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TGF-β1 induces proteinase-activated receptor 2 (PAR2) expression in endometriotic stromal cells and stimulates PAR2 activation-induced secretion of IL-6

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BACKGROUND: Proteinase-activated receptor 2 (PAR2) is a G-protein-coupled receptor that is activated by several serine proteases. PAR2 activation in endometriotic stromal cells (ESCs) has been implicated in the development of endometriosis but the regulatory mechanism of PAR2 expression in ESC is unknown. Our objective was to study the mechanism by which PAR2 expression may be regulated in endometriotic lesions.

METHODS: Primary cultures of ESCs were treated with transforming growth factor-β (TGF-β) 1, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and the expression of PAR2 was examined by real-time quantitative PCR. ESCs pretreated with or without TGF-β1 were treated with PAR2 agonist peptide (PAR2AP) and the secretion of the pro-endometriotic cytokine, IL-6, was measured using a specific enzyme-linked immunosorbent assay. Effects of TGF-β type I inhibitor, SB431542, and PAR2 small interfering RNA (siRNA) on the TGF-β1 stimulation of PAR2 gene expression and PAR2AP-induced IL-6 secretion were also evaluated. To study intracellular signaling, effects of inhibitors of mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) and of Smad4 siRNA on the TGF-β1-induced PAR2 gene expression were studied.

RESULTS: Only TGF-β1, but neither TNF-α nor IL-1β, increased gene expression of PAR2. Activation of PAR2 with PAR2AP increased the secretion of IL-6 from ESCs. As expected, TGF-β1 pretreatment dose-dependently enhanced the PAR2AP-induced increase in IL-6 secretion from ESCs. Treatment of ESCs with the TGF-β type I inhibitor, SB431542, inhibited both TGF-β1-stimulation of PAR2 gene expression and PAR2AP-induced IL-6 secretion. Transfection of ESCs with PAR2 siRNA produced a similar inhibition of IL-6 secretion. The TGF-β1-induced increase in PAR2 gene expression was repressed by inhibition of p38 MAPK, p42/44 MAPK or PI3K, but not by knockdown of Smad4 expression.

CONCLUSIONS: In view of significant roles of PAR2 and IL-6 in endometriosis, the TGF-β1-induced increase in PAR2 expression may be an elaborate mechanism that augments the progression of the disease.

Key words: endometriosis / TGF-β1 / proteinase-activated receptor / interleukin-6

Introduction

Endometriosis is defined by the presence of viable endometriotic tissue outside the uterus and remains an incompletely understood disease. Endometriosis adversely affects the health of women of reproductive age, causing pain and infertility (Momoeda *et al.*, 2002; Osuga *et al.*, 2002). Although numerous studies have been conducted on the pathophysiology of the disease, its mechanism of progression is poorly understood. Multiple lines of evidence indicate that endometriosis is a chronic inflammatory disease and both immune and inflammatory responses contribute to the development of the disease. The immune and inflammatory responses are induced by interactions of

endometriosis-associated immune cells with endometriotic cells through various inflammatory substances, such as cytokines, chemokines, proteases, prostaglandins and growth factors. A number of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin (IL)-1, IL-6, IL-8 and IL-17, are suggested to play important roles, such as cell proliferation and angiogenesis, in promoting the disease (Lebovic *et al.*, 2001; Osuga, 2008; Osuga *et al.*, 2008, 2011).

Proteinase-activated receptor 2 (PAR2) is a G-protein-coupled receptor that is activated by cleavage within its extracellular N-terminal domain (Macfarlane *et al.*, 2001). We have previously reported that PAR2 activation stimulates the secretion of IL-6 and IL-8 in endometriotic stromal cells (ESCs) (Hirota *et al.*, 2005a). This response suggests that PAR2 has a functional role in endometriosis-associated inflammation. Moreover, PAR2 activation induces proliferation of ESCs, indicating that PAR2 activation may directly relate to the growth of the endometriotic lesion (Hirota *et al.*, 2005a). In a mouse model of endometriosis, both the number and the total weight of endometriotic lesions were significantly decreased in the PAR2-deficient mice compared with the wild-type mice. Interestingly, concentrations of IL-6 and monocyte chemotactic protein-1 were decreased in the peritoneal fluid and the serum of the PAR2-deficient mice, suggesting alleviated inflammation in the peritoneal cavity of the mice (Osuga *et al.*, 2008). These findings underscore the possible pivotal role of PAR2 in endometriosis. In the eutopic endometrium, PAR2 expression is increased during the menstrual phase (Hirota *et al.*, 2005b), which might contribute to the implantation of endometrial fragments in the retrograde menstruation to the peritoneum. PAR2 activation is induced by proteinases from neutrophils and mast cells, which are both observed in endometriotic tissues. PAR2 is also activated by the coagulation product, TF/VIIa, (Molino *et al.*, 1997; Uehara *et al.*, 2002, 2003), which could be formed by the local bleeding that is often observed in endometriosis.

Despite the observed effects of PAR2 in endometriosis, the regulation of PAR2 expression in endometriotic tissues remains unknown. TNF-α and IL-1β both increase PAR2 expression in neurons (Noorbakhsh *et al.*, 2005), whereas TGF-β increases PAR2 expression in fibroblasts (Materazzi *et al.*, 2007). Interestingly, TNF-α, IL-1β and TGF-β are all implicated in the development of endometriosis. In the present study, we found that TGF-β1, but neither IL-1β nor TNF-α, increased the gene expression of PAR2 in ESCs. This finding prompted us to investigate further the TGF-β1-induced expression of PAR2 in endometriosis. Therefore, we examined the effect of TGF-β1 on PAR2 activation-induced IL-6 secretion in ESCs. IL-6 is a representative pleiotropic cytokine involved in the development of endometriosis (Witz, 2000; Salmassi *et al.*, 2008). We also studied the possible intracellular mechanism by which TGF-β1 increases PAR2 expression in ESCs.

Materials and Methods

Patients and samples

Endometriotic tissues were obtained from patients with ovarian endometriomas undergoing laparoscopy. The diagnosis of endometriosis was confirmed by histopathological examination. Laparoscopic excision of ovarian endometriomas was performed as follows. After inspection of the pelvis, the ovary was freed from any adhesions. The endometrioma cyst wall was stripped away from the normal ovarian tissue gently and completely. Endometriotic tissue samples obtained from the excised endometrioma cyst wall were transported to the laboratory in DMEM/Ham's F12 medium (DMEM/F12; Invitrogen, Rockville, MD, USA) on ice under sterile conditions. All of the women had regular menstrual cycles, and none had received hormonal treatment for at least 6 months before surgery. This experimental procedure was approved by the Institutional Review Board of the University of Tokyo and signed informed consent for the use of the endometriotic tissues was obtained from each woman.

Isolation and culture of human ESCs

Isolation and culture of human ESCs were conducted as described previously (Hirota *et al.*, 2005a,c; Hirata *et al.*, 2008). Briefly, fresh endometriotic tissues collected in sterile medium were rinsed to remove blood cells and then were minced into small pieces and incubated in DMEM/F-12 containing type I collagenase (0.25%; Sigma, St Louis, MO, USA) and deoxyribonuclease I (15 U/ml; Takara, Tokyo, Japan) for 120 min at 37°C. The resulting dispersed endometriotic cells were separated by filtration through a 100 and 70 μm nylon cell strainers (BD, Franklin Lakes, NJ, USA). ESCs in the filtrate were collected by centrifugation and resuspended in phenol-red free DMEM/F-12 containing 5% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. ESCs were seeded in a 100 mm culture plate and kept at 37°C in a humidified 5% CO_2–95% air atmosphere. At the first passage, the cells were plated into 12- or 48-well culture plates (BD) at a density of 2×10^5 cells/ml. The purity of the ESC population was more than 95%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin, CD45 and von Willebrand factor.

