Decidualization modulates a signal transduction system via protease-activated receptor-1 in endometrial stromal cells

Running title: Modulation via PAR-1 in decidualized endometrium

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Abstract

PROBLEM: Decidual cells are thought to be involved in the maintenance of pregnancy. We conducted the present study to evaluate the cellular function of endometrial stromal cells (ESCs) transitioning to decidualization.

METHODS OF STUDY: Normal endometrial specimens were obtained from premenopausal patients who had undergone hysterectomies for subserosal leiomyomas. Decidualization of the ESCs (DSCs) were induced by incubating subconfluent cells in media containing MPA and db-cAMP. We first analyzed the expression profile of protease-activated receptor-1 (PAR-1) between ESCs and DSCs. To investigate the intracellular signal transduction system in the DSCs, we incubated cells with thrombin receptor activator peptide 6 (TRAP-6). The levels of IL-8, MCP-1, MMP-1, and VEGF in the culture medium were measured by ELISAs. The activation of the MAP kinase signaling pathway was detected by a western blot analysis. The activation was evaluated for the expression of p21.

RESULTS: PAR-1 receptor expression is upregulated in DSCs. The productions of chemokine and MMP-1 increased in the DSCs with the addition of TRAP-6. The activity of both the ERK-1 and ERK-2 isoforms was increased by 5–15 min. after TRAP-6 treatment. p70 S6 kinase showed the strongest expression after 1 hr. p21 was strongly observed in ESCs compared to the DSCs.

CONCLUSIONS: Our results suggest that cell function is changed by decidualization in association with increasing PAR-1 expression. The up-regulation of PAR-1 may have some influence on pregnancy in the decidua.

Key words: human endometrium, decidua, PAR-1, chemokine, implantation

Introduction

In human reproduction, the attachment, adhesion and invasion of the embryo to the uterine endometrium are recognized as important events called to implantation. As the decidualization of the endometrium is a critical event for reproduction,[1] decidual tissues are thought to contribute to the implantation and thus to establishment of pregnancy with the differentiation of the blastocyst,[2] the secretion of hormones or other factors,[3] trophoblast invasion,[4] and the protection of the conceptus from maternal immune rejection.[5]

It has been reported that decidualization is triggered by sex steroid hormones in the secretory phase of the menstrual cycle.[6] The endometrial stromal cells (ESCs) are morphologically differentiated to the relatively large, rounded, polygonal, or epithelioid-like, secretory decidual cells, consequently developing into loose or sponge-like tissues in the decidualization process.[7,8] Morphological changes and the production of several physiological substances such as prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) are induced from decidualized cells. It was reported that these findings of intracellular morphological changes including the dilatation of the rough endoplasmic reticulum, multilayering, the formation of gap junctions, and an increased size of Golgi complexes were characterized in *in vitro* decidualized stromal cells (DSCs), and these findings mimic the *in vivo* decidualization.[9] However, the mechanisms underlying the change of the cellular characteristics from ESC to DSC remain unknown.

Proteinase-activated receptors (PARs) is a peptide receptor with the prototype,[10-12] which carries its own ligand. The ligand remains hidden until it is revealed by a selective cleavage of the amino-terminal exodomain of PAR-1. PAR-1 is activated by thrombin with binding to its aminoterminal exodomain and cleaving them to unmask a

new amino terminus, which serves as a tethered peptide ligand, that binds to the receptor body to provide transmembrane signaling. The synthetic peptide SFLLRN, which mimics the first six amino acids of the new amino terminus unmasked by receptor cleavage, functions as an agonist for PAR-1 and activates the receptor without the proteolysis of thrombin.[11,13,14] We reported that several growth factors and chemokine are produced via PAR-1 activation in human endometrium.[13,14] We hypothesized that the role of PAR-1 in decidualized cells is important to our understanding of the physiological conditions in human reproduction. In order to investigate the intracellular signal transduction systems at work in DSCs via PAR-1, the production of interleukin (IL)-8, monocyte chemoattractant protein (MCP)-1, matrix metalloproteinase (MMP)-1, or vascular endothelial growth factor (VEGF) in response to thrombin receptor activator peptide 6 (TRAP-6; a PAR-1 agonist) was evaluated in the presence of U0126 (a specific MEK inhibitor) or $_{D}$-phenylalanyl-1-proplyl-$_{L}$-arginine chloromethyl ketone (PPACK; a PAR-1 antagonist).

