tients is sustained despite progestin therapy.[3,6] The prevailing hypothesis is that an enhanced estrogenic influence and/or reduced P_4 responsiveness leads to endometriosis.[3,12,14] Therefore, an animal model presenting more than normal estrogenic activity with suboptimal P_4 responsiveness will be valuable for delineating the etiology of endometriosis, because all human studies focus on this disease when it is already in progress or established, precluding studies on its initiation. Mice missing FKBP52 fulfill this purpose, because these null females show uterine specific P_4 resistance as described below.

The immunophilin cochaperone FK506-binding protein 4 (FKBP52) is a key component of the mature PR complex. Functionally mature steroid hormone receptors including that of PR consist of a receptor monomer, a 90-kDa heat shock protein (Hsp90) dimer, the cochaperone p23, and one of the four cochaperones containing a tetratricopeptide repeat that binds to Hsp90.[15] FKBP52 is one such cochaperone that binds both Hsp90 and PR to stabilize the complex for optimal P_4 binding to PR and subsequent transcriptional activation.[15] Basal PR responsiveness, however, persists in the absence of FKBP52.[16] We have recently shown that *Fkbp52* deficient female mice with normal PR expression and P_4 levels show reduced uterine PR responsiveness with more than normal estrogenic influence, leading to implantation failure.[16] Implantation and full-term pregnancy, however, can be rescued with excess P_4 supplementation, depending on the genetic background of mice.[17] Thus, FKBP52 deficiency confers endometrial P_4 resistance during pregnancy.

Because serum ovarian hormone levels during the menstrual cycle in women with endometriosis are similar to those without endometriosis, it is possible that reduced P_4-PR signaling, rather than reduced P_4 levels, is a major contributing factor for P_4 resistance in endometriosis. Unlike $PR^{-/-}$ mice with no P_4-PR signaling, $Fkbp52^{-/-}$ mice with basal uterine P_4-PR responsiveness are perhaps a more physiologically relevant model to study the role of P_4 resistance in endometriosis.[16] Using $Fkbp52^{-/-}$ females, we show here that FKBP52 deficiency promotes the growth of endometriotic lesions with increased inflammation, cell proliferation and angiogenesis. These findings in mice corroborate our observations of down-regulation of FKBP52 expression in eutopic endometria and ectopic lesions of women with endometriosis compared to endometria of women without endometriosis. Together, these findings provide evidence that reduced levels of FKBP52 contribute to decreased P_4 responsiveness in furthering the development of endometriosis.

Materials and Methods

Mice

Fkbp52 null mice were originally established on a C57BL/6/129SvJ background[18] and then backcrossed with CD1 mice to the F10 generation.[17] $Flk1^{lacZ+/-}$ transgenic mice were originally generated on a C57BL/6J/Sv129 background and backcrossed to CD1 background to the F10 generation.[19] CD1 $Fkbp52^{-/-}/Flk1^{lacZ+/-}$ (knockout [KO]; Flk^{KO}) mice were generated by crossing $Fkbp52^{+/-}/Flk1^{lacZ-/-}$ males and $Fkbp52^{+/-}$ females. $Fkbp52^{+/+}/Flk1^{lacZ-/-}$ (wild-type [WT]; $Flk1^{WT}$) littermates were used as control. Mice were housed and used in the present investigation in accordance with the National Institutes of Health and institutional guidelines on the care and use of laboratory animals.

Mouse Endometriosis Model

There is evidence that estrogen promotes growth of endometriotic lesions in ovariectomized mice.[20] However, estrogen doses used in the study were much higher than physiological levels. *Fkbp52* null mice have normal estrous cycles and the status of ovarian hormones during early pregnancy is comparable to WT littermates (data not shown). Because we have shown that $Fkbp52^{-/-}$ uteri during pregnancy have more than normal estrogenic influence due to uterine P_4 resistance,[17] we were able to circumvent ovariectomy or estrogen treatment in our current study of endometriosis. Induction of endometriosis was performed according to the method previously published with some modifications.[20,21] Seven to ten-week-old female mice were used for endometriosis induction. Vaginal smears of all mice were examined daily at least 7 days before inoculation. Mice in diestrus were selected to be used for donor and recipient mice. Donor mice were sacrificed, and 0.8 ml PBS was injected into the peritoneal cavity, and peritoneal fluid (donor/control) was collected. Then, uterine horns were removed and weighed. One piece of uterine tissue was kept in 10% neutral-buffered formalin as a control (donor) for immunostaining. The remaining uterine tissue was placed into a dish and minced using a surgical knife. Fragments suspended in 0.6 ml PBS were injected with an 18-gauge needle through the abdominal wall just below the umbilicus into the peritoneal cavity of recipient mice with the ratio of 1 donor to 2 recipients. All procedures were performed under aseptic conditions.

Fourteen days postinjection, recipient mice were sacrificed, their peritoneal cavities washed with 0.8 ml PBS, and peritoneal fluid collected. Then, uteri (eutopic) and endometriotic implants (ectopic) were removed and weighed. Tissues were fixed in 10% neutral-buffered formalin for histological analysis and immunostaining, or in 0.2% paraformaldehyde solution for lacZ staining. Each sample of peritoneal fluid was centrifuged, and the supernatant was kept at −80°C until enzyme-linked immunosorbent assay.

