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.

Acknowledgements

We thank Dr Heather M. Martinez for her helpful discussion and critical reading of the manuscript. We thank Dr Yasufumi Shimizu, Dr Hiroshi Motoyama and Dr Toshihiro Kawamura (Denentoshi ladies' clinic), Dr Kenichi Tatsumi (Umegaoka women's clinic), Dr Susumu Tokuoka (Tokuoka women's clinic), Dr Ryo Matsuoka (Tokyo Hitachi hospital) and Dr Ryukichi Ogawa (Ogawa clinic) for their supporting our study. We also thank Mr Ryuji Nojiri and Mr Yoshitsugu Funatsu (Takinogawa clinic) and Mr Mitsuru Harako (Teikyo University Mizonokuchi hospital) for their technical assistance.

Conflict of interest: none declared.

Funding

This work was supported by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan and Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology.

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Lymphocytes in Endometriosis

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Keywords
B lymphocyte, endometriosis, natural killer cell, T lymphocyte

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Submitted April 12, 2010;
accepted May 6, 2010.

Citation
Osuga Y, Koga K, Hirota Y, Hirata T, Yoshino O, Taketani Y. Lymphocytes in Endometriosis. Am J Reprod Immunol 2011; 65: 1–10

doi:10.1111/j.1600-0897.2010.00887.x

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

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

the follicular phase, where retrograde endometrial cells should be destroyed by NK cells.[72]

Factors Modulate NK Cells Cytotoxic Activity in Endometriosis

Given the impaired NK cells cytotoxic activity in both systemic and local setting, the next interest was the cause of this dysfunction. Studies have demonstrated the presence of inhibiting factors against NK cells in sera of patients with endometriosis.[73,74] As for PF, Oosterlynck et al. found that PF taken from patients with endometriosis had greater suppressive effect on NK cells cytotoxicity compared to PF from healthy women,[75] suggesting the presence of substances which suppress NK cells cytotoxic activity. In this context, it is notable that the level of free IL-12p40, which functions as an antagonist of IL-12, was higher in PF from endometriosis compared to healthy PF.[76] Because IL-12 induces cytotoxicity of NK cells, it is possible that free IL-12p40 is one of factors that exist in the endometriotic PF and suppress NK cells cytotoxic activity.

The next question is the source of these suppressive factors. In this regard, supernatants of cultured endometriosis tissues was found to have suppressive effects on the cytotoxity of NK cells.[77] In addition, supernatants of cultured eutopic endometrial stromal cells taken from women with endometriosis had more inhibitory effect on NK cells cytotoxity than those from without the disease.[78] These findings suggest that substances derived from ectopic and/or eutopic endometrium of women affected with endometriosis have a high potential to suppress NK cell cytotoxic activity, despite these substances have not been fully identified.

Altered NK Cells Inhibitory Receptors in Endometriosis

In order to control their excess cytotoxic activity to the target cell, NK cells are expressing inhibitory receptors. Killer cell inhibitory receptors (KIRs) are representative inhibitory receptors, which recognize class 1 MHC molecules on target cells and control NK cells' cytotoxicity against the target. Expressions of KIR3DL1, KIR2DS1 and KIR2DL1 was significantly elevated in the peritoneal NK cells of women with advanced-stage endometriosis compared with controls.[79] Likewise, the percentage of NK cells that express KIR2DL1 was significantly higher in PF and peripheral blood of women with endometriosis.[80,81] Such increased KIRs expression in NK cells in endometriosis may also explain the decreased NK cells cytotoxity in women affected with endometriosis.

Impact of Surgical/Medical Therapy on of NK Cells Function

Whether surgical and/or medical treatments could alter NK cells activity was an interesting concern. Surgical resection of endometriosis did not improve NK cells activity,[82] which implies that the deficiency in NK cells seen in endometriosis is primary but not secondary. In contrast to surgical treatments, GnRHa treatment increased NK cells activity[83,84] and NK cells number[47] in peripheral blood. Interestingly, low NK cells activity during GnRHa treatment and follow-up period was significantly associated with high recurrence rate.[83] These findings suggest that NK cells dysfunction seems a cause but not a consequence of endometriosis, and hormonal treatments can improve NK cells function and thereby prevent the development of endometriosis.

