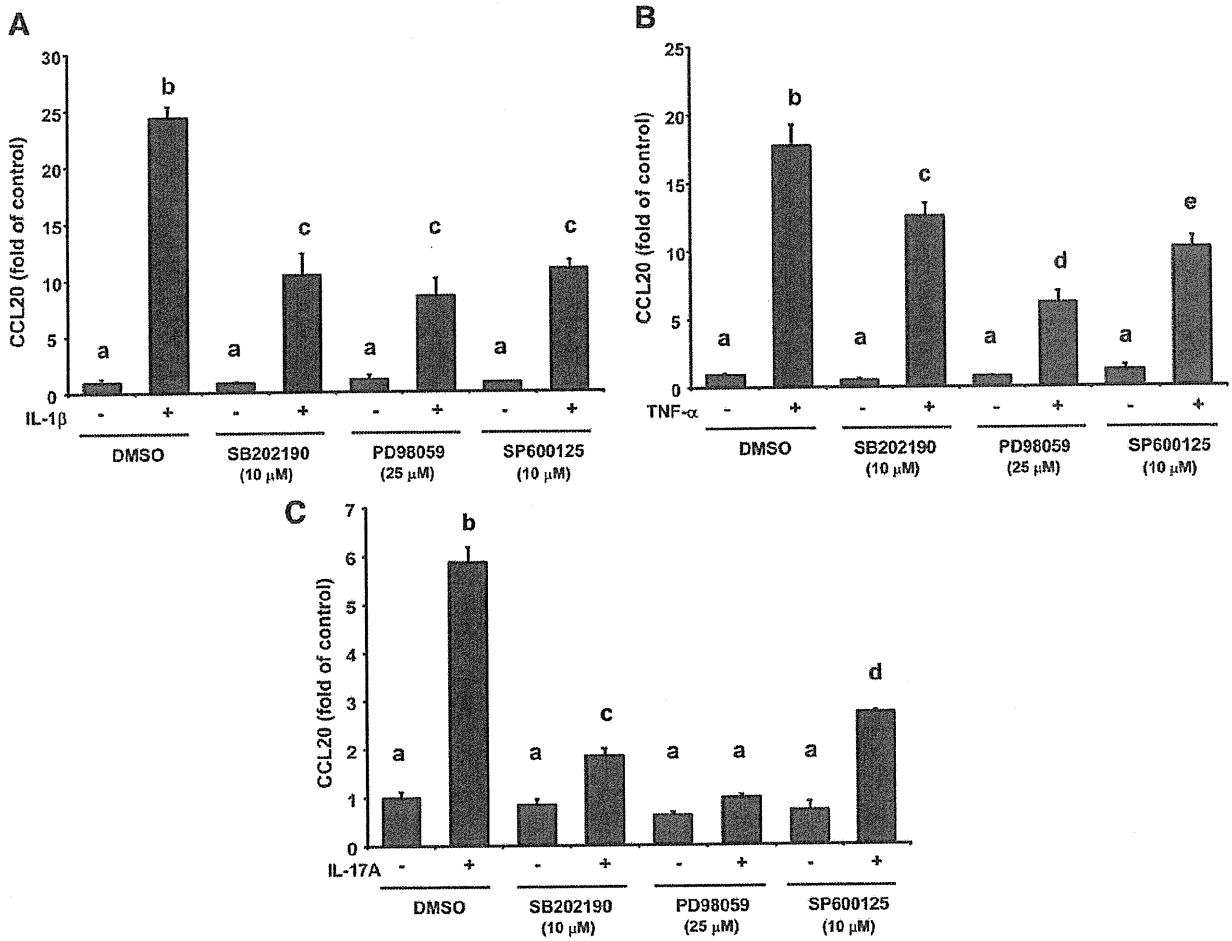

FIG. 5. The effect of MAPK inhibitors on IL-1β- (A), TNF-α- (B), and IL-17A-induced (C) CCL20 secretion in ESCs. ESCs were pretreated with or without inhibitors of p38-MAPK (SB202190), p42/44-MAPK (PD98059), and SAPK/JNK (SP600125) for 1 h and stimulated with IL-1β (1 ng/ml), TNF-α (1 ng/ml), or IL-17A (10 ng/ml) for 24 h. The conditioned medium was collected and assayed for CCL20 concentration using a specific ELISA. Values are expressed as the mean ± SEM of five identical cultures. The data are representative of four independent experiments. *Different letters* denote significant differences between the groups ($P < 0.05$). DMSO, Dimethylsulfoxide.

MAPKs have been shown to inhibit several responses of ESCs implicated in the progression of endometriosis, such as IL-6 and IL-8 secretion induced by IL-1β (26), IL-8 secretion induced by IL-17A (5), and cell proliferation induced by IL-4 and proteinase-activated receptor 2 activation (8, 27). These findings indicate that phosphorylation of these MAPKs in ESCs play an important role in the development of the disease. Therefore, our finding that inhibitors of these three kinases suppressed CCL20 production by IL-1β, TNF-α, and IL-17A suggests an essential role for these kinases in the pathophysiology of endometriosis and also points to a potential target for endometriosis therapy. Interestingly, an inhibitor of p38-MAPK has been shown to suppress the development of endometriosis in a mouse model, in which suppression of CCL20 might have been involved (28).

In searching for treatment strategies for endometriosis, it is encouraging to note that studies in mice have shown that neutralization of CCR6 hindered development of experimental autoimmune encephalomyelitis and rheumatoid arthritis, two conditions in which Th17 cells are suggested to play a critical role in the pathogenesis of the disease (15, 29, 30). Based on our present findings, antagonism of CCR6 would suppress the progression of endometriosis by inhibiting migration of Th17 cells. Although further studies are needed, the present study proposes that antichemokine therapy could be a novel approach for the treatment of endometriosis.

The expression of CCL20 and CCR6 in eutopic endometrium is an interesting issue related to the present study. CCL20 is expressed in an endometrial epithelial cell line and cultured eutopic endometrial cells (31). Therefore, the CCL20/CCR6 system might play a role in the eutopic endometrium, although few Th17 cells are present there (5). It would also be interesting to study the CCL20/CCR6 system in endometriotic lesions other than endometrioma.

In summary, the present study suggests that Th17 cells are recruited to endometriotic tissues by CCL20 secreted

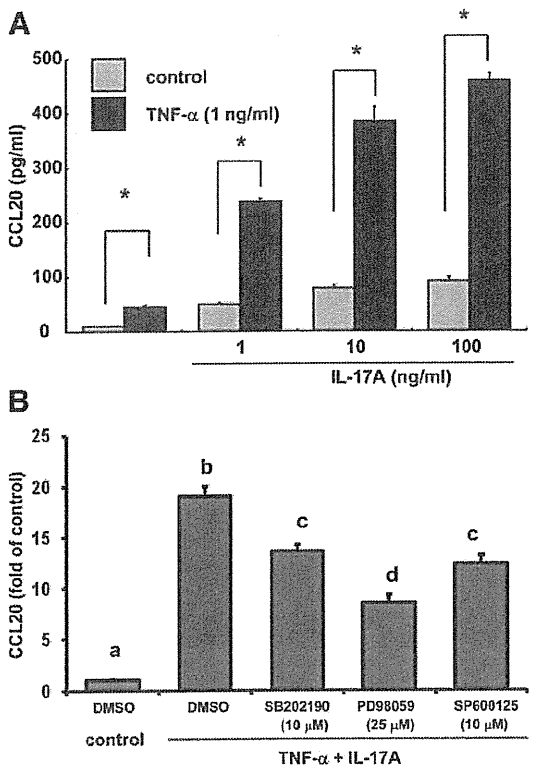

FIG. 6. A, The effect of IL-17A on TNF-α-mediated CCL20 secretion from ESCs. ESCs were treated with IL-17A or TNF-α or both in combination for 24 h. The conditioned medium was collected and assayed for CCL20 concentration using a specific ELISA. Values are expressed as the mean ± SEM of five identical cultures. The data are representative of four independent experiments. *, Significant difference from control, $P < 0.001$. B, The effect of MAPK inhibitors on CCL20 secretion from ESCs induced by a combination of TNF-α and IL-17A. ESCs were pretreated with or without inhibitors of p38-MAPK (SB202190), p42/44-MAPK (PD98059), and SAPK/JNK (SP600125) for 1 h and stimulated with a combination of TNF-α (1 ng/ml) and IL-17A (10 ng/ml) for 24 h. The data are representative of three separate experiments using samples from different patients. *Different letters* denote significant differences between the groups ($P < 0.05$). DMSO, Dimethylsulfoxide.

from ESCs. The inflammatory milieu of the tissue may up-regulate CCL20 expression through IL-1β, TNF-α, and IL-17A, subsequently augmenting Th17 cell migration and development of endometriosis.