The purpose of the present study was to clarify the physiological role of PAR-1 in the regulation of the endometrial secretion of chemokines, MMP-1 and VEGF in DSCs.

Materials and Methods

Reagent

The cell culture medium, Dulbecco's modified eagle medium (DMEM) was purchased from Nissui (Tokyo), and fetal bovine serum (FBS) was purchased Sigma Chemical (St. Louis, MO, USA). Hank's balanced solution was purchased from Gibco-BPL (Gaithersburg, MD). Collagenase (type I), and DNase, medroxyprogesterone acetate (MPA), and dibutyryl-cyclic adenosine monophosphate

(db-cAMP) were also purchased from Sigma Chemical. Thrombin receptor activator peptide 6 (TRAP-6, SFLLRN) and D-phenylalanyl-1-proplyl-L-arginine chloromethyl ketone (PPACK) were obtained from Bachem (Bubendorf, Switzerland), and U0126 (MEK inhibitor) was obtained from Promega (Madison, WI).

Cell Culture

Normal endometrial specimens were obtained from six premenopausal patients (aged 39-49 years) who had undergone hysterectomies for subserosal leiomyomas. All of the patients had regular menstrual cycles, were multiparous, and were considered to be healthy with the exception of the uterine leiomyoma. None of the patients was taking any medication before the operation, and they had shown no uterine bleeding. All of the specimens were classified as midsecretory phase (days 19 to 21 of the menstrual cycle) on the basis of the results of a standard histologic examination of endometrial tissues.

Normal ESCs were separated from epithelial glands by collagenase digestion of the tissue fragments as described[15] with slight modification. Briefly, tissues were cut into 2–3-mm pieces and incubated with collagenase (200 IU/ml) and DNase (150 μg/ml) in Hanks balanced solution with stirring for 2 hr at 37°C. The suspension was filtered through a 150-μm wire sieve to remove mucus and undigested tissues. The filtrate was passed through an 80-μm wire sieve, which allowed the stromal cells to pass through while intact glands were retained. After being washed three times with serum-free DMEM, the cells were transferred to culture flasks (Nalgene Nunc, Rochester, NY) at a density of 1×10^6 cells/ml in DMEM supplemented with 10% heat-inactivated FBS with penicillin (100 IU/ml; Gibco-BPL) and streptomycin (100 mg/ml; Gibco-BPL).

The culture medium was replaced every 3 days. After two passages (10–12 days after isolation) using standard methods of trypsinization, the cells, which were >99%

126 pure as analyzed by immunocytochemical staining, along with antibodies to vimentin
127 (V9; Dako, Copenhagen, Denmark), cytokeratine (Dako), factor VIII (Dako) and
128 leukocyte common antigen (2B11+PD7/26; Dako), were used for the experiments. The
129 cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air at 100% humidity.
130 This study was approved by the institutional review board of Oita University, and
131 written informed consent was obtained from all patients.
132
133 *Decidualization of human ESCs in vitro*
134 The decidualization of ESCs (DSCs) was induced by incubating subconfluent
135 ESCs in DMEM supplemented with 2.5% heat-inactivated FBS containing 100 nmol/L
136 MPA and 0.5 mmol/L db-cAMP for long-term (16 days) as described (n=6)[16]. Briefly,
137 MPA was dissolved in ethanol and db-cAMP was dissolved in filtered distilled water
138 that had been added to the media before use. The final concentration of ethanol in the
139 media never exceeded 0.1% (v/v). Phase contrast microscopy was used to verify
140 morphological changes associated with differentiation *in vitro* in response to MPA and
141 db-cAMP. The decidualization of ESCs was indicated by an elevation of prolactin in the
142 culture medium. PRL was measured by a Unicel Dxi 800 immunoassay system
143 (Beckman Coulter; CA). The sensitivity of the assay for PRL was 0.1 ng/ml.
144
145 *Reverse Transcription (RT) and Quantitative Real-Time Polymerase Chain Reaction*
146 *(PCR) Analysis*
147 Cells were grown to confluence in a 60-mm dish (Nalgene Nunc). Before being
148 assayed, the supernatant was replaced with fresh serum-free medium. Total RNA was
149 extracted from ESCs and DSCs with an RNeasy minikit (Qiagen, Hilden, Germany)
150 (n=3). RT was performed using Reverse Transcription System (Promega). One