In a rat model of endometriosis, dienogest, a new progestin for treatment of endometriosis, increased the NK cells activity of PF.[85] Similarly, danazol increased NK cells numbers in peripheral blood and PF.[86] These drugs might also improve NK cells function in women affected with endometriosis, although human data are not currently available.

Taken together, the involvement of NK cells in the pathology of endometriosis can be concluded by following way. Impaired NK cells cytotoxic activity may be a primary cause of development of endometriosis, by allowing endometrial cells escape from their attacks. However, the established disease further modulates NK cells cytotoxic activity, which enhances the disease progress. Hormonal therapy may improve the NK cells function and this may contribute to the control of disease.

Summary

A large volume of evidence indicates that immune cells in lymphoid lineage play significant roles in endometriosis. Generally, it appears that immune activities that are supposed to reject eutopic endometrial cells and/or established endometriotic cells are suppressed in women with endometriosis, although whether this status is cause or result of endometriosis is not still clear. In addition to their

direct effects on rejecting endometriosis, immune cells contribute to the development of endometriosis by inducing inflammatory reactions and proliferation of endometriotic cells. These findings support therapeutic strategies for endometriosis by modulating specific immune cell functions. Further studies in this field are warranted to elucidate the pathogenesis of endometriosis and develop novel approach to improve health care for women confronting endometriosis.

Acknowledgment

This study is partly supported by grants from the Ministry of Health, Labour and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology.

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Reprod Med Biol (2011) 10:1–7
DOI 10.1007/s12522-010-0072-3

REVIEW ARTICLE

The function of bone morphogenetic proteins in the human ovary

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Received: 3 December 2010 / Accepted: 9 December 2010 / Published online: 6 January 2011

Abstract The gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), are of particular importance in ovarian physiology. However, FSH receptors and LH receptors are not expressed until the secondary follicle stage, indicating that initiation of follicular growth is independent of the gonadotropins. Among many intra-ovarian growth factors, many studies have shown that bone morphogenetic proteins (BMPs) play pivotal roles in regulating the early phases of follicular growth. The BMP system induces the gonadotropin system by modulating gonadotropin receptors in early-stage follicles. Interestingly, the BMP system also prevents precocious maturation of the follicle by suppressing luteinization. Signals provoked by the preovulatory LH surge eliminate BMPs, enabling luteinization to progress. Thus, the BMP system and the gonadotropin system seem to cooperate in regulating follicular development, maturation, and luteinization.

Keywords Bone morphogenetic protein (BMP) · Human ovary · Folliculogenesis · Premature ovarian failure (POF)

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Introduction

To advance the study of reproductive medicine, it is necessary to understand the mechanisms of folliculogenesis, oocyte maturation, ovulation, and corpus luteum formation. There is no question that the gonadotropin hormones (follicle-stimulating hormone, FSH, and luteinizing hormone, LH), which are extra-ovarian factors, are important throughout this process. In addition to these extra-ovarian factors, intra-ovarian factors are also essential to ovarian function. In particular, the bone morphogenetic protein (BMP) family has attracted increasing attention recently in this field.

BMP was discovered by Urist et al. [1], as a factor which promotes bone formation. BMPs are structurally classified as members of the transforming growth factor (TGF)-β superfamily. To date, more than 20 members of the BMP family have been identified in various species [2]. In addition to bone, BMPs are expressed in a variety of tissues and regulate growth, differentiation and apoptosis [2, 3]. BMPs expressed in the ovary have been the subject of much research. To understand the role of BMPs in the ovary, we must first examine the role of extra-ovarian factors. During the follicular phase, estrogen production in the ovary is induced by FSH stimulation, while progesterone production is suppressed. However, in-vitro studies have shown that FSH induces estrogen and progesterone production in granulosa cells simultaneously. The reason for this discrepancy between the in-vivo and in-vitro findings has been unclear. Shimasaki et al. reported that in the presence of FSH, BMP-4 and 7, derived from theca cells, augment estrogen production, while suppressing progesterone production in rat granulosa cells. Accordingly, BMPs are recognized as a luteinizing inhibitor in granulosa cells [4]. Moreover, Dong et al. [5] observed

arrest of folliculogenesis at the primary follicle stage in GDF-9 knockout mice. These studies opened a new field of study of the BMP system in the mammalian ovary. In the last decade, numerous studies have been published, and there are many excellent reviews about BMPs in the ovary [6–8]. In this review, we will discuss the function of BMPs, focusing on the effect of BMPs on human granulosa cells.