Acknowledgments

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Bone Morphogenetic Protein-2 (BMP-2) Increases Gene Expression of FSH Receptor and Aromatase and Decreases Gene Expression of LH Receptor and StAR in Human Granulosa Cells

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Keywords
BMP, folliculogenesis, FSH receptor, HCG, LH receptor, luteinization, ovary

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Problem
A growing body of evidence indicates that bone morphogenetic protein (BMP) cytokines play a key role in female fertility in mammals. BMP-2 is known to be expressed in the ovary of many species. In the present study, we examined the expression and function of BMP-2 in the human ovary.

Method of Study
BMP-2 mRNA expression in the human ovary was evaluated by *in situ* hybridization. Human granulosa cells were obtained from *in vitro* fertilization patients. Human granulosa cells were cultured with recombinant BMP-2 or human chorionic gonadotrophin (HCG), followed by RNA extraction.

Results
BMP-2 expression was detected in granulosa cells of antral follicles but not of corpus luteum. The *in vitro* study showed that BMP-2 induced follicular stimulating hormone (FSH) receptor and aromatase expression, while decreasing luteinizing hormone (LH) receptor and steroidogenic acute regulatory protein expression in human granulosa cells. HCG decreased gene expression of BMP-2 and increased BMP and activin membrane-bound inhibitor (BAMBI), an antagonist of BMP-2.

Conclusion
Expression and disappearance of BMP-2 might contribute to folliculogenesis and luteinization by regulating gonadotropin receptor expression in human granulosa cells. HCG can modulate BMP-2 function by controlling BMP-2 and BAMBI expression.

Introduction

Proper transition from follicular growth to luteinization is an essential event in the ovulatory follicle for an achievement of pregnancy. In a phase of follicular growth, granulosa cells (GC) express follicular stimulating hormone (FSH) receptor and proliferate under the stimulation of FSH.[1] When the follicle approaches ovulation, the luteinizing hormone (LH) receptor is increasingly expressed in GC. The increased LH

receptor makes GC sensitive to LH surge that cues luteinization of these cells. Progesterone, produced by luteinized GC, sustains the initiation and maintenance of pregnancy. Any perturbation of these events, such as premature luteinization and luteinization failure, can impair reproduction.[2,3]

A growing body of evidence indicates that bone morphogenetic protein (BMP) cytokines, members of the TGF-ß superfamily, play a key role in female fertility in mammals.[4,5] Each molecule shows a spatiotemporally different expression and a specific effect in the ovary. For example, we have reported that BMP-7 and BMP-6 are expressed in theca cells and GC, respectively, in the human ovary, and both substances increase FSH receptor and decrease LH receptor expression in GC.[6,7] Others have demonstrated that BMP-15 and GDF-9 are expressed in oocytes and that mutation of these genes leads to folliculogenesis arrest in human.[8]

Given the importance of BMP cytokines in the human ovary, comprehensive studies of BMP family are needed to understand ovarian physiology and pathology. Other than BMP cytokines described earlier, BMP-2 is also thought to play a role in the ovary. In rat, BMP-2 is expressed in GC, and its expression decreases after ovulation.[4] BMP-2 suppresses progesterone production of GC in sheep[9] and rat,[10] and human granulosa cell-like tumor cell line, KGN cell.[11] According to these findings, we hypothesized that BMP-2 might play a role in regulating luteinization.

To address the roles of BMP-2 in follicular growth and luteinization in the human ovary, we studied its expression and its effect on the expression of FSH receptor, LH receptor, aromatase, and steroidogenic acute regulatory protein (StAR) in human GC. To understand the function of BMP-2, we also studied the expression of BMP and activin membrane-bound inhibitor (BAMBI), a molecule that resembles the type-I receptor and interferes with BMP-2.

Materials and methods

Reagents and Materials

Hyaluronidase, fetal bovine serum (FBS), Dulbecco's minimum essential medium (DMEM)/Ham's F12 (F12) and antibiotics (mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma (St. Louis, MO, USA). Recombinant human BMP-2 was purchased from R&D Systems (Minneapolis, MN, USA). Human chorionic gonadotrophin (HCG) was purchased from Mochida (Tokyo, Japan).

Collection of Ovarian Tissues and *In situ* Hybridization for BMP-2

Tissue specimens of human ovaries were obtained under signed informed consent from seven women (age range, 28–40 years old) who underwent salpingo-oophorectomy for a treatment of uterine cervical cancer. All patients had normal ovarian cycles prior to the surgery, and no histological abnormality was observed in ovarian tissues. Among seven patients, four were in follicular phase and three were in luteal phase. The experimental procedure was approved by the institutional review board. Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks. *In situ* hybridization was performed using an ISHR Starting kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. To prepare the digoxigenin (DIG)-labeled RNA anti-sense probes for BMP-2, a primer set (NM_001200: 1022–1041 and 1298–1279) was used. Sense probe hybridization was used as a control for a background level.

Cell Culture of Human Luteinized GC

The method to obtain and culture human luteinized GC was described previously.[6,12] Briefly, follicular fluids with luteinized GC were aspirated from patients undergoing oocyte retrieval for *in vitro* fertilization (IVF). The clinical indications for IVF in these patients were primarily male factor or tubal factor infertility. Patients with ovarian dysfunction were excluded from the study. The experimental procedures were approved by the institutional review board, and signed informed consent for use of GC was obtained from each patient. All of the follicular aspirates from each patient were mixed and centrifuged at $200 \times g$ for 5 min, resuspended in PBS with 0.2% hyaluronidase, and incubated at 37°C for 30 min. The suspension was layered onto Ficoll-Paque and centrifuged at $150 \times g$ for 20 min. The GC were collected from the interphase, washed with PBS, and cultured in DMEM/F12 media supplemented with 5% FBS and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 250 ng/mL amphotericin B) for 15 min at 37°C, to remove contaminating macrophage cells from GC. Using this method, GC were remained in the supernatant while macrophages were attached to

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the culture dish. The collected GC were cultured in DMEM/F12 containing 5% FBS and antibiotics in 12-well plates at a density of 2×10^5 cells/mL and kept at 37°C in a humidified 5% CO_2/95% air environment for 5 days. All of the GC used for the experiments were precultured for 5 days prior to treatments to allow the GC to regain sensitivity to FSH stimulation.[13] Media were changed at 48 -hr intervals. To evaluate the effect of BMP-2, human GC were cultured with or without BMP-2 (100 ng/mL) for 24 hr. To investigate the effect of HCG, GC were cultured with HCG (10 IU/mL) for up to 48 hr. Recombinant BMP-2 was dissolved in 0.1% BSA + 4 mM HCl as a vehicle. The same amount of vehicle was used for a control.