Treatment of ESCs

When the ESC culture reached 70–80% confluence in 1 or 2 days, media were removed and replaced with fresh media containing 2% charcoal-stripped FBS and antibiotics. After culturing for an additional 12 h, the cells were ready for use in the experiments. To examine the effect of cytokines on PAR2 gene expression, ESCs were incubated with TGF-β1 (10 ng/ml), IL-1β (10 ng/ml) and TNF-α (10 ng/ml) (all cytokines were from R&D Systems, Minneapolis, MN, USA) for 6 h. We used PAR2 agonist peptide (PAR2AP; SLIGKV, BACHEM, Bubendorf, Switzerland) for the activation of PAR2 (Hirota *et al.*, 2005a). PAR2AP comprised the unmasked amino-terminal peptide of PAR2 cleaved by the activating proteinases. To examine the effect of TGF-β1 on IL-6 secretion induced by PAR2AP, ESCs were pretreated with TGF-β1 (10 ng/ml) for 24 h and then incubated with PAR2AP (30 μM) for 24 h. To examine the effect of inhibition of type I TGF-β receptor on gene expression of PAR2, ESCs were incubated with or without SB431542 (10 μM) (Calbiochem, La Jolla, CA, USA), and with TGF-β1 (10 μg/ml) for 6 h. To examine the effect of SB431542 on PAR2AP-induced IL-6 secretion, ESCs were pretreated with or without SB431542 (10 μM) and with TGF-β1 (10 ng/ml) for 24 h and then incubated with PAR2AP (30 μM) for 24 h. To see the effect of inhibitors of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) on TGF-β1-induced gene expression of PAR2, ESCs were pretreated with SB202190 (10 μM), PD98059 (25 μM), SP600125 (10 μM) or LY294002 (20 μM) [inhibitors of p38 MAPK, p42/44 MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and PI3K, respectively; Calbiochem] for 30 min before treatment with TGF- β for 6 h.

Small interfering RNA

The small interfering RNA (siRNA) constructs used were obtained as ON-TARGET plus SMART pool PAR2 (L-005095-00-0005) and Smad4 (L-003902-00-0005) from Dharmacon (Lafayette, CO, USA). The non-targeting siRNA control, ON-TARGET plus siCONTROL non-targeting pool (D-001810-10-05), was also obtained from Dharmacon. Cells were transfected with 30 nmol/l siRNA for 24 h in Opti-MEM I using Lipofectamine RNAi max according to the manufacturer's protocol. After transfection, the medium was removed and replaced with fresh medium containing 5% charcoal-stripped FBS and antibiotics for 24 h. The cells were then treated with TGF-β1 and PAR2AP as described above.

RNA extraction, RT and real-time quantitative PCR

RNA extraction, RT and real-time quantitative PCR were performed as described previously (Takemura *et al.*, 2007; Hirata *et al.*, 2008). Total RNA was extracted from cultured ESCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR and data analysis were performed using a LightCycler (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. Expression of PAR2 and Smad4 mRNA was normalized to RNA loading for each sample using human glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mRNA as an internal standard. The PAR2 primers chosen (sense, 5′-CTGCATCTGTCCTCACTGGA-3′; antisense, 5′-ACAGAG AGGAGGTCAGCCAA-3′) amplified a 181 bp fragment. The Smad4 primers chosen (sense, 5′-TGGCTGGTCGGAAAGGATTT-3′; antisense, 5′-ACTGGCAGGCTGACTTGTGG-3′), amplified a 431 bp fragment. The PCR conditions were as follow: for PAR2, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 10 s; for Smad4, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 18 s; for GAPDH, 30 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 18 s. All of the PCR experiments were followed by melting curve analysis.

Measurement of IL-6 protein

The concentration of IL-6 in the conditioned media was measured using a specific ELISA kit (R&D Systems). The sensitivity of the assay was 3.12 pg/ml, and the intra- and inter-assay coefficients of variation were less than 5%.

Statistical analysis

Data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons, or Student's *t*-test, appropriately. A value of $P < 0.05$ was considered significant.

Results

Effects of TGF-β1, TNF-α and IL-1β on gene expression of PAR2

TGF-β1 increased gene expression of PAR2, whereas neither TNF-α nor IL-1β affected PAR2 expression (Fig. 1A). The TGF-β1-induced PAR2 expression was dose-dependent between 1 and 10 ng/ml, the increase being significant from 1 ng/ml (Fig. 1B).

Effects of TGF-β1 on PAR2AP-induced IL-6 secretion

Although PAR2AP alone increased IL-6 secretion by 2.8-fold, TGF-β1 pretreatment dose-dependently enhanced PAR2AP-induced IL-6 secretion, with a total increase of 9.8-fold observed at 10 ng/ml TGF-β1 (Fig. 2).

Effects of SB431542 and PAR2 siRNA on TGF-β1-induced gene expression of PAR2 and on TGF-β1 stimulation of PAR2AP-induced IL-6 secretion

The TGF-β1 type I inhibitor, SB431542, suppressed the TGF-β1-induced expression of PAR2 (Fig. 3A). SB431542 also suppressed the TGF-β1 stimulation of PAR2AP-induced IL-6 secretion (Fig. 3B). Knockdown of PAR2 expression using PAR2 siRNA remarkably reduced PAR2 mRNA levels (Fig. 4A). Similar to the treatment with SB431542, PAR2 siRNA treatment inhibited the TGF-β1 stimulation of PAR2AP-induced IL-6 secretion (Fig. 4B).

Effects of MAP kinase inhibitors, PI3K inhibitor and Smad4 siRNA on TGF-β1-induced gene expression of PAR2

The Smad pathway, several MAPK pathways and PI3K pathway are typical intracellular signaling pathways activated by TGF-β1. To examine whether these pathways are involved in TGF-β1-induced gene expression of PAR2, ESCs were treated with MAPK inhibitors and Smad4 siRNA. The p38 MAPK, p42/44 MAPK and PI3K inhibitors (SB202190, PD98059 and LY294002 respectively) significantly

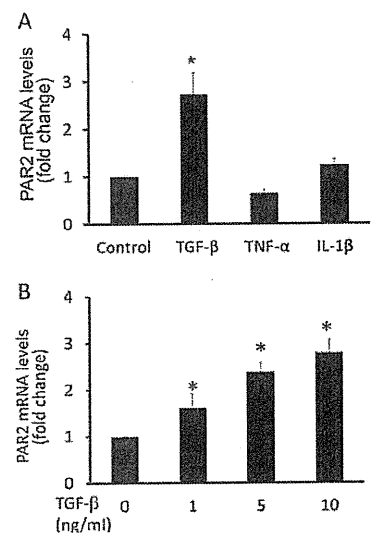

Figure 1 TGF-β1-induced increase in PAR2 mRNA expression in ESCs. (**A**) ESCs were cultured with TGF-β1 (10 ng/ml), TNF-α (10 ng/ml) and IL-1β (10 ng/ml) for 6 h. (**B**) ESCs were cultured with different concentrations of TGF-β1 for 6 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. Data are the mean ± SEM of six (A) and five (B) independent experiments using different ESCs. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. *$P < 0.05$ versus control.

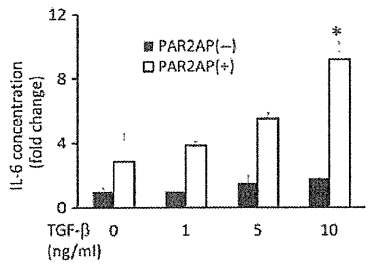

Figure 2 TGF-β1-induced increase in PAR2AP-induced ESC secretion of IL-6. ESCs were pretreated with TGF-β1 (0, 1, 5 and 10 ng/ml) for 24 h and subsequently incubated with or without 30 μM PAR2AP for 24 h. At the end of the incubation period, the conditioned medium was collected and assayed for IL-6 by ELISA. The values are presented as the mean ± SEM of four separate cultures. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. *$P < 0.05$ versus ESCs stimulated with PAR2AP but without TGF-β1 pretreatment. The result is representative of three repeated experiments using samples from three different women.

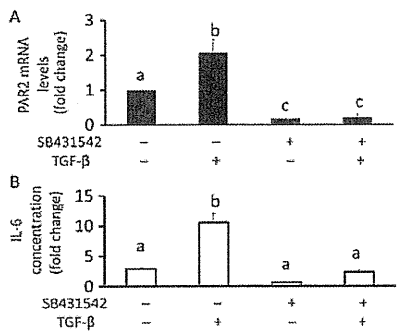

Figure 3 Effects of SB431542 on the TGF-β1-induced increase in PAR2 mRNA expression and in PAR2AP-induced IL-6 secretion from ESCs. (**A**) ESCs were cultured with or without SB431542 (10 μM) and TGF-β1 (10 ng/ml) for 24 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. The values are presented as the mean ± SEM of three independent experiments. The data were analyzed by Student's *t*-test. (**B**) ESCs were pretreated with or without SB431542 (10 μM) and TGF-β1 (10 ng/ml) for 24 h and subsequently incubated with PAR2AP (30 μM) for 24 h. At the end of the incubation period, the conditioned media were collected and assayed for IL-6 by ELISA. The values are presented as the mean ± SEM of four separate cultures. Data are shown as the fold change in IL-6 concentrations in ESCs not pretreated with SB431542 and TGF-β1 and not stimulated with PAR2AP. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. The result is representative of three repeated experiments using samples from three different women. (A and B) Different letters denote significant differences between groups ($P < 0.05$).

diminished TGF-β1-induced PAR2 gene expression (Fig. 5A). In contrast, neither the SAPK/JNK inhibitor (SP600125) nor treatment with Smad4 siRNA had any effect on TGF-β1-induced PAR2 gene expression, although Smad4 siRNA markedly decreased gene expression of Smad4 (Fig. 5B).