Expression and processing of BMPs

The BMP cytokines known to be expressed in the ovary are BMP-2, 3, 4, 6, 7, and 15 and GDF-9 [6]. In situ hybridization using rat ovaries revealed that BMP-2 is expressed in granulosa cells, BMP-3, 3b, 4, and 7 are expressed in theca cells, and BMP-6 is expressed in granulosa cells and oocytes. Interestingly, GFD-9 and its closest homolog, BMP-15, also known as GDF-9B, are expressed exclusively in oocytes [9, 10]. In the human ovary the expression pattern of these BMPs is virtually identical with that in rat ovary [11–13] (Table 1). BMPs are translated as large preproproteins composed of a signal peptide, proregion, and mature domain [14, 15]. After removal of the signal peptide, the proproteins undergo dimerization. Dimerized proproteins are cleaved by a proteolytic enzyme to generate the biologically active dimeric mature protein. The proregion is known to be important in the processing of the proprotein. This is evident from mutations in the *BMP15* gene which are associated with premature ovarian failure (POF) phenotype [16, 17]. All of these mutations have been described in the heterozygous state and are located in the part of the gene encoding the proregion. It has been demonstrated that proper post-translational processing of the BMP proprotein is critical to normal female fertility [18, 19].

BMP signaling

BMPs and GDFs utilize Type I and II receptors. The type I receptors for BMPs are ALK-2 (ActR-I), ALK-3 (BMPR-IB), and ALK-6 (BMPR-IA), and the type II receptors are BMPR-II, ActR-II, ActR-IIB. GDF, activins, and TGF-β utilize ALK-4 (ActR-IB), ALK-5 (TβR-I), and ALK-7 as Type I receptors and BMPR-II, ActR-II, ActR-IIB, and TβR-II as type II receptors. Upon binding of the ligand, the type II receptor transphosphorylates the type I receptor, after transphosphorylation of a set of intracellular substrate signaling proteins, receptor-regulated Smads (R-Smads). These R-Smads include Smad-1, 2, 3, 5, and 8. Activated R-Smads interact with Smad-4, then translocate to the nucleus to regulate the expression of target genes [20]. Activation of receptors by BMP ligands phosphorylates R-Smad-1/5/8, and activation of receptors by GDFs, activins, and TGF-β result in phosphorylation of R-Smad-2/3 [21]. Type I and II receptors are expressed in oocytes, granulosa cells, and theca cells [6].

The function of BMPs in the ovary

In this section, we summarize the role of BMPs during gonadal development and during each stage in the cycling ovary (folliculogenesis, ovulation, and luteinization).

Gonadogenesis

BMPs are involved in gonadal development during embryogenesis. This has been demonstrated in BMP-4 knockout mice, in which a defect in primordial germ cell (PGC) formation was observed. Even heterozygous mutant mice for BMP-4 had significantly fewer PGC [22]. A severe defect of PGC formation has also been observed in BMP-2, BMP-8b, and BMP-7 null mice [23–25]. Moreover, mice lacking ALK-2, Smad-1, and Smad-5, which are modulators of BMP signals, had fewer PGC [26–28]. Therefore, the BMP system is important in ovarian development by maintaining the number of PGC.

Folliculogenesis

Folliculogenesis involves a series of sequential steps in which a growing follicle develops to the ovulatory stage. The major steps in folliculogenesis include the primordial/primary transition, the primary/secondary transition, and selection of dominant follicles. Folliculogenesis is accompanied by a precise spatial and temporal regulation of BMP expression.

Table 1 The location and biological actions of BMP molecules in the human granulosa cell

	Location	FSHR	LHR	Inhibin α	Inhibin/activin βA	Inhibin/activin βB	AMH
BMP-2	Granulosa cell	↑	↓	→	↑	↑	↑
BMP-6	Granulosa cell oocyte	↑	↓	→	↑	↑	↑
BMP-7	Theca cell	↑	↓	→	↑	↑	↑
BMP-15	Oocyte	↑	↓	→	↑	↑	↑

Primordial follicle stage

In the early stages of folliculogenesis, in which follicles do not express FSH receptor (FSHR) [29], the mechanism of follicular growth is poorly understood. Although activins are known to be important factors in follicle growth in the early stages [30], the regulation of activins in the follicles at this stage is not well understood. Our study revealed that oocytes of primordial follicles strongly express BMP-6 protein [11]. The pattern of expression suggested that BMP-6 might be involved in human folliculogenesis. Moreover, in-vitro experiments demonstrated that BMP-6 increased the mRNA expression of inhibin/activin β subunits in granulosa cells [11]. It is possible that an oocyte-derived factor, BMP-6, might be an inducer of activins, regulating folliculogenesis in the primordial stages.