151 microgram of total RNA was reverse transcribed in a 20-μL volume. The quantitative
152 real-time-PCR was carried out in triplicate with a LightCycler 480 (Roche Diagnostics,
153 Penaberg, Germany) according to the manufacturer's instructions. The PCR primers
154 were purchased from Applied Biosystems (Carlsbad, CA) with F2R (for PAR-1, Assay
155 ID: Hs00169258_ml), CDKN1A (for p21, Assay ID: Hs00355782_ml), IL-8 (for IL-8,
156 Assay ID: Hs00174103_ml), CCL2 (for MCP-1, Assay ID: Hs00234140_ml), CLGN
157 (for MMP-1, Assay ID: HSS101765), FLT1 (for VEGF, Assay ID: HSS103744) or
158 GAPDH (for GAPDH, Assay ID: VHS40322). The expressions of mRNA were
159 normalized to RNA loading for each sample using GAPDH mRNA as an internal
160 standard. These expression levels were calculated by the ΔΔCT method.

161 For the assessment of the inhibitory effects of PPACK or U0126, these reagents
162 were added with/without thrombin, and the ESCs/DSCs were collected for 8 hr after
163 treatment. For the assessment of IL-8, MCP-1, MMP-1 or VEGF, we performed
164 quantitative real-time PCR, and a data analysis.

165

166 *Measurement of chemokine, proMMP-1 and VEGF levels*

167 Cells were grown to confluence in a 100-mm dish (Nalgene Nunc). Before being
168 assayed, the supernatant was replaced with fresh serum-free medium. The
169 concentrations of IL-8, MCP-1, Pro-MMP- 1 and VEGF in supernatant were measured
170 in triplicate using specific enzyme-linked immunosorbent assay (ELISA) kits
171 (Quantikine; R&D Systems, Minneapolis, MN) (n=3). In the experiments, the
172 supernatant was replaced with fresh serum-free medium containing 100 μM TRAP-6.
173 The supernatant was collected at 24 hr after treatment and stored at −80°C until assay.
174 For the clarification of the inhibitory effects of PPACK and those of U0126, these
175 reagents were added with TRAP-6 and the supernatant was collected for 24 hr after

176 treatment.

177 The sensitivity of the assays were as follows: for IL-8, 3.5 pg/ml; for MCP-1, 1.7
178 pg/ml; for Pro-MMP-1, 0.021 ng/ml; and for VEGF, 9.0 pg/ml. The inter- and intra-
179 assay coefficients of variance for IL-8 were 8.1% and 4.7%; for MCP-1, 5.9% and
180 5.9%; for pro-MMP-1, 8.8% and 5.2%; and for VEGF, 8.5% and 6.5%.

181

182 *Protein preparation of ESCs/DSCs and western immunoblotting analysis*

183 For the investigation of the intracellular signal transduction systems in ESCs/DSCs,
184 prepared cells were plated on a 100-mm dish in 10 ml of culture medium with 10% FBS
185 and cultured until the cells were fully confluent. The intracellular signal transduction
186 system was detected by a western blot analysis as described (n=3).[14] The supernatant
187 was replaced with fresh serum-free culture medium containing TRAP-6. At the end of
188 the culture period, ESCs/DSCs were washed with cold phosphate-buffered saline (PBS)
189 without calcium or magnesium, harvested, pelleted, and lysed in ice-cold buffer
190 containing 10 mM Hepes (pH 7.9), 10 mM KCL, 0.1 mM EDTA (pH 8.0), 0.1 mM
191 EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, and 0.3 μg/ml
192 leupeptin.