Discussion

In the present study, we demonstrated that TGF-β1, but neither TNF-α nor IL-1β, increased gene expression of PAR2. TGF-β1 dose-dependently increased the secretion of IL-6 in PAR2AP-stimulated ESCs. SB431542, an inhibitor of the TGF-β receptor, inhibited the TGF-β-induced increase in gene expression of PAR2 in ESCs and the TGF-β1-augmented IL-6 secretion from PAR2AP-stimulated ESCs. Likewise, PAR2 siRNA inhibited the TGF-β1-induced increase in gene expression of PAR2 in ESCs and the TGF-β1-augmented

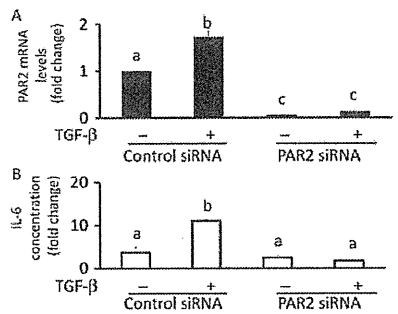

Figure 4 Effects of PAR2 siRNA on TGF-β1-induced increase in PAR2 mRNA expression and in PAR2AP-induced ESC secretion of IL-6. (**A**) ESCs were transfected with 30 nmol/l PAR2 siRNA or negative control siRNA for 24 h. After transfection, the medium was removed and replaced with medium containing 5% charcoal-stripped FBS and antibiotics for 24 h. Thereafter, ESCs were cultured with or without TGF-β1 (10 ng/ml) for 24 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. The values are presented as the mean ± SEM of three independent experiments using different ESCs. The data were analyzed by Student's *t*-test. (**B**) ESCs were transfected with 30 nmol/l PAR2 siRNA or negative control siRNA for 24 h. After transfection, the medium was removed and replaced with the medium containing 5% charcoal-stripped FBS and antibiotics for 24 h. Thereafter, ESCs were pretreated with or without TGF-β1 (10 ng/ml) for 24 h and subsequently incubated with PAR2AP (30 μM) for 24 h. At the end of the incubation period, the conditioned medium was collected and assayed for IL-6 by ELISA. The values are presented as the mean ± SEM of four separate cultures. Data are shown as fold changes in IL-6 concentrations in ESCs pretreated with control siRNA but without TGF-β1, and not stimulated with PAR2AP. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. The result is representative of three repeated experiments using samples from three different women. (A and B) Different letters denote significant differences between groups ($P < 0.05$).

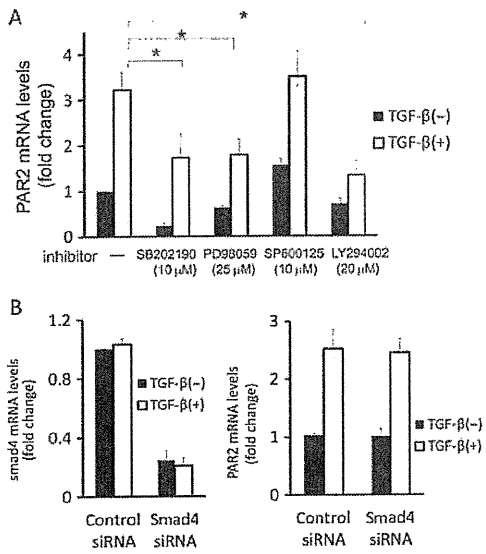

Figure 5 TGF-β1-induced expression of PAR2 mRNA in ESCs treated with inhibitors of MAPKs or PI3K, or Smad4 siRNA. (**A**) ESCs were treated with inhibitors of p38 MAPK (SB202190), p42/44 MAPK (PD98059), SAPK/JNK (SP600125) or PI3K (LY294002) for 30 min and stimulated with TGF-β1 (10 ng/ml) for 6 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. Data are the mean ± SEM of nine independent experiments using different ESCs from nine patients. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. $^*P < 0.05$ versus ESC treated without the inhibitors but with TGF-β1. (**B**) ESCs were transfected with 30 nmol/l Smad4 siRNA for 24 h. After transfection, media were removed and treated with or without TGF-β1 (10 ng/ml) for 6 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for Smad4 and PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2 and Smad4. Data are the mean ± SEM of three independent experiments using different ESCs from three women. The data were analyzed by Student's *t*-test.

IL-6 secretion from PAR2AP-stimulated ESCs. SB202190, a p38 MAPK inhibitor, PD98059, a p42/44 MAPK inhibitor, and LY294002, a PI3K inhibitor, suppressed the TGF-β1-induced gene expression of PAR2. Suppression of Smad4 expression by the siRNA had no effect on TGF-β1-induced gene expression of PAR2.

It is interesting that neither IL-1β nor TNF-α increased gene expression of PAR2 in ESCs, compared with the stimulation by both molecules of PAR2 expression in neurons, osteoarthritis chondrocytes and osteoblasts (Noorbakhsh *et al.*, 2005, Xiang *et al.*, 2006; Boileau *et al.*, 2007; Amiable *et al.*, 2009). We observed that TGF-β1 increased PAR2 expression in ESCs, which is consistent with TGF-β1 stimulation of PAR2 expression in human dermal fibroblasts (Materazzi *et al.*, 2007). Therefore, PAR2 expression appears to be differentially regulated in different cell types.

TGF-β may play multiple roles in different stages of the progression of endometriosis (Omwandho *et al.*, 2010). The present study demonstrates a new role of TGF-β1 in the development of endometriosis, in the induction of PAR2 expression in ESCs. The increase in PAR2 expression consequently enhanced IL-6 secretion from PAR2AP-stimulated ESCs. Given that the proteinases and the coagulation product that activate PAR2 are present in endometriotic tissues, this sequence may partly explain the increased expression of IL-6 in both endometriotic tissue and in peritoneal fluid of women with endometriosis (Salmassi *et al.*, 2008; Velasco *et al.*, 2010). The elevation in TGF-β levels in endometriotic tissues is therefore likely to contribute to this sequence of events (Tamura *et al.*, 1999; Komiyama *et al.*, 2007).

IL-6 is a multifunctional cytokine that is involved in numerous immunological and proliferative responses in endometriosis (Witz, 2000). In particular, IL-6 increases aromatase activity, haptoglobin production and hepatocyte growth factor production in endometriotic cells and/or endometriotic cells (Piva *et al.*, 2001; Khan *et al.*, 2005; Velasco *et al.*, 2006; Sharpe-Timms *et al.*, 2010). IL-6 is also known to stimulate the proliferation of ESCs (Khan *et al.*, 2005). These findings indicate that IL-6 stimulates the progression of endometriosis via various events such as cell proliferation, angiogenesis and immunomodulation. Therefore, the TGF-β1-stimulated increase in PAR2 expression and the resulting increase in IL-6 is a possible mechanism by which TGF-β1 can amplify PAR2-mediated disease progression. This hypothesis is also consistent with the previous finding that PAR2 activation stimulates the proliferation of ESCs (Hirota *et al.*, 2005a,b,c). In addition, increased IL-6 production via a TGF-β1-stimulated increase in PAR2 expression might contribute to endometriosis-associated infertility, because IL-6 is suggested to be a causative factor for infertility in endometriosis (Gomez-Torres *et al.*, 2002, Yoshida *et al.*, 2004; Deura *et al.*, 2005).

The effect of TGF-β1 on PAR2 expression might provide a novel insight in the pathogenesis of endometriosis. Immune cells are an important component of endometriotic tissues and are involved in the development of the disease. In particular, our recent studies suggest that Th2 cells and Th17 cells contribute to disease progression by inducing inflammation and cell proliferation (Hirata *et al.*, 2008; OuYang *et al.*, 2008, 2010). The differentiation of Th cells is under the strict control of cytokines, with Th17 cells being induced from naïve Th cells by TGF-β1 in combination with IL-6. Without IL-6, naïve Th cells will differentiate into regulatory T cells under the influence of TGF-β1 (Miossec *et al.*, 2009). Therefore, we hypothesize that TGF-β1 stimulates the environment to aid the development of Th17 cell by increasing PAR2 activation-induced secretion of IL-6 in endometriotic tissues. This process could be potentiated by enhancement of PAR2 activation by the proteolytic enzymes produced by neutrophils activated by Th17 cells (Miossec *et al.*, 2009). In this way, TGF-β1 may co-operate with Th17 cells to stimulate disease progression. Further studies are warranted to corroborate the notion.