Human Tissue

The following tissues were obtained from 80 women undergoing laparoscopy: 1) endometrial tissues of women without endometriosis (endometriosis-free endometrium, $n = 40$), 2) endometrial tissues of women with endometriosis (eutopic endometrium, $n = 40$), 3) endometriotic tissues of women with endometriosis (ectopic endometrium; ovarian endometriosis, $n = 32$: peritoneal endometriosis, $n = 8$: deep-infiltrating endometriosis, $n = 4$). All women underwent laparoscopy for pain, infertility or other benign gynecological disorders during the period of

2006 to 2007. Endometriosis was diagnosed laparoscopically and confirmed histologically. Lesions of deep-infiltrating endometriosis were defined as those deeper than 5 mm beneath the peritoneal surface according to the previous study by Cornillie et al.[22] Forty women (aged 35.8 ± 6.2 years; mean ± SD) were diagnosed with endometriosis, and 40 women (aged 37.2 ± 6.5 years) had no endometriosis. Severity of endometriosis was determined according to the revised American Society for Reproductive Medicine classification. Among 40 women with endometriosis, 17 and 23 women were classified as stage 3 and stage 4, respectively. Forty endometriosis-free endometria and 40 eutopic endometria were collected from 40 different women without and with endometriosis, respectively. Eight of 44 endometriotic lesions were obtained simultaneously from four women, and rest of them from 36 different individuals. All subjects had regular menstrual cycles without any hormonal treatment for at least 6 months before surgery. Endometrial samples were dated according to the women's menstrual history and standard histological criteria by Noyes et al.[23] Tissues were fixed for histology and immunohistochemistry and snap-frozen for RNA isolation. The experimental procedures were approved by the institutional review board of University of Tokyo (IRB number 324), and signed informed consent for use of tissues was obtained from each woman.

Immunohistochemistry

Immunostaining of Ki-67, cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), PR, FKBP52, and estrogen receptor (ER) α was performed in 10% neutral-buffered formalin-fixed and paraffin-embedded sections (5 μm) of human and mouse tissues as previously described.[24] Antibodies specific to Ki-67 (Thermo Fisher Scientific, Fremont, CA), COX-2 (Cayman Chemical, Ann Arbor, MI), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), PR (Zymed Laboratories, Carlsbad, CA), FKBP52 (kindly given by David F Smith, Mayo Clinic), and ERα (Santa Cruz Biotechnology) were used. A Histostain-Plus kit (Zymed Laboratories) was used to visualize specific antigens. Brown deposits indicate sites of positive immunostaining.

Aberrant proliferation in ectopic lesions is a characteristic of endometriosis, with ectopic cells growing outside the normal hormonal regulation. In fact, the pattern of cell proliferation in ectopic lesions differs from estrogen- and P_4-governed proliferation of eutopic endometria.[25,26] The proliferative status of mouse ectopic lesions due to FKBP52 deficiency was assessed by Ki-67 immunohistochemistry. For Ki-67 quantification, five high-powered fields per respective section were analyzed microscopically. The percentage of the total cells staining for Ki-67 was calculated.

COX-2 and VEGF are widely accepted markers of endometriosis and are associated with its pathophysiology.[27,28] To assess whether there is any similarity between our mouse model of endometriosis with that of humans, immunostaining of COX-2 and VEGF was performed in uterine sections from donors (controls), as well as in sections from eutopic endometria and ectopic lesions retrieved from reciprocal transplantation of endometrial tissue in mice. Estrogen is a key factor in development of endometriosis, and the levels of ER and PR expression modulate estrogenic effects on various cells and tissues. To evaluate the contribution of these hormone receptors to our mouse model of endometriosis, immunostaining of PR and ERα was also performed. In addition, immunostaining of COX-2, VEGF, and PR in human endometriosis was performed to compare with the results of those in our mouse model of endometriosis.

FKBP52 immunostaining was performed in both human and mouse tissues. The intensity of staining was analyzed by a semiquantitative method, H-scoring.[29] H-score was calculated by the following equation: H-score = $\sum P_i(i + 1)$ where i is the intensity of staining with a value of 0, 1, 2, or 3 (negative, weak, moderate, or strong, respectively) and P_i is the corresponding percentage of the cells. Five high-powered fields per respective section were analyzed microscopically. Stromal cells in mice and both epithelial and stromal cells in humans were evaluated.

LacZ Staining and Quantification of Microvessel Density

LacZ staining and quantification of vessel density were performed as previously described.[30,31] Briefly, tissues were fixed in 0.2% paraformaldehyde for 24 hours followed by infusion in 30% sucrose at 4°C overnight. Tissues were then embedded in optimal cutting temperature compound and snap-frozen. Frozen sections were mounted onto glass slides and stained overnight at 37°C using β-galactosidase as a substrate. Sections were counterstained with eosin. Blue deposits indicate sites of positive staining. Endometriotic lesion areas occupied by lacZ-stained blood vessels were quantified. Six sections per lesion were randomly selected, digital images were obtained, and measurements were made using the Scion Image (Scion Corporation, Frederick, MD). The percentage of area occupied by lacZ-positive vessels was measured for each section.