193 The cell lysate was centrifuged for 10 min at 3000*g* in order to pellet the nuclei.
194 The protein content was determined using a microbicinchoninic acid assay (Pierce,
195 Rockford, IL) with BSA as a standard. The lysate was mixed with loading buffer (200
196 mM Tris-HCl, pH 7.9, 7% SDS [w/v], 30% glycerol [v/v], 15% 2-mercaptoethanol [v/v],
197 0.75% bromophenol blue [w/v]) and heated at 95°C for 10 min. For each sample, 10 μg
198 or 20 μg of protein was applied per lane. The blotted membranes were blocked in PBS
199 containing 5% skim milk (Difco, Detroit, MI) for 1 hr at room temperature and washed
200 with three changes of Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6)

buffer containing 0.05% Tween 20 for 10 min at room temperature.

The blotted membranes were then incubated and reacted overnight with 1:200-diluted primary antibody (anti-human thrombin R antibody: #sc-13503, Santa Cruz Biotechnology, Santa Cruz, CA; or anti-human p21 antibody: #2947, Cell Signaling Technology, Beverly, MA), 1:1,000-diluted primary antibody (anti-human phosphor-p44/42 MAP kinase antibody: #4370, Cell Signaling Technology; anti-human ERK1/2 antibody: #sc-135900, Santa Cruz Biotechnology; anti-human phospho-p70 S6 kinase antibody: #9205, Cell Signaling Technology; anti-human p70 S6 kinase antibody: MAB8962, R&D Systems or 1:5,000-diluted primary anti-human GAPDH antibody (#sc-47724, Santa Cruz Biotechnology) in TBS containing 5% BSA at 4°C.

After the membranes were washed with three changes of TBS containing 0.05% Tween 20, the blotted membranes were incubated and reacted with 1:5,000-diluted peroxidase-conjugated secondary antibody (anti-rabbit immunoglobulin or anti-mouse immunoglobulin) or 1:10,000-diluted peroxidase-conjugated secondary antibody (anti-mouse immunoglobulin) in TBS containing 5% BSA for 1 hr at room temperature. After washing with three changes of TBS containing 0.05% Tween 20, Amersham ECL (GE Healthcare, Buckinghamshire, England) was added to the blotted membranes and reacted for 1 min. The membranes were then covered with plastic wrap and exposed to X-ray film (GE Healthcare) for 10 sec to 60 min.

In vitro wound repair assay

In vitro wound repair assays were performed as described.[17,18] Cells were grown to confluence in a 60-mm dish (Nalgene Nunc). Before being assayed, the supernatant was replaced with fresh serum-free medium. The monolayer was scratched with a 2-mm cell scraper without damaging the dish surface. Immediately after a lesion was created, areas

for evaluation were chosen, and parallel samples were incubated for 48 hr with/without MPA and db-cAMP or TRAP-6. The incubation time was determined by background experiments.

The cells were stained with Diff-Quik solution (Sysmex, Kobe, Japan). Areas with lesions were photographed, and the wound repair was assessed by calculating the area in square micrometers between the lesion edges with ImageJ, ver. k1.45 software. Values are expressed as a percentage of the area repaired within 48 hr under untreated conditions.

Statistical analysis

Data are presented as the mean ± SD of triplicate samples and are presented as percentages relative to the corresponding controls, as the mean ± SD values were analyzed by Bonferroni-Dunn test. A level of $p<0.05$ was considered significant. The confidence intervals with p-values for multiple statistical analyses are at the 95% level.

Results

The accumulation of PRL for decidualization of ESC

To investigate the production of PRL for decidualization of ESCs, the supernatant was measured at 16 days after treatment. The kinetics of PRL secretion by stromal-decidual cells under each passage was detected. The concentration of PRL in the culture media of unstimulated ESCs was below the detection limit of the assay. The date are expressed as the mean ± SD of triplicate samples from three separate representative experiments (controls [mean ± SD]: 0, 0, and 0 ng/ml; db-cAMP and MPA: 0, 52.0±6.5, and 95.7±3.6 ng/ml, on days 0, 8 and 16, respectively). The PRL production stimulated by db-cAMP and MPA was significantly increased compared to

the controls ($p<0.01$). DSCs have verified the differentiation of elongated, fibroblast-like mesenchymal cells in the ESCs to rounded, epithelioid-like cells (data not shown).

