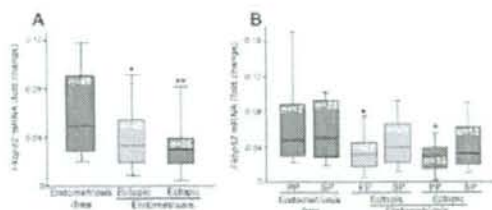

Figure 7. Reduced Fkbp52 mRNA levels in eutopic and ectopic human endometria. Fkbp52 mRNA levels in endometriosis-free, eutopic and ectopic human endometrium (**A**), and their relation to the phase of the menstrual cycle (**B**). Boxes represent the distance between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes denote the median. Whiskers represent the 10th percentile at the lower limit and the 90th percentile at the upper limit. Ectopic endometrium is derived from ovarian endometriosis. **A:** $^*P < 0.05$ and $^{**}P < 0.01$ compared with control endometrium. **B:** $^*P < 0.05$ compared with endometriosis-free endometrium of the same menstrual phase; Mann-Whitney *U*-test.

endometria compared to endometriosis-free endometria, the signal intensity was even lower in ectopic lesions (Figure 8, A). As expected, PR immunostaining in endometriosis-free PP endometria was higher than SP, but the signal intensity was remarkably low in eutopic endometria or in ectopic lesions regardless of the cycle stage (Figure 8B).

COX-2 expression was low in endometriosis-free endometria in both PP and SP, but appeared higher in eutopic endometria primarily at SP. Ectopic lesions also had higher levels of COX-2 (Figure 8C). In contrast, VEGF immunostaining appeared to be of similar intensity in control, eutopic endometria, and ectopic lesions regardless of the cycle stage (Figure 8D). These results show that our genetic mouse model of endometriosis recapitulates similar read-outs as those found in human endometriosis, specifically reduced levels of FKBP52 and PR expression and increased levels of COX-2.

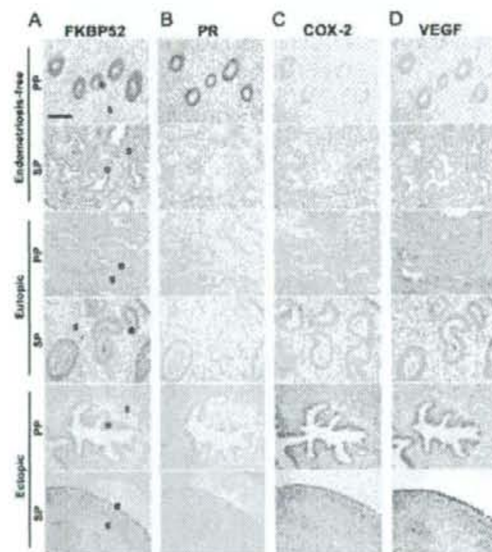

Figure 8. Reduced PR expression with increased COX-2 and VEGF expression is observed in human ectopic endometria. Representative immunostaining of FKBP52 (**A**), PR (**B**), COX-2 (**C**), and VEGF (**D**) in endometriosis-free, eutopic and ectopic human endometria at proliferative (PP) and secretory (SP) phases. Serial sections of the same sample are displayed in each horizontal row. *e*, epithelium; *s*, stroma. Scale bar = 200 μm.

Discussion

Progestin therapy is commonly used to treat endometriosis-related symptoms including pain. In fact, progestin treatment is considered one of the most useful therapies for alleviating endometriosis-related pain. Still, some women are unresponsive to the treatment.[3,6] Therefore, it has long been suspected that some patients with endometriosis have P_4 resistance. The recent development of genetic and molecular approaches allows for the examination of underlying causes of P_4 resistance. Effects of P_4 are primarily mediated by PR, which has two isoforms, PR-A and PR-B. Studies in PR-A and PR-B null mice have shown that these two isoforms are expressed in a cell-specific manner and function differently.[50,51] PR-A is essential for normal ovarian and uterine functions including ovulation, implantation and decidualization.[50] In contrast, PR-B is critical for normal mammary gland development.[51] Given that stromal cells of human endometria contain predominantly PR-A,[52] it is assumed that functional FKBP52-PR-A signaling is crucial for normal endometrial events. PR-A expression is substantially decreased in endometriotic lesions compared with eutopic endometria with non-detectable PR-B expression.[11] In our present study, we also found reduced PR expression in endometriosis of both mice and humans. These findings suggest that decreased PR expression is one reason for P_4 resistance. However, our results of reduced FKBP52 expression in ectopic lesions also suggest that FKBP52 deficiency contributes to attenuated PR signaling in endometriosis.

Molecular and cellular interactions between the ectopic endometria and peritoneal surface are crucial for the development of ectopic lesions. Existing endometriotic models are heterologous, using human endometrial tissues transplanted in immunocompromised mice. Our homologous mouse model provides the opportunity to study immunological aspects of endometriosis. Because immunophilin FKBP52 has properties of an immunological factor, our homologous model could also be a valuable system to study the immunological functions of FKBP52 in endometriosis.

In our *Fkbp52* null mouse model, we transplanted minced endometria from donor mice into the peritoneal cavity of recipient mice to simulate the aspect of human endometriosis that results from retrograde endometrial shedding into the peritoneal cavity during menstruation. The ability of transplanted endometrial tissues to form ectopic lesions depends not only on the state of the donor endometrium, but also on the peritoneal environment of the recipient. We evaluated the effects of loss of *Fkbp52* on cooperation of endometrial tissues with peritoneal receptivity. Our results show that loss of *Fkbp52* in both the donor and recipient mice is required to

significantly increase the number and growth of lesions, suggesting that FKBP52 deficiency in both the donor endometrium and recipient peritoneal cavity is involved for optimal development of endometriosis. It is not yet understood why and how the lack of FKBP52 in the peritoneum bolsters endometriotic growth, but it is possible that FKBP52 has other functions independent of PR.[18,53–55]

Many studies have shown anti-proliferative roles of PR in the uterus.[7,56] In fact, a previous study using *PR* null mice found that estrogen is a primary factor that determines the size of ectopic lesions.[8] This study also found that the anti-proliferative effects of P_4 on estrogen-mediated lesion growth are mediated by PR. Our findings are consistent with this study, because deletion of *Fkbp52* that reduces P_4-PR signaling, leads to enhanced endometriotic growth with increased proliferation. However, the novelty of our present study is that we provide genetic evidence in mice that even a reduced responsiveness to P_4-PR signaling encourages endometriotic growth; the complete absence of uterine P_4-PR signaling is not an absolute requirement.

Endometrial angiogenesis is normally regulated by estrogen and P_4. Estrogen stimulates proliferation of human endometrial endothelial cells *in vitro*,[57,58] but inhibits angiogenesis in mouse uteri *in vivo*.[30] In contrast, P_4 inhibits estrogen-induced proliferation of human endometrial endothelial cells *in vitro*,[57,58] but stimulates angiogenesis in mouse endometria *in vivo*.[30] These findings suggest that uterine endothelial cells respond to ovarian steroids differently in extrauterine sites, and/or that heterogeneous cell types of the uterus respond differently to these hormones in a dynamic manner. Here we present genetic evidence that more Flk1-positive blood vessels enter *Fkbp52* null ectopic lesions than WT lesions. Because these lesions are mainly composed of stromal cells with minimal contribution from the epithelial component, we propose that this change in cell composition may alter the angiogenic response to ovarian hormones. This may explain then why reduced P_4 responsiveness due to FKBP52 deficiency promotes migration of endothelial cells into endometriotic lesions.

Our study also used *Flk1*$^{lacZ+/-}$ mice to explore donor versus recipient induced angiogenesis. We found that ectopic lesions recruited blood vessels for angiogenesis from the recipient. We have previously shown that while estrogen attenuates angiogenesis in the mouse uterus, P_4 promotes this process in the uterus.[30] Here we found that while FKBP52 deficiency confers reduced angiogenesis in eutopic endometria due to enhanced estrogenic influence resulting from reduced P_4-PR signaling, loss of FKBP52 enhances angiogenesis in ectopic lesions. The question then is why does the loss of *Fkbp52*, associated with decreased P_4-PR signaling, promote angiogenesis in ectopic lesions? One possibility is that there is an angiogenic switch in endometrial tissues with regard to steroid hormone responsiveness depending on the site (eutopic versus ectopic). Further studies are warranted to address this issue.

The establishment of a new blood supply is essential for survival of endometriotic lesions and development of endometriosis. VEGF is a heparin-binding angiogenic growth factor, the most potent mediator of angiogenesis.[58] It is a strong endothelial mitogen/survival factor and an inducer of vascular permeability. VEGF binds to a family of tyrosine kinase receptors, particularly Flt1 (VEGFR1) and Flk1 (VEGFR2). Flk1 is the major transducer of VEGF signals that induce migration and proliferation of endothelial cells.[59] To date, evidence indicates that VEGF is involved in the pathophysiology of endometriosis.[28,46,47] We show here that VEGF is expressed in ectopic lesions in our mouse model of endometriosis, and that VEGF expression is increased in *Fkbp52* null ectopic lesions compared to WT. There is evidence that ER along with its cofactors binds to the VEGF promoter to promote its transcription in human endometrial cells.[60] Moreover, estrogen induces VEGF secretion in human endometrial stromal cells, an induction that is suppressed by progestin.[26] We propose that in the absence of FKBP52, P_4-PR signaling is reduced and P_4 cannot inhibit estrogen-induced VEGF secretion, resulting in excess VEGF in ectopic lesions and increased angiogenesis.