Reverse Transcription and Quantitative Real-time PCR Analysis

Total RNA was extracted from GC, using the RNA-easy minikit (Qiagen, Hilden, Germany). Reverse transcription was performed using Rever Tra Dash (TOYOBO, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a 20-μL volume. For

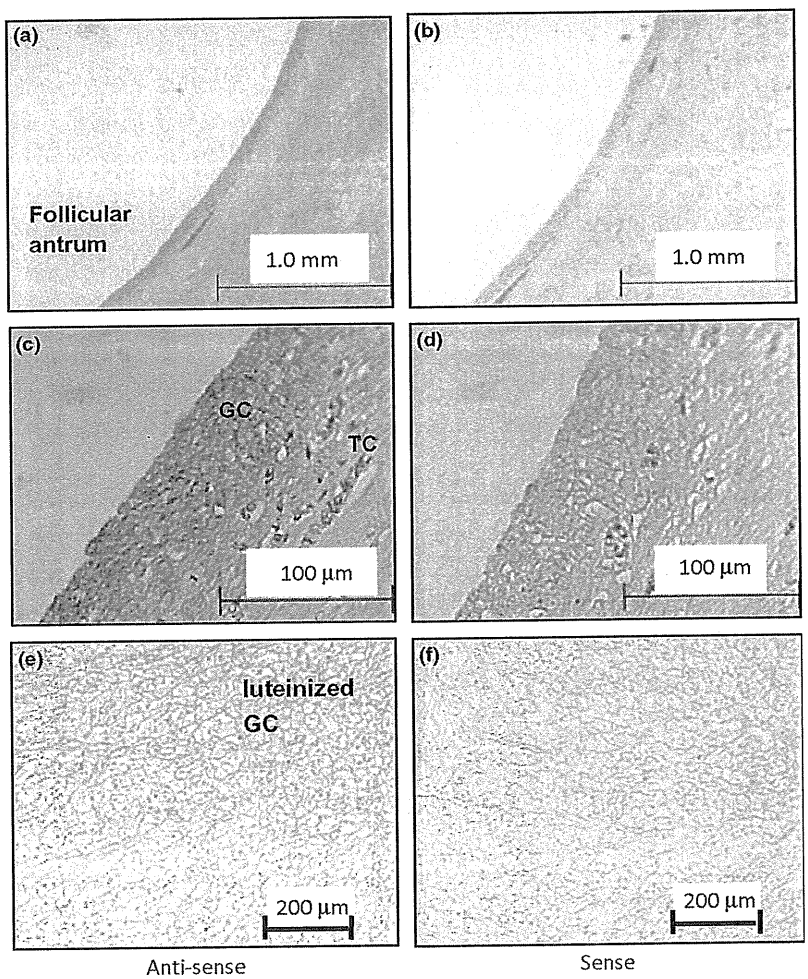

Fig. 1 *In situ* hybridization of bone morphogenetic protein-2 (BMP-2) mRNA of human ovary. Normal human ovaries were examined for BMP-2 mRNA expression. Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks. *In situ* hybridization was performed using the digoxigenin (DIG)-labeled RNA anti-sense probes for BMP-2 or sense probe for negative control. Representative data from seven specimens were shown. (a–d): antral follicle, the follicular size was 10 mm in diameter (e and f): corpus luteum; (a, c, and e): BMP-2 anti-sense probe, (b, d, and f): sense control; (a and b): lower magnification (×40), (c–f): higher magnification (×200). GC: granulosa cell layer, TC: theca cell layer.

the quantification of various mRNA levels, real-time PCR was performed using LightCycler (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. PCR primer sets were designed to span intron to discriminate PCR products that might arise from possible chromosomal DNA contaminants. The primer sequences were as follows, BMP-2 (NM_001200: 1022–1041 and 1298–1279), BAMBI (NM_012342: 711–730 and 984–965), StAR (NM_000349: 171–190 and 551–532), FSH receptor (NM_000145: 174–196 and 510–492), LH receptor (NM_000233:747–767 and 981–962), and aromatase (NM_031226: 1864–1883 and 2105–2086), GAPDH (NM_002046: 628–648 and 1079–1060). PCR conditions were as follows, BMP-2: 43 cycles of 95°C for 10 s, 65°C for 10 s, and 72°C for 12 s, BAMBI: 31 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 11 s, StAR: 35 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 15 s, aromatase: 30 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 10 s. After amplification, melting curve analysis was followed. PCR conditions for FSH receptor and LH receptor and GAPDH were described in elsewhere.[6,7] The expression of each mRNA was normalized by GAPDH mRNA.

Statistical Analysis

All results are shown as mean ± SEM of data from at least three separate experiments, each performed in triplicate. Data were analyzed by Student's t-test for paired comparison and one-way ANOVA with post hoc test for multiple comparisons using STATVIEW software (SAS Institute Inc., Cary, NC, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Localization of BMP-2 in the Human Ovary

The expression of BMP-2 mRNA in the human ovary was examined by *in situ* hybridization. BMP-2 mRNA was barely detectable in GC of primordial, primary, and secondary follicles (data not shown) but detected in GC of antral follicles. Follicular size of antral follicles was from 2.5 mm to 10 mm in diameter. The representative data of an antral follicle with 10 mm in diameter were shown (Fig. 1a–d). In the corpus luteum, BMP-2 mRNA expression in GC was extremely low (Fig. 1e–f).

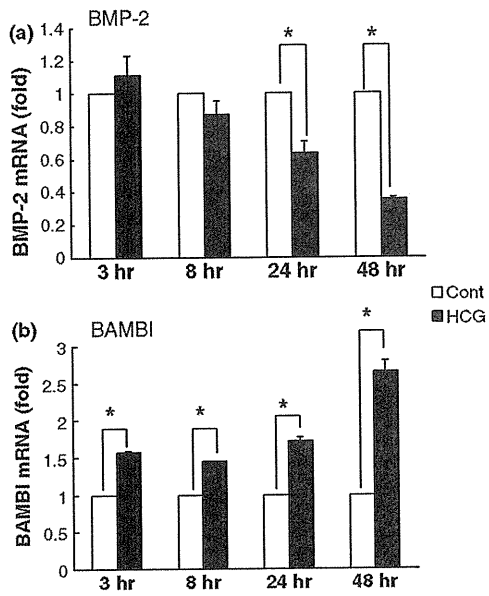

Fig. 2 Effect of HCG on bone morphogenetic protein-2 (BMP-2) (a), and BMP and activin membrane-bound inhibitor (BAMBI) (b) mRNA expression. The human granulosa cells (GC) were cultured with human chorionic gonadotrophin (HCG, 10 IU/mL) for 3–48 hr. Total RNA was extracted from the GC and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels. Data were normalized to GAPDH mRNA levels. Representative data from three different experiments were represented as mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. $^*P < 0.05$ (versus control).

The Effect of HCG on BMP-Related Molecules in GC

To investigate the effect of HCG on BMP-2 and BAMBI mRNA in human GC, cells were cultured with HCG (10 IU/mL) for 3–48 hr (Fig. 2). Notably, HCG significantly decreased BMP-2 mRNA expression from 24 hr onward. On the other hand, HCG significantly increased BAMBI mRNA expression from 3 hr onward.

The Effect of BMP-2 on Folliculogenesis-Related Molecules in GC

In human GC, BMP-2 (100 ng/mL) significantly increased FSH receptor and aromatase mRNA expression (Fig. 3a,e), while BMP-2 significantly decreased LH receptor and StAR mRNA expression (Fig. 3b,c). BMP-2 decreased the expression of StAR in a dose-dependent manner (Fig. 3d)

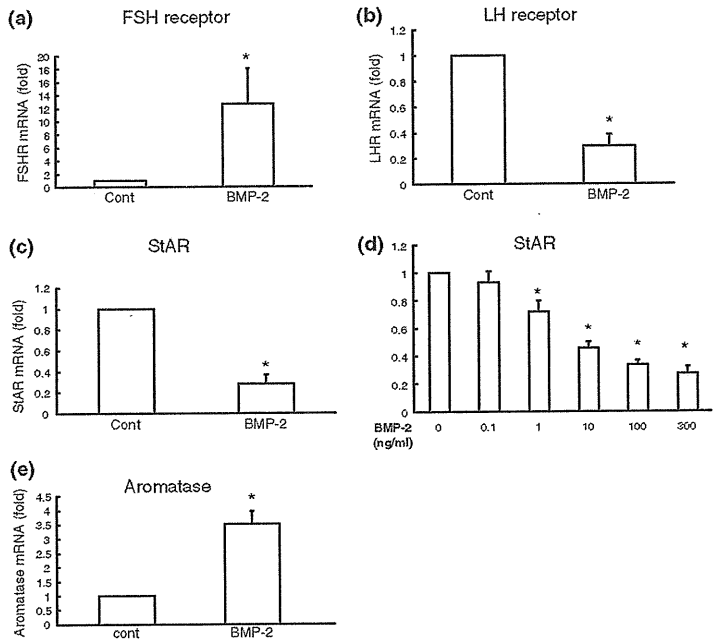

Fig. 3 Effect of bone morphogenetic protein-2 (BMP-2) on FSH receptor (a), LH receptor (b), StAR (c, d) and aromatase (e) mRNA expression. The human granulosa cells (GC) were cultured with BMP-2 (a–c, e: 100 ng/mL, d: 0–300 ng/mL) for 24 hr. Total RNA was extracted from the GC and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. *$P < 0.05$ (versus control).

