-chemokine receptor-1 (CCR1) is a member of the G-protein-coupled receptor family, expressed on many inflammatory cells, and binds several members of the chemokine family such as chemokine (C-C motif) ligand (CCL)3, 5, 15 and 23 (Berahovich et al., 2005). As a major pro-inflammatory chemokine receptor, CCR1 contributes to the accumulation of inflammatory cells in

the pathogenesis of many chronic inflammatory diseases (Repeke et al., 2010; Lebre et al., 2011). CCL5 expression in eutopic endometrium and CCR1 expression in plasma are increased in women with endometriosis (Hornung et al., 1997; Agic et al., 2007; Fang et al., 2009; Wang et al., 2010), suggesting an important role of CCR1 in the pathogenesis of this disease. Adenomyosis and endometriosis are often considered to be related conditions, although there are many differences in their pathogenesis and developmental events. Recently, we found an increased CCR1 protein expression in lesions from women with deeply infiltrating endometriosis (DIE), which was not correlated with the stage of the menstrual cycle (Yang et al., 2013). In this study we determined expression of CCR1 in eutopic endometrium of adenomyosis and its correlation with the clinical symptoms.

Materials and methods

Patients

Between June 2010 and November 2011, we recruited 30 women undergoing hysterectomy for adenomyosis because of dysmenorrhea and/or menorrhagia (N=30, mean age: 45.0 years; range: 38-49 years), and 15 women undergoing hysterectomy for cervical intraepithelial neoplasia (CIN) III (N=15, mean age: 44.3 years; range: 41-49 years). There were no significant differences between groups with regard to age or parity (P>0.05). However, the number of gravidity and abortion, and percentage of dysmenorrhea and menorrhagia were all significantly higher in cases compared to controls (P<0.01). Body mass index (BMI) was significantly higher in the control group than the case group (P<0.05, Table1). All operations were performed in the proliferative phase of their menstrual cycles in case and control groups. All subjects had a regular menstrual cycle, which was confirmed by menstrual dating and subsequent histological examination. None of women had received any hormone therapy or an intrauterine device for at least three months before surgery. A diagnosis of adenomyosis was confirmed by postoperative histological examination. Women with endometriosis, leiomyoma and endometrialpolyps were excluded.

Evaluation of dysmenorrhea and menorrhagia

The severity of dysmenorrhea was determined preoperatively using a standardized questionnaire with a visual analogue scale (VAS). The pain scale was subdivided into 10 grades. 'No pain' was indicated at the left side of the scale and 'the maximum pain you could imagine' at the right side of the scale (Vercellini et al., 2006). Twenty eight women with adenomyosis (28/30, 93.3%) complained of dysmenorrhoea. Two women with CIN III, (2/15, 13.3%) complained of dysmenorrhea.

In this study, we defined 'menorrhagia' as using more than five pads per day. In the adenomyosis group, 12/30 (40.0%) had menorrhagia; in the CIN III group, 2/15 (13.3%) had menorrhagia. In the study group, it was graded as minimal (5-7 pads, n=4), moderate (7-9 pads, n=5) and severe (>9 pads, n=3), respectively.

Tissue Collection

We collected two endometrial samples from all subjects with and without adenomyosis immediately after surgery. One specimen was fixed immediately in 10% neutral-buffered formalin for 24 hours for immunohistochemistry before processing and embedded in paraffin according to a standard protocol. The remaining specimen was immersed in liquid nitrogen for real-time quantitative polymerase chain reaction detecting system (qPCR) and western blot analysis before storing at -80 degrees. Written informed consent was obtained from all participants in the study. The Institutional Review Board of the Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, China approved this study.

Immunohistochemical staining

Tissue blocks were prepared and sectioned at 4 μm. Routine deparaffinization and rehydration procedures were performed. The sections were incubated with anti-rabbit CCR1 primary antibody (1:1000, ab89055, Abcam Ltd, HK) for 2 hours at room temperature. After washing with phosphate-buffered saline (PBS), the sections were incubated with secondary antibody (Goat anti-rabbit, GK500705, Gene Biotech Company, Shanghai, China) at room temperature for 1 hour. After washing with PBS again, the sections were treated with diaminobenzidine (Gene Biotech Company, Shanghai, China) and counterstained with hematoxylin, dehydrated, and mounted on a mounting medium. The primary antibody was replaced by PBS as a negative control. All slides were analyzed by two blinded observers.