Expression of PAR-1 mRNA and protein secretion in cultured ESCs and DSCs

To investigate the expression of PAR-1 in cultured ESCs/DSCs, the expression of PAR-1 mRNA and protein secretion were evaluated. PAR-1 mRNA was detected in the human ESCs and DSCs under each passage by the RT-PCR analysis (Fig. 1A). According to the real-time quantitative PCR analysis, PAR-1 mRNA and protein expression levels on day 8 after decidual stimulation appeared to be higher than those on day 0. At 16 days after stimulation, the expression of PAR-1 was higher in the decidualized stromal cells (Fig. 1B).

TRAP-6 induced mRNA expression and protein secretion of chemokine, MMP-1 and VEGF in DSCs

To investigate the intracellular signal transduction system at work in DSCs, the production of chemokine, MMP-1, and VEGF in response to TRAP-6 was evaluated in the presence of U0216 or PPACK. Before being assayed, the decidualization of ESCs was induced by incubating subconfluent ESCs in media containing 100 nmol/L MPA and 0.5 mmol/L db-cAMP for 16 days. The supernatant was replaced with fresh serum-free medium at day 16. Chemokine, MMP-1 and VEGF mRNA was detected in the DSCs during incubation with/without TRAP-6, U0126 or PPACK for 8 hr by the RT-PCR analysis (Fig. 2A–D). Protein secretion was detected during incubation for 24 hr by ELISA (Fig. 2E–H). The concentrations of IL-8, MCP-1, proMMP-1, and VEGF in the culture medium without DSCs were each below the level of detection. Increases

in IL-8, MCP-1, and MMP-1 mRNA and the concentration of protein were evident during incubation with TRAP-6. An increase in the VEGF concentration was not evident. The TRAP-6-induced mRNA expression and the protein secretion of IL-8, MCP-1 and MMP-1 was decreased by treatment with U0126 and PPACK.

TRAP-6 induced MAP Kinase phosphorylation in DSCs

To investigate PAR-1's role in MAP kinase activation, we performed a western immunoblot analysis to determine the signal transduction pathways during stimulation with TRAP-6 (100 μM). The activity of both the ERK-1 and ERK-2 isoforms was increased by treatment with TRAP-6 for 5-15 min, but the increase was attenuated thereafter (Fig. 3A). TRAP-6 caused a rapid, time-dependent phosphorylation of both the p44 and p42 isoforms of ERK.

TRAP-6 induced p70 S6 kinase phosphorylation in DSCs

To investigate PAR-1's role in p70 S6 kinase activation, we performed a western immunoblot analysis to determine the signal transduction pathways during stimulation with TRAP-6 (100 μM). The activity of p70 S6 kinase in decidual stromal cells was increased at 1 hr, but the increase was attenuated 8 hr later (Fig. 3B).

Detection of in vitro wound repair by treatment ESCs/DSCs with/without TRAP-6.

The effects of TRAP-6 on the motility with ESCs/DSCs were assessed by an *in vitro* wound repair assay. The concentration of PRL in the culture media of unstimulated ESCs for 48 hr of incubation was below the detection limit of the assay. The PRL production stimulated by db-cAMP and MPA was significantly increased compared with that of the controls ($p<0.01$) (data not shown). After 48 hr, $64.2 \pm 14.1\%$ of the

wounded area was repaired by DSCs with TRAP-6, whereas 22.6 ± 16.5% of the wounded area was repaired by ESCs with TRAP-6. As shown in Figure 4A,B, the wound repair of the DSCs was significantly enhanced compared to that of the ESCs.