Enzyme-Linked Immunosorbent Assay

There is evidence that several cytokines and growth factors are increased in peritoneal fluids of women with endometriosis and associated with its pathophysiology.[32,33] Among the up-regulated factors, levels of monocyte chemotactic protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted (RANTES) and VEGF are elevated by estrogen.[34–37] To assess the contribution of FKBP52 deficiency to excessive estrogenic effects, concentrations of MCP-1, RANTES, and VEGF in mouse peritoneal fluid were measured by respective enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN) according to the manufacturer's protocol. Absorbance was read at 450 nm with an ELx800 automated microplate reader (BIO-TEK, Winooski, VT).

Reverse Transcription and Quantitative PCR

Total RNA was isolated from human tissues using Isogen (Nippongene, Toyama, Japan). Reverse transcription (RT)

and quantitative PCR were performed as previously described.[38] The RT reaction was performed using ReverTra Ace-α (Toyobo, Osaka, Japan). Quantitative PCR was performed in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using FastStart DNA Master Plus SYBR Green (Roche Diagnostics GmbH). The following primers were used: *Fkbp52*, sense, 5'-AGATGACAGCCGAGGAGATG-3'; antisense, 5'-AATTTGTCCTTGCGATCCAG-3'; *Gapdh*, sense, 5'-ACCACAGTCCATGCCATCAC-3', antisense, 5'-TCCACCACCCTGTTGCTGTA-3'. Fkbp52 expression was normalized to RNA loading for each sample using Gapdh mRNA as an internal standard. Standardization of data was performed by subtracting the signal threshold cycles of Gapdh from that of Fkbp52. Each PCR product was purified with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany), and identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical Analysis

Mann-Whitney *U*-test was used to compare expression levels of *Fkbp52* mRNA in human tissues. All other data were analyzed using unpaired Student's *t*-test and analysis of variance with posthoc analysis. $P < 0.05$ was accepted as statistically significant.

Results

FKBP52 Deficiency Promotes Endometriotic Growth and Cell Proliferation in a Newly Established Mouse Model

Using *Fkbp52*$^{-/-}$ mice, we examined the effects of FKBP52 deficiency on endometriosis and the relative roles of donor versus recipient FKBP52 in this disease using reciprocal transplantation of endometrial tissue minces within the peritoneum. All donor and recipient mice were synchronized at the diestrus stage to have their hormonal milieu comparable. Our reciprocal transplantation protocol was as follows: WT recipient to WT donor (WT-WT), *Fkbp52*$^{-/-}$ donor to *Fkbp52*$^{-/-}$ recipient (KO-KO), WT donor to *Fkbp52*$^{-/-}$ recipient (WT-KO), and *Fkbp52*$^{-/-}$ donor to WT recipient (KO-WT). We used age-matched groups, and there were no significant dif-

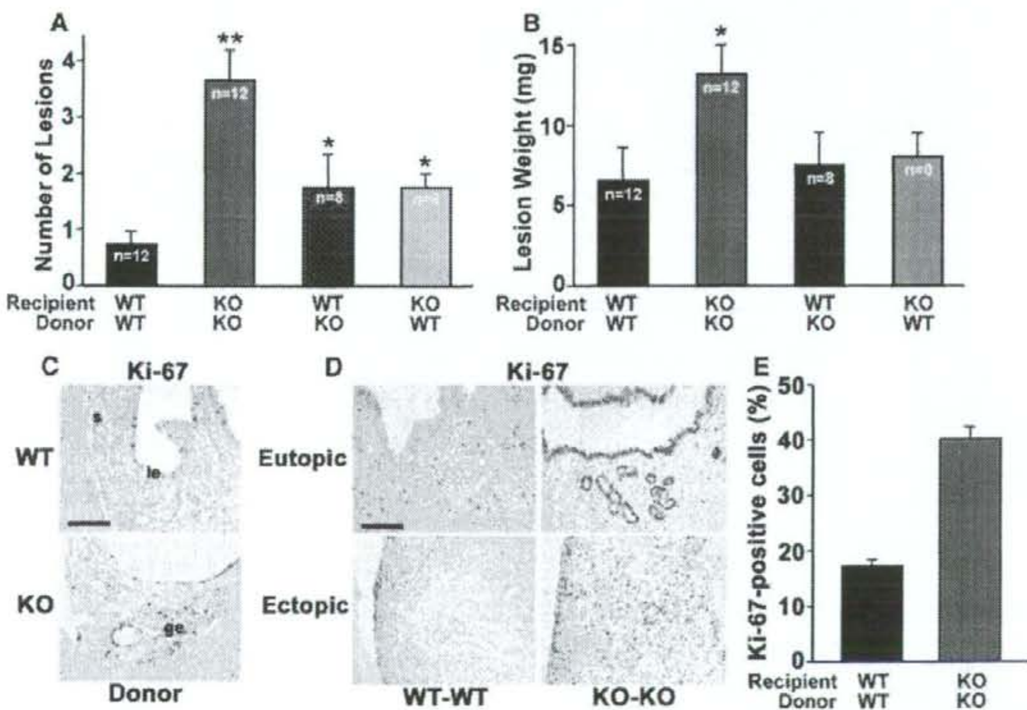

Figure 1. FKBP52 deficiency promotes growth of ectopic endometrial lesions in mouse model of endometriosis. The number (**A**) and total weight (**B**) of ectopic lesions in mouse endometriosis is shown. Both the number and weight of ectopic lesions in *Fkbp52*$^{-/-}$ recipient mouse with injection of *Fkbp52*$^{-/-}$ endometrium (KO-KO) is significantly higher than those in WT recipient mouse with injection of WT donor endometrium (WT-WT). Values are mean ± SEM. $^{*}P < 0.05$ compared with WT-WT group, $^{**}P < 0.0001$ compared with WT-WT group, analysis of variance. Immunohistochemistry of Ki-67 in (**C**) WT and KO donor endometrium (control) and (**D**) eutopic and ectopic endometrium of WT-WT (left column) and KO-KO (right column). ge, glandular epithelium; le, luminal epithelium; s, stroma. Scale bar = 200 μm. **E:** The percentage of Ki-67-positive stromal cells in ectopic endometrium of WT-WT and KO-KO group. The values (Ki-67-positive stromal cells/total stromal cells, %) are presented as mean ± SEM of five different lesions. $^{*}P < 0.05$, unpaired Student's *t*-test.