Primary follicle to secondary follicle transition

Primary follicles express BMP-6, BMP-15, and GDF-9 in oocytes [6, 10]. Recent study has shown that, although litter size is reduced in BMP-6 null mice compared with controls, folliculogenesis is not impaired [31]. In humans, mutation of the *GDF-9* [32] or *BMP-15* [16, 17] genes results in arrest of folliculogenesis at the primary stage. Thus, we will focus on the role of GDF-9 and BMP-15 during this stage of folliculogenesis.

Studies in GDF-9-deficient mice have demonstrated that folliculogenesis is blocked at the primary/secondary follicle transition stage [5]. The granulosa cells of primary follicles in GDF-9 null ovaries showed decreased expression of proliferation markers compared with control ovaries, suggesting that the transition between primary and secondary follicles is mediated in part through GDF-9-induced granulosa cell proliferation. In addition, follicles from GDF-9 null ovaries lack supporting theca cells [5]. Therefore, GDF-9 regulates proliferation or differentiation of granulosa and theca cells [33, 34]. Interestingly, inhibin A is upregulated in the granulosa cells of primary follicles in GDF-9 null ovaries [5], and GDF-9/inhibin A double-null ovaries contain follicles beyond the primary stage [34].

Several groups have confirmed that BMPs, including GDF-9, can induce inhibin/activin β subunit expression in human granulosa cells [11–13, 35]. In the early stage of folliculogenesis, normal granulosa cells express only a small amount of inhibin α. Therefore, it is expected that activin A and B, which strongly induce granulosa cell proliferation [36], are preferentially produced. Inhibins can suppress the effects of activins and BMPs at the receptor level [37, 38]. In the early stages of folliculogenesis, the finely tuned balance between inhibins, activins, and BMPs may be important [34].

It has long been recognized that the absence of one X chromosome in patients with gonadal dysgenesis (i.e. Turner syndrome) is observed in a significant proportion of women presenting with primary amenorrhea because of ovarian failure [39]. Thus, regions of the X-chromosome were thought to contain ovarian determinant genes [40]. Because the BMP-15 gene is located on the X-chromosome [9], and several cases of BMP-15 mutation have been reported in POF patients, BMP15 may be the first identified ovarian determining gene on the X chromosome [40]. In monoovulatory species, for example ewes, mutations of the BMP-15 gene lead to arrest of folliculogenesis [41]. On the other hand, in mice, polyovulatory animals, deletion of BMP-15 had no effect on folliculogenesis [42], suggesting that the relative importance of BMP-15 during follicular development differs among species. An in-vitro transfection system of 293 human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cell lines demonstrated that recombinant human and sheep BMP-15 are processed in this system, although mouse BMP-15 is not produced [18]. Thus, it has been proposed that mouse BMP-15 is not produced during folliculogenesis. As expected, in vivo, mouse oocytes barely express BMP-15 protein during folliculogenesis [43]. As described above, GDF-9 mutation or deletion results in arrested folliculogenesis in mice, humans, and sheep. In contrast with recombinant mouse BMP-15, recombinant mouse GDF-9 produced by in-vitro cell transfection is readily processed and secreted as its mature GDF-9 protein [44]. Likewise, recombinant human GDF-9 [45] and recombinant ovine GDF-9 [46] are also readily processed.