Estrogen is also known to up-regulate expression of several cytokines in endometriosis. MCP-1 and RANTES are well-known chemokines whose concentrations are high in peritoneal fluid of women with endometriosis[61,62] and whose secretions are promoted by estrogen.[34,35] In human endometriotic stromal cells, inflammatory stimuli induce secretion of these chemokines, and estrogen enhances their secretion.[34,35] *In vitro* studies in human endometrial stromal cells have shown that adding P_4 does not suppress estrogen-induced expression of these chemokines,[34,35] suggesting P_4 resistance in endometriotic cells. Our present findings that *Fkbp52* null mice with endometriotic lesions have enhanced levels of chemokines in their peritoneal fluids corroborates well with reduced PR and FKBP52 expression and unchanged ERα expression in ectopic lesions.

COX-2, an inducible enzyme that synthesizes prostaglandins, is known to play an important role in both immunological and angiogenic responses during endometriosis.[63–65] Because inflammatory stimuli, growth factors, and cytokines induce COX-2, it is possible that the immune environment imposed by the developing endometriotic lesions in the face of reduced P_4-PR signaling in the absence of FKBP52 induces COX-2 expression, which also could contributes to up-regulation of VEGF expression.

There is very limited information linking FKBP52 with disease processes. To our knowledge, this is the first report showing that down-regulation of FKBP52 is associated with a human disease. In contrast, FKBP52 overexpression is observed in breast and prostate cancer.[66–68] In fact, the immunosuppressant drug FK506, which binds immunophilins FKBP51 and FKBP52, inhibits androgen receptor activity in prostate cancer cells.[67] Likely, FK506 is considered to affect other steroid receptor activities.[69,70] Although there is no report that FK506 influences the incidence and development of endometriosis, it may be prudent to address this issue, because endometriosis is considered an immunological disorder.[71,72]

In conclusion, our novel mouse model of endometriosis shows that the deletion of FKBP52 promotes the growth of endometriotic lesions with increased angiogenesis recruited from the recipient. Moreover, we demonstrate that FKBP52 expression is decreased in eutopic and ectopic endometria of women with endometriosis compared with endometria of women without endometriosis. After our genetic studies on the role of FKBP52 in uterine biology and pregnancy in mice were reported,[16,17] a study reported that FKBP52 levels are reduced in eutopic endometria of baboons subjected to experimental endometriosis.[73] Collectively, these findings suggest that endometriosis induced by FKBP52 deficiency is a conserved phenomenon and FKBP52 is not only physiologically but also pathologically critical molecule for female reproductive functions.

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Novel Therapeutic Strategies for Endometriosis: A Pathophysiological Perspective

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Key Words
Endometriosis · Pathophysiology · GnRH analogues · T-cell immune response

Abstract
It has previously been considered that the therapeutic effects of gonadotropin-releasing hormone (GnRH) analogues on endometriosis could be explained by the suppression of estrogen levels due to pituitary downregulation; however, recent research on the pathogenesis of endometriosis suggests that these effects may be exerted by multiple mechanisms. These include the inhibition of ovulation, which results in a reduction in the exposure of endometriotic lesions to midkine, a growth factor present in ovarian follicular fluid that is thought to be involved in the proliferation of endometriotic cells and development of endometriosis. Also the inhibition of bleeding induced by GnRH analogue therapy can reduce the exposure of endometriotic lesions to thrombin, which is produced in the process of coagulation. Thrombin and its specific receptor, protease-activated receptor 1 (PAR1), are important factors in inflammation and cell proliferation and may be involved in the pathophysiology of endometriosis. Abnormal uterine contractions have been observed in women with endometriosis, and it is thought that the resulting mechanical stretch might stimulate the production of pro-inflammatory mediators, such as interleukin-8 (IL-8), as has been observed in studies with endometrial stromal cell cultures. The inhibition of uterine contractions by GnRH analogue therapy, in particular during menstruation, would block the mechanical stress on the endometrium and ultimately inhibit the development of endometriosis. Alongside the recent revolutionary progress in T-cell immunology, it has been argued that the development of endometriosis is associated with an abnormal T-cell function, and the existence of a 'T-cell immune network' is hypothesized to explain the etiology of the disease.

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Introduction

Endometriosis, the presence of endometrium-like glands and stroma outside the uterus, is a common, poorly understood, and extremely debilitating benign gynecological condition affecting mainly women of reproductive age. The etiology and pathophysiology of endometriosis are not well understood but continue to be investigated. Currently, no cure exists for the disease and treatment is directed to medical suppression, surgical excision, and alleviation of symptoms. Optimal therapeutic strategies are still the subject of considerable research.

This paper will provide a pathophysiological perspective on novel therapeutic targets in endometriosis and the role and underlying mechanism of action of gonadotropin-releasing hormone (GnRH) analogues in this disease

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based on data from the latest preclinical studies. It will also review recent advances in our understanding of the role of T cells in the development of endometriosis.

Mechanism of Action of GnRH Analogues

It has previously been considered that the therapeutic effect of GnRH analogues on endometriosis can be explained by the suppression of estrogen levels due to pituitary downregulation; however, recent investigations into the pathogenesis of endometriosis suggest that the effect of GnRH analogues on endometriosis is exerted by multiple mechanisms. When considering possible alternative therapeutic mechanisms of action of GnRH analogues in endometriosis, other than hypoestrogenism, it is important to note that during GnRH analogue therapy the following phenomena, generally observed during normal menstrual cycles, are not seen:

- Retrograde menstruation
- Ovulation
- Focal bleeding
- Uterine contraction

These phenomena may provide clues to the possible underlying pathophysiological effects of these agents.

Retrograde Menstruation

Retrograde menstruation describes retrograde flow of endometrial tissue through the fallopian tubes into the peritoneal cavity. A study was conducted by Bulletti and colleagues to evaluate differences between patients with and without eutopic endometrium in the recurrence of ectopic endometriotic implants [1]. Endometrial ablation was performed on 14 women out of a total of 28 who were treated laparoscopically for endometriosis and recurrence of the disease was evaluated 24 months later. It was found that no recurrence occurred in those who had undergone laparoscopy plus endometrial ablation, whereas 9 out of 14 patients exhibited recurrence in the laparoscopy-only group ($p < 0.001$). These findings support a role for eutopic endometrium in the recurrence of endometriosis through tubal dissemination of endometrial debris and implantation of endometrial cells into the abdomen. In addition, they suggest that the absence of menstruation, as it occurs during GnRH analogue therapy, inhibits the development of endometriosis.

Anovulation

Although retrograde menstruation is an important factor in the development of endometriosis, it is not the whole story since it is known to occur in many women, but not all of them go on to develop endometriosis. It has therefore been suggested that the eutopic endometrium and the peritoneal environment of women with endometriosis may have different pathophysiological and biochemical properties compared with those of women without endometriosis.

Preovulatory follicles have a large antrum filled with follicular fluid that contains many bioactive substances. During ovulation, follicular fluid runs out of the follicle and diffuses into the peritoneal fluid. This fluid has an important role in nurturing endometriotic lesions and contains a range of cytokines and other growth factors, including the protein midkine, which has been suggested to be involved in the development of endometriosis.

Midkine is a basic, low-molecular-weight, nonglycosylated protein that is a member of the heparin-binding growth factor family and has been identified as the product of a retinoic acid-responsive gene. Although its expression is restricted to certain tissues in the adult, it is strongly induced during oncogenesis, inflammation and tissue repair. Midkine is known to have pleiotropic effects, including the induction of cell proliferation, cell migration, angiogenesis and fibrinolysis. Interestingly, midkine is known to be present at high concentrations in follicular fluid and is suggested to play important roles in folliculogenesis [2].

A study has been undertaken to compare the concentrations of midkine in the peritoneal fluid of women without or with endometriosis and those receiving GnRH analogue treatment [3]. Using a specific enzyme immunoassay, it was observed that midkine concentrations in the peritoneal fluid of women with advanced endometriosis (stages II, III and IV; r-ASRM score ≥ 6) were significantly higher (median: 1.21 ng/ml) than those of women without endometriosis or with stage I endometriosis (r-ASRM score ≤ 5; 0.96 ng/ml, $p < 0.05$). In contrast, the midkine concentrations observed in the peritoneal fluid of women undergoing GnRH agonist therapy were significantly lower than those of the other groups ($p < 0.001$) (fig. 1).

When midkine concentrations in peritoneal fluid were compared according to the menstrual phase, those in the luteal phase were significantly higher than those in the follicular phase, irrespective of the severity of endometriosis [3] (fig. 2). One possible explanation of this is that during ovulation, follicular fluid, which we know contains high concentrations of midkine, runs out of the follicle and diffuses into the peritoneal fluid, which may partly explain the higher concentrations in peritoneal fluid during the luteal phase compared with the follicular phase.

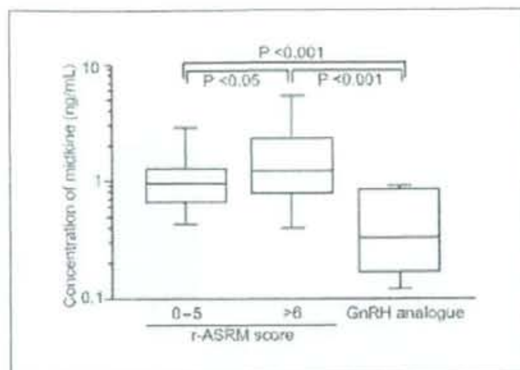

Fig. 1. Midkine concentrations in the peritoneal fluid of women without or with endometriosis and those receiving GnRH agonist treatment [3].