(0–300 ng/mL). The dose-dependent changes by BMP-2 were also observed in FSH receptor, aromatase, and LH receptor expression (data not shown).

Discussion

In the present study, we found that BMP-2 mRNA was expressed in GC of antral follicles whereas its expression was almost undetectable in the corpus luteum. In the functional analysis using human GC, HCG decreased BMP-2 mRNA but increased BAMBI mRNA. BMP-2 up-regulated FSH receptor and aromatase mRNA, whereas it down-regulated LH receptor and StAR mRNA.

LH receptor is a key factor in the ability of GC to undergo luteinization.[14,15] StAR, which mediates translocation of cholesterol from the outer to the inner mitochondrial membrane, is one of the rate-limiting factors in progesterone production.[16] Accordingly, the present finding that BMP-2 suppresses the gene expression of these molecules indicates that BMP-2 may play a role as an anti-luteinization factor in the ovary. In contrast, the finding that BMP-2 up-regulated FSH receptor and aromatase gene expression suggests that it promotes follicular growth. Given the expression of BMP-2 exclusively seen in antral follicles, it is plausible that BMP-2 promotes follicular growth and prevents precocious luteinization by regulating the expression of these molecules in GC. On the other hand, the vanishing of BMP-2 expression in the corpus luteum would facilitate luteinization.

In view of BMP-2 effects on GC shown in the present study, the regulation of BMP-2 expression in GC seems to have an impact on the appropriate transition from the growing follicle to the corpus luteum. Our finding that HCG suppressed BMP-2 expression in cultured GC suggests that BMP-2 expression might be attenuated in GC in response to the LH surge *in vivo*. The remarkable decrease in BMP-2 expression in the corpus luteum is consistent with this finding. In this context, it should also be noted that HCG induced expression of BAMBI in GC. BAMBI has a structural feature that resembles type-I receptors but lacks the intracellular serine/threonine kinase domain. BAMBI can compete with

the type-I receptors for ligand binding and inhibit the signaling of the TGF-βs, activins, and BMPs, including BMP-2.[17] Taken together, it is speculated that the LH surge not only inhibits BMP-2 expression but increases BAMBI expression in GC to extinguish the effects of BMP-2 that impede the establishment of the corpus luteum. In particular, BAMBI may inhibit the initial phase of BMP-2 effect in the corpus luteum because the increase in BAMBI expression by HCG was observed earlier than the decrease in BMP-2 expression.

Although gonadotropin receptors play important roles in ovarian physiology, the precise mechanism of their regulation remains unclear. It has been reported that the expression of human FSH receptor and LH receptor of corpus luteum were about 50% and 700%, respectively, compared to those of preovulatory follicle.[1,18] The expression pattern and the function of BMP-2 shown in the present study might explain in part the mechanism of gonadotropin receptor transition and imply possible involvement of BMP-2 in early luteinization and luteinization failure in infertile patients.

It is also interesting to note the possible role of BMP-2 in ovarian hormone production. As for production of progesterone, BMP-2 present in GC in growing follicles reduces the expression of StAR and thus in turn suppresses the production of progesterone. In contrast, in luteinized GCs, the absence of BMP-2 may stimulate the expression of StAR and thus results in an induction of progesterone synthesis. As for estradiol, BMP-2 in growing follicles induces aromatase which subserves the production of estradiol while the absence of BMP-2 in luteinized GC may result in a reduction of estradiol synthesis. However, it is interesting to refer an observation that once GC gain LH receptor, GC can produce not only progesterone but also estradiol by LH/HCG stimulation.[19] Given this observation as well as our finding that the reduction of BMP-2 in luteinizing GC enhance their LH receptor expression, we could speculate that BMP-2 contributes to the maintenance of estradiol and progesterone production in luteinizing GC by diminishing its own expression. Taken together, BMP-2 may control the steroid synthesis within GC via modulating local enzymes as well as endocrine systems.

BMP family members are important for folliculogenesis in many species. It is known that oocytes express BMP-6, -15 and GDF-9; GC express BMP-2 and -6; theca cells express BMP-4 and -7.[4,5] There is a growing recognition that various BMP cytokines contribute to folliculogenesis by inhibiting luteinization of GC.[4] We have reported that BMP-6 and -7 attenuated LH receptor expression in human GC.[6,7] Interestingly, animals that suffer from an abnormality in BMP signaling exhibit a precocious maturation of ovarian follicles, resulting in ovulation of follicles which are smaller than those of their wild type counterparts.[2,3] Therefore, it can be hypothesized that malfunction of BMPs that inhibit luteinization may lead to inadequate follicular development and impaired fertility.

In summary, the present findings suggest that BMP-2 expressed in GC promotes follicular growth while BMP-2 inhibits early luteinization. Our findings also suggest that both disappearance of BMP-2 and upregulation of BAMBI by LH surge facilitate luteinizations. These observations imply that BMP-2 is involved in a successful transition from the growing follicle to the corpus luteum and that BMP system and gonadotropin system might cooperate with mutual communication to regulate an ovarian function.

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Author's contributions

O.Y. and Y.O. participated in the study design; J.S., E.N., and Y.H. executed; K.K analyzed; O.Y. and Y.O. drafted the manuscript; and T.Y., O.N., and Y.T. involved in the critical discussion

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ORIGINAL ARTICLE *Gynaecology*

Decreased pregnancy rate is linked to abnormal uterine peristalsis caused by intramural fibroids

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BACKGROUND: The relationship between fibroids and infertility remains an unsolved question, and management of intramural fibroids is controversial. During the implantation phase, uterine peristalsis is dramatically reduced, which is thought to facilitate embryo implantation. Our aims were to evaluate (i) the occurrence and frequency of uterine peristalsis in infertile women with intramural fibroids and (ii) whether the presence of uterine peristalsis decreases the pregnancy rate.

METHODS: Ninety-five infertile patients with uterine fibroids were examined using magnetic resonance imaging (MRI). Inclusion criteria were as follows: (i) presence of intramural fibroids, excluding submucosal type; (ii) no other significant infertility factors (excluding endometriosis); and (iii) regular menstrual cycles, and MRI performed at the time of implantation (luteal phase day 5–9). The frequency of junctional zone movement was evaluated using cine-mode-display MRI. After MRI, patients underwent infertility treatment for up to 4 months, and the pregnancy rate was evaluated prospectively.

RESULTS: Fifty-one patients fulfilled the inclusion criteria, and 29 (57%) and 22 (43%) patients were assigned to the low (0 or 1 time/3 min) or high frequency (≥2 times/3 min) uterine peristalsis group, respectively. Endometriosis incidence was the same in both groups. Ten out of the 29 patients (34%) in the low-frequency group achieved pregnancy, compared with none of the 22 patients (0%) in the high-frequency group ($P < 0.005$). Comparing pregnant and non-pregnant cases, 4 of 10 patients (40%) and 9 of 41 patients (22%), respectively, had endometriosis (not significant).

CONCLUSIONS: A higher frequency of uterine peristalsis during the mid-luteal phase might be one of the causes of infertility associated with intramural-type fibroids.

Key words: uterine fibroma / cine magnetic resonance imaging / uterine peristalsis / infertility / intramural fibroids

Introduction

Uterine fibroids are the most common solid pelvic tumors found in women, and are estimated to occur in 20–50% of women, with increased frequency during the late reproductive years (Verkauf, 1992). Despite this impressive epidemiological burden, the majority of fibroids are asymptomatic and do not require treatment (Somigliana *et al.*, 2007).

In fertility treatment, it is generally accepted that the anatomical location of a uterine fibroid (submucosal, intramural or subserosal), is an important factor in determining the treatment plan (Donnez and Jadoul, 2002). If the fibroids are of the submucosal type, they can be effectively resected with a hysteroscope, which is a less invasive surgical technique. On the contrary, intramural or subserosal lesions should be treated by laparotomy or laparoscopy (Donnez and Jadoul, 2002). However, in some cases myomectomy leads to surgical complications and adhesion formation. In the case of intramural fibroids, patients are required to cease fertility treatment for several months following surgery to allow the uterine scars to heal. However, even with these precautions, scarring has been known to

cause uterine rupture during labor or pregnancy (Somigliana *et al.*, 2007). Moreover, whereas meta-analyses consistently showed a detrimental effect of submucosal but not subserosal fibroids on treatment outcomes, conclusions regarding intramural lesions have been conflicting (Donnez and Jadoul, 2002; Somigliana *et al.*, 2007). Therefore, management of intramural fibroids continues to be difficult and discordant. To address this problem, we decided to examine the mechanisms through which fibroids may influence fertility.