Expression of p21 mRNA and protein secretion in cultured ESCs and DSCs

To investigate the expression of p21 in cultured ESCs/DSCs, the expression of p21 mRNA and protein secretion were evaluated. p21 mRNA was detected in the human ESCs and DSCs under each passage by our RT-PCR analysis. According to the real-time quantitative PCR analysis, in the ESCs, p21 mRNA expression levels on day 4 appeared to be higher than those on day 0 (Fig. 5A). Regarding the protein secretion, the expression of p21 was not significantly different between the ESCs and DSCs on day 8 after stimulation. At 12 days after stimulation, the expression of p21 was higher in the non-decidualized stromal cells (Fig. 5B).

Discussion

A human decidualization model was established in *in vitro* studies.[19-21] In the present study, we identified the increase of PAR-1 expression, which reduced the cell senescence condition by not increasing the expression of p21 in DSCs compared to ESCs, which are non-decidualized cells. The chemokine expression and cell migration in DSCs are modulated via PAR-1, its activation by the tethered ligand, and the activation of ERK1/2 or P70 S6 K is probably involved in this response. The effect of TRAP-6 was blocked with U0126 or PPACK, an inhibitor or antagonist. In the functioning of decidualized cells, cell condition is modulated by any factors. The production of other physiological substances such as MMP-1 and VEGF can also be regulated. In order to investigate the modulation of PAR-1 in DSCs, endometrial cells

were cultured for 16 days with decidualization. Consequently, the expression levels of PAR-1 mRNA and protein secretion was significantly higher on day16.

In DSCs, IL-8 and MCP-1 are produced via PAR-1 activation. The expression of chemokines in endometrial tissues has been described.[14-16] We also demonstrated that in ESCs, chemokines such as IL-8, GROα and MCP-1 are modulated via PAR-1 and that this modulation involves MAP kinase[14]. PARs are identified members of G protein-coupled receptors (GPCRs), multiple studies indicate that stimulation of many GPCRs also leads to the rapid activation of the ERK pathway [22,23] involving G protein-coupled signal transduction, i.e. the activation of phospholipase C (PLC), the generation of inositol 1,4,5-trisphosphate (InsP3), increase of intracellular Ca2+ and activation of protein kinase C (PKC). It has been reported that stimulation by thrombin activates ERK in several cell types [24,25]. The MAPK pathway exerts a positive feedback on the p70S6K pathway, leading to an increased chemotaxis response in terminally differentiated cells [26]. However, the production of chemokines from DSCs has not been reported, to our knowledge. The distribution of immune cells has been shown to differ between the proliferative phase and secretory phase.[21] Various types of immune cells including immunosuppression cells were detected in decidua.[27] The chemokines induce attractants of leukocytes such as immune cells. PAR-1 acts after catalysis by enzyme such as thrombin. Instead of enzyme catalysis, tethered ligand has been added. It mimics the same effect of enzyme catalysis. In order to clarify the direct effect of the inhibitor with TRAP-6-stimulated cells and compare the both inhibitor U0126 and PPACK, U0126 and PPACK were added with TRAP-6.

These cells may support the maintenance of a pregnancy. It has been recognized that the distribution of immune cells could provide clues to the immunological perturbation in cases of unexplained recurrent miscarriage in decidual tissues. Many

factors are shown to correlate with unexplained recurrent miscarriage, but it has been difficult to unravel these factors. It was indicated that NK cells and T cells contribute to partially cause abortion/miscarriage in mice and humans.[27]

After morphological changes of DSC, the significance of the ability of decidua to potentiate the pregnancy process is unknown. It was reported that increased blood flow, stromal edema, and enhanced vascular permeability occur in the secretory phase of endometrium.[28] As a consequence, the transudation of substances such as VII/VIIa and Xa into the stromal component results the generation of thrombin. The affection of thrombin in the stromal component via PAR-1 has been speculated.[13,14] In light of the trophoblastic invasion into the endometrium it was speculated that some local bleeding may occur from the implantation site with unhealed because of the increasing flow of maternal blood into the lacunar spaces. As thrombin has been known to activate PAR-1, the roles of PAR-1 may be particularly important in the context of these phenomena. MMPs could influence decidual cell migration. They play an important role in decidual tissue remodeling involving various biological processes including angiogenesis, trophoblast invasion, and wound repair. MMPs are often regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). It was suggested that the MMP/TIMP ratio often determines the extent of extracellular matrix protein degradation and tissue remodeling.[29] On the other hand, our present findings indicate that the expression of p21 was decreased in decidual change. This result suggests that reducing p21 may prevent the senescence of DSCs in order to maintain a pregnancy including placental function. Cell migration is also increased in DSCs. The activation of cell migration involves PAR-1. We confirmed the following points: [1] The expression of chemokines involving PAR-1, and [2] the expressions of cell cycle-related protein in decidual cells. These findings reveal the hormone-mediated mechanisms of decidualization in eutopic

endometrium.