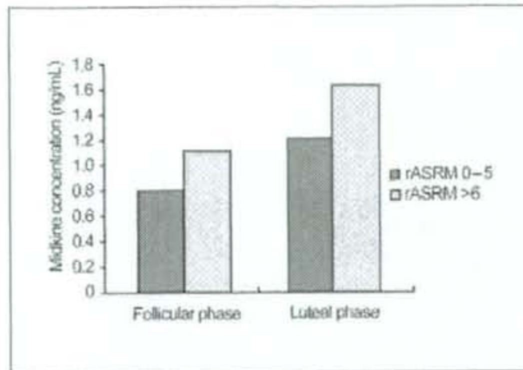

Fig. 2. Midkine concentrations in peritoneal fluid during the follicular and luteal phases of the menstrual cycle [3]. Median midkine concentration in the follicular fluid: 265 ng/ml.

The effect of midkine on the proliferation of cultured endometriotic stromal cells (ESC) has also been examined using a BrdU incorporation assay [3]. ESC were treated with midkine at different concentrations for 24 h. BrdU incorporation into DNA was significantly increased by midkine at concentrations of 100 and 1,000 ng/ml.

Overall, these results suggest that midkine may play a role in the stimulation of endometriotic cell proliferation during the development of endometriosis. In addition, it can be speculated that the inhibition of ovulation that results with GnRH analogue therapy would also block the exposure of endometriotic lesions to midkine, and subsequently suppress the proliferation of endometriotic cells and the development of endometriosis.

Absence of Focal Bleeding

Bleeding often occurs at the site of endometriotic lesions and blood clots can often be observed in endometrial cysts. Thrombin, which is produced in the process of coagulation, and its specific receptor, protease-activated receptor 1 (PAR1), are important factors in inflammation and cell proliferation and are thought to be involved in the pathophysiology of endometriosis. It has also been suggested that the inhibition of bleeding caused by GnRH analogue therapy can reduce the exposure of endometriotic lesions to thrombin and thus suppress the development of endometriosis.

PAR is a member of a group of seven transmembrane G protein-coupled receptors. As a result of the activation of PARs, proteases such as thrombin and trypsin cleave at a point within the extracellular domain and thereby unmask a new amino terminus that functions as a tethered ligand to bind back to the receptor. This property of PARs has allowed researchers to perform studies using specific agonists comprised of the amino terminal peptides to study individual PARs. To date, four PARs have been identified and characterized. PAR1, PAR3 and PAR4 are activated by thrombin; PAR2 is activated by trypsin, mast-cell tryptase, and neutrophil serine proteases. PAR1 is a typical thrombin-activating receptor and can be activated by the PAR-1 agonist peptide SFLLRN (Ser-Phe-Leu-Leu-Arg-Asp).

In view of the emerging concept of thrombin and its specific receptor, PAR1, as important players in inflammation and cell proliferation, studies have been performed to investigate whether thrombin and PAR1 might be involved in the pathophysiology of endometriosis, using a primary cell culture system of endometriotic tissues [4].

PAR1 expression was detected in cultured ESC. It was also observed that the addition of thrombin and SFLLRN to ESC cultures for 2 h significantly increased the gene expression of interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and cyclooxygenase-2 (COX-2), important proinflammatory mediators in endometriosis. Thrombin also increased the expression of tissue factor (TF), an upstream molecule of the coagulation cascade, in ESC. Furthermore, thrombin and SFLLRN were found to stimulate the proliferation of ESC.

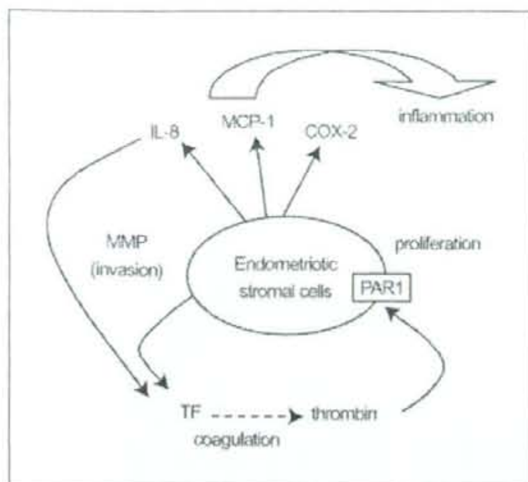

Fig. 3. The possible mechanism of action of thrombin in the development of endometriosis based on studies undertaken in endometriotic stromal cells.

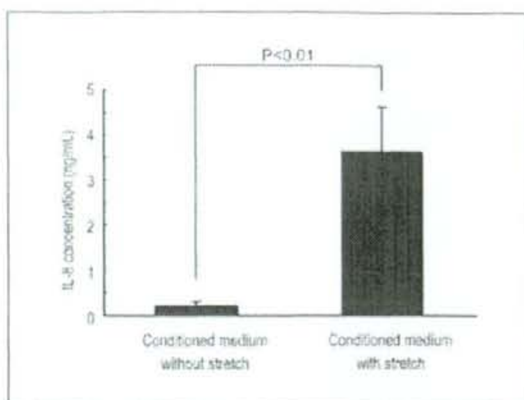

Fig. 4. Effect of conditioned medium obtained from endometrial stromal cells (EmSC) cultured with and without stretch on IL-8 secretion from EmSC [5]. Reproduced with permission from Harada et al. [5] .

Matrix metalloproteinases (MMPs) are essential for extracellular matrix remodeling and may contribute to the development of endometriosis. Production of pro-MMP-9 and MMP-2 were also increased by thrombin in ESC. In this case, addition of the agonist SFLLRN did not produce a similar increase, suggesting the effect is not mediated by PAR1.

Based on the studies undertaken in ESC, the potential role of thrombin in endometriosis is summarized in figure 3. Thrombin, produced in the process of coagulation, activates PAR1 and stimulates the expression of MCP-1, IL-8, COX-2 and TF in endometriotic cells. Thrombin also stimulates the proliferation of endometriotic cells via PAR1 and increases the production of MMP via a different mechanism. The increased TF levels may further activate the coagulation cascade to produce thrombin, which subsequently enhances the production of the pro-inflammatory molecules, in a positive feedback loop, ultimately stimulating the development of endometriosis.

Thus, it is speculated that the inhibition of bleeding elicited by GnRH analogues would also block the exposure of endometriotic lesions to thrombin, and subsequently suppress the inflammation, proliferation and invasion of endometriotic cells. As a result, the development of endometriosis would be inhibited.

Absence of Uterine Contractions

Abnormal uterine contractions have been observed in women with endometriosis. It is believed that mechanical stretch induces biochemical changes in cells, and thus it has been postulated that the mechanical stretch exerted by uterine movement might stimulate the production of biochemical mediators in endometrial cells.

To investigate the effects of mechanical stretch in the endometrium, EmSC were cultured on flexible-bottomed culture plates, and cyclic stretch (25% elongation) was applied in serum-free conditions at a rate of two cycles per minute using a computer-operated cell tension system [5]. Cyclic stretch was found to increase the gene expression of IL-8 in EmSC and the secretion of IL-8 from EmSC.

Subsequent experiments also revealed some interesting characteristics. The authors investigated whether stretch-induced IL-8 expression in EmSC was regulated by autocrine/paracrine factors. They used conditioned medium obtained from EmSC cultured with cyclic stretch and added this to EmSC cultured under stationary conditions [5]. It was observed that the conditioned medium obtained from EmSC cultured with stretch induced a significant increase in IL-8 secretion and IL-8 mRNA expression from stationary-cultured EmSC, suggesting that some autocrine/paracrine mediators are involved in this phenomenon (fig. 4).

The effect of ovarian hormones on mechanical stretch-induced IL-8 production in EmSC was also investigated.

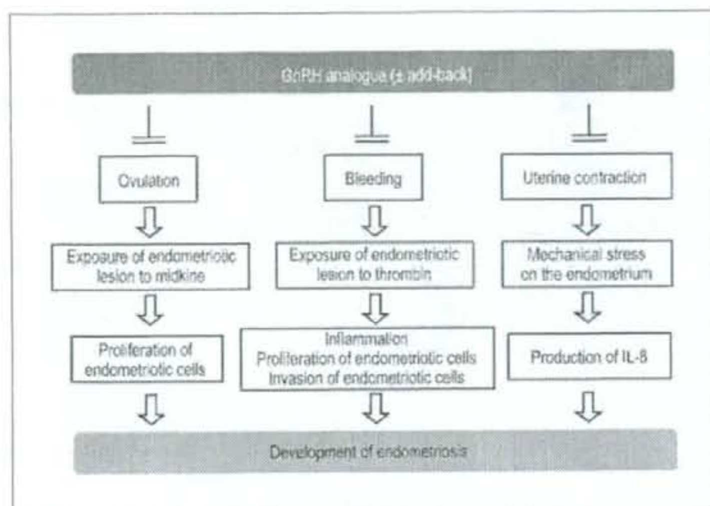

Fig. 5. Summary of the proposed effects of GnRH analogues on the inhibition of endometriosis.

The addition of progesterone, with or without estradiol, significantly suppressed stretch-induced IL-8 secretion from EmSC, whereas estradiol alone did not show any significant effects.

These findings imply that uterine movement has an impact on endometrial physiology and pathology by stimulating the production of a biochemical mediator(s) in the endometrium. It can be speculated that the inhibition of uterine contractions, particularly associated with menstruation, by GnRH analogues would also block the mechanical stress on the endometrium and subsequently suppress the production of IL-8. Ultimately, this would result in inhibition of the development of endometriosis.

In summary, based on these findings, the effect of GnRH analogues can be attributed to various events that result in the inhibition of the development of endometriosis (fig. 5). It is anticipated that these effects would be the same, regardless of whether estrogen add-back therapy was employed alongside GnRH analogue therapy.