Assessment of immunochemical staining

CCR1 expression in endometrium was classified

Table 1. Patients' characteristics in case and control groups.

Variables	Cases (n=30)	Controls (n=15)	P value
Age (years)	45.0±4.4	44.3±5.1	0.169
Gravidity	3.37±1.09	2.40±0.63	0.003*
Parity	1.06±0.25	1.27±0.46	0.069
Induced abortion	2.30±1.09	1.13±0.74	0.001**
Body mass index	23.29±3.03	25.36±2.53	0.024*
Dysmenorrhea	93.33% (28/30)	13.33% (2/15)	0.000**
Menorrhagia	40.00% (12/30)	13.33% (2/15)	0.066

#*: P<0.05; **: P<0.001.

according to the grading system described by Ota and Tanaka (1997). Although the immunohistochemistry showed that staining in endometrial glands was different from that in stroma in both groups, we analyzed and compared staining in the endometrial cells, including glands and stroma between groups. Scores corresponding to the percentages of stained cells were defined as follows: 0 for no documented positive staining cells; 1 for 25% positive staining cells; 2 for >25% and <50%; and 3 for >50%. In terms of stain intensity, the following scores were designated: 0 for no documented staining; 1 for weak; 2 for moderate; and 3 for high. CCR1 expression was represented as the sum of both scores.

Reverse transcription and quantitative PCR

For RT-PCR, total RNA of samples were isolated using Trizol reagent (Gibco, USA). One μg RNA was reverse transcribed in a 20 μL reaction (DRR037A, TaKaRa Biotechnology Co., Ltd,). PCR cycles and conditions for denaturation, annealing, and elongation included 35 cycles, 1 min at 94°C, 30s at 55°C, and 30s at 72°C, respectively. Ten μL PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, photographed and saved.

Triplicate samples containing cDNA were prepared as described above, and quantitative PCR was conducted on an ABI7000 thermal cycler (Biosystems). The reactions were performed in a total volume of 20 μL, containing 2 μL cDNA sample (DRR041A, TaKaRa Biotechnology Co., Ltd,). The cycling conditions consisted of a denaturation step at 95°C for 20 sec, followed by 40 cycles at 95°C for 15 sec, a 30 sec annealing step at 60°C. The cycle time (Ct) was chosen to determine the amount of gene products present in each sample using SDS Version 2.3 software (Applied Biosystems). The average Ct value was calculated from triplicate wells for each sample with each primer set (Duplicate samples varied by <0.5 Ct). The relative gene expressions expressed as $2^{-\Delta Ct}$ values, ΔCt calculated by subtraction of the Ct value for ß-actin primers from the Ct value for target gene primers. ß-actin was chosen as the housekeeping gene to normalize the gene expression levels. The primer sequences of CCR1 (forward, 5'-AAGGGCTTGGACTCAAGCAA GA-3', reverse 5'-TGGAGCCCACAGTCACCACTAC-3') and ß-actin (forward, 5'-TGGCACCCAGCACAAT GAA-3', reverse 5'- CTAAGTCATAGTCCGCCTA GAAGCA-3') were produced by Sangon Biotech Co., Ltd. Shanghai, China.