TGF-β utilizes multiple signaling pathways to stimulate different cells. Smad4 is essential for TGF-β signal transduction (Prud'homme, 2007), but suppression of Smad4 expression by siRNA did not inhibit the TGF-β1-induced increase in PAR2 expression in ESCs. In contrast, a p38 MAPK inhibitor and a p42/44 MAPK inhibitor suppressed the effect of TGF-β1. The present findings indicate that the activation of

p38 MAPK and p42/44 MAPK is involved in TGF-β1-induced increase in PAR2 expression in ESCs. In this context, it appears to be somewhat paradoxical that IL-1β and TNF-α did not increase PAR2 expression, although these molecules activate p38 MAPK and p42/44 MAPK in ESCs (Yoshino *et al.*, 2004). Presumably, the activation of p38 MAPK and p42/44 MAPK is necessary but not sufficient for increasing PAR2 expression in ESC, though the precise mechanism is unknown at present. Another notable point of our finding is that the effect of p38 MAPK and p42/44 MAPK inhibitors on PAR2 expression was not great, which indicates that other pathways may also have an effect on TGF-β1-induced PAR2 expression. In this sense, it is interesting that a PI3K inhibitor also inhibited TGF-β1-induced increase in PAR2 expression in ESCs. The PI3K pathway may play a complementary role to the MAPKs in the TGF-β1-induced response.

Since TGF-β stimulates disease progression, it should be a therapeutic target of endometriosis. A variety of agents that interfere with TGF-β signaling, including neutralizing antibodies, soluble receptors, antagonists and antisense nucleotides, have been developed to treat diseases in which TGF-β is a crucial pathogenic factor (Gordon and Blobe, 2008). Indeed, some of these drugs are undergoing clinical trials. The present study suggests that inhibitors of p38 MAPK and p42/44 MAPK could be candidate drugs for the treatment of endometriosis, given that they repress TGF-β1-induced PAR2 expression. This action by a p38 MAPK inhibitor may partly explain our previous finding that the inhibitor diminished endometriosis in a mouse model (Yoshino *et al.*, 2006).

In the present study, we evaluated the effect of TGF-β1, but neither TGF- β2 nor TGF- β3, on PAR2 expression in ESCs. As TGF- β2 and TGF- β3 have been reported to be increased in endometriotic tissues (Tamura *et al.*, 1999), it would be interesting also to study the effect of these molecules.

In summary, the present study demonstrated that TGF-β1 enhanced PAR2 expression and, as a consequence, increased PAR2-activation-induced IL-6 secretion from ESCs. In light of the multiple roles of PAR2 in promoting the development of endometriosis, TGF-β1 may accelerate disease progression by up-regulating PAR2 expression.

Authors' roles

A.S., Y.O. and O.Y. participated in study design and manuscript drafting. A.S., Y.O. and M.T. participated in execution and analysis. A.S., Y.O., T.H., Y.H., K.K., M.H., Y.T., T.Y. and Y.T. participated in critical discussion.

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診療

子宮筋腫により誘導される子宮内膜の異常蠕動様運動は妊娠率を低下させる

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子宮筋腫のうち，特に筋層内子宮筋腫が不妊症を引き起こす機序はいまだよくわかっておらず，その取り扱いに苦慮することが多い。正常子宮内膜は着床期に蠕動様運動を低下させることで胚の着床を促すことが知られている。動画MRIを用いて子宮内膜の蠕動様運動の評価を行い，筋層内子宮筋腫が着床期の子宮内膜蠕動様運動に与える影響と妊孕性への関与について検討を行った。筋層内子宮筋腫を合併し，かつ，卵管因子・重度の男性因子のない51例の挙児希望患者を対象とした。黄体期5～9日目に，動画MRI検査を行った。子宮内膜蠕動様運動が3分間で2回未満（低頻度群）および2回以上（高頻度群）の2群に分け，MRI撮影後4カ月間の妊娠率を前方視的に比較した。群分けの基準は，これまでの検討で子宮筋腫を有さない症例では子宮内膜蠕動様運動が3分間で2回未満であることによる。低頻度群は29名（57%），高頻度群は22名（43%）であり，子宮筋腫の個数および最大径に関して差を認めなかった。MRI撮影後，低頻度群は29名中10名（34%）が妊娠に至ったのに対し，高頻度群での妊娠例はなかった（0%，$p<0.005$）。本来では子宮内膜の蠕動様運動を認めない着床期において，筋層内子宮筋腫を合併する不妊患者の約4割に子宮内膜の異常蠕動様運動を認めた。子宮筋腫により誘導される子宮内膜の異常蠕動様運動は妊娠率を低下させる可能性が示唆された。

はじめに

子宮筋腫は生殖年齢女性の20～50%が罹患しており[1]，日常臨床においてよく遭遇する疾患であるが，大半の症例は特に症状がないことから治療を要しない[2]。しかし，特に不妊治療との関連となると，子宮筋腫の解剖学的位置，すなわち粘膜下，筋層内，漿膜下のいずれに存在するかより，その取り扱いは大きく異なる[3]。粘膜下子宮筋腫の場合，子宮内腔への突出率が高ければ，子宮鏡下に低侵襲手術を行うことができる。一方で，筋層内・漿膜下を手術する際，腹壁からのアプローチが必要となる[3]。特に筋層内子宮筋腫症例は，手術による正常筋層へのダメージを考慮し，子宮筋腫核出術後に不妊治療の一時中止および，分娩時には，子宮破裂のリスクを避けるために帝王切開が推奨されることが多く[2]，患者への負担が多いことが挙げられる。子宮筋腫の位置と妊娠率に関するメタアナリシスによると，粘膜下子宮筋腫は明らかに妊娠率を低下させ，漿膜下子宮筋腫は妊娠率を低下させないことが判明している一方で，筋層内子宮筋腫が妊娠そのものに与える影響は一定の見解が得られていない[2][3]。すなわち，筋層内子宮筋腫の不妊症治療という観点からの取り扱いには苦慮することが多い。そこで，われわれ

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は筋層内子宮筋腫が不妊症に与える影響について，そのメカニズムの観点から検討することとした。

これまで，筋層内子宮筋腫は，着床に悪影響を与えること[4]，その機序として子宮における異常収縮を誘導する可能性が提唱されてきた[2)5)]。しかし，この仮説を実際に検証する報告はこれまでなされていない。着床期，子宮内膜の蠕動様運動は減少することが知られており，このことが着床に重要な現象であると考えられている[6)~8)]。近年のMRI撮像技術の進歩により，数秒間隔での連続撮影が可能となった。同一部位で連続撮影を行うことで，動画画像(Cine MRI）を構築することができるようになり，心臓をはじめとする各種臓器での応用が始まっている。Togashiらは同法を用いて，子宮内膜の蠕動様運動が描出できることを報告しており，着床期における子宮内膜の蠕動様運動の頻度は3分間で0~1回と報告している[6)]。本研究は筋層内子宮筋腫が着床期の子宮内膜蠕動運動に与える影響とその妊孕性への関与について検討を行った。