ferences in uterine and body weights of donor and recipient mice (data not shown). In each group, ectopic endometrial lesions formed in the peritoneum, omentum and perivesical fat tissues, intestine, and liver. On gross examination, these lesions resembled red, yellow, or white inflamed tissues (Supplemental Figure 1, A–D, see *http://ajp.amjpathol.org*). They did not appear cystic as observed in previous studies that used estrogen administration to induce endometriosis.[20,21] Histologically, lesions were comprised primarily of stromal cells with a small number of epithelial cells; this is also the characteristic of human endometriosis. Hemorrhage and hemosiderin depositions, normally seen in human endometriosis,[39] were also found in these ectopic lesions (Supplemental Figure 1E, see *http://ajp.amjpathol.org*). The KO-KO group had a significantly higher number of lesions with significantly higher weights of the lesions than the other groups (Figure 1, A and B). While the WT-KO and KO-WT groups had a higher number of lesions than the WT-WT, weights of the lesions of these groups were comparable (Figure 1, A and B). These results show that deletion of *Fkbp52* in both the donor and recipient produces more robust endometriotic lesions.

Previous studies have shown that while proliferative activity is reduced in endometriotic lesions compared with eutopic endometria,[40] red endometriotic lesions, known to be relatively active, have high proliferative activity compared with black lesions, which are more established and matured.[25,41] These findings suggest that the status of lesions correlate with the mitogenic activity. While the proliferative status was comparable between WT and KO donor tissues (Figure 1C), we observed a remarkable increase in Ki-67-positive cells in ectopic lesions of the KO-KO group compared with those of the WT-WT group (Figure 1D). Indeed, quantification of Ki-67-positive cells showed that proliferation was significantly higher in the KO-KO ectopic lesions compared to those of WT-WT (Figure 1E). These findings suggest that FKBP52 deficiency aggravates endometriotic lesions with cell proliferation.

COX-2 and VEGF, known endometriosis markers, were expressed in ectopic lesions of both WT-WT and KO-KO groups, although their expression was more intense in the KO-KO group (Figure 2, A and B). These results are consistent with previous studies in both humans and mouse models.[42–45] Because COX-2 and VEGF are known regulators of uterine angiogenesis,[30,31] our observed increased stromal expression of COX-2 and VEGF primarily in KO-KO ectopic lesions led us to next question whether events of angiogenesis differ in the absence of FKBP52.

FKBP52 Deficiency Induces Angiogenesis in Murine Endometriotic Lesions

It is well-recognized that angiogenesis plays a key role in the development of endometriosis.[46,47] We have previously shown that ovarian estrogen and P_4 govern normal endometrial angiogenesis in mice.[30] This observation, together with our present finding of COX-2 and VEGF expression in ectopic lesions led us to hypothesize that decreased P_4-PR signaling observed in *Fkbp52* null uteri

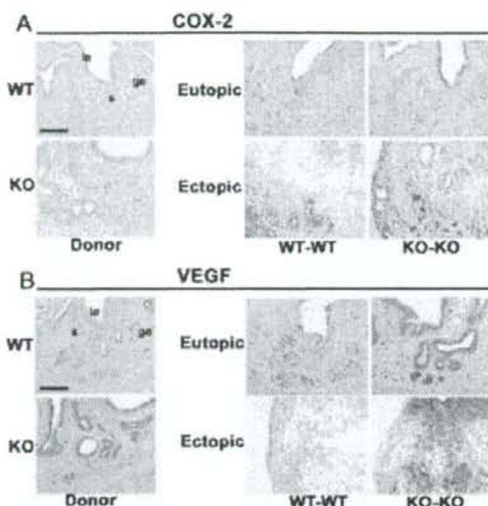

Figure 2. COX-2 and VEGF expression are increased in mouse endometriosis. **A:** COX-2 immunohistochemistry in donor (WT or KO), and eutopic and ectopic endometria of both WT-WT and KO-KO. **B:** VEGF immunohistochemistry in donor (WT or KO), and eutopic and ectopic endometria of both WT-WT and KO-KO. ge, glandular epithelium; le, luminal epithelium; s, stroma. Scale bar = 200 μm.