The T-Cell Immune Response in Endometriosis

For many years it was believed that the T-helper (Th) cell family comprised only Th1 and Th2; however, recent studies have revealed that naïve Th cells are differentiated into Th1, Th2, Th17 and regulatory T cells (T-reg). Each Th cell has been shown to secrete different cytokines; for example, Th1 secretes interferon-γ, Th2 secretes IL-4, Th17 secretes IL-17 and T-reg secretes transforming growth factor-β [6].

It has been argued that endometriosis is an inflammatory disease and its development is associated with an abnormal T-cell function [7]. Enhanced IL-4 expression has been reported in patients with endometriosis and this study clearly demonstrated that IL-4 concentration was significantly higher in ectopic endometrium compared with eutopic endometrium [8]. In view of these findings it can be hypothesized that IL-4 may have a direct effect on endometrial cells. Other studies have demonstrated alternations in the T-helper cytokine profile and in lymphocyte activation at the systemic and local levels in women with endometriosis [9]. Further investigations into the role of IL-4 have confirmed the presence of a high number of IL-4 positive cells in the endometrial tissue. In addition, IL-4 has been shown to dose-dependently stimulate the proliferation of endometrial cells [10].

A major role for the cytokine IL-17 has now been described in various models of immune-mediated tissue injury, including organ-specific autoimmunity in the brain, heart, synovium and intestines, allergic disorders of the lung and skin, and microbial infections of the intestines and the nervous system. The Th17 pathway is thought to be responsible for causing and sustaining tissue damage

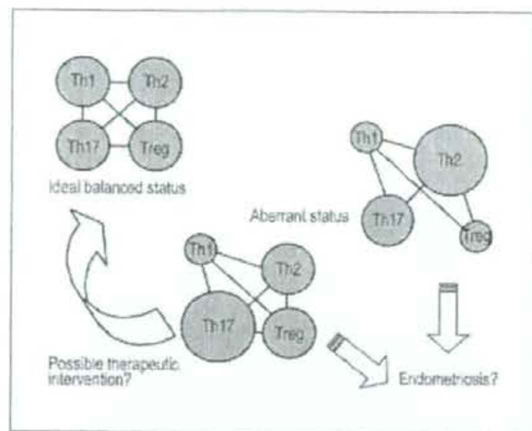

Fig. 6. The T-cell immune network hypothesis.

in these situations. It has also been shown that the Th1 pathway antagonizes the effects of the Th17 pathway.

The presence of Th17 cells has also been demonstrated in the peritoneal fluid of women with endometriosis and IL-17-positive cells have been found in endometriotic tissues [11]. To further investigate the role of IL-17 in the development of endometriosis, studies were undertaken to determine the role of IL-17 on IL-8 production, COX-2 expression, and cell proliferation of cultured ESC. IL-17 was found to enhance IL-8 secretion from ESC in a dose-dependent manner, and this was suppressed by anti-IL-17 receptor antibodies. IL-17 also enhanced the expression of COX-2 mRNA and the proliferation of ESC, as determined by increased BrdU incorporation.

Therefore, it can be concluded that IL-17 exerts proinflammatory and mitogenic effects in endometriotic tissues and may play a role in the development of endometriosis.

A recent publication by Fairbanks et al. found that patients with severe endometriosis have higher IL-12 levels irrespective of IL-18 levels in peritoneal fluid [12]. Based on these data, they claimed that, in endometriosis, an alternative pathway is involved in induction of the Th1 immune response.

Based on all these data, one hypothesis to explain the etiology of endometriosis could be the existence of a 'T-cell immune network' (fig. 6). Th1, Th2, Th17 and T-reg cells interact with each other, via cytokines or cell–cell contact, in a network. In the normal situation these pathways are balanced, but any aberrations or imbalances in these pathways or the cytokines they produce may allow endometriosis to develop. Possible future therapies might include agents targeted to restore this imbalance.

Conclusions

Data from the latest preclinical studies has shown that, in addition to their central action of suppressing estrogen levels by downregulation of the pituitary, GnRH analogues also have several other direct beneficial physiological effects on endometrial cells that result in inhibition of the development of endometriosis. Recent data have demonstrated the importance of the T-cell immune response in the etiology of this disease and may help in the development of future therapeutic agents.

Post-Presentation Discussion

Dr. Kahn, Nagasaki University, Japan: You described a positive correlation between estradiol and midkine in follicular fluid, and that midkine concentrations decrease after treatment with a GnRH analogue in peritoneal fluid of women with endometriosis. Did you also measure midkine levels in peritoneal fluid and follicular fluid after GnRH treatment with add-back therapy?

Dr. Osuga: During GnRH treatment it is very difficult to obtain follicular fluid from patients, so we only examined follicular fluid from women undergoing in vitro fertilization.

Dr. Kahn, Nagasaki University, Japan: You showed some very interesting data about the effect of mechanical stress, and the suppression of uterine contractions with GnRH analogue therapy. Estrogen has a positive correlation with levels of prostaglandin, which is known to influence a range of immune cells. Did you measure the correlation between endogenous prostaglandin levels and decreased production of IL-8 after GnRH therapy?

Dr. Osuga: We did not measure this, but it would be a very interesting experiment to do, as uterine contractions are partially under the control of estrogen.

Dr. Kitawaki, Kyoto University of Medicine, Japan: What do you propose is the mechanism of action by which GnRH analogues reduce the levels of midkine?

Dr. Osuga: During GnRH treatment ovulation does not occur. During the usual menstrual cycle at the time of ovulation follicular fluid leaks out into the peritoneal cavity and diffuses into the peritoneal fluid. Molecules,

such as midkine, dissolved in the follicular fluid have an important influence on the peritoneal environment: midkine levels are 200 times higher in follicular fluid than in peritoneal fluid. During GnRH therapy, since ovulation does not occur, follicular fluid does not leak out into the peritoneal cavity, so midkine will not have its proliferative effect on endometrial tissue.

Disclosure Statement

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Forum Minireview

Basic and Translational Research on Proteinase-Activated Receptors: Proteinase-Activated Receptors in Female Reproductive Tissues and Endometriosis

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Abstract. During the menstrual cycle, dynamic morphological changes are observed in the ovarian follicle and the endometrium. These changes are associated with the onset of the inflammatory response in which many proteinases play various roles. Thrombin-induced activation of PAR_1 (proteinase-activated receptor 1) stimulates the production of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) in human granulosa cells, suggesting a possible role for PAR_1 in the ovulatory process. In the endometrium, PAR_2 expression increases during the menstrual period. PAR_2 activation induces IL-8 production and cell proliferation in human endometrial stromal cells. PAR_1 also stimulates proinflammatory cytokine production in human endometrial stromal cells. Thus, the PARs may be important in directing the dynamic changes of the endometrium. PARs also appear to play a role in endometriosis, a common gynecological disease, since activation of PAR_1 and PAR_2 induces the secretion of inflammatory cytokines and the proliferation of stromal cells in endometriotic lesions. Taken together, PARs appear to play diverse roles in the human reproductive organs.

Keywords: ovary, endometrium, endometriosis, proteinase-activated receptor (PAR), reproduction

Introduction

During the menstrual cycle, dynamic morphological changes are observed in the ovarian follicle and the endometrium. These changes, which are pivotal in reproduction, are associated with the onset of the inflammatory response. Endometriosis, an endometrium-related disease, is also accompanied by inflammatory responses. Emerging findings have suggested that proteinase-activated receptors (PARs) play important roles in these inflammatory reactions. PARs are seven-transmembrane G protein-coupled receptors that are activated by proteinases. For example, thrombin can activate PAR_1, PAR_3, and PAR_4, while trypsin can activate PAR_1, PAR_2, and PAR_4. Cleavage of the PARs occurs within the extracellular N-terminal domain, thereby unmasking a new amino terminus, and the cleaved N-terminal domain functions as a tethered ligand by binding back to the receptor (1, 2). In this review, the findings on the role of PARs in female reproductive tissues and endometriosis are presented, and the pathophysiological significance of PARs in these tissues is discussed.

PAR_1 in human luteinized granulosa cells

Ovulation is an inflammation-like process in which a mature ovarian follicle ruptures and discharges an oocyte that participates in reproduction. At ovulation, various morphological changes are observed in the follicle, including the extravasation of erythrocytes and fibrin deposition in the extracellular space of the follicular wall and in the follicular fluid. After expulsion of the oocyte, a fibrin clot forms in the remnant antral cavity. These findings suggest an involvement of thrombin, a proteinase essential for fibrin formation, in the ovulatory process. Indeed, the generation of thrombin and its functional activity in the follicular fluid has been

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demonstrated (3).

Granulosa cells form the inner layer of the ovarian follicle wall. These cells undergo luteinization at ovulation and subsequently become a component of the corpus luteum. To address the possible role of thrombin in ovulation, the expression of PAR_1 in human luteinized granulosa cells, as well as the effects of thrombin and a PAR_1 agonist on the production of inflammation-related molecules in luteinized granulosa cells, was investigated (4). Luteinized granulosa cells were collected at the time of oocyte pick-up from patients undergoing in vitro fertilization. Expression of PAR_1 mRNA was detected in luteinized granulosa cells by RT-PCR analysis. Thrombin and SFLLRN (Ser-Phe-Leu-Leu-Arg-Asp), a PAR_1-agonist peptide, stimulated the production of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) in cultured luteinized granulosa cells. The stimulatory effects of thrombin were inhibited by inhibitors of thrombin [hirudin and L-prolyl-L-arginine chloromethyl ketone (PPACK)] and the protein kinase C inhibitor calphostin C. As IL-8 and MCP-1 are typical chemoattractants for leukocytes, these findings suggest involvement of PAR_1 in leukocyte infiltration into the extravascular spaces in the ovulatory follicle. In addition, thrombin and SFLLRN stimulated the gelatinase activities of luteinized granulosa cells, the effect of both being inhibited by hirudin and PPACK. Therefore, in luteinized granulosa cells, PAR_1 may also participate in the dissolution of the granulosa cell basement membrane and fragmentation of the extracellular matrix of the follicular wall that precedes rupture of the follicle.