Although the mechanism by which fibroids may reduce fertility is uncertain, it is believed that fibroids might interfere with embryo implantation (Richards *et al.*, 1998). This detrimental effect on implantation may be mediated by the occurrence of abnormal uterine contractility (Fujiwara *et al.*, 2004; Somigliana *et al.*, 2007), but as far as we know there have been no comprehensive studies regarding intramural fibroids, infertility and uterine contractility.

During the implantation phase, it is well known that uterine peristalsis is dramatically reduced, which is thought to aid in implantation of the embryo in the endometrium (Fanchin *et al.*, 1998; Togashi, 2007; Fanchin and Ayoubi, 2009). Recent major changes in ultrafast magnetic resonance imaging (MRI) techniques have enabled acquisition of serial images, at intervals of only a few seconds. The cine mode display (cine MRI) of these sequential images enables direct visualization of uterine contractility (Togashi, 2007). Using a cine MRI display, we have confirmed that no uterine corporal peristalsis was noted in the healthy volunteers during the mid- and late-luteal phases (Orisaka *et al.*, 2007). However, in the pilot study, we have also revealed that three out of five patients who have intramural fibroids showed uterine peristalsis during the time period of the implantation window (luteal phase day 5–9) (Orisaka *et al.*, 2007). The aims of this study are to evaluate the following: (i) the occurrence and frequency of uterine peristalsis in infertile women with intramural uterine fibroids; and (ii) whether the presence of uterine peristalsis decreases the pregnancy rate.

Materials and Methods

A total of 95 patients with uterine fibroids who desire pregnancy were examined by MRI between September 2008 and October 2009 at four hospitals (Teikyo University Mizonokuchi hospital, Keio University, Fukui University and Takinogawa clinic) after obtaining approvals from the ethics committee at each institute. Among 95 subjects, 51 fulfilled the following inclusion criteria: (i) they had intramural fibroids without submucosal type; (ii) in advance of the MRI test, all patients underwent screening for infertility factors at each hospital; (iii) MRI was performed during the time of the implantation window (luteal phase day 5–9).

Patients had no other significant infertility factors (excluding endometriosis) in the screening test, i.e. anovulation, corpus luteum insufficiency, tubal disease or abnormal semen analysis of the partner. In detail, patients had regular menstrual cycles of about 28 days and basal levels of serum FSH, LH and prolactin on menstrual cycle day 3–5 were within the normal range (criteria: FSH 3.5–12.5 mIU/ml, LH 2.4–12.6 mIU/ml and prolactin 4.9–29.3 ng/ml). Serum estradiol and progesterone concentrations in the mid-luteal phase were above 100 pg/ml and 10 ng/ml, respectively. Patients showed no tubal obstruction in the hysterosalpingography test. Sperm concentration of the partner was above 20×10^6/ml (World Health Organization, 1992). After the screening tests, the functional status of the ovaries was monitored using a basal body temperature (BBT) chart. An analysis of BBT graphs was carried out, where a rise in temperature of at least 0.2°C above the preceding 6 days (and occurring in <48 h) which is sustained for at least 11 days would indicate that ovulation had occurred (Ayres-de-Campos *et al.*, 1995). All patients included in this study showed unequivocal biphasic cycles in their BBT chart. We designated the day showing an elevated temperature at least 0.2°C as luteal phase day 1. The implantation window (luteal phase day 5–9) was judged retrospectively using the BBT chart (judged by gynecologists O.Y., M.O., H.O., H.A.).

By routine MRI study, the information retrieved included the location, number and size of fibroids. Also the presence of endometriosis and a distorted uterine cavity was examined. The conditions for cine MRI have been described elsewhere (Orisaka *et al.*, 2007). MRI studies were performed using a 1.5-T magnet unit (MRI machine from Siemens Medical Systems at Takinogawa clinic or from GE Healthcare at Teikyo University, Keio University and Fukui University) with a six channel array coil. Under quiet respiration, a total of 30 serial images were obtained by single-shot fast spin-echo sequence [repetition time (TR)/echo time (TE) = 6000/78 ms, field of view = 240 mm, slice thickness = 10 mm, matrix = 256 × 256], every 6 s for 3 min in the mid-sagittal plane of the uterus. All images in one study were summated into one image and displayed sequentially on the cine mode display at 250 ms intervals. Subsequently, conventional axial and sagittal T2 weighted images (T2WIs, TR/TE = 4000–4720/90–111 ms) and axial T1WIs (TR/TE = 400–550/7.0–8.5 ms) were obtained using fast spin-echo techniques to detect endometriosis and uterine fibroids. One radiologist (T.H.) interpreted the images, without knowledge of the patients' menstrual cycle. Evaluated points included (i) perception of movement of the junctional zone on the cine mode display, (ii) frequency of that movement, if perceivable, (iii) the presence or absence of endometriosis and (iv) the location and number of uterine fibroids. Patients were divided into two groups based on the frequency of uterine peristalsis; <2 times/3 min (low-frequency group) and ≥2 times/3 min (high-frequency group), as described (Togashi, 2007). After receiving MRI, the patients underwent treatment for infertility at each hospital for up to 4 months. Briefly, ovulation induction was performed without use of drugs (natural cycle), or with clomiphene citrate or hMG for 2–3 courses, respectively. Clomiphene citrate (50–100 mg) was started on cycle day 5 for 5 days. HMG (75–150 mIU) was administered on cycle day 3 and continued according to the ovarian response. Depending on the previous ovarian response or the treatment history at a previous hospital, an appropriate treatment was chosen. The size of follicles was checked frequently using transvaginal ultrasound until the diameter of the leading follicle reached 18 mm or greater, and the timing of ovulation was estimated. In some cases, hCG at a dose of 5000 IU was administered. Intrauterine insemination (IUI) was performed when motile sperm concentration was $<20 \times 10^6$/ml. Luteal phase support was not provided.

Data for age, period of infertility, number of fibroids and maximum diameter of fibroids in different groups were expressed as median with minimum–maximum range and compared using the Mann–Whitney *U*-test (Statcel software). Additional patient information and results were analyzed by 2 × 2 contingency table analysis. Statistical significance was set at $P < 0.05$.

Results

The distribution of patients, as categorized by peristalsis frequency, is shown in Table I. Among 51 infertility patients harboring intramural fibroids, 29 (57%) and 22 (43%) patients were assigned to the low- and high-frequency group of uterine peristalsis, respectively. Clinical characteristics of patients in both groups are presented in

Table I The distribution of women with infertility categorized by frequency of uterine peristalsis (per 3 min).

Peristalsis frequency (/3 min)	Number of Patients (total 51)
0	19
1	10
2	1
3	6
4	10
5	3
6	2

Table II Patients with intramural-type fibroids were divided into two groups, based on the frequency of uterine peristalsis; <2 times/3 min (low-frequency group) and ≥2 times/3 min (high-frequency group).

	Low-frequency peristalsis	High-frequency peristalsis	
Patients (number)	29	22	
Age (years)	36 (29–41)	37 (29–41)	Median (min–max range) N.S.
Infertility period (month)	24 (3–84)	24 (4–108)	Median (min–max range) N.S.
Infertility (number of patients)			
Primary	20	17	
Secondary	9	5	N.S.
History of IVF (number of patients)			
No	24	18	
Yes	5	4	N.S.

Clinical characteristics of both groups are shown.
N.S., not significant.

Table II: the data are comparable for age, gravida, infertility period and the ratio of patients undergoing IVF treatment.

The MRI study showed that the endometriosis morbidity, the number of fibroids, the maximum diameter of fibroids and ratio of patients having a distorted uterine cavity were the same in both groups (Table III). Uterine fibroids were located only in the corpus uteri and fundus uteri. There was no case of isthmic and cervical fibroma.

After receiving MRI, 6 out of 29 patients in the low peristalsis group and 6 out of 22 in the high peristalsis group underwent hMG treatment, while others had natural cycles (timed intercourse or IUI) or clomiphene citrate treatment (Table IV). IUI was performed in 9 out of 29 patients and 4 out of 22 patients in the low and high peristalsis groups, respectively.