would lead to altered angiogenesis in ectopic lesions, contributing to the pathophysiology of the disease. To address this, we used *Fkbp52*$^{+/+}$/*Flk1*$^{lacZ+/-}$ (*Flk1*^{WT}) and *Fkbp52*$^{-/-}$/*Flk1*$^{lacZ+/-}$ (*Flk1*^{KO}) mice. *Flk1*$^{lacZ+/-}$ mice express β-galactosidase as a read-out for Flk1 promoter activity in newly formed endothelial cells.[48] Using this model, we were able to separately examine donor-derived and recipient-derived angiogenesis in ectopic lesions and evaluate the contribution of donor versus recipient derived blood vessels. We divided mice into the following four groups for reciprocal transplantation of minced endometrial tissues within the peritoneal cavity: WT donor to *Flk1*^{WT} recipient (WT-*Flk1*^{WT}), *Flk1*^{WT} donor to WT recipient (*Flk1*^{WT}-WT), *Flk1*^{KO} donor to *Fkbp52*$^{-/-}$ recipient (*Flk1*^{KO}-KO) and *Fkbp52*$^{-/-}$ donor to *Flk1*^{KO} recipient (KO-*Flk1*^{KO}). We found lacZ-stained Flk1-positive blood vessels in ectopic lesions of WT-*Flk1*^{WT} and KO-*Flk1*^{KO} groups, but not in those of *Flk1*^{WT}-WT or *Flk1*^{KO}-KO (Figure 3, A & B). These results show that endometriotic lesions arising from transplanted uterine tissues recruit blood vessels from the recipients for their growth. The microvessel density in ectopic lesions of the KO-*Flk1*^{KO} group was higher than those in WT-*Flk1*^{WT} group, showing that *Fkbp52* deletion also promotes angiogenesis (Figure 3C). The angiogenic status in eutopic endometria is shown in Supplemental Figure 2, (see *http://ajp.amjpathol.org*). *Fkbp52* null recipient endometria (*Flk1*^{KO}) with higher than normal estrogenic influence, had less new vessels (marked by lacZ staining) than that of WT with normal hormonal status. This is consistent with our previous findings showing that estrogen negatively regulates uterine angiogenesis in mice.[30] However, increased angiogenesis observed in ectopic lesions of *Fkbp52* null

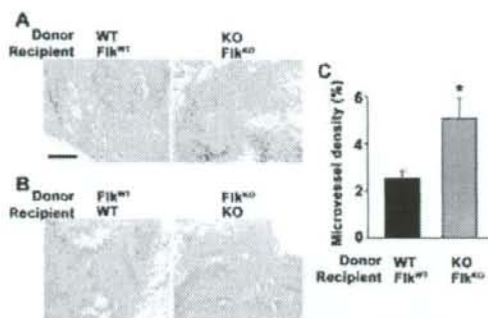

Figure 3. FKBP52 deficiency promotes angiogenesis in ectopic lesions in mouse models of endometriosis. **A** and **B:** LacZ staining of Flk1-expressing blood vessels in mouse endometriotic lesions. Observed blood vessels are derived from the recipient (**A**) but not from the donor (**B**). Scale bar = 200 μm. **C:** Microvessel density in endometriotic lesions of WT-*Flk1*^{WT} and KO-*Flk1*^{KO} group. Values are presented as mean ± SEM of five different lesions. $^{*}P < 0.05$, unpaired Student's *t*-test.

mice suggests that the angiogenic environment in ectopic lesions is different from that of eutopic endometria.

PR and FKBP52, but Not ERα, Expression Is Reduced in Mouse Endometriosis

Immunohistochemistry results show reduced PR expression in both WT-WT and KO-KO ectopic lesions compared with eutopic and donor (control) endometria (Figure 4A). These results in mice are in accord with previous findings of reduced PR expression in human endometriosis.[11,25,26] We also observed that FKBP52 levels were reduced in ectopic lesions of WT-WT samples with only a few stromal cells showing expression (Figure 4B & Supplemental Figure 3A, see *http://ajp.amjpathol.org*). Our results showing exacerbated endometriotic lesion growth in *Fkbp52*$^{-/-}$ mice and decreased FKBP52 expression in WT endometriotic lesions suggest that down-regulation of PR or FKBP52 potentially contributes to P_4 resistance and the pathogenesis of endometriosis. ERα expression in ectopic lesions of KO-KO group was nearly identical to those of WT-WT, and the intensity of stromal ERα expression in ectopic lesions appeared similar to that of eutopic and donor endometria (Figure 4C).

Peritoneal Levels of MCP-1 Are Increased in Mice with Endometriotic Lesions

Because our findings suggest that FKBP52 deficiency confers more than normal estrogenic effects, we hypothesized that concentrations of MCP-1, RANTES, and VEGF in mouse peritoneal fluids would increase. Indeed, enzyme-linked immunosorbent assay results show that levels of MCP-1, RANTES, and VEGF in endometriotic peritoneal fluids (recipients) were significantly increased compared with those found in controls (donors) (Figure 5A–C). MCP-1 levels in KO-KO endometriotic peritoneal fluid were also significantly higher than those of WT-WT (Figure 5A). While the levels of RANTES and VEGF in KO-KO endometriotic samples also increased compared to WT-WT, these levels did not reach statistical significance ($P = 0.081$ and 0.138, respectively) (Figure 5, B and C).