1．方　法

本研究は各施設の倫理委員会の承認を得て行った。2008年9月から2009年10月までに子宮筋腫を有する不妊症例に対して4病院（帝京大学医学部附属溝口病院，慶應義塾大学病院，福井大学医学部附属病院，滝の川クリニック）にてMRI検査を受けた95名を対象とした。このうち，本研究の適応症例として，①粘膜下病変を有さない筋層内子宮筋腫症例，②子宮筋腫以外に不妊症原因のないこと。具体的には排卵，黄体機能，卵管，パートナーの精子所見に重度な異常を認めないこと，③月経周期が順調であり，基礎体温表でのフォローを行い，MRIを高温期の5~9日目，いわゆるimplantation windowに撮影した。95名のうち，この基準に合う症例は51名であった。通常のMRI検査を行い，子宮筋腫の数および最大径，子宮内膜の変形の有無，子宮内膜症病変の有無を検討した。その際のMRI撮影条件は以下のとおりである。なお，MRIは1.5テスラ装置を用いた。T1強調像：(TR/TE＝400~550/7.0~8.5 msec)，T2強調像：(T2WIs，TR/TE＝4000~4720/90~111 msec）子宮内膜の動画MRIの条件はOrisakaらによって報告されている方法を用いた[9)]。通常の呼吸下に6秒ごとに3分間，計30枚の子宮内膜部位のMRIをsingle-shot fast spin-echo（SSFSE）法にて撮影した（TR/TE＝6000/78 msec，FOV＝240 mm，slice thickness＝10 mm，matrix＝256×256)。得られた画像はcineモードにて高速再生した。読映は放射線科医師1名が判定した。判定のポイントとして，①子宮内膜周囲に存在する正常筋層，いわゆるjunctional zoneに運動があるか否か，また運動がある場合は3分間における蠕動様運動の回数として表記，②子宮筋腫の個数とその位置，および③子宮内膜症の有無を主に子宮内膜症性卵巣嚢胞の有無にて判別した。動画MRI検査の結果により，子宮内膜蠕動様運動が3分間で2回未満（低頻度群）および2回以上（高頻度群）の2群に分け，MRI撮影後4カ月間の妊娠率を比較した。群分けの基準は，Togashiらの報告，およびわれわれのこれまでの検討で子宮筋腫を有さない症例では子宮内膜蠕動様運動が3分間で2回未満であることによる[6)]。MRI撮影後4カ月間，通常の不妊治療を行い，妊娠に関して前方視的に検討した。基本的に自然周期ないしclomiphene citrate（CC）による治療を2~3コース行い，妊娠に至らない場合はhuman menopausal gonadotropin（hMG）療法を2コース程度行った。卵胞の最大径が18 mmに成長した段階で，hCG注射（5,000単位）に切り替え排卵を誘導した。基本的に排卵時期を予測し，性交時期を指示したが，特にパートナーの運動精子濃度が20×10^6/m*l*以下の場合は人工授精を行った。黄体ホルモン補充療法は行わなかった。

子宮内膜蠕動様運動の低頻度群および高頻度群間の患者年齢，不妊期間，子宮筋腫の個数および最大径はmeans±SDで示し，両群間の比

表2 患者背景

	低頻度群	高頻度群	
患者数（人）	29	22	
年齢	36（29～41）	37（29～41）	中間値（最小～最高値） 有意差なし
不妊期間（月）	24（3～84）	24（4～108）	中間値（最小～最高値） 有意差なし
原発性・続発性不妊（患者数） 原発性 続発性	 20 9	 17 5	 有意差なし
体外受精の既往（患者数） なし あり	 24 5	 18 4	 有意差なし

子宮内膜蠕動運動が3分間で2回未満（低頻度群）および2回以上（高頻度群）の2群に分け，患者背景を比較した。

表1 蠕動様運動回数別の患者分布

蠕動様運動回数（/3 min）	患者数（全51名）
0	19
1	10
2	1
3	6
4	10
5	3
6	2

動画MRI法（Cine MRI法）を用いて子宮内膜の蠕動様運動を計測した。3分間当たりの蠕動様運動回数別による患者分布。これまでの報告から，3分間で2回以上の蠕動様運動は高頻度と考えられる。

較はMann-Whitney's U-test（Statcel software）を用いた。その他の指標については患者数で示し，χ^2検定にて2群間の比較を行った。

2．結　果

MRI検査を受けた子宮筋腫を有する不妊症95例のうち，上記条件に適合した対象患者は51名であった。51名を子宮内膜の蠕動様運動回数別に分類した（表1）。3分間に蠕動様運動を0ないし1回認めた低頻度群，および2回以上を認めた高頻度群の症例数はそれぞれ，29例（57%）と22例（43%）であった。両群の患者背景を表2に示す。患者年齢，不妊期間，原発性不妊と続発性不妊の割合，体外受精既往の割合に差を認めなかった。MRI検査で得られた所見に関して表3に示す。子宮内膜症の罹患率，子宮筋腫の個数および最大径，子宮筋腫により子宮内膜の変形をきたしている症例数の割合は，両群間で差を認めなかった（表3）。子宮筋腫の個数と最大径はそれぞれ，低頻度群で2.8個，53 mm（中間値），高頻度群では3.5個，58 mm（中間値）であった。なお，子宮筋腫の位置は子宮体部もしくは子宮底部であり，子宮狭部や頸部筋腫症例は認めなかった。低頻度群29名中6名，および高頻度群22名中6名がHMG療法を受け，残りは自然排卵もしくはクロミフェン療法を受けた。また，パートナーの精液所見が不良（運動精子$<20\times10^6$/ml）により，低頻度群29名中9名，および高頻度群22名中4名が人工授精を受けた（表4）。MRI撮影後の4カ月間，低頻度群29名中10名（34%）が妊娠したのに対し，高頻度群22名中妊娠例は0名であった（表3）（0%，$p<0.005$）。

妊娠症例10名の内訳は，7例が自然排卵および3例がクロミフェン療法を受けていた。また，10例中，1例が人工授精による妊娠であった（表4）。

表3 両群間のMRI所見と妊娠率

	低頻度群	高頻度群	
患者数（人）	29	22	
子宮内膜症 なし あり	 22 7	 16 6	 有意差なし
子宮筋腫の個数	2.8±2.8	3.5±3.0	有意差なし
子宮筋腫の最大径（mm）	53±17	58±21	有意差なし
子宮内腔の変形をきたした症例 なし あり	 14 15	 12 10	 有意差なし
MRI後の妊娠例 症例数（%）	 10（34%）	 0（0%）	 p<0.005

子宮内膜蠕動様運動が3分間で2回未満（低頻度群）および2回以上（高頻度群）の2群に分け，MRI所見およびMRI撮影後4カ月間に妊娠した症例を示した。
N. S.: not significant

表4 MRI撮影後の不妊治療の詳細と妊娠症例数

低頻度群			
排卵誘発法		患者数	妊娠例
自然	タイミング指導	14	7
	人工授精	5	0
クロミフェン	タイミング指導	2	2
	人工授精	2	1
hMG	タイミング指導	4	0
	人工授精	2	0
高頻度群			
排卵誘発法		患者数	妊娠例
自然	タイミング指導	11	0
	人工授精	3	0
クロミフェン	タイミング指導	2	0
	人工授精	0	0
hMG	タイミング指導	5	0
	人工授精	1	0

3．考　察

子宮内膜は蠕動様運動を呈し，この運動の頻度および方向は月経周期によって大きく変動することが知られている[8]。特に排卵期および月経期に子宮内膜蠕動様運動の頻度が大きい。排卵期は子宮頸部から底部に向かって運動することで，精子を汲み上げる働きをしていることが考えられている。また，月経期には子宮底部から頸部方向に運動することで，月経血を子宮内腔より排出する作用があると考えられている。一方で着床期には，子宮内膜の蠕動様運動はほとんどみられなくなる[6)9)]。そして運動を抑制することで，胚の子宮内膜への着床を促していることが予想されている[10]。これら子宮内膜の蠕動様運動は女性ホルモンにより制御されており，エストロゲンは運動亢進を，プロゲステロンは運動抑制に寄与することが知られている[11]。

Fanchinらは子宮に異常を認めない不妊症患者を対象に超音波断層装置を用いた検討を報告している。彼らはIVF-ET患者の胚移植時の子宮内膜蠕動様運動の回数と妊娠率を比較し，蠕動様運動回数が少ないほど妊娠率が高くなるとしている[7)8)]。彼らの報告は，implantation window（黄体期day 5～day 9）時点での検討ではなく，黄体期2日目での検討ではあるが，子宮内膜の蠕動様運動頻度が高いと妊娠率が低下することの理由として，同運動が胚を子宮腔から

押し出すことが推測される[7]。Orisakaらはcine-MRIを用いた検討で，子宮に異常を認めない正常コントロールでは着床期に子宮内膜蠕動様運動を認めなかったのに対し，筋層内子宮筋腫を有する患者の中には異常運動を呈する症例があることを報告している[9]。

子宮筋腫，特に筋層内子宮筋腫が不妊症に与える影響はよくわかっておらず，また不妊症の観点からは，その取り扱いに苦慮することが多い。われわれは子宮筋腫により誘導される子宮内膜の異常蠕動様運動に着目し，これらが不妊症患者に対し悪影響を与えているかを検討した。MRI検査を着床期に行ったところ，51例中22例（43.1%）と半数以下の症例で，本来は子宮内膜に蠕動様運動を認めない時期に異常運動を認めた。さらに，MRI検査後に前方視的に妊娠について調査を行ったところ，興味深いことに蠕動様運動を高頻度に認めた22例中に妊娠症例を認めなかった（0%）のに対し，低頻度群では29例中10例（34.5%）に妊娠を認めた。低頻度群と高頻度群を比較したところ，子宮筋腫の個数，最大径，内腔の変形をきたしている患者の割合に差がなかった（表3）。また，妊娠例（10例）と非妊娠例（41例）に分けて同様に比較したところ，子宮筋腫の個数，最大径，内腔の変形をきたしている患者の割合に差を認めなかった（データは示さず）。