Table III Patients with intramural-type fibroids were divided into two groups, based on the frequency of uterine peristalsis; <2 times/3 min (low-frequency group) and ≥2 times/3 min (high-frequency group).

	Low-frequency peristalsis	High-frequency peristalsis	
Patients (number)	29	22	
Endometriosis (number of patients)			
No	22	16	
Yes	7	6	N.S.
Number of fibroid	2.8 ± 2.8	3.5 ± 3.0	N.S.
Maximum diameter (mm)	53 ± 17	58 ± 21	N.S.
Deformed uterine cavity (number of patients)			
No	14	12	
Yes	15	10	N.S.
Pregnancy			
number of patients (%)	10 (34%)	0 (0%)	$P < 0.005$

Magnetic resonance imaging (MRI) findings and pregnancy rates within 4 months after MRI study are shown.
N.S., not significant.

Ten out of 29 patients (34%) achieved pregnancy in the low-frequency group within 4 months, while none of the 22 patients (0%) in the high-frequency group achieved pregnancy ($P < 0.005$) during the same 4-month period. All conceptions were achieved with non-IVF techniques. As shown in Table IV, seven and three patients achieved pregnancy with natural cycle and clomiphene citrate treatment, respectively. One out of 10 pregnant cases utilized IUI, and others became pregnant with timed natural intercourse.

Discussion

It is well described that the direction and frequency of uterine peristalsis significantly varies during the cycle phases (Fanchin and Ayoubi, 2009). Uterine peristalsis is active during the periovulatory and menstrual phase, and the direction is cervix to fundus during the periovulatory phase and fundus to cervix during the menstrual phase. However, during the luteal phase, uterine peristalsis is barely observed (Togashi 2007, Orisaka *et al.*, 2007; Togashi, 2007). These results support the concept that uterine peristalsis is related to uterine function, namely such activities as sperm transport, embryo implantation and discharge of menstrual blood (Zervomanolakis *et al.*, 2007). With ultrasonography, Fanchin *et al.* examined the uterine peristalsis of infertile patients who do not have uterine abnormalities (Fanchin *et al.*, 1998; Fanchin and Ayoubi, 2009) and demonstrated a negative correlation between the frequency of uterine peristalsis on the day of embryo transfer and pregnancy outcome. Although they recorded uterine peristalsis on luteal phase day 2, not the implantation window (luteal phase day 5–9), they did show that high-frequency

Table IV The distribution of fertility treatment and pregnancy outcome in 51 patients: ovulation induction was performed without drugs (natural cycle), and with clomiphene citrate or hMG.

Ovulation induction	Patients (number)	Pregnancy (number)
Low-frequency group		
Natural		
Timed intercourse	14	7
IUI	5	0
Clomiphene citrate		
Timed intercourse	2	2
IUI	2	1
HMG		
Timed intercourse	4	0
IUI	2	0
High-frequency group		
Natural		
Timed intercourse	11	0
IUI	3	0
Clomiphene citrate		
Timed intercourse	2	0
IUI	0	0
HMG		
Timed intercourse	5	0
IUI	1	0

When motile sperm concentration was $<20 \times 10^6$/ml, intrauterine insemination (IUI) was performed. Data are shown as the number of patients in the low (<2 times/3 min) and high (≥2 times/3 min) frequency uterine peristalsis groups.

endometrial waves on the day of embryo transfer appear to affect the IVF-embryo transfer outcome in a negative manner, perhaps by expelling embryos from the uterine cavity (Fanchin *et al.*, 1998). In a previous study using cine MRI, we found that during the time of the implantation window, although no corporal contractions were noted in healthy volunteers, some patients with intramural-type fibroids exhibited uterine peristalsis (Orisaka *et al.*, 2007).

A critical and still unsolved question is the relationship between fibroids and infertility. Management of the intramural-type fibroid is very controversial in the field of reproductive medicine (Donnez and Jadoul, 2002; Somigliana *et al.*, 2007). Here, we focused on the occurrence of abnormal uterine contractility caused by intramural fibroids, and examined whether this has a detrimental effect on the pregnancy rate in infertility patients. We found that less than half of the patients with intramural fibroids exhibited abnormal uterine peristalsis during the mid-luteal phase. Interestingly, in the high-frequency peristalsis group, no patients achieved pregnancy, while one-third of the patients in the low peristalsis group achieved pregnancy. Comparing the low- and high-frequency peristalsis groups, there is no difference in the number of fibroids, the maximum diameter of the fibroids and the incidence of a deformed uterine cavity (Table III). Also, when comparing pregnant ($n = 10$) and non-pregnant cases ($n = 41$), no difference was found in the number of fibroids, the maximum diameter of the fibroids and the incidence of a deformed uterine cavity (data not shown).

The relationship between abnormal peristalsis and fibroids (i.e. deformation of uterine cavity, number and size) has been unclear. As estrogen induces peristalsis (Mueller *et al.*, 2006), aromatase expression in fibroids (Bulun *et al.*, 2005), which might result in elevated tissue estrogen concentration, could be a contributory factor. Further study is needed to examine this hypothesis.

Endometriosis is one of the most important factors of infertility (Maruyama *et al.*, 2000). In the present study, when comparing pregnant ($n = 10$) and non-pregnant cases ($n = 41$), 4 out of 10 patients (40%) and 9 out of 41 patients (22%) had endometriosis, respectively, and the difference was not significant. Meanwhile, the endometriosis morbidity was comparable between low and high peristalsis groups (Table III). This finding implies that endometriosis has little or no impact on uterine peristalsis at the time of the implantation window, whereas others have found that uterine peristalsis was suppressed during the periovulatory phase in patients with endometriosis (Kido *et al.*, 2007).

We utilized MRI technology to detect uterine peristalsis. With ultrasonography, it is difficult to clearly detect the endometrium because of deformation caused by fibroids. Furthermore, pressing the uterus with a transvaginal transducer may induce uterine contraction (Lesny *et al.*, 1998). Thus, the cine MRI method is favorable for evaluating patients with fibroids.

In the present study, we demonstrate that abnormal uterine peristalsis in the presence of intramural fibroids could be one of the reasons for a decreased pregnancy rate in these patients. Studies are warranted to investigate if myomectomy for patients in the high peristalsis group is a constructive method to normalize uterine peristalsis.

Authors' roles

O.Y., T.H., M.O., H.A., S.O., M.H., H.H., T.F. contributed to the study design, O.Y., T.H., M.O., H.A., S.O., M.F., H.O., Y.S., O.N. executed the study, O.Y., Y.O. performed the analysis, O.Y., Y.O., M.O., S.O. contributed toward drafting the manuscript and H.A., M.H., F.K., Y.Y., Y.T. involved in critical discussion.

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Conflict of interest: none declared.

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Lymphocytes in Endometriosis

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Endometriosis is a disease characterized by the presence of endometriotic tissue outside the uterine cavity. Although its pathogenesis remains to be elucidated, immune status is suggested to play an important role in the initiation and the progression of the disease. In particular, immune cells in lymphoid lineage that comprised T and B lymphocytes and natural killer cells play essential roles in determining either accept or reject survival, implantation, and proliferation of endometrial and endometriotic cells. Numerous studies have shown aberrant functions of these immune cells in women with endometriosis. The abnormalities include reduced activity of cytotoxic T cells and NK cells, cytokine secretion by helper T cells, and autoantibody production by B lymphocytes. These alterations are suggested to be induced by various manners and promote the disease. Understanding of these immune aspects in endometriosis is thus expected to benefit the treatment of the disease.

Introduction

Endometriosis is an enigmatic disease that impairs the health of women in reproductive age, by causing pain and infertility.[1,2] Although numerous studies have been conducted to elucidate its etiology, only a small part has been understood. Based on accumulated evidences, the most widely accepted hypothesis is that endometriosis originates from shed endometrium that refluxes in the peritoneal cavity during menstruation. However, retrograde menstruation is seen in most women. This brings a question why only a portion of women develops endometriosis.

Many factors have been suggested to play a role in its pathogenic process: to permit and promote survival, implantation, and proliferation of the endometrial cells.[3] These factors include bioactive molecules, such as hormones, growth factors, cytokines and prostaglandins, as well as various type of cells that present in the endometriosis lesion such as immune cells, endometrial epithelial cells, stromal cells, and vascular endothelial cells.