PARs in the endometrium

Decidualization is a process of endometrial differentiation that is essential for successful implantation of the embryo and maintenance of pregnancy. Decidualization occurs under the influence of progesterone secreted from the corpus luteum. Interestingly, the tissue factor content in endometrial stromal cells is increased in the decidualization process, and therefore, thrombin may play a role in the decidualized endometrium. Thrombin-induced PAR_1 activation increases VEGF expression in decidualized endometrial stromal cells (5). Thus, PAR_1, via up-regulation of VEGF, may be involved in angiogenesis and vascular permeability in the decidualized endometrium. Furthermore, PAR_1 activation by thrombin increases the production of IL-8, matrix metalloproteinase (MMP)-1, and active MMP-2 in endometrial stromal cells (6, 7), implying multiple roles of PAR_1 in the endometrium.

Expression of PAR_2 mRNA in the human endometrium is increased during the menstrual phase and in early pregnancy (8). This finding is consistent with a remarkable increase in the number of leukocytes in the endometrium during menstruation since PAR_2-activating proteinases are secreted by the resident leukocytes such as mast cells and neutrophils. In vitro, the PAR_2-agonist peptide (PAR_2AP, Ser-Leu-Ile-Gly-Lys-Val) stimulates IL-8 production in both endometrial epithelial cells and endometrial stromal cells (8). PAR_2AP also stimulates the mRNA expression of stem cell factor, a known activator of mast cells, in endometrial stromal cells and protein expression of activated MMP-7, an epithelial cell-specific matrix metalloproteinase, in endometrial epithelial cells. These findings indicate the involvement of activated PAR_2 in upregulating molecules important for endometrial remodeling in the tissue modification process during the menstrual cycle. In addition, PAR_2AP significantly increased the incorporation of 5-bromo-2'-deoxyuridine into DNA in endometrial stromal cells. This mitogenic effect underscores the possible involvement of PAR_2 in repair of the endometrium undergoing shedding during menstruation. Recent studies have demonstrated that the tissue factor-FVIIa complex activates PAR_2. Such signaling is an emerging role for tissue factor (9). Since tissue factor is produced in the endometrium, it is currently speculated that tissue factor-stimulated PAR_2 also plays a role in the endometrium.

Possible relevance of PARs to the pathogenesis of endometriosis

Endometriosis, defined by the presence of viable endometriotic tissue outside the uterus, is an enigmatic disease. It impairs the health of women of reproductive age, causing pelvic pain and infertility. Implantation and growth of endometrial cells from the overflow of menstrual blood into the peritoneal cavity is a widely accepted hypothesis for the pathogenesis of endometriosis, in which peritoneal inflammation is thought to play a pivotal role (10). PAR_1 and PAR_2, which play roles in many inflammatory events, might be involved in the development of the disease. In addition, the disease is characterized by recurrent ectopic bleeding, and the resultant generation of thrombin is expected to activate PAR_1. Mast cells and neutrophils present in endometriotic tissues might activate PAR_2 by producing specific proteinases. The expression of PAR_1 and PAR_2 in endometriotic cells, as well as the effects of PAR-activating molecules on these cells, has, therefore, been investigated.

Possible function of PAR_1 in endometriotic cells

Using endometriotic stromal cells from surgically

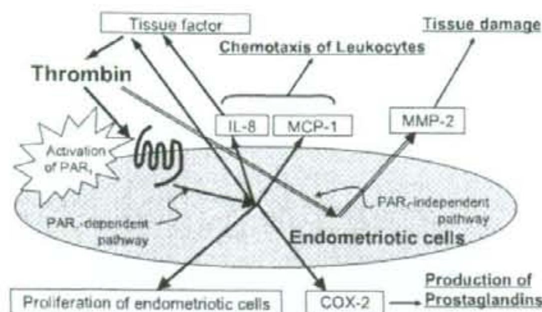

Fig. 1. Possible roles of thrombin in endometriosis. Thrombin stimulates production of IL-8, MCP-1, tissue factor, and COX-2 via PAR_1 activation. Thrombin also stimulates proliferation of endometriotic stromal cells via PAR_1 activation. Thrombin stimulates production of MMP-2 in a PAR_1-independent manner. In combination, these events may stimulate the development of endometriosis.

removed endometrioma (11), several findings have been obtained (Fig. 1). PAR_1 mRNA was expressed in endometriotic stromal cells. Thrombin and SFLLRN increased the mRNA expression of IL-8, MCP-1, and cyclooxygenase-2 (COX-2) and the protein secretion of IL-8 and MCP-1 in endometriotic stromal cells. The concurrent addition of the thrombin inhibitor PPACK inhibited the thrombin-induced secretion of IL-8 and MCP-1. Since MCP-1, IL-8, and prostaglandins are involved in the pathogenesis of endometriosis, PAR_1 is thought to function in the development of endometriosis. IL-8, as well as thrombin, stimulated the expression of tissue factor in endometriotic stromal cells. The increased level of tissue factor may stimulate the coagulation cascade to produce thrombin, which subsequently enhances IL-8 production through PAR_1 activation in endometriotic lesions. Viewed this way, PAR_1 activation could link inflammation with coagulation, thus conferring self-sustaining mechanisms for the progression of endometriosis. Moreover, thrombin and SFLLRN stimulate the proliferation of endometriotic stromal cells. Therefore, thrombin-induced PAR_1 activation might be involved in the pathophysiology of endometriosis, stimulating inflammatory responses of endometriotic cells and their mitogenic activity. In contrast to PAR_1-dependent upregulation of MMP-2 by thrombin in endometrial stromal cells (6), MMP-2 production was increased by thrombin without PAR_1 activation in endometriotic stromal cells (11). Thrombin may stimulate the development of endometriosis both dependently and independently of PAR_1.

Possible function of PAR_2 in endometriotic cells

Activation of PAR_2 stimulated the proliferation of endometriotic stromal cells and the secretion of IL-6 and IL-8 from these cells in a dose-dependent manner (12). Since IL-8 is a chemoattractant of neutrophils, it can be speculated that PAR_2 activation in endometriotic stromal cells may promote the migration of neutrophils via IL-8 secretion. Neutrophils can secrete PAR_2-activating proteinases, and thus PAR_2 activation may cause self-perpetuating inflammation at endometriotic lesions. In addition, activation of PAR_2 stimulated the phosphorylation of mitogen-activated kinases (MAPKs), such as p38 MAPK, p42/44 MAPK, and the stress-activated protein kinase/*c*-jun N-terminal kinase; and this finding may imply pleiotropic functions of PAR_2 in endometriotic tissues. Activation of PAR_1 and PAR_2 in endometriotic stromal cells may, therefore, result in the pathophysiology observed in endometriosis by inducing the growth and inflammation of endometriotic lesions.

Endometriosis model in PAR_2 deficient mouse

In view of the possible significance of PAR_2 in the establishment of endometriosis demonstrated in the in vitro study, an in vivo study was performed using PAR_2-deficient mice (kindly provided by Kowa Co., Ltd., Tokyo). A mouse model of endometriosis was developed according to the method previously described (13). Both the number and the total weight of endometriotic lesions were significantly decreased in the PAR_2-deficient mice compared to the wild type mice (Fig. 2A). Concentrations of IL-6 and MCP-1 were decreased in the peritoneal fluid and the serum of the PAR_2-deficient mice (Fig. 2B), suggesting alleviated inflammation in the peritoneal cavity of the mice. These findings indicate that PAR_2 is involved in the development of experimental endometriosis in mice and that the anti-inflammatory environment in PAR_2-deficient mice might hinder the progress of the disease. Combined with the findings of the in vitro study, it can be argued that PAR_2 would be a target for the treatment of endometriosis.

Conclusion

PARs may play diverse roles in female reproductive

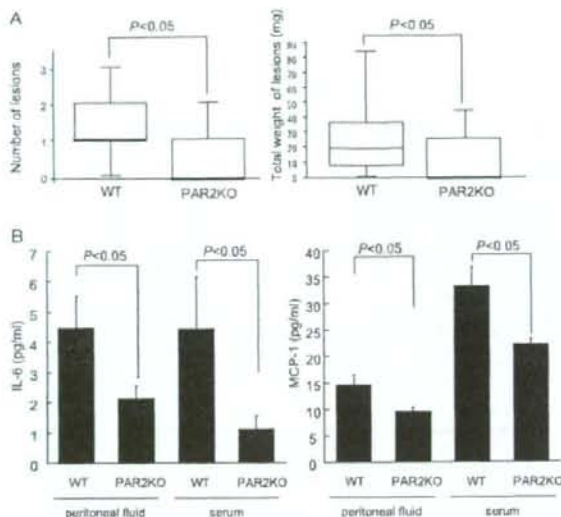

Fig. 2. Endometriotic lesions and cytokines in peritoneal fluid in a model of endometriosis using PAR$_2$-deficient mice. Number and total weight of endometriotic lesions (A) and concentrations of IL-6 and MCP-1 in peritoneal fluids and serum (B) are shown. Endometriosis was induced in wild type (WT, n = 20) and PAR$_2^{-/-}$ (PAR$_2$KO, n = 21) C57BL/6 mice by injecting syngenic endometrial fragments into the peritoneal cavity. Three weeks later, the mice were sacrificed and endometriotic lesions in the peritoneal cavity were measured. At the same time, peritoneal fluid and serum were collected from each mouse. IL-6 and MCP-1 concentrations in the peritoneal fluid and serum were measured using specific ELISA. Boxes represent the distances between the first (25%) and third (75%) quartiles, the horizontal lines in the boxes represent the medians, and the whiskers represent the 10th percentile at the lower limit and the 90th percentile at the upper limit (A). Values are shown as the mean ± S.E.M. (B).

tissues and endometriosis. Their roles appear to be essential for normal physiological events in reproduction, such as ovulation, endometrial changes, and menstruation, as well as the pathogenesis of endometriosis. Further understanding of the function of PARs in these tissues is necessary for the development of reproductive medicine.