Secondary follicle and antral follicle

As primary follicles progress to the secondary stage, theca cell layers develop and follicles are served by one or two arterioles, terminating in an anastomotic network just outside the basal lamina [47]. The physiological importance of this event is emphasized by the fact that the follicle gains access to factors circulating in the blood, for example FSH. At this time, granulosa cells develop the ability to respond to FSH, because FSHR is expressed. Consequently, FSH stimulates the follicle to develop to the antral stage. Thus, it is important to elucidate the mechanism responsible for regulation of FSHR expression. At this stage, follicles express BMP-6, BMP-15, and GDF-9 in oocytes, BMP-2 and BMP-6 in granulosa cells, and BMP-4 and BMP-7 in theca cells [6]. Several factors, for example activins [48], FSH [49], cyclic adenosine monophosphate (cAMP) stimulants, and cAMP analogs [50], are known to modulate the synthesis of FSHR mRNA in granulosa cells. We found that BMP-2, BMP-6, BMP-7, and BMP-15 induced FSHR mRNA expression in human granulosa cells

(Table 1) [11–13] (partly unpublished results), which suggested that BMPs enhance folliculogenesis by promoting the expression of FSHR. Our observation that BMPs increased mRNA levels not only of FSHR, but also of inhibin/activin β subunits, led us to examine the possibility that the increase in FSHR mRNA might be mediated by an increase in activin protein synthesis. However, SB-431542, which inhibits activin signaling but does not affect BMP-7 signaling, failed to suppress BMP-7-induced FSHR mRNA expression, suggesting that BMPs and activin-A induce FSHR expression in a different pathway [12]. Orisaka et al. [51] reported that knockdown of GDF-9 in rat follicles suppresses FSH receptor expression in granulosa cells, suggesting that GDF-9 is a positive regulator of FSHR. Moreover, there are reports that BMPs inhibit the FSH receptor [52, 53]. It is likely that the effect of BMPs on FSHR expression is not uniform and may be species-specific.

We also found that BMP-2, BMP-6, BMP-7, and BMP-15 inhibited LH receptor (LHR) expression in human granulosa cells (Table 1) [11–13] (partly unpublished results). LHR is involved in luteinization of antral granulosa cells [54, 55]. During folliculogenesis, it is important to prevent granulosa cells from undergoing precocious luteinization. Pangas et al. [56] reported that in an ovarian conditional knockout mice for Smad-4, which is a common SMAD for TGF-β superfamily signaling, granulosa cells underwent premature luteinization and expressed higher levels of LHR and lower levels of FSHR compared with those from control mice. During the follicular phase, it is thought that BMPs regulate folliculogenesis by suppressing LHR expression in granulosa cells, whereas FSH induces LHR expression in these cells. These effects of BMP may be important in controlling the timing of follicle development during the period of endometrial receptivity for embryo implantation.

Selection of dominant follicles

Recruitment, or engagement of a cohort of follicles into further growth, does not guarantee ovulation, because growing follicles are vulnerable to atresia [47]. Healthy follicles will be selected during the subsequent process. In the human ovary we found that BMP-6 protein was strongly expressed in granulosa cells of healthy antral follicles [11]. In contrast, BMP-6 protein was only weakly expressed in granulosa cells of atretic follicles. This expression pattern of BMP-6 observed in our study is consistent with the finding that inhibin/activin β subunits are expressed in healthy follicles, but not in the similarly sized atretic follicles [30]. These results indicate that BMP-6 might be an important factor in selection of the follicle. In addition, in our in-vitro experiment, BMP-6 increased mRNA expression of inhibin/activin β subunits in granulosa cells. Serum FSH concentration decreases in the latter half of the follicular phase. Therefore, the sensitivity of follicles to FSH during this period is critical and determines whether the follicle becomes atretic or dominant [29]. In view of our finding that BMPs increased the expression of FSHR, follicles with high BMP protein expression may be more likely to survive the decrease in serum FSH, enabling these follicles to progress to the dominant stage.

Another intra-ovarian factor, anti-mullerian hormone (AMH), is produced by granulosa cells of the growing antral follicles in the human ovary [57]. Accelerated depletion of follicles is observed in female mice lacking AMH. This effect is a reflection of the inhibitory effect of AMH on recruitment of primordial follicles into the growing pool [58]. Although it is reported that FSH and estradiol down-regulate AMH expression in granulosa cells [59], no stimulants of AMH expression have yet been identified. We found that BMPs (BMP-2, 6, 7, and 15) increased expression of AMH [11] (unpublished results). Thus, we hypothesize that BMPs in the healthy growing follicles up-regulate AMH expression, which, in turn, suppresses growth of the surrounding primordial follicles, thereby preserving the ovarian reserve (Fig. 1).