今回の検討で，どの条件の筋層内子宮筋腫が子宮内膜の異常蠕動様運動を引き起こすかについて，子宮内腔を変形させるタイプ，個数，最大径などの条件では明らかにすることはできなかった。上述したようにエストロゲンは子宮内膜の蠕動様運動を亢進させる作用がある[11]。子宮筋腫にはエストロゲン産生に重要な酵素であるアロマターゼが発現している[12]ことから，子宮筋腫によっては局所のエストロゲン濃度が亢進することで，蠕動様運動を誘導しているのかもしれない。さらなる検討が必要である。

今回，われわれはMRIを用いて子宮内膜の蠕動様運動の評価を行った。超音波断層法では，子宮筋腫の存在により，子宮内膜を正確に描出することが困難なことが多い。また，経腟プローブはその刺激により子宮収縮を誘導することから，この分野の検討には不向きだとされている[13]。すなわち，特に子宮筋腫を有する患者の子宮内膜の運動評価にはMRI法が優れているといえる。

本研究において，われわれは子宮筋腫により誘導される子宮内膜の異常蠕動様運動が妊娠を妨げている一要因になりうる可能性を示した。

おわりに

本研究は不妊症の観点から子宮筋腫の手術を受けるべきか悩んでいる患者の一助になる可能性があり現在，子宮筋腫核出術が異常蠕動様運動の改善につながる否かについて検討中である。また，同法は着床の評価法としても有用であるかもしれない。例えば，一般的に体外受精において採卵周期よりも自然周期のほうが，着床率が高いことが知られている。この理由として採卵周期の高エストロゲン状態が子宮内膜の異常蠕動様運動を誘導することで，着床を妨げているのかもしれない。今後，同法を用いることで，不妊症の評価が発展することが期待される。

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ORIGINAL ARTICLE

Alternative strategies to in vitro fertilization/intracytoplasmic sperm injection treatment for aged infertile women

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Abstract
Purpose This study aimed to maximize the chance of pregnancy and provide an optimal protocol for infertile female patients of advanced reproductive age as an alternative to in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment.
Methods We retrospectively analyzed medical records of 432 infertile women aged ≥38 at the beginning of the treatment. Stepwise non-IVF/ICSI treatments using timed intercourse or intrauterine insemination, with or without controlled ovarian stimulation, were adopted for all patients. In this population, we extracted 8 representative infertility factors and examined these effects on fertility rate by calculating clinical pregnancy rate.
Results The prognosis for infertile women possessing at least one of the three factors, 'advanced female age (≥42 years)', 'endometriosis/adenomyosis', and 'tubal infertility' was apparently poor because only 5 out of 155 women were able to conceive (1.02% per cycle). In contrast, 95 patients without the four factors, 'advanced female age', 'endometriosis/adenomyosis', 'tubal infertility', and 'male infertility', were more likely to conceive (9.14% per cycle).
Conclusions Fertility centers can offer appropriate protocols for non-IVF/ICSI treatment and establish guidelines for aged infertile patients by examining infertility factors and considering their combinations.

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Keywords Advanced reproductive age · Clinical pregnancy rate · ICSI · Infertility · IVF

Introduction

Although age-related fertility decline is widely accepted [1], recent trends have led to alterations in the pattern of childbearing in developed countries, with more people now delaying reproduction into their late 30s and 40s [2]. Historical observations show that fertility decreases by nearly 50% at the age of 40 compared with females younger than 25 and reaches 0% by the age of 47–48 [3]. Considering the steady and irreversible decline in ovarian reserve, it seems appropriate that older patients are often counseled to undergo in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) to maximize the per-cycle chances of pregnancy; however, many couples may not have access to these technologies for financial, social, psychological or other reasons, or may not wish to pursue them [2, 4]. As an alternative to IVF/ICSI, the stepwise approach using ovarian stimulation with clomiphene citrate (CC) or human menopausal gonadotropin (hMG) combined with timed intercourse (TI) or intrauterine insemination (IUI) has been commonly utilized as a means to increase the fertility rate of subfertile couples with patent fallopian tubes. This stepwise non-IVF/ICSI treatment is possibly efficient because the observed clinical pregnancy rate (CPR) per cycle is 4–9% using CC [5], and 8–23% using hMG [6]. However, the efficacy of non-IVF/ICSI is postulated to be questionable for aged patients since the likelihood of fertility decreases significantly after the age of 37 [5, 7, 8]. In addition, the duration of non-IVF/ICSI treatment is important for successful IVF/ICSI, because female age is the most significant factor affecting the success of IVF/ICSI [3, 9].

The objective of this retrospective study is to determine the crucial factors that affect the age-related decline in CPR, and to optimize the treatment protocols for infertile advanced reproductive age women whether these women necessitate IVF/ICSI treatment or non-IVF/ICSI treatment according to the evidence-based policies [10].

Materials and methods

Collection of data

The medical records of 372 infertile female patients who had undergone a total of 1392 non-IVF/ICSI treatment cycles from 1 January 2000 to 28 February 2010 at The University of Tokyo Hospital were reviewed retrospectively. During this period, general trends of treatment strategy for infertile couples remained unchanged. Of 432 women aged ≥38 at the start of the infertility treatment cycle, we excluded 60 patients: 39 were lost during the follow-up period and 21 were excluded for unsuccessfully treated recurrent fetal loss. Our research covered the process of achieving first pregnancy after the beginning of infertility treatment and we registered the number of cycles for each ovarian stimulation method with TI or IUI. This study was reviewed and approved by the Human Ethical Committee of The University of Tokyo Hospital.

Outcome measures

The endpoint, clinical pregnancy, was defined as the detection of a gestational sac by transvaginal ultrasound. To detect the factors that affect CPR in these aged patients, we considered 8 representative factors that are generally examined prior to the infertility treatment. These include (1) 'advanced female age' defined as ≥42 years at the time of the first visit, in view of the report that CPR per embryo transfer decreased from 26% to 13% from age 40 to 44 [11] ($n = 75$), (2) 'endometriosis/adenomyosis' defined as a past or present history of endometriosis and/or adenomyosis, based on clinical images ($n = 57$), (3) 'tubal infertility' defined as an obstructive fallopian tube, based on hysterosalpingography, hysterofiberscope, or past tubal surgery ($n = 49$), (4) 'male infertility' defined as sperm abnormality, based on semen analysis [12] ($n = 208$), (5) 'decrease in ovarian reserve' defined as serum follicle-stimulating hormone level >10 mIU/mL in the early follicular phase ($n = 147$), (6) 'primigravida' ($n = 213$), (7) 'uterine fibroid' defined as a past or present history of uterine fibroid, based on clinical images ($n = 131$), and (8) 'positive serum Chlamydia antibodies' defined as serum Chlamydia antibody immunoglobulin (Ig)A and/or IgG > 0.9 cut-off index ($n = 64$). Eighteen females, including 70 cycles, had none of these 8 factors. We stratified these 8 groups into 24 subgroups by three methods of ovarian stimulation, namely, unstimulated, CC, and hMG cycle. After identifying these factors, we further selected all patterns of their combination to identify the patients' characteristics that are most difficult to conceive by calculating CPR. In this process, the difference of ovarian stimulation methods was not considered and we measured the overall CPR.

Statistical analysis

Categorical variables were compared using chi-squared test, and multivariate logistic regression analysis was used to test for correlations between the 8 factors and the occurrence of pregnancy since various factors overlapped in some cases. Calculations were performed using JMP version 5.1.

Results

The overall outcomes of the 8 groups

The 1392 cycles of non-IVF/ICSI treatment yielded 54 pregnancies and overall CPR per cycle was 3.88%. Of these 54 pregnancies, a live birth rate per cycle was 2.16% (30/1392) and 5 patients were lost during the follow-up period in the first trimester. The CPR of the 24 subgroups is shown in Table 1. There was no successful pregnancy in the 4 subgroups, (1) 'advanced female age; treatment, CC-TI/IUI', (2) 'advanced female age; treatment, hMG-TI/IUI', (3) 'endometriosis/adenomyosis; treatment, hMG-TI/IUI', and (4) 'tubal infertility; treatment, hMG-TI/IUI'.