FKBP52 Expression Is Reduced in Human Endometriosis

The results described above using *Fkbp52* null mice as an endometriosis model demonstrate that reduced P_4-PR

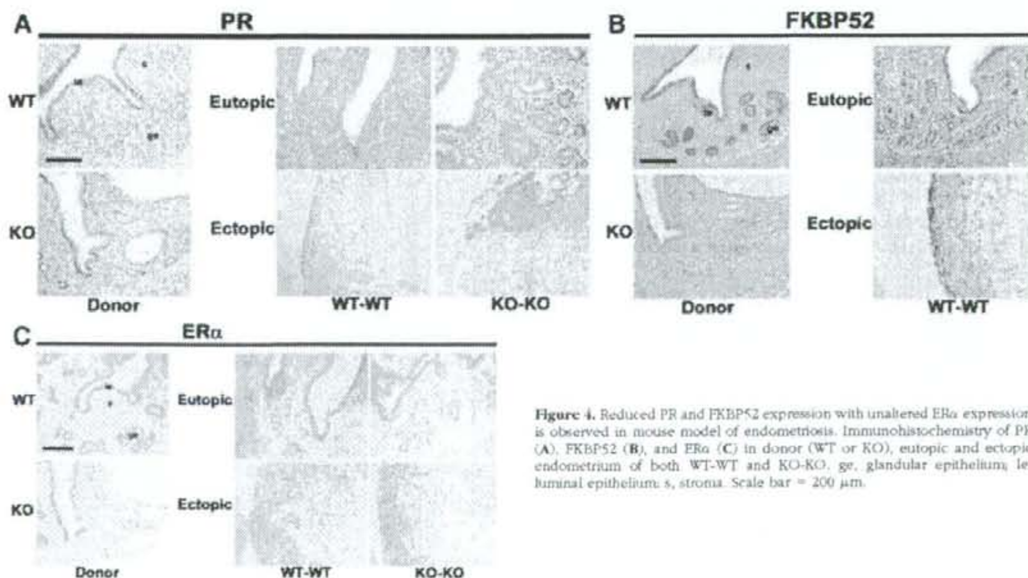

Figure 4. Reduced PR and FKBP52 expression with unaltered ERα expression is observed in mouse model of endometriosis. Immunohistochemistry of PR (**A**), FKBP52 (**B**), and ERα (**C**) in donor (WT or KO), eutopic and ectopic endometrium of both WT-WT and KO-KO. ge, glandular epithelium; le, luminal epithelium; s, stroma. Scale bar = 200 μm.

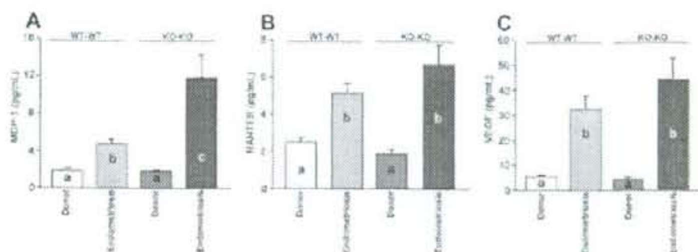

Figure 5. Increased cytokine levels in endometriotic peritoneal fluid of *Fkbp52*$^{-/-}$ mice with endometriosis. Concentrations of MCP-1 (**A**), RANTES (**B**), and VEGF (**C**) in peritoneal fluid of mouse endometriosis. Control peritoneal fluid (donor); endometriotic peritoneal fluid (endometriosis) is from the recipient. Letters within the bars indicate statistical significance (a vs b, $P < 0.05$; b vs c, $P < 0.0005$; analysis of variance).

signaling facilitates the development of endometriosis. We speculated that FKBP52, because of its conserved role as a PR cochaperone, is also critical in the pathogenesis of human endometriosis. Thus, we examined its expression in human endometria of women with and without endometriosis (eutopic and endometriosis-free endometria, respectively) and in human endometriotic lesions ($n = 5$ separate individuals in each stage of the menstrual phase). Results from three representative individuals in each menstrual phase are shown (Figure 6, A–C). Immunostaining detected FKBP52 in stromal and epithelial components of both endometriosis-free and eutopic endometria, although immunoreactive FKBP52 was less intense in eutopic than endometriosis-free endometria, especially in stromal cells (Figure 6, A–B and Supplemental Figure 3B, see *http://ajp.amjpathol.org*). Interestingly, levels of FKBP52 were also remarkably low in ectopic lesions, showing only weak staining in epithelial cells (Figure 6C). There were no striking differences in FKBP52 expression between tissues in proliferative (PP) or secretory phases (SP) among the three groups. Because there is evidence that ovarian, peritoneal and deep-infiltrating endometriotic lesions each have different pathogeneses[49] and because our samples were of ovarian endometriosis, we also confirmed reduced FKBP52 expression in ectopic tissues of peritoneal and deep-infiltrating endometriosis (Supplemental Figure 4, see *http://ajp.amjpathol.org*).

The immunohistochemical studies were complemented by quantitative expression levels of Fkbp52 mRNA in human endometrial and endometriotic samples. Fkbp52 mRNA levels in eutopic endometria and ectopic lesions were significantly lower than endometriosis-free endometria, confirming our immunostaining results (Figure 7, A). We subdivided the data to compare differences in Fkbp52 mRNA levels between PP and SP of each group. We found that Fkbp52 mRNA levels in eutopic endometria and ectopic lesions were significantly decreased in PP, but only moderately in SP compared with endometriosis-free endometria at these phases (Figure 7B).