Ovulation

As described above, no defect in follicular development was observed in BMP-15 null mice; they were, however, still sub-fertile. The ovulation rates of these mice are reduced because of defects in the ovulation process [42]. We found that the functional BMP-15 mature protein is barely detectable in mice oocytes, except for those in the

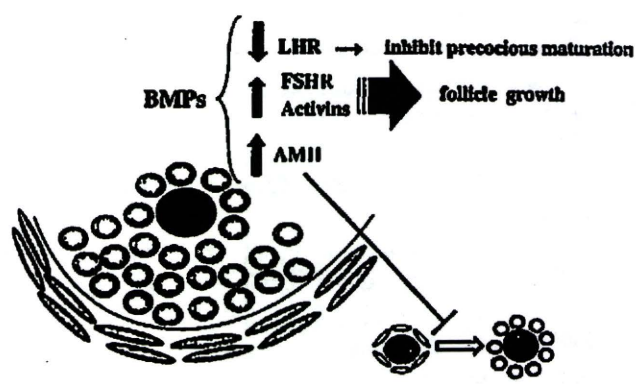

Fig. 1 The function of BMPs in the regulation of folliculogenesis. BMPs induce activins and FSHR, which promote follicle development. On the other hand, BMPs inhibit LHR, preventing granulosa cells from precocious luteinization. BMPs also induce AMH, which suppresses the recruitment of the surrounding primordial follicles

preovulatory follicles after LH/hCG-induced meiotic maturation [43]. Furthermore, mature mouse BMP-15, processed at the time of ovulation, might induce cumulus expansion [43]. In addition to BMP-15, GDF-9 is known to be a cumulus expansion-enabling factor (CEEF) [60]. Recently, EGF-like growth factors have been shown to be important for ovulation. Su et al. [61] reported that GDF-9 and/or BMP-15 is/are required for EGF receptor expression by cumulus cells.

Luteinization

After ovulation, residual granulosa cells undergo luteinization and the follicle is transformed to corpus luteum. Progesterone, produced by luteinized granulosa cells, sustains the initiation and maintenance of pregnancy. If embryo implantation does not occur, the corpus luteum stops synthesizing progesterone and degenerates. LH/hCG has a central role in the maintenance of the function of corpus luteum [62]. StAR, which mediates translocation of cholesterol from the outer to the inner mitochondrial membrane, is one of the rate-limiting factors in progesterone production [63]. Any perturbation of these events, for example luteinization failure, can impair reproduction [64]. We confirmed that BMPs (BMP-2, 6, 7, and 15) suppressed the gene expression of LHR and StAR in human granulosa cells, indicating that BMP might be an anti-luteinizing factor in the ovary [11–13] (partly unpublished results). Because BMP-6 and BMP-15 are derived from oocytes, it is reasonable to conclude that release of the oocyte at ovulation results in spontaneous luteinization of granulosa cells [6]. We found that BMP-2 mRNA was expressed in human granulosa cells of antral follicles, whereas its expression was almost undetectable in the corpus luteum [13]. The vanishing of BMP-2 expression in the corpus luteum might facilitate luteinization. It should also be noted that hCG induced expression of BMP and activin membrane-bound inhibitor (BAMBI) in granulosa cells [13]. BAMBI inhibits dimerization of type-I receptors, thereby inhibiting BMP signaling [65]. Therefore, the BMP system, which serves as an inhibitor of luteinization, is down-regulated after ovulation.

BMPs play crucial roles in controlling folliculogenesis and luteinization in the human ovary. During the gonadotropin-independent phase, BMPs are expressed in follicles and contribute to the initiation of follicular growth by inducing activin expression in granulosa cells. With growth of the follicle, suppression of LHR by BMPs prevents granulosa cells from precocious luteinization, which is essential for follicle growth. In turn, BMPs increase AMH expression to suppress recruitment of the surrounding primordial follicles, thereby preserving the ovarian reserve. Because BMPs induce FSHR expression in human granulosa cells, sufficient FSHR enables follicles to fully respond to serum FSH to support follicular growth. With continuous stimulation of FSH, LHR is dramatically induced through FSHR signaling to respond to the LH surge [47], and eventually induce ovulation (Fig. 1). After ovulation, LH/hCG suppresses BMPs directly or indirectly to maintain corpus luteum function. In conclusion, complicated ovarian physiology is achieved through harmonious cooperation of the BMP and gonadotropin systems, throughout the ovarian cycle.

Acknowledgments We thank Dr Heather M. Martinez for her helpful discussion and critical reading of the manuscript. This work was supported in part by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology.

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