Among these factors, immune cells have been noted to play crucial roles in either rejecting or accepting refluxed endometrial cells. In addition to their direct functions, immune cells have been also suggested to contribute to the disease development by secreting various cytokines that control cell proliferation, inflammation, angiogenesis and so on. Indeed, a variety of immune cells such as T and B lymphocytes, natural killer cells, macrophages and mast cells have been demonstrated to be present in endometriotic lesion, indicating their potential roles in the disease.

These immune cells are classified into either lymphoid lineage or myeloid lineage. In this review, we will focus on the lymphoid lineage, i.e. T and B lymphocytes and natural killer cells. We will not touch the myeloid lineage in this article, and one may refer excellent review articles listed later.[4,5]

T Lymphocytes

T lymphocytes are classically classified into cytotoxic T cells or helper T cells. Cytotoxic T cells are capable

of destroying a specific target by cytotoxic mechanism, and helper T cells transmit signals from antigen-presenting cells and enhance further immune response. Recently, new paradigm for helper T-cell classification has been introduced: Th1, Th2, Th17 and regulatory T cells, and researcher for endometriosis have also embraced such new premise in order to understand the pathogenesis of this disease. Here, we review studies for endometriosis with regard to T-lymphocyte functions.

When Cytotoxic T Lymphocytes do not Respond to Autologous Endometrium, Endometriosis Develops

Several studies demonstrated that defective T-lymphocyte response to autologous endometrial cells was associated with endometriosis. Classical experiments using rhesus monkey showed that intradermal injection of autologous endometrium induced less number of lymphocytes infiltration to the injected site in animals affected with endometriosis compared to healthy animals.[6] In human as well as rhesus monkey, the lymphocyte proliferative response to autologous endometrial cells was decreased in women with endometriosis.[6–8] The cytotoxicity of T lymphocytes against autologous endometrial cells was also reduced in women with endometriosis.[9] Given these observations, attempts have been made to correct the defect of T-lymphocyte cytotoxicity against autologous endometrium as a therapeutic strategy for endometriosis. Indeed, the defect of T-lymphocyte cytotoxicity was corrected by stimulating peripheral blood lymphocytes with recombinant interleukin (IL)-2,[10] implying therapeutic potential of IL-2 for endometriosis. Consistently, IL-2 treatment decreased the size of endometriosis-like lesion with greater number of lymphocytes recruited to the lesion in the rat model of endometriosis.[11]

Another mechanism by which endometriotic cells are able to escape from immune surveillance of cytotoxic T lymphocyte is attributable to FasL expressed by endometriotic cells. FasL induces apoptosis of lymphocytes by binding to its receptor, Fas, expressed on lymphocytes. Therefore, cells that are expressing high FasL may cause apoptosis of surrounding lymphocytes and thereby escape from lymphocytes response. Interestingly, FasL expression in endometrial stromal cells are induced by IL-8 and CCL2,[12,13] cytokines/chemokines known to be increased in serum and peritoneal fluid (PF) of women with endometriosis.[14–18] Indeed, Jurkat cells (T lymphocyte cell line) underwent apoptosis when they were cocultured with endometrial stromal cells that had been pretreated with IL-8 or CCL2.[12,13] Similarly, the level of soluble FasL, which also induces apoptosis for Fas-expressing cells, was increased in PF of women with advanced stages of endometriosis,[19] also explaining the escape of endometriotic cells from peritoneal lymphocytes response. Taken together, PF of women with endometriosis may have a potential to induce apoptosis of cytotoxic T lymphocytes, directly or indirectly via stimulating endometriotic cells and contribute to the survival of endometriosis.

Helper T-Cell Activity is Decreased in Endometriotic PF

Besides cytotoxic T lymphocytes, characterized as $CD8^+$ T cells, helper T cells or namely $CD4^+$ T cells are further diminished in their activity in PF from patients with endometriosis. Classic studies looking at CD4:CD8 ratio showed that the ratio was decreased in endometriotic PF.[20–22] In addition, although the total concentration of $CD4^+$ T cells were shown to be high,[23] the activated status of $CD4^+$ T cells as well as $CD8^+$ T cells were decreased in endometriotic PF.[21,24] These findings indicate that activation of helper T cells is suppressed in PF of patients with endometriosis. In this context, one study showed that THP1 cells (monocytic cell line), when cultured in the presence of endometriotic PF, decreased their expression of MHC class II and CD80/CD86, molecules that stimulate T-cell activation. This indicates that putative substances exist in endometriotic PF and these may affect on antigen-presenting cells (monocyte lineages) and thereby diminish helper T-cell activation. One of the candidates of these substances is IL-10, because IL-10 neutralization was shown to abrogate the effect.[25] Consistently, high concentration of IL-10 was associated with decreased activated $CD4^+$ T cells in endometriotic PF.[26]

Presence of T Lymphocytes in Endometriotic Lesion

T lymphocyte is one of two major leukocyte subpopulations in endometriotic tissues along with macrophage.[27] The number of total T lymphocytes as well as that of activated T lymphocytes was shown to be increased in ectopic endometrium compared to

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eutopic endometrium,[28,29] whereas one study failed to detect any differences.[30] As a specific subgroup of T lymphocytes, gamma delta T lymphocytes were demonstrated in the stroma of endometriotic tissues, although its function remains to be elucidated.[31]

Th1 Cells and Th2 Cells

Nearly two decades ago, a new classification for helper T cells, namely Th1 cells and Th2 cells was introduced. Th1 cells produce large quantities of interferon-γ (IFN-γ) and induce delayed hypersensitivity reactions, activate macrophages and defense against intracellular pathogens. Th2 cells, on the other hand, produce IL-4, and induce immunoglobulin (Ig) E production, recruit eosinophils at inflammation and help clear parasitic infections. Since this new paradigm was introduced, immunologists were enthusiastic about explaining various physiological and pathological conditions by looking at the balance between Th1 and Th2 cells in both systemic and local environment.

This paradigm has also been applied for endometriosis study. IL-4 and IL-10 were shown to be upregulated in peripheral lymphocytes in women with endometriosis. Increased IL-4 expression is also seen in lymphocytes in endometriotic tissues and in PF.[32,33] On the other hand, production of IFN-γ was reduced in peripheral lymphocytes in endometriosis.[34,35] Likewise, production of IFN-γ in peritoneal cells[33] and IFN-γ concentrations in PF were decreased in endometriosis.[36,37] Another study showed an increase in ratios of IL-4/IFN-γ, IL-4IL-2, IL-10/IFN-γ and IL-10/IL-2 in PF of endometriosis.[38] All these findings indicate that Th1/Th2 balance is shifted toward Th2 in endometriosis, with one exceptional study showing a shift toward Th1 when limited to early stage endometriosis.[39]

Despite these observations, the effect of Th2-skewed immune response on the pathogenesis of endometriosis has been poorly understood. In order to address this issue, we conducted a couple of experiments. First, we found that a substantial number of IL-4-positive Th2 cells were present in endometriotic tissues. We then asked the effect of IL-4 on endometriosis and revealed that IL-4 increased proliferation of cultured endometriotic stromal cells. This effect was synergized with TNFα and was mediated by multiple mitogen-activated protein kinases.[40] IL-4 also increased a secretion of eotaxin from endometriotic stromal cells. Immunohistochemical analysis showed that eotaxin-positive cells colocalized with IL-4-positive cells and accumulated around the blood vessels in the endometriotic tissue.[41] Because eotaxin is a potent chemoattractant for Th2 cells, these findings indicate the presence of a positive feedback loop in which IL-4 and eotaxin co-operatively enhance Th2 immune response in endometriosis tissues.

Th17 Cells and Regulatory T (Treg) Cells

In very recent years, however, the Th1/Th2 dogma has been challenged by the introduction of two other subsets of T cells: Th17 cells and regulatory T (Treg) cells.