Reduced PR and FKBP52 Levels with Increased COX-2 and VEGF Expression Is Observed in Human Endometriosis

Our *Fkbp52* null mouse model of endometriosis shows decreased PR expression with increased COX-2 and VEGF expression in ectopic lesions. We therefore examined whether similar expression patterns occur in human endometriosis. Immunostaining of PR, COX-2 and VEGF in human tissues showed that these expression patterns are conserved between mice and humans. While the intensity of FKBP52 immunostaining was lower in eutopic

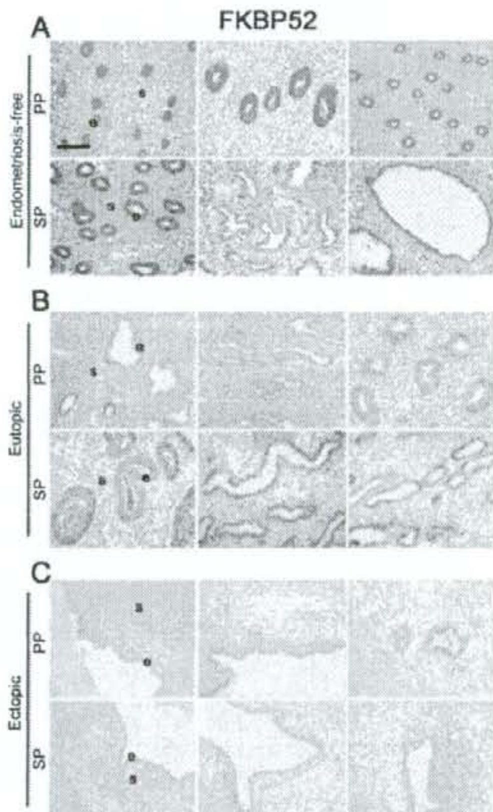

Figure 6. FKBP52 expression is reduced in human eutopic and ectopic endometria. Representative FKBP52 immunostaining in human endometria of women without endometriosis (endometriosis-free) (**A**), or of women with endometriosis: eutopic (**B**) and ectopic (**C**). Each column represents a different individual. Each panel is a representative photograph of five samples of endometriosis-free, eutopic and ectopic human endometria in each menstrual phase. Ectopic endometrium is derived from ovarian endometriosis. PP, proliferative phase; SP, secretory phase; e, epithelium; s, stroma. Scale bar = 200 μm.

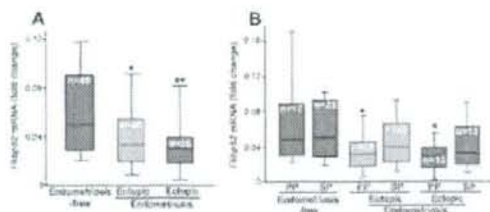

Figure 7. Reduced Fkbp52 mRNA levels in eutopic and ectopic human endometria. Fkbp52 mRNA levels in endometriosis-free, eutopic and ectopic human endometrium (**A**), and their relation to the phase of the menstrual cycle (**B**). Boxes represent the distance between the first (25%) and third (75%) quartiles, 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.

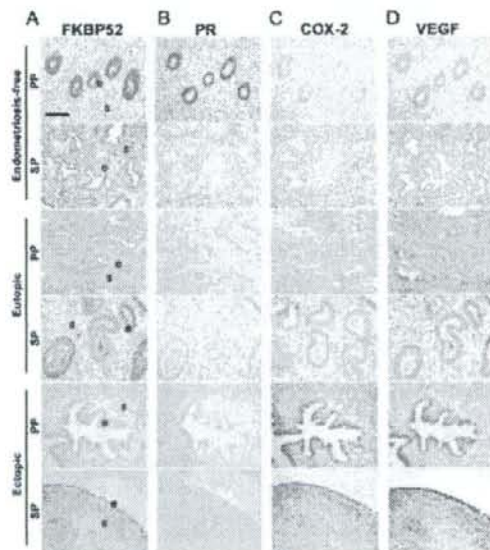

Figure 8. Reduced PR expression with increased COX-2 and VEGF expression is observed in human ectopic endometria. Representative immunostaining of FKBP52 (**A**), PR (**B**), COX-2 (**C**), and VEGF (**D**) in endometriosis-free, eutopic and ectopic human endometria at proliferative (PP) and secretory (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.[6] 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.[59] 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.[36] 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.

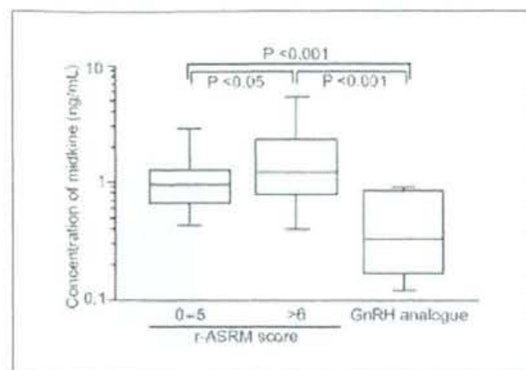

Fig. 1. Midkine concentrations in the peritoneal fluid of women without or with endometriosis and those receiving GnRH agonist treatment [3].

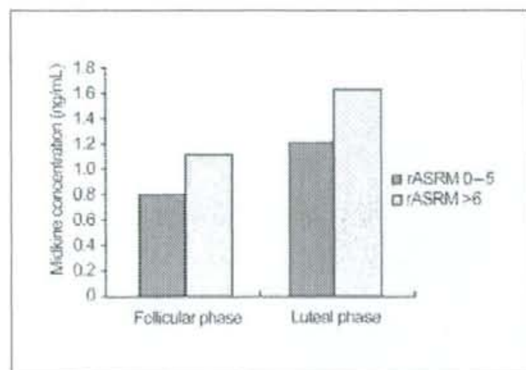

Fig. 2. Midkine concentrations in peritoneal fluid during the follicular and luteal phases of the menstrual cycle [3]. Median midkine concentration in the follicular fluid: 265 ng/ml.