The overall outcomes of the 8 groups, including the number of patients who had the 8 factors, CPR per cycle and the number of pregnant patients in each group with P values are summarized in Table 2. We found that the 4 groups, 'advanced female age', 'endometriosis/adenomyosis', 'tubal infertility' and 'male infertility', exhibited extremely poor outcomes because the CPR per cycle was less than 2%. However, among the 4 factors, 'endometriosis/adenomyosis' and 'tubal infertility' were not significantly different by multivariate analysis. Only 2 females were able to conceive after non-IVF/ICSI treatment in the 3 groups, 'advanced female age', 'endometriosis/adenomyosis' and 'tubal infertility', although the treatment cycle in each group was >100 cycles. On the other hand, the factor 'decrease in ovarian reserve' showed a significant difference, although CPR per cycle was 2.55%.

CPR in each combination of the 4 factors

To indicate the efficacy and predict the outcomes at the beginning of non-IVF/ICSI treatment, each combination of

Table 1 CPR per cycle of 24 subgroups divided by ovarian stimulation protocols

		Age	EM/AM	Tube	Male	Ovary	Primigravida	Fibroid	Chlamydia
No	CPR	2.22% (2/90)	1.18% (1/85)	1.28% (1/78)	1.70% (7/411)	1.65% (4/243)	2.00% (9/451)	1.74% (5/287)	6.61% (8/121)
	P value[a]	0.56	0.25	0.30	0.0061	0.082	0.012	0.061	0.077
CC	CPR	0% (0/97)	1.12% (1/89)	2.13% (1/47)	0.75% (2/268)	2.65% (6/226)	4.36% (13/296)	6.59% (12/182)	7.23% (6/83)
	P value[b]	0.014	0.075	0.37	<0.0001	0.044	0.60	0.16	0.53
hMG	CPR	0% (0/46)	0% (0/24)	0% (0/21)	5.15% (5/97)	4.94% (4/81)	3.70% (3/81)	3.92% (2/51)	8.33% (2/24)
	P value[c]	0.0037	0.31	0.34	0.22	0.38	0.079	0.90	0.19

We stratified 392 patients (1392 cycles) into 24 subgroups by 8 infertile factors and 3 methods of ovulation stimulation. Remarkably, there was no successful pregnancy in the 4 subgroups

No no stimulation, *Age* advanced female age, *EM/AM* past or present history of endometriosis/adenomyosis, *Tube* tubal infertility, *Male* male infertility, *Ovary* decrease in ovarian reserve, *Fibroid* uterine fibroid, *Chlamydia* positive serum Chlamydia antibodies

[a, b, c] *P* value was calculated using chi-squared test. In each subgroup, we compared the patients with above listed factor with the patients without the factor

Table 2 Overall CPR per cycle in the 8 groups

Infertility factors	CPR with the following factor	CPR without the following factor	*P* value in univariate analysis	*P* value in multivariate analysis
Women aged ≥42	0.86% (2/233)	4.49% (52/1159)	0.0088	0.013
Endometriosis/adenomyosis	1.01% (2/198)	4.36% (52/1194)	0.024	0.11
Tubal infertility	1.37% (2/146)	4.17% (52/1246)	0.097	0.19
Male infertility	1.80% (14/776)	6.49% (40/616)	<0.001	<0.001
Decrease in ovarian reserve	2.55% (14/550)	4.75% (40/842)	0.037	0.048
Primigravida	3.01% (25/830)	5.16% (29/562)	0.043	0.13
Uterine fibroid	3.65% (19/520)	4.01% (35/872)	0.74	0.93
Serum Chlamydia antibodies	7.02% (16/228)	3.26% (38/1164)	0.045	0.059

The effects of 8 infertile factors on CPR were evaluated by univariate and multivariate analysis. Four groups exhibited extremely poor outcome, <2% CPR per cycle, and three groups showed significant statistical differences

Table 3 Cumulative CPR in each combination of the 4 factors

Analyzed factors	Patients with at least one factor in the following combination			Patients without any factor in the following combination		
	Number of pregnancies	CPR per cycle	CPR per patient	Number of pregnancies	CPR per cycle	CPR per patient
A/E	4	1.02%/391	3.25%/123	50	5.00%/1001	20.1%/249
A/T	4	1.14%/351	3.54%/113	50	4.80%/1041	19.3%/259
A/M	16	1.80%/887	6.58%/243	38	7.52%/505	29.5%/129
E/T	3	0.96%/311	3.06%/98	51	4.72%/1081	18.6%/274
E/M	15	1.74%/862	6.58%/228	39	7.36%/530	27.1%/144
T/M	16	1.93%/829	6.93%/231	38	6.75%/563	27.0%/141
A/E/T	5	1.02%/488	3.23%/155	49	5.42%/904	22.6%/217
A/E/M	17	1.77%/962	6.51%/261	37	8.60%/430	33.3%/111
A/T/M	18	1.93%/931	6.90%/261	36	7.81%/461	32.4%/111
E/T/M	16	1.78%/901	6.45%/248	38	7.74%/491	30.6%/124
A/E/T/M	18	1.80%/988	6.50%/277	36	9.14%/394	37.9%/95

To analyze each combination of the 4 factors that showed extremely poor outcome for CPR, we divided the patients into two groups: patients with at least one of the analyzed factors and patients with none of the analyzed factors. Four groups with only female factors exhibited approximately 1% CPR per cycle

A advanced female age, *E* past or present history of endometriosis/adenomyosis, *T* tubal infertility, *M* male infertility

the aforementioned 4 factors was analyzed. We divided the patients into two groups: the patients with at least one of these four factors, and the others without these factors (Table 3). Of these 11 combinations, the 4 groups without 'male infertility', indicating the 4 groups with only female factors, showed approximately 1% CPR per cycle, whereas CPR improved as the number of excluded factors increased. For example, 95 patients without these 4 factors showed 9.14% CPR per cycle.

Discussion

Associated with a trend to postpone childbearing and first birth in developed countries [2], there is an increase in visits to the infertility clinic for aged women who have an intrinsic decrease in fertility with advancing age. Although a woman will not reach menopause until about 50 years of age, her effective childbearing period may stop almost a decade earlier; however, they should be candidates for an expedited infertility work-up. In order to maximize the chance of pregnancy, some aged women are possibly obliged to choose IVF/ICSI or to abandon their entire treatment because of the extremely poor prognosis for conception [3, 7, 8], while others can expect relatively good prognosis. The optimization of treatment protocols for infertility in advanced reproductive age women is crucial and fertility centers should develop evidence-based policies to guide decisions about treating couples with very poor prognoses [10]. Previously, many literatures reported prediction models using various parameters such as female age, duration of infertility, male factor, unilateral tubal obstruction, cervical factors, endometriosis, uterine anomalies, and increasing number of cycles. Female age is one of the most important factors among these determinants [5, 6, 8]. Our data focused only on advanced age women who underwent non-IVF/ICSI treatment. 95 patients who had none of the following 4 factors, 'advanced female age', 'endometriosis/adenomyosis', 'tubal infertility', and 'male infertility' achieved relatively good CPR, 9.14% per cycle and 37.9% per patient in our study. Although our study was retrospective and our sample size was limited, our data clearly indicate that clinicians could distinguish between poor and good prognostic infertile women easily by considering the combination of poor prognostic factors. For instance, 41.7% (155/372) of patients who had one of the three factors, 'advanced female age', 'endometriosis/adenomyosis', or 'tubal infertility', had difficulty conceiving by non-IVF/ICSI treatment in our study population because CPR was only 3.23% per patient, while the others could expect over 20–30% CPR per patient (Table 3). This result indicates the efficacy of non-IVF/ICSI treatment for selected advanced age women, and gynecologists should take the patient's background into account prior to commencing therapy [3, 7, 10].

In conclusion, extraction of poor prognostic factors and consideration of their combinations can provide appropriate protocols for advanced reproductive age patients.