Western Blot

The frozen tissues were disintegrated (25-50 mg) on ice in lysis buffer (RIPA, Beyotime, Shanghai, China). After centrifuging at 18,000g for 5 minutes at 4°C, the supernatants were collected and total concentration of protein was determined by BCA protein assay kit (Beyotime, Shanghai, China). 50 μg of total protein was separated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then electrotransferred onto polyvinylidene diflouride membrane (Pall Life Sciences, NY). After blocking in 5% non-fat skimmed milk for 1 hour at room temperature, the membranes were incubated with anti-CCR1 antibody as described above, and anti-GAPDH antibody (1:1000, sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were further incubated for one hour with a secondary antibody against goat IgG and labelled with horse-radish peroxidase (#7074, #7076, Cell Signalling Technology, USA). The immune-complexes were detected by ECL detection kit (Biological Industries, The State of Israel). Relative protein levels were quantified on band volume with respect to GAPDH expression as assessed by Image Quant TL7.0 software.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software package (v5.01; GraphPad Prism Software Inc, San Diego, CA). The continuous variables were expressed as mean ± standard deviations (SDs). Mann-Whitney test was conducted for numerical variables analysis. Categorical variables were analyzed by Fisher's exact test. Pearson correlation analysis was used to determine the correlations between CCR1 protein levels and VAS score in women with adenomyosis. Univariate and multivariate regression ananlysis were used to determine the correlations between CCR1 protein levels and the clinical characteristics. $P<0.05$ was considered a significant difference.

Results

Immunoreactivity of CCR1 in endometrium in women with and without adenomyosis

CCR1 localized in the glandular and stromal cells of endometrium, and was detected in all subjects (Fig. 1). Scores of CCR1 expression in the glandular and stromal cells of endometrium were 4.89±1.06 in women with adenomyosis, and 2.21±1.16 in women without adenomyosis, respectively. Scores of CCR1 expression in endometrial cells were significantly higher in women with adenomyosis than those without adenomyosis ($P<0.001$, Fig. 2).

CCR1 mRNA levels in endometrium in women with and without adenomyosis

To further confirm differences between CCR1 expression in endometrium in women with and without adenomyosis, we determined CCR1 mRNA in endometrium using qPCR analysis. The qPCR shows that levels of CCR1 mRNA in the endometrium were significantly increased in women with adenomyosis

(0.00043±0.00028) compared to women without adenomyosis (0.00019±0.000062, P>0.05; Fig. 3).

CCR1 protein expression in endometrium using western blot analysis in women with and without adenomyosis

Based on observations of mRNA and immunoreactivity levels of CCR1, we further conducted Western blot analysis to confirm CCR1 protein expression in endometrium of both groups. Western blot showed a specific 36 kDa band for CCR1 (Fig. 4A). CCR1 protein levels were significantly higher in endometrium of women with adenomyosis (1.66±0.79 vs 0.56±0.13, P<0.001) (Fig. 4B).

Correlations of CCR1 expression in endometrium and clinical symptoms in women with adenomyosis

Pearson correlation analysis showed that CCR1 protein levels in the endometrium were positively