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.

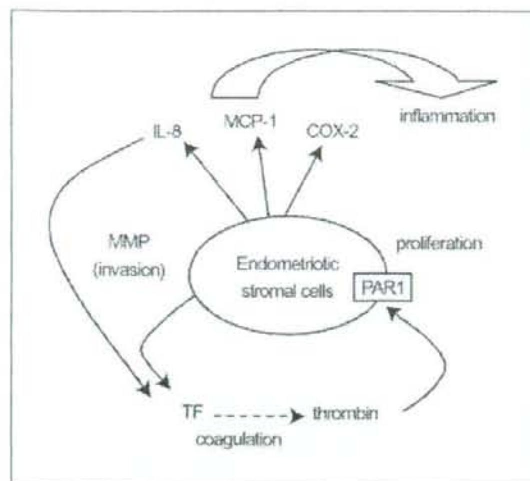

Fig. 3. The possible mechanism of action of thrombin in the development of endometriosis based on studies undertaken in endometriotic stromal cells.

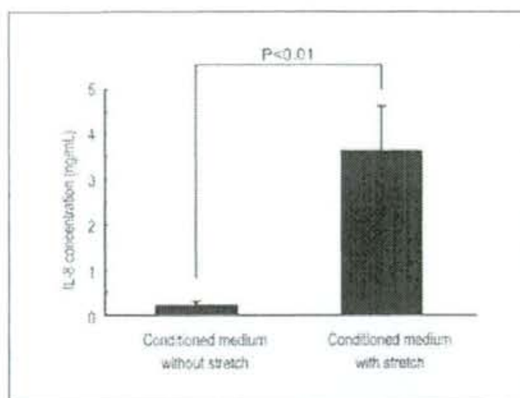

Fig. 4. Effect of conditioned medium obtained from endometrial stromal cells (EmSC) cultured with and without stretch on IL-8 secretion from EmSC [5]. Reproduced with permission from Harada et al. [5] .

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.

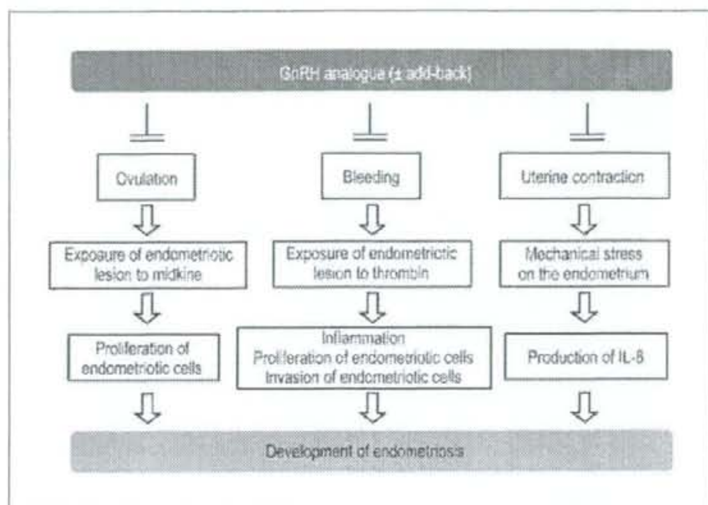

Fig. 5. Summary of the proposed effects of GnRH analogues on the inhibition of endometriosis.

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

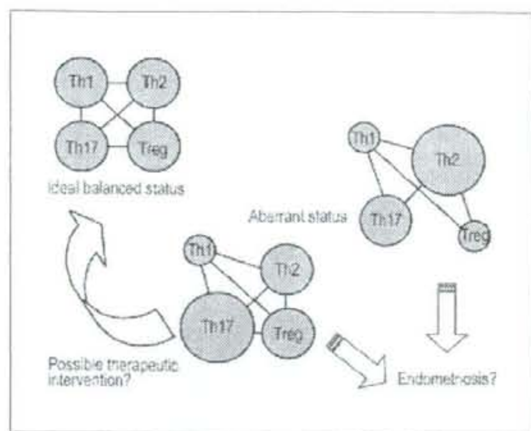

Fig. 6. The T-cell immune network hypothesis.

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

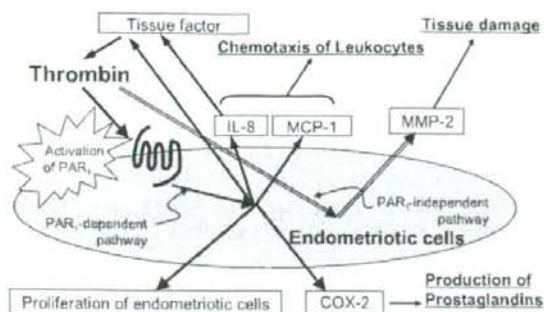

Fig. 1. Possible roles of thrombin in endometriosis. Thrombin stimulates production of IL-8, MCP-1, tissue factor, and COX-2 via PAR$_1$ activation. Thrombin also stimulates proliferation of endometriotic stromal cells via PAR$_1$ activation. Thrombin stimulates production of MMP-2 in a PAR$_1$-independent 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