Acknowledgments

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Interleukin (IL)-17A Stimulates IL-8 Secretion, Cyclooxygensase-2 Expression, and Cell Proliferation of Endometriotic Stromal Cells

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IL-17A is secreted from Th17 cells, a discovery leading to revision of the mechanism underlying the role of Th1/Th2 in the immune response. Strong evidence suggests that immune responses associated with inflammation are involved in the pathogenesis of endometriosis. In the present study, we first demonstrated that the presence of Th17 cells in peritoneal fluid of endometriotic women by flow cytometric analysis and IL-17A-positive cells in endometriotic tissues by immunohistochemistry. To investigate the role of IL-17A in the development of endometriosis, we then studied the effect of IL-17A on IL-8 production, cyclooxygensase-2 expression, and cell proliferation of cultured endometriotic stromal cells (ESCs). IL-17A enhanced IL-8 secretion from ESCs in a dose-dependent manner. The IL-17A-induced secretion of IL-8 from ESCs was suppressed by anti-IL-17 receptor A antibodies or inhibitors of p38 MAPK, p42/44 MAPK, and stress-activated protein kinase/c-Jun N-terminal kinase. Addition of TNFα synergistically increased IL-17A-induced IL-8 secretion from ESCs. IL-17A also enhanced the expression of cyclooxygensase-2 mRNA and proliferation of ESCs. IL-17A may play a role in the development of endometriosis by stimulating inflammatory responses and proliferation of ESCs. (*Endocrinology* 149: 1260–1267, 2008)

ENDOMETRIOSIS, DEFINED BY the presence of viable endometriotic tissue outside the uterus, is an enigmatic disease. Implantation and growth of endometrial cells from the overflow of menstrual blood into the peritoneal cavity is a widely accepted hypothesis for the pathogenesis of endometriosis. Although retrograde menstruation is observed in most women, only a fraction develop endometriosis. Multiple lines of evidence suggest that inflammation and immune responses play a pivotal role in the pathogenesis of endometriosis (1, 2).

Recent expeditious understanding of Th17 cells substantially revised the conventional Th1/Th2 hypothesis of T cell immunology (3–5). Th17 cells, along with Th1 and Th2 cells, differentiate from naïve T cells. Interferon-γ and IL-4 are specific cytokines secreted from Th1 and Th2 cells, respectively. IL-17A is a representative cytokine secreted from Th17 cells. IL-17A, a disulfide-linked homodimeric glycoprotein consisting of 155 amino acids, has been described in various immune responses and inflammation (6).

Elevated levels of inflammatory substances and cells in the peritoneal fluid (PF) of women with endometriosis is highly indicative of pelvic cavity inflammation (7). A recent study demonstrated that increases in the level of IL-17A in PF correlate with the severity of endometriosis and infertility associated with this disorder (8).

In view of the emerging significance of IL-17A in a novel paradigm in immunology, we investigated the role of IL-17A in endometriosis. In the present study, we first examined presence of IL-17A immunoreactive cells in endometriotic tissues. In particular, we demonstrated Th17 cells in peritoneal fluid mononuclear cells (PFMCs). Thereafter, we studied effects of IL-17A on endometriotic stromal cells (ESCs). To this end, we mainly measured production of IL-8 in ESCs because IL-8 is a possible key player in endometriosis. It has been reported that IL-8 concentrations are increased in PF of endometriotic women, and IL-8 stimulates proliferation, matrix metalloproteinase activity, invasive capability, Fas ligand protein expression, and adhesion capability of endometrial stromal cells (9–11). We also examined cooperative effect of IL-17A to TNFα, another proinflammatory cytokine important for the disease (12). Finally, we studied direct proliferative effect of IL-17A on ESCs.

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; COX, cyclooxygenase; ESC, endometriotic stromal cell; FBS, fetal bovine serum; GAPDH, glyceraldehyde dehydrogenase; IL-17RA, antihuman IL-17 receptor; JNK, c-Jun N-terminal kinase; PF, peritoneal fluid; PFMC, PF mononuclear cell; PMA, phorbol 12-myristate 13-acetate; SAPK, stress-activated protein kinase.

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Materials and Methods

Reagents and materials

Human recombinant IL-17A, TNFα, goat antihuman IL-17A, goat IgG control, mouse antihuman IL-17 receptor (IL-17RA) and mouse IgG1 isotype control were purchased from R&D systems (Minneapolis, MN). Antibodies of human CD3, CD4, and mouse IgG1 isotype control and Goldistop were purchased from BD Bioscience (San Jose, CA). Antihuman IL-17A antibody and isotype control IgG1 were purchased from eBioscience (San Diego, CA). Antibodies of human p38 MAPK, phospho

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p38 MAPK, p42/44 MAPK, phospho p42/44 MAPK, stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and phospho SAPK/JNK were purchased from Cell Signaling Technology (Beverly, MA). MAPK inhibitors SB202190, PD98059, and SP600125 were from Calbiochem (La Jolla, CA). Collagenase was obtained from WAKO (Osaka, Japan). The antibiotic mixture of penicillin, streptomycin, amphotericin B phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Sigma (St. Louis, MO). Charcoal-stripped fetal bovine serum (FBS) was from Hyclone (Logan, UT). DMEM/Ham's F12 (DMEM/F12) and deoxyribonuclease I were from Invitrogen (Rockville, MD).

Patients and samples

Endometriotic tissues and PF were obtained from patients with ovarian endometriomas undergoing laparoscopy. The severity of the disease was determined according to the revised American Society for Reproductive Medicine classification. The final diagnosis was confirmed by histopathological examination. Laparoscopic excision of ovarian endometrioma was performed as follows. After inspection of the pelvis, the ovary was freed from any adhesions. The cyst wall of endometrioma was stripped away from the normal ovarian tissue gently and completely. Endometriotic tissue were obtained from the excised cyst wall of ovarian endometrioma and transported to the laboratory in DMEM/F12 on ice under sterile condition. The PF was obtained from the patients, who were diagnosed as stage III or IV. All patients had regular menstrual cycles, and none had received hormonal treatment for at least 6 months before surgery. The tissues collected under sterile conditions were processed for the generation of primary cell cultures. The peritoneal fluid was collected under sterile conditions before any manipulative procedure. PFMCs were collected as previously described (13, 14). Briefly, the collected PF was centrifuged at $200 \times g$ for 5 min, and the supernatants were removed. The cell pellet was resuspended in PBS, layered onto Ficoll-Paque (Amersham Biosciences, Piscataway, NJ), and centrifuged at $150 \times g$ for 30 min. PMFCs were recovered from the interface.

The experimental procedures were approved by the Institutional Review Board of the University of Tokyo and signed informed consent for use of the endometriotic tissue was obtained from each patient.

Immunohistochemistry

Paraffin-embedded specimens were sliced at a 5-μm thickness. These slide sections were deparaffinized and rehydrated. Antigens were retrieved by buffer at 98 C. Endogenous peroxidase was blocked by incubation for 20 min with a solution of 1% hydrogen peroxidase. Immunohistochemical tissue labeling was performed using the avidin-biotin peroxidase methods. After blocking with normal rabbit serum (Vector Laboratories, Burlingame, CA), the sections were incubated with 1 μg/ml anti IL-17A antibody or goat IgG for 60 min at room temperature and incubated with avidin-biotin peroxidase complex (Vectastain Elite; Vector Laboratories), according to the manufacture's instructions. The pattern of immunoreactivity was visualized using Vector VIP (Vector Laboratories) as substrate. All sections were counterstained with hematoxylin and evaluated under a light microscope.

Flow cytometric analysis

PFMCs were resuspended in 10% FBS RPMI 1640 medium. The cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 5 h in the presence of Goldistop. Cells were firstly stained extracellularly with anti-CD3 and anti-CD4 antibodies, then fixed and permeabilized with Perm/Fix solution (eBioscience), and finally stained intracellularly with anti-IL-17A antibody. Samples were analyzed using FACSCalibur (BD Bioscience) and Cell Quest Pro (BD Bioscience).

Isolation and culture of ESCs

The isolation and culture of human ESCs were performed as described previously (15, 16). Fresh endometriotic tissue collected in sterile medium was rinsed to remove blood cells. The tissue was minced into small pieces and incubated in phenol-red free DMEM/F12 containing type I collagenase (0.25%) and deoxynuclease I (15 IU/ml) for 120 min at 37 C. The resultant dispersed endometriotic cells were separated by filtration through a 100-μm nylon cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ) and 70 μm nylon cell strainer. Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in phenol-red free DMEM/F-12, plated onto 100-mm dishes (Iwaki, Asahi technology Co., Tokyo, Japan), and allowed to adhere at 37 C for 12 h. At the first passage, the cells were plated into six-well plates at 4×10^5 cells/well, 12-well plates at 2×10^5 cells/well, or 48-well plates at 1×10^5 cells/well. Once the cells reached confluence, in 2 or 3 d, they were used for experiments. The purity of ESCs was greater than 95%, according to positive cellular staining for vimentin and negative cellular staining for cytokeratin or CD45, CD68, and von Willebrand factor.