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特集 専門家に聞く子宮筋腫Q＆A－子宮温存を目指して

5. 子宮筋腫合併不妊を妊娠に導くには

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要旨

子宮筋腫合併不妊症例に対し，治療方針を決定する上で重要な検討事項として，①子宮筋腫が本当に不妊の原因となっているか，②子宮筋腫を核出することにより，どの程度の妊孕性改善が見込まれるか（特に患者の年齢，卵巣予備能を考慮），③不妊治療を優先した場合，また筋腫に対する手術を優先した場合の各々の利点，欠点について，十分な説明を受け，患者が納得しているか，が挙げられる．なお，筋腫が不妊の原因かどうかの判断が難しい場合，特に高年齢の不妊症例に対しては，積極的にARTを行い，筋腫が着床障害の原因となっているかを確認し，できれば凍結胚を確保した上で手術に踏み切る．あるいは，手術のための時間のロスを見越して，最初から採卵，良好胚の全凍結を行い，手術を行う方法もある．

KeyWords: Myomectomy, infertility, ART

はじめに

近年の晩婚化，晩産化に伴い，不妊外来を高齢（35歳以上，特に40歳以上）の患者が受診することは，日常しばしば見受けられるようになった．またこれらの年齢層は子宮筋腫の好発年齢とも重なることから，初診時に子宮筋腫を認め，手術と不妊治療のどちらを優先させるか，対応に苦慮することも多い．ここでは，子宮筋腫合併不妊症例を妊娠に導くために，考慮すべき点について述べたい．

子宮筋腫が不妊の原因となるか

子宮筋腫の手術と不妊治療のどちらを優先させるかを決める最も大きな要素は，子宮筋腫が不妊原因となっているかどうかである．一般に，粘膜下筋腫や，内腔変形を伴う筋層内筋腫が着床障害の原因となっていることはエビデンスが得られている[1]が，漿膜下筋腫や内腔変形を伴わない筋層内筋腫が不妊の原因となるかどうかについては議論の余地がある．Oliveiraらは，こうした筋腫を有する245名の症例に対し生殖補助医療（ART）を行い，位置，数にかかわらず4 cm以上の筋腫を有する症例では対照群に比較して妊娠率が低下した，と報告している[2]．また，Prittsらのメタ解析によれば，筋層内筋腫では妊孕性が低下し，流産率が増加する傾向にあったが有意差はなく，また筋腫核出術がこれらを改善するか否かについてもエビデンスは得られなかったとしている．また，漿膜下筋腫は妊孕性には影響しないが流産率が増えたと報告している[1]．

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また，妊娠成立の有無のみならず，妊娠継続～出産に至るまでのプロセスに筋腫が影響するか否かについても検討が必要である．Oliveらは，子宮筋腫が妊娠に与える影響として，帝王切開率の上昇，胎位異常，産褥出血，胎盤遺残，子宮内胎児発育遅延，早産，前置胎盤，胎盤早期剥離を挙げている[3]．なお，Yoshinoらは，cine-mode-display MRIを不妊治療前に行うことにより，子宮筋腫により妊孕性が低下する症例を抽出しえた，としており[4]，今後の検討が注目される．

いずれにしろ，子宮筋腫が不妊の原因となっているか否かをみるためには，超音波検査，子宮鏡，MRIを行い，子宮内腔に対する影響を調べる．筋腫が不妊の原因と考えられる場合は，手術療法を優先し，筋腫の位置，大きさ，数に応じて，また施設の設備，術者の経験に照らし合わせて，子宮鏡下筋腫核出術(TCR)，腹腔鏡下筋腫核出術(LM)，腹腔鏡補助下筋腫核出術(LAM)，開腹子宮筋腫核出術のいずれかを選択する．

一般に術前のGnRHアゴニスト投与は，筋腫の縮小効果が期待でき，術中の出血軽減につながること，月経停止により貧血改善に寄与することから，推奨されているが，症例の年齢などからできるだけ早期の手術が望ましく，また貧血を認めず，術中の出血がそれほど多量にならずに手術可能と判断し，かつ，施設において手術が早期に施行できる場合には，GnRHアゴニスト投与を省略することも考慮する．

術後の避妊期間は，以前は半年～1年とすることが一般的であったが，近年の高齢不妊症例の増加により，施設によっては避妊期間を短くしているところもある．当科では，TCR後の避妊期間は3カ月間，それ以外の術式については，34歳以下は6カ月間，35歳以上の症例については3か月間としているが，避妊期間とその後の妊娠中の合併症(特に子宮破裂の発症率)の関連については，明確なエビデンスはないため，今後の大規模な検討が必要である．

筋腫が明らかに不妊の原因とはいえない場合は，あとに述べる手術による妊孕性低下の可能性も考慮して，不妊治療を先行する．34歳以下の卵巣予備能の保たれた症例に対しては，ほかに不妊因子がなければ原因不明不妊と同様に扱ってよいと思われるが，35歳以上(特に40歳以上)の高齢不妊症例では，子宮筋腫が着床障害の原因となっているかどうかを早期に確認する必要があり，早期のART導入をすすめる．年齢にかかわらず，良好胚の単一胚移植を2回以上行って妊娠に至らない場合は，子宮筋腫による妊孕性低下の可能性を考え，筋腫核出術をすすめる．なお，特に高齢不妊症例においては，手術待ち時間，GnRH投与期間，術後避妊期間による時間のロスを考慮し，凍結保存胚を確保しておくことが望ましい．なお，手術先行が望ましいと判断される場合でも，高齢不妊症例に対しては，手術前に採卵，良好胚の凍結を行った上で手術を行う選択もある[5]．

子宮筋腫を核出することにより，どの程度の妊孕性改善が見込まれるか

子宮筋腫が不妊の原因と想定される場合，次に問題となるのは筋腫が不妊原因の中のどれだけの割合を占めているか，すなわち手術によりどの程度の妊孕性改善が見込まれるか，である．ここで重要なことは，不妊が主訴である場合は，手術優先の方針であっても卵管因子，男性因子など他のスクリーニング検査を必ず行っておくべきである．たとえば男性因子の検索によりsever male factorのため卵細胞質内精子注入法(ICSI)が必須と考えられる場合は，手術による卵管因子への影響をそれほど考慮する必要がなくなる，ということになるし，また術後すぐのART導入を予定している高齢症例では，卵

巣予備能に関する検索は必須であり，術前の凍結胚保存を行うことで，手術は行ったがその後ARTを行ってもまったく良好胚ができない，といった問題が回避できる．

術前のARTで良好胚ができない場合や，症例が43歳以上の場合は，手術により全体的な妊孕性改善を期待することは困難と考える．最終的には患者のインフォームドチョイスによるが，手術に対する過度な期待をもたせないよう努めるべきである．

不妊治療を優先した場合，また筋腫核出術を優先した場合の各々のリスク・ベネフィットについて，十分な説明を受け，患者が納得しているか

不妊の主訴の有無によらず，前医にて子宮筋腫を指摘された場合，医師より手術をすすめられ，または患者本人が手術を希望し，手術可能な施設を紹介受診してくることは多いが，特に手術適応が明らかな場合，手術のリスクについて十分な説明のないまま，手術直前の説明の際にトラブルとなることがある．こうしたトラブルを回避するため，東京大学附属病院女性診療科・女性外科では，子宮筋腫核出術を予定する症例に対して，生殖医療・内視鏡手術担当医が以下の事項を文書により説明するよう，義務付けている．

1．手術のリスク

①腹部に傷が残る（LM＜LAM＜開腹）
②手術に伴う，合併症，副作用の可能性
③術後の癒着により，不妊となる可能性（開腹術，多発筋腫の場合に，よりリスクが高い）
④筋腫の位置によっては，核出，縫合の際に卵管の通過性が悪くなり，不妊となる可能性
⑤術後妊娠した場合，妊娠中の子宮破裂のriskがある
⑥子宮破裂の予防のために，帝王切開の可能性が高くなる（術者の判断により，経腟分娩が不可能となることもある）．

2．経過観察とした場合のリスク

①筋腫が不妊の原因となっている可能性
②筋腫により月経過多となっている場合は，症状増悪
③筋腫が増大した場合，腹腔鏡下手術が不可能となる
④筋腫が増大した場合，周囲臓器の圧迫により腫瘤感，頻尿，便秘が増悪，また静脈圧迫による血栓症，尿管圧迫による水腎症を起こす可能性
⑤妊娠した場合，筋腫による変性痛，胎児発育遅延，流早産，胎盤早期剝離，分娩の障害，子宮収縮不全による出血多量をきたす可能性がある
⑥子宮筋腫と術前に診断されていても，まれに術後に悪性腫瘍と診断されることがあり，経過観察中に病期が進行する可能性がある

子宮筋腫合併不妊症例に対する治療方針

以上のことを踏まえた上で，子宮筋腫合併不妊症例に対する治療方針をまとめた．

1．卵巣予備能が十分ある症例

筋腫が明らかに不妊原因と考えられる場合は手術先行．明らかな不妊原因でないと考えた場合は，ほかに不妊因子がなければ原因不明不妊として不妊治療先行．ARTにより，良好胚移植を2回以上行っても妊娠に至らない場合は筋腫核出術を考慮．基本的に術前GnRH投与を行うが，筋腫が小さく，術中出血が少ないと見込まれる場合は省略可能．術後3カ月～6カ月の避妊期間後，不妊治療を行う．

2．卵巣予備能が低下した症例

筋腫が明らかに不妊原因と考えられる場合は手術先行．早期の手術が可能であればGnRHを省略することも考慮する．手術待ち期間，筋腫および卵巣の位置関係によっては，手術前に採