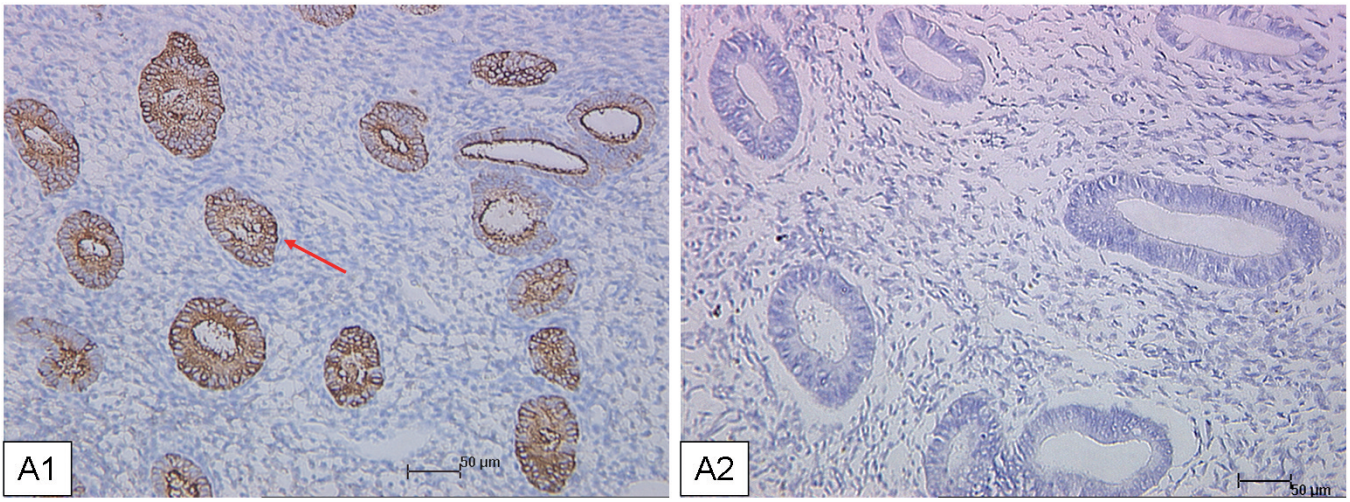

Fig. 1. CCR1-immunoreactive staining in endometrium in women with adenomyosis. **A1.** Strong CCR1-immunoreactive staining in endometrium (red arrows) from a woman with adenomyosis. **A2.** Negative control of CCR1-immunoreactive staining in endometrium from a woman with adenomyosis. Scale bar: 50 μm.

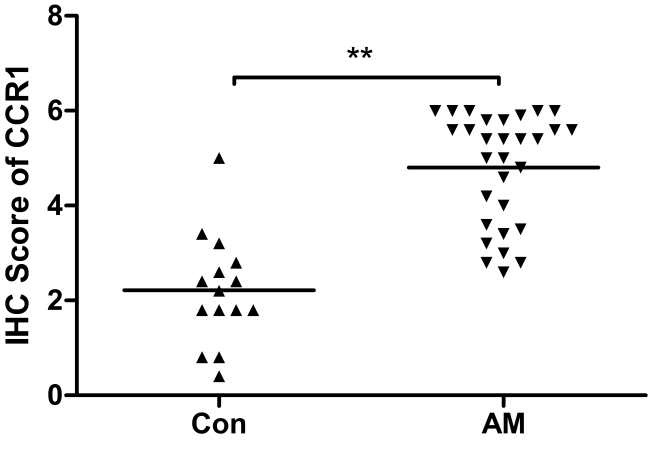

Fig. 2. Comparison of endometrial CCR1-immunoreactive expression scores between women with and without adenomyosis. (Con), Controls (women without adenomyosis). (AM), Adenomyosis (women with adenomyosis). **P<0.001. Compared with women without adenomyosis, CCR1-immunoreactive expression scores in endometrial glands and stroma were both significantly higher in women with adenomyosis.

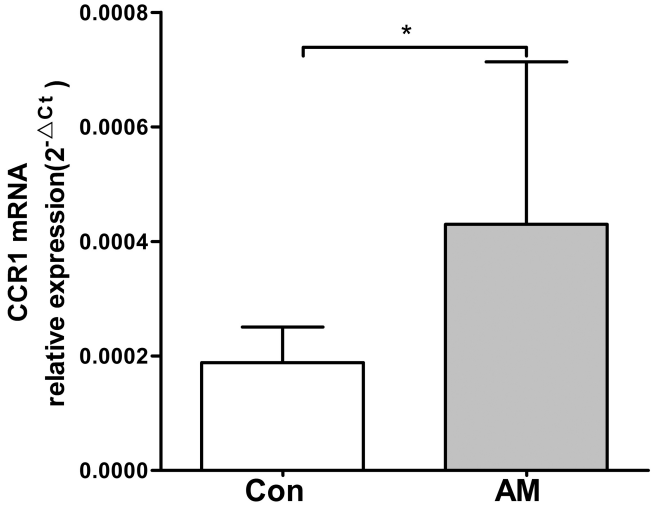

Fig. 3. Endometrial CCR1 mRNA levels in women with and without adenomyosis. (Con), Controls (women without adenomyosis). (AM), Adenomyosis (women with adenomyosis). *P<0.05. CCR1 mRNA expression in endometrium significantly increased in women with adenomyosis compared to women without adenomyosis.

Table 2. Correlations of CCR1 protein levels and clinical variables in women with adenomyosis.

Clinical variables	CCR1 protein levels					
	Univariate regression analysis			Multivariate regression ananlysis		
	r^2	r	P value	R^2	R	P value
Age	0.054	0.232	0.215			
Gravidity	0.149	0.386	0.035*	0.241	0.491	0.957
Parity	0.001	0.032	0.891			
Induced Abortion	0.147	0.383	0.037*	0.237	0.487	0.914
BMI	0.011	0.105	0.575			
Dysmenorrhea	0.584	0.764	<0.001**	0.592	0.769	0.000**
Hypermenorrhea	0.043	0.207	0.270			

*P<0.05; **P<0.001.