Treatment of the cells

First, to examine the effect of IL-17A on IL-8 production, the cells were incubated for 24 h in 5% FBS DMEM/F12 medium with varying doses of IL-17A. Second, to examine the effect of the anti-IL-17RA antibody, ESCs were preincubated in 5% FBS DMEM/F12 with the antibody for 30 min and then stimulated with 10 ng/ml IL-17A for 24 h. Third, to evaluate the effect of IL-17A on MAPK phosphorylation in ESCs, the cells were incubated with 5% FBS media with IL-17A (10 ng/ml) for different time periods. Fourth, to evaluate the effect of MAPK inhibitors, the cells were preincubated with each MAPK inhibitor for 1 h before the addition of IL-17A and then incubated for 24 h. Fifth, to evaluate the synergic effect of IL-17A and TNFα on IL-8 secretion, the cells were stimulated with varying doses of IL-17A (1–100 ng/ml) with or without TNFα (1 ng/ml). Finally, for time-course experiments examining the expression of IL-8 and cyclooxygenase (COX)-2 mRNA, ESCs were incubated with 5% FBS medium with IL-17A (10 ng/ml) for different time periods up to 24 h.

RNA extraction, reverse transcription, and PCR of IL-17A, IL-17RA, IL-8, and COX2

We extracted total RNA from endometriotic tissues and PFMCs by the acid guanidinium-phenol-chloroform method using ISOGEN (Nippongene, Toyama, Japan). Using an RNeasy minikit (QIAGEN, Hilden, Germany), we extracted total RNA from ESCs cultured in a 12-well plate. One microgram of total RNA was reverse transcribed in a 20-μl vol using an RT-PCR kit (TOYOBO, Osaka, Japan). Standard PCR was performed using Rever Tra Dash (TOYOBO) according to the manufacturer's instructions. Human glyceraldehyde dehydrogenase (GAPDH) primers (TOYOBO) were used as a positive control for RNA levels. Primer pairs for IL-17A, IL-17RA, IL-8, and COX2 used in PCR are shown in Table

TABLE 1. Primer pairs used for PCR analysis

mRNA		Oligonucleotide sequences (5′–3′)	Size (bp)
IL-17	Sense	ACTCCTGGGAAGACCTCATTGG	462
	Antisense	GGCCACATGGTGGACAATCG	
IL-17R	Sense	ACACCCAACAAGGAGACCTG	430
	Antisense	ATTCGTTCCACAGGGTGAAG	
IL-8	Sense	ACTTCCAAGCTGGCCGTGGCTCTCTTGGCA	295
	Antisense	TGAATTCTCAGCCCTCTTCAAAAACTTCTC	
COX2	Sense	TTCAAATGAGATTGTGGGAAAATTGCT	306
	Antisense	AGATCATCTCTGCCTGAGTATCTT	
GAPDH	Sense	ACCACAGTCCATGCCATCAC	452
	Antisense	TCCACCACCCTGTTGCTGTA	

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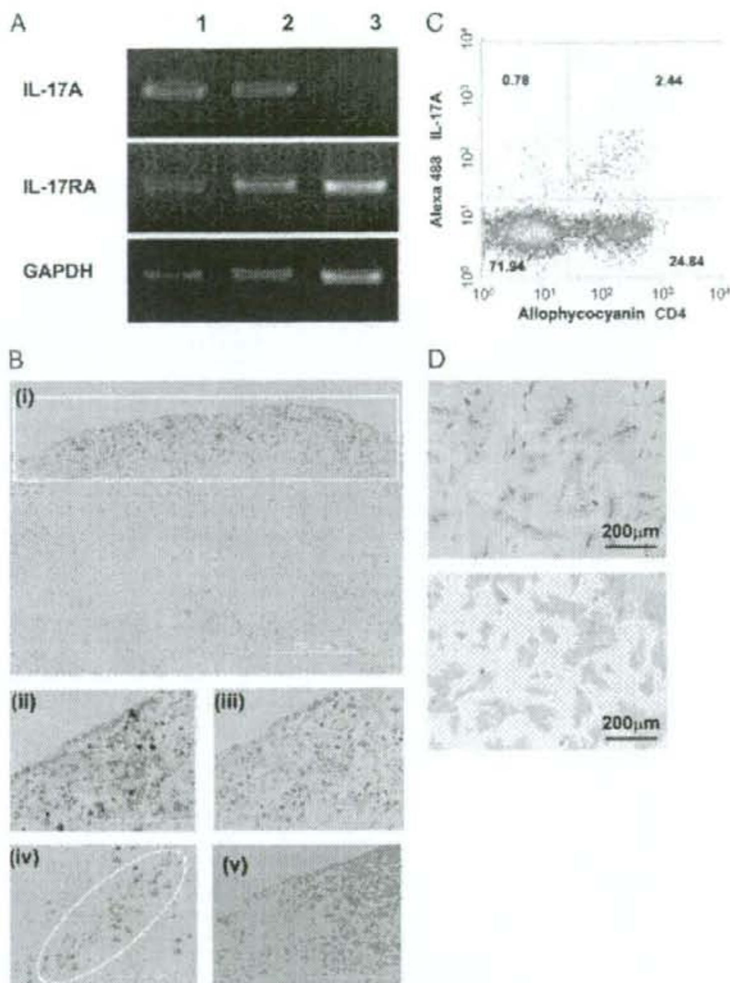

FIG. 1. A, Expression of IL-17A and IL-17RA mRNA in endometriotic tissues (lane 1), PFMCs (lane 2), and ESCs (lane 3). Endometriotic tissues, PFMCs, and ESCs without stimulation were analyzed by RT-PCR. B, Expression of IL-17A in the cyst wall of endometrioma (i, ii, iii, and iv) and ovary (v). Sections were immunostained with antihuman IL-17A antibody (i, ii, iv, and v) or goat IgG (iii). IL-17A-positive cells were detected in the stroma immediately beneath epithelium (i, *white rectangular area*). *Arrowheads* indicate IL-17A-positive cells in the stroma immediately beneath epithelium (ii). IL-17A-positive cells (*arrowheads*) were detected at the site of hemosiderin deposits (iv, *white circle*). No IL-17A-positive cells were detected in ovarian surface (v). Magnification, ×100 (i), ×400 (ii and iii), and ×200 (iv and v). *Scale bars*, 200 μm (i, iv, and v); 100 μm (ii and iii). C, Th17 cells in PFMCs of patients with endometriosis. PFMCs were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) and labeled with an antibody specific to lymphocytes (CD3 and CD4) and IL-17A. Th17 cells were CD4 positive and IL-17A positive. The data shown are representative of four separate experiments. D, Immunocytochemistry of IL-17RA in ESCs. Cultured ESCs were immunostained with anti-IL-17RA antibody (*upper photo*). *Lower photo* shows the control with mouse IgG1 isotype. Magnification, ×100.

1. PCR conditions for amplification were 30 cycles (for IL-17RA, IL-8, COX-2, and GAPDH) or 35 cycles (for IL-17A) at 98 C for 10 sec, 60 C for 2 sec, and 74 C for 14 sec. Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN), and the identity of PCR products was confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Real-time quantitative PCR

Real-time quantitative PCR was performed as reported previously (17). To assess IL-8 and COX2 mRNA expression, real-time quantitative PCR and data analysis were performed using Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). Expression of IL-8 and COX2 mRNA was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. The primers for IL-8 and COX2 were the same as those used for standard PCR. PCR conditions were as follows: for IL-8, 40 cycles at 95 C for 10 sec, 66 C for 10 sec, 72 C for 11 sec; for COX2, 30 cycles at 95 C for 10 sec, 66 C for 10 sec, 72 C for 13 sec; for GAPDH, 30 cycles at 95 for 10 sec, 64 C for 10 sec, 72 C for 18 sec. All PCR conditions were followed by melting curve analysis.

Counting cell numbers

Cell counting was performed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instruction.

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay

BrdU incorporation assay was performed as reported previously (15, 18, 19). The effects of IL-17A and IL-8 on the proliferation of ESCs was examined by measuring BrdU incorporation into DNA using the Biotrak cell proliferation ELISA system (Amersham Biosciences) according to the manufacture's instructions. Briefly, ESCs were seeded into a 96-multiwell plate (Becton Dickinson) at a density of 5×10^4 cells/well in 100 μl of the culture medium. After 24 h, cells were stimulated with IL-17A or IL-8 for 48 h. Then 10 μl BrdU solution were added and incubated at 37 C for an additional 2 h. After removing the culture medium, the cells were fixed and the DNA denatured by the fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in the newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read

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at 450 nm in the DigiScan microscope reader (ASYS Hitech GmbH, Eugendorf, Austria).

Immunocytochemistry

ESCs were cultured in 16-well chamber slides (Nunc, Naperville, IL) in a humidified 5% CO_2-95% air environment and allowed to grow to approximately 50% confluence. The cells were fixed with cold methanol/acetone at −20 C for 20 min, washed twice with PBS, blocked for 20 min with 5% bovine serum in PBS, and incubated with an anti-IL-17RA antibody (10 μg/ml in 1.5% BSA in PBS) or IgG2b mouse IgG isotype control for 40 min at room temperature. After three washes with PBS, the slides were incubated with peroxidase-conjugated secondary antibody (goat antimouse Envision plus; Dako, Glostrup, Denmark) for 30 min at room temperature. Staining was detected with the diaminobenzidine chromogen after 3 min. All slides were counterstained with hematoxylin and evaluated under a light microscope.