Th17 cells preferentially produce IL-17, but not IFN-γ or IL-4. Th17 cells can rapidly initiate an inflammatory response mainly by recruitment, activation, and migration of neutrophils. The involvement of Th17 cells has been suggested in various chronic inflammatory diseases.[42] This novel notion has also been embraced for understanding of the pathogenesis of endometriosis. Recently, we demonstrated the presence of Th17 cells in PF of endometriosis. We further showed that IL-17 stimulates endometriosis stromal cells proliferation, their IL-8 and cyclooxygensase-2 expression.[43] Another group reported the presence of IL-17 in endometrial cyst fluid and found that the level was high in aromatase positive endometriosis.[44]

Treg cells are specialized subpopulation of T lymphocytes that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens.[45] In eutopic endometrial tissues, Treg cells are significantly decreased during secretory phase in women without endometriosis, whereas the decrease was not seen in women with the disease. It was proposed that the preserved Treg cells seen in women with endometriosis decrease the ability of newly recruited immune cell populations to effectively recognize and target endometrial antigens during menstruation, allowing survival and implantation of shed endometrial cells.[46]

The Effect of Hormonal Therapy on T Lymphocyte Population

Suppression of estrogen such as GnRH analog (GnRHa) therapy is a strategy widely used for treatment of endometriosis. This is based on an idea that

estrogen promotes the proliferation of endometriotic cells. However, the suppression of estrogen is also known to alter immune status and thereby further contributes to the therapeutic effect on endometriosis. GnRHa treatment was reported to increase total T lymphocytes number in peripheral blood and T-lymphocyte activity in peripheral blood and PF.[47,48] It was also demonstrated that peripheral lymphocytes taken from patient with endometriosis showed decreased IFN-γ production and increased IL-4 production when they were stimulated by PHA, but this abnormality was corrected after the patient had been treated by GnRHa.[34] Similarly, IFN-γ concentrations was decreased in PF with endometriosis and the decrease was normalized in GnRHa-treated population.[36] These findings indicate that hormonal therapies for endometriosis not only effect directly on the endometriotic cells but also alter the immunological environment and thus in turn contribute to the control of endometriosis.

T Lymphocytes in Animal model of Endometriosis

Baboon is widely used as an animal model for endometriosis. This animal develops endometriosis spontaneously, but one can also induce endometriosis experimentally by seeding autologous eutopic endometrium in the peritoneal cavity. In peripheral blood, the percentage of activated $CD4^+$ T cells was increased in baboons with both spontaneous and induced endometriosis. In PF, however, the increase of $CD8^+$ T cells was only seen in spontaneous but not in induced endometriosis, suggesting that alterations in PF leukocyte may be causative but not consequence of endometriosis.[49]

As new paradigms have been introduced in general immunology, the study for endometriosis has been kept up them and the premise has been changed accordingly. However, as seen in other pathological situation, none of these paradigms can solely explain for all pathologies of endometriosis. Further studies are required to understand the complex interaction of immune cells in the pathogenesis of this disorder.

B Lymphocytes

B lymphocytes and autoantibody

B lymphocytes are responsible for humoral immune response, principally producing antibodies against antigens. In the pathogenesis of endometriosis, they have been suggested to play roles by secreting autoantibodies. An increase in B lymphocyte reactivity in women with endometriosis was first suggested in 1980.[50] The same year, another study indicated that women with endometriosis had deposits of C3 and IgG in the endometrium and low total complement level in sera, suggesting an autoimmune response with local activation and consumption of complement factors by the antigen-antibody complex.[51] Thereafter, many researchers have been focused on the role of B lymphocytes in the pathogenesis of endometriosis, particularly autoimmune responses via two major types of autoantibody: antibodies specifically response to the endometrium and antibodies that is commonly observed in various autoimmune disorders.

Autoantibody Specific to the Endometrium

Wild and Shivers first demonstrated the presence of anti-endometrial antibodies in sera of women with endometriosis by indirect immunofluorescence.[52] Likewise, Fernandez-Shaw et al.[53] demonstrated that anti-endometrial antibodies were detected more frequently in sera from women with endometriosis than in those from unaffected women. Immunohistochemical examination revealed that anti-endometrial antibodies bounded to the glandular component of ectopic and eutopic endometrium.[53–55] A western blotting analysis further demonstrated that autoantibodies reacted with endometrial membrane proteins, and that the immunoreactivity was increased with the progress of endometriosis.[56] Marthur et al. identified IgG and IgA autoantibodies against endometrial tissue not only in sera and but also in cervical and vaginal secretions of women with endometriosis. They found that the exact antigen to which autoantibodies react were transferrin and alpha 2-HS glycoprotein exists in the endometrium.[57–59] A following study identified the glycotope Thomsen-Friedenreich (T) antigen (Gal beta1-3GalNAc) in 2-HS glycoprotein and carbonic anhydrase as a common carbohydrate epitope for the response.[60]

Autoantibody Commonly Observed in Autoimmune Diseases

Autoantibodies that are frequently found in patient with various autoimmune diseases such as antinuclear antibodies, antiDNA antibodies, and antiphosp-

holipid antibodies have also been observed in women with endometriosis. In one study, of 31 patients with endometriosis, 64.5% exhibited IgG autoantibodies and 45.2% demonstrated IgM autoantibodies to at least one of 16 antigens investigated.[61] This suggests that endometriosis is associated with abnormal polyclonal B-cell activation, a classic characteristic of autoimmune disease. The association between autoantibody and endometriosis may also explain endometriosis-related infertility, as these antibodies might bind to not only the endometrium but also embryos and sperms. However, whether the autoantibody response plays a primary role in disease pathogenesis or if it is an epiphenomenon is still to be determined.

B Lymphocytes in Endometriosis

Whereas many studies have shown aberrant productions of autoantibodies in endometriosis, contradictory reports are found with regard to the number and the function of B lymphocytes in endometriosis. Badawy et al.[62] measured erythrocyte antibody complement binding capacity and found an increased number of B lymphocytes in PF and peripheral blood from patients with endometriosis. On the contrary, Gagne et al.[63] reported that the number of B lymphocyte in peripheral blood was lower in women with endometriosis over healthy control, while Antsiferova et al.[32] found no such difference. As for the function of B lymphocytes, a study demonstrated that amount of IgG and IgA produced by peritoneal cells was increased in women with endometriosis,[64] suggesting the increased activity of B lymphocytes in endometriosis. The increase in serum concentration of soluble CD23, which is produced from activated B lymphocytes, in patients with endometriosis[65] also indicates enhanced activation of B lymphocytes. In contrast, IgG2 production by circulating B cells stimulated with polyclonal B-cell activators was decreased in women with severe endometriosis, which may imply B-cell dysfunction in advanced endometriosis.[66]

B-1 Cells

In contrast to aforementioned classic studies that analyzed B lymphocytes as a homogeneous population, recent studies have classified B lymphocytes into subclasses. One of the subclasses of B lymphocytes is B-1 cells, which is known to undergo self-renewal in the periphery and is involved in innate immune response. Classically, immunostaining study showed that very few B lymphocytes were present in endometriotic lesions.[27] However, a recent elaborate analysis using flow cytometry demonstrated that the number of B-1 cells as well as total B lymphocytes was significantly elevated in endometriosis tissues compared with eutopic endometrium.[32] In addition, women affected with endometriosis showed significantly higher B-1 cell populations in PF than did women without the disease.[67] In this context, it is intriguing to introduce a recent study which showed endometriotic lesions were characterized by the presence of abundant plasma cells that were suggested to be derived from B-1 cells.[68]

Taken together, there is no doubt that B lymphocyte is responsible for producing autoantibody, both specific and non-specific to the endometrium, and thus in turn contributes to the pathogenesis of endometriosis. However, further studies are required to determine characteristic roles of particular subclasses of B cells and their interaction with other immune cells which may further modulate local and systemic immune environment.

NK Cells

NK Cells Cytotoxic Activity is Reduced in Endometriosis

In general, NK cells are responsible for rejection of tumors or cells infected by microbe. NK cells destroy target cells by releasing small cytoplasmic granules of proteins that induces apoptosis. A possible link between NK cells and endometriosis was initially arisen from a study which showed NK cells in peripheral blood have an ability to destroy endometrial cells.[69] This finding suggested a hypothesis that NK cells may keep clearing regurgitated endometrial cells in the peritoneal cavity, and reduction in NK cells cytotoxic activity may cause development of endometriosis.

Indeed, succeeding studies demonstrate that NK cells cytotoxic activity is reduced in endometriosis. Several investigators found that the cytotoxic ability of NK cells against endometrium was diminished in peripheral blood of women with endometriosis.[69–71] In addition, the reduction was correlated with the severity of the disease.[72] The reduction of cytotoxic activity of NK cells was also observed in PF with endometriosis.[24,72] The reduction was pronounced in