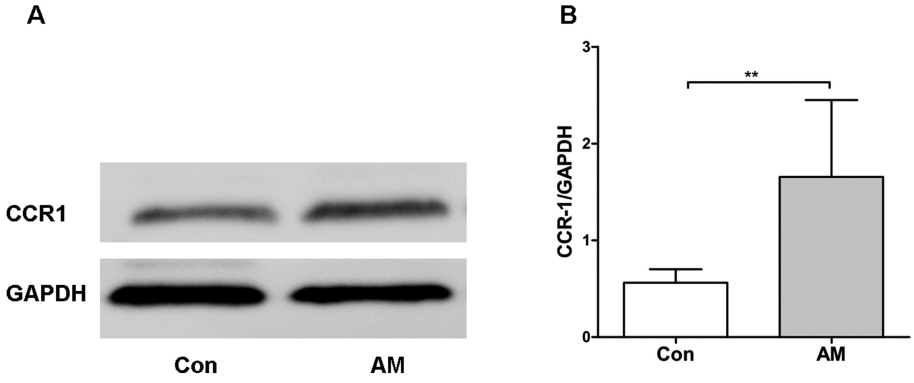

Fig. 4. Endometrial CCR1 protein levels in women with and without adenomyosis. (Con), Controls (women without adenomyosis). (AM), Adenomyosis (women with adenomyosis). **A.** Western blot analysis in endometrium in women with and without adenomyosis. **B.** Comparison of endometrial CCR1 protein levels between women with and without adenomyosis *P<0.001. Western blot showed a specific band (36 kDa) for CCR1, and the levels of endometrial CCR1 protein expression were significantly higher in women with adenomyosis compared to women without adenomyosis.

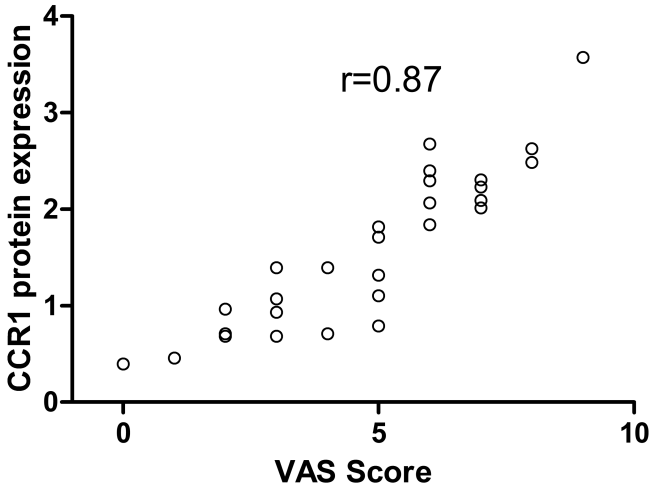

Fig. 5. Correlation between CCR1 protein levels and VAS scores in women with adenomyosis. Pearson correlation analysis showed that the levels of endometrial CCR1 protein expression were significantly correlated with VAS scores (r=0.87, P<0.001).

correlated with VAS score in women with adenomyosis (r=0.87, P<0.001; Fig. 5), but the correlations between the protein levels of CCR1 in endometrium and the severity of menorrhagia did not reach statistical significance (P>0.05). To further determine the correlation of CCR1 protein levels and the clinical characteristics, such as gravidity, parity, induced abortion rate, BMI, dysmenorrhea and menorrhagia, we first performed univariate regression analysis. Significant correlations of CCR1 protein levels were observed with gravidity (r^2=0.149, P=0.035), induced abortion rate (r^2=0.147, P=0.037) and dysmenorrhea (r^2=0.584, P<0.001). For these significant factors, a stepwise multiple regression analysis was further performed. Results revealed that only dysmenorrhea had a significant correlation with CCR1 protein levels in endometrium (R^2=0.592, R=0.769, P<0.001; Table 2).