Western blotting

Cultured cells in 6-well plates were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue. The lysates were further diluted with lysis buffer to give a final concentration of 1 mg total protein per milliliter. Samples were resolved using 10% SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane and incubated with rabbit antibodies to p38 MAPK (1:1000), phospho-specific p38 MAPK (1:1000), p42/44 MAPK (1:1000), phospho-specific p42/44 MAPK, SAPK/JNK (1:1000), phospho-specific SAPK/JNK (1:1000) as primary antibodies, and antirabbit horseradish peroxidase antibody (1:1000) as a secondary antibody. Immune complexes were visualized by use of the ECL Western blotting system (Amersham Biosciences).

Measurement of IL-8

The concentration of IL-8 in conditioned media was measured using a specific ELISA kit (Genzyme/Techne, Minneapolis, MN). The sensitivity of the assay was 15.6 pg/ml. The intraassay and interassay coefficients of variation were less than 5%.

Statistical analysis

Data were evaluated using ANOVA with Scheffé's *post hoc* analysis for multiple comparisons and Student's *t* test for two groups. $P < 0.05$ was accepted as statistically significant.

Results

Expression of IL-17A and IL-17RA mRNA in endometriotic tissue, PFMCs, and ESCs

The expression of IL-17A mRNA was detected in endometriotic tissues and peritoneal cells, but not in ESCs, by standard RT-PCR analysis. The expression of IL-17RA mRNA was detected in endometriotic tissues, PFMCs, and ESCs (Fig. 1A).

In vivo expression of IL-17A in the endometriotic lesion

As shown in Fig. 1B, the presence of immunoreactive IL-17A was detected in the cyst wall of endometrioma. Intense IL-17A immunoreactive cells were localized in the stroma immediately beneath epithelium and at the site of hemosiderin deposits. No IL-17A immunoreactivity was visualized in endometriotic epithelial cells. Few IL-17A-positive cells were observed in the endometrium of proliferative and secretary phases (data not shown). No IL-17A-positive cells were detected in ovarian surface. No staining was observed when normal goat IgG was used as a primary antibody.

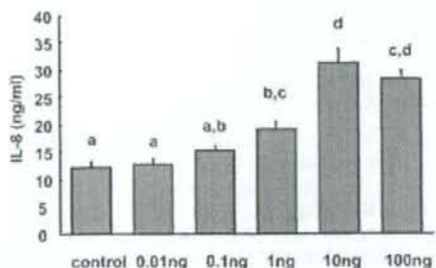

FIG. 2. IL-17A stimulates IL-8 secretion in ESCs. ESCs were cultured in 5% FBS with different doses of IL-17A for 24 h. Concentration of IL-8 in the conditioned medium was measured using a specific ELISA. Values are the mean ± SEM of pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The result is representative of nine separate experiments using samples from different patients.

Th17 cells in PFMCs of patients with endometriosis

The presence of IL-17A-producing cells was evaluated by flow cytometry on T cells (CD3+ cells) from PFMCs after stimulation with PMA and ionomycin. As shown in Fig. 1C, IL-17A-producing T cells were detected in PFMCs of patients with endometriosis. We also found IL-17A-producing T cells were predominantly CD4+ T cells.

Expression of IL-17RA protein in ESCs

The presence of immunoreactive IL-17RA was demonstrated in ESCs (Fig. 1D). No staining was observed when mouse IgG1 was used as a primary antibody.

Effects of IL-17A on IL-8 secretion by ESCs

As shown in Fig. 2, IL-17A at 1 ng/ml and higher significantly enhanced the secretion of IL-8 from ESCs. The maximum effect was observed with IL-17A at 10 ng/ml. The magnitude of increase

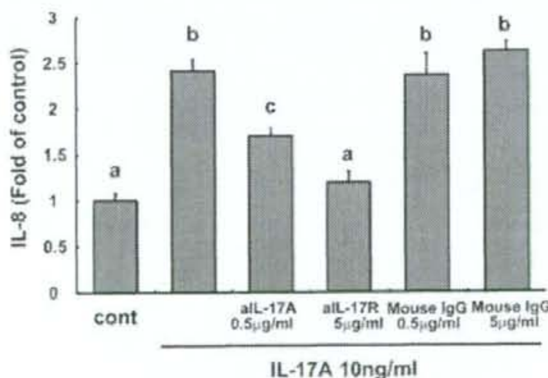

FIG. 3. Effect of anti-IL-17RA antibody (aIL-17R) on IL-17A-induced IL-8 secretion by ESCs. ESCs were preincubated in 5% FBS medium with or without mouse IgG1 and aIL-17R for 30 min and then stimulated with or without IL-17A (10 ng/ml) for 24 h. Concentrations of IL-8 in the conditioned media were measured using a specific ELISA. All value are expressed as the mean ± SEM of pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The result is representative of four separate experiments using samples from different patients. Cont, Control.

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with 10 ng/ml IL-17A varied between patients from 1.8- and 5.3-fold, with a median increase of 3.5-fold (n = 9).

Effect of anti-IL-17RA antibody on IL-17A-induced IL-8 secretion in ESCs

Treatment with the neutralizing antibodies for IL-17RA significantly diminished the IL-17A-induced increase in IL-8 secretion in a dose-dependent manner, whereas the control IgG had no effect (Fig. 3).

Effect of IL-17A on MAPK phosphorylation in ESCs

The phosphorylation of three MAPKs (p42/44 MAPK, p38 MAPK, and SAPK/JNK) by IL-17A was determined in cultured ESCs (Fig. 4A). An increase in MAPK phosphorylation was apparent after 5–15 min. Phosphorylation levels reached a maximum after 5 min for P42/44 MAPK and after 15 min for p38 MAPK and SAPK/JNK, respectively.

Effect of MAPK inhibitors on IL-17A-induced IL-8 secretion

The intracellular mechanism of IL-17A-induced secretion of IL-8 by ESCs was investigated by examining the effect of MAPK inhibitors. As shown in Fig. 4B, the addition of inhibitors for p38MAPK, p42/44 MAPK, and SAPK/JNK significantly diminished IL-17A-induced IL-8 secretion.

Synergic effects IL-17A and TNFα on IL-8 secretion in ESCs

We chose TNFα as a representative proinflammatory cytokine known to induce IL-8 secretion from ESCs (12). TNFα together with IL-17A triggered IL-8 secretion above the combined levels generated by each stimulus alone (Fig. 5). This synergistic effect was apparent when TNFα (1 ng/ml) was combined with 1 ng/ml IL-17A, and maximal synergy was obtained at the highest dose of IL-17A tested (100 ng/ml).

Effect of IL-17A on the expression of IL-8 mRNA and COX2 mRNA in ESCs

Time-course experiments were conducted to examine the effect of IL-17A on the expression of IL-8 mRNA and COX2 mRNA in ESCs (Fig. 6A). Real-time quantitative PCR anal-

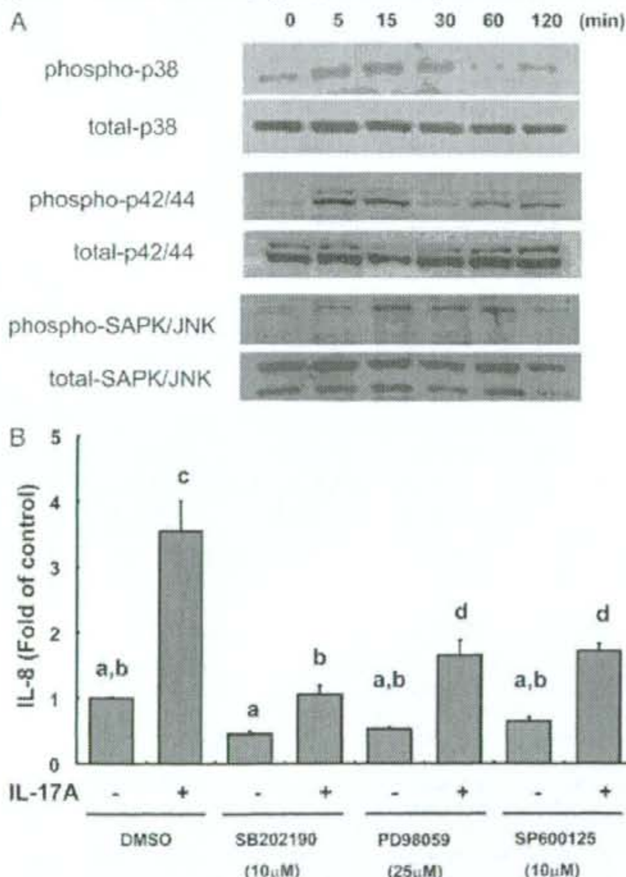

FIG. 4. Involvement of MAPKs in IL-17A induced IL-8 secretion from ESCs. A, phosphorylation of p38 MAPK, p42/44 MAPK, and SAPK/JNK induced by IL-17A in ESCs. ESCs were incubated with IL-17A (10 ng/ml) for the indicated time (0–120 min). Cell lysates were assayed for phosphorylated p38 MAPK (phospho-p38), total p38 MAPK (total-p38), phosphorylated p42/44 MAPK (phospho-p42/44), total p42/44 (total-p42/44), phosphorylated SAPK/JNK (phospho-SAPK/JNK), and total SAPK/JNK (total-SAPK/JNK) by Western blotting. The data are representative of four independent experiments. B, Effects of MAPK inhibitors on IL-17A-induced IL-8 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-17A (10 ng/ml) for 24 h. The conditioned medium was collected and assayed for IL-8 concentration using a specific ELISA. All values are expressed as the mean ± SEM of pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The data are representative of four independent experiments. DMSO, Dimethylsulfoxide.

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