Discussion

In our study, up-regulation of CCR1 mRNA and protein levels was observed in eutopic endometrium in women with adenomyosis. Previous studies have

demonstrated immunologic impairment in the development of adenomyosis, which induces increased local cytokines in the endometrium, such as interferon-α, interferon-γ, tumour necrosis factor-α, and IL-1ß (Lebovic et al., 2001; Leyendecker et al., 2009).

There are many *in vivo* and *in vitro* studies of chemokine and cytokine expression in endometriosis, which is always regarded as being closely related to adenomyosis. Up-regulated CCR1 may result in secretion of pro-inflammatory factors such as TNF-α, TGFß-1 and IL-6 in other inflammatory diseases (Fifadara et al., 2009). *In vivo* and *in vitro* experiments have also suggested that ligand/receptor interactions are blocked, which can result in inhibiting chemotaxis and reducing inflammation (Volin et al., 1998; Braunersreuther et al., 2010). CCR1 has a high affinity for CCL5, induced by many pro-inflammatory cytokines (TNF-α, IL-ß and IFN-γ), and contributes to self-propagating recruitment of inflammatory cells in endometriosis (Lebovic et al., 2004). IL-1ß combined with estradiol may induce a synergistic stimulatory effect on CCL5 expression by endometriotic cells in endometriosis (Akoum et al., 2002). It is possible that local inflammation contributes to the disruption and growth of endometrial tissues, while infiltrative growth of endometrial tissues may further aggravate the chronic inflammatory process. There may be a positive feedback loop that stimulates continuing alterations of inflammation and progression of disease in adenomyosis.

Our findings are consistent with recent reports that eutopic endometrium in patients with adenomyosis is biologically different from normal endometrium. There are several reports describing endometrial anomalies in aberrant local cytokine production (Sotnikova et al., 2002), aromatase activity (Yamamoto et al., 1993), abnormal innervation (Zhang et al., 2010), angiogenesis (Li et al., 2006; Kang et al., 2009), and altered apoptosis and proliferation in eutopic endometrium (Yang et al., 2007). The evidence suggests that eutopic endometrium expressing CCR-1 may play a role in the pathogenesis of adenomyosis.

Dysmenorrhea and menorrhagia are important symptoms in patients with adenomyosis, though the absence of effective therapy is a major dilemma in the treatment of adenomyosis. Local inflammation in the endometrium may be responsible for clinical symptoms associated with adenomyosis, such as dysmenorrhea and menorrhagia. In our study, CCR1 protein expression in eutopic endometrium significantly increased with the severity of dysmenorrhea in patients with adenomyosis. This suggests that CCR1 may also become an attractive target for the diagnosis and treatment of adenomyosis. This is further supported by clinical applications of CCR1 inhibitors in the other inflammatory-related diseases (Wieser et al., 2009). Recent evidence reveals proinflammatory chemokines may be modulators in the management of neuropathic pain (Scholz and Woolf, 2007; Gao and Ji, 2010). CCR1 has been shown to participate in various biological processes, and blocking ligand/receptor has been considered as a good therapeutic target (Canavese et al., 2010). CCL5 gene expression and protein secretion can be suppressed through NF-kappa B pathway (Wieser et al., 2005) or PPAR-gamma ligands (Pritts et al., 2003) *in vitro* studies of endometriotic stromal cells, which may be beneficial for the treatment of endometriosis-associated symptoms. The clinical effectiveness of chronic progesterone treatment may be attributed to inhibition of CCL5 production and suppression of inflammatory responses in endometriosis (Zhao et al., 2002). With high af?nity for chemokines, CCR1 antagonist treatment can reduce the severity of experimental autoimmune myocarditis by suppressing ERK1/2 and JNK activities and inhibiting cytokine expression (Futamatsu et al., 2006).

In conclusion, we have detected CCR1 mRNA and protein expression in eutopic endometrium of patients with adenomyosis. There were significantly increased levels of CCR1 mRNA and protein in eutopic endometrium in adenomyosis that correlated with the degree of dysmenorrhea in patients with adenomyosis. These findings suggest a possible role for CCR1 in the pathogenesis of adenomyosis-associated dysmenorrhea.

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