Our present findings combined with published data regarding chemokine production via PAR-1 in ESCs provide supportive evidence that PAR-1 expression is regulated by hormonal regulation, and its activation enhanced the chemokine production. The breaking of the balance of lymphocytes was reported to lead to the failure of pregnancy. The balance of dendritic cells is disrupted in interferon-gamma (IFN-γ)-induced abnormal pregnancy, which is accompanied by a reduction of regulatory T cells.[30] In addition, the Th1/Th2 and decidual NK1/decidual NK ratios were higher in women with unexplained recurrent miscarriage compared to women with normal pregnancies.[31] The findings of immunological dysfunction described recently may underlie the pathological formation of implantation failure as well as miscarriage.[27,30,31] The pathophysiological situation of miscarriage remains unclear in cases other than fetal chromosomal anomalies or maternal disorders. The present research field may contribute to the resolution of the problems of unknown miscarriage.

A limitation of our study is that we conducted only *in vitro* experiments. One of the remaining questions is whether these conditions are present within the decidual tissues *in vivo*. Moreover, the perturbation of this mechanism could cause miscarriage. Further examinations concerning the *in vivo* conditions are needed.

In conclusion, our findings demonstrate a new role for PAR-1 in *in vitro* decidualization with increased chemokine expression, cell migration with metalloproteinase expression, and reduced cell senescence condition. Further studies are necessary to elucidate the role of decidualization.

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Figure legends

Figure 1

The expression levels of PAR-1 mRNA and protein secretion over 16 days. ESCs were incubated with/without db-cAMP and MPA for the indicated times. **A:** Total RNA was extracted from the cells and subjected to real-time PCR to determine the PAR-1 levels.

B: The protein was assayed by western blot analysis. The bands of PAR-1 and GAPDH were detected at molecular weights 50 and 37 kD, respectively. Data were normalized to GAPDH mRNA/protein levels. According to the real-time quantitative PCR analysis, the PAR-1 mRNA and protein expression levels on day 8 after decidual stimulation appeared to be higher than those on day 0. At 16 days after stimulation, the expression of PAR-1 was higher than those on day 0 in the decidualized stromal cells ($p<0.01$). Representative data from three different experiments (n=3) are shown as the mean ±SEM relative to an adjusted value of 1.0 for the mean value of the each control. mRNA and protein: $^{**}p<0.01$, $^{*}p<0.05$ vs. day 0. Open bars are control and closed bars mean being added db-cAMP (0.5mM) and MPA(100nM)

Figure 2

The chemokine, MMP-1, and VEGF mRNA and protein levels in DSCs

Cultured DSCs were stimulated with TRAP-6 for 8 hr. Total RNA was extracted from the cells and subjected to real-time PCR to determine IL-8 (**A**), MCP-1 (**B**), MMP-1 (**C**), and VEGF (**D**) mRNA levels. Increases in IL-8, MCP-1, an MMP-1 mRNA were evident during incubation with TRAP-6. An increase in the VEGF concentration was not evident. The treatment of DSCs with the TRAP-6-induced production of IL-8, MCP-1 and MMP-1 was decreased by treatment with U0126 and PPACK. VEGF mRNA was decreased by treatment with U0126. The protein level showed similar results. When DSCs were treated with TRAP-6 and U0126 or PPACK, the productions of IL-8 (**E**), MCP-1 (**F**) and MMP-1 (**G**) VEGF (**H**) were significantly decreased. $^{\#\#}p<0.01$ vs. control. $^{**}p<0.01$, $^{*}p<0.05$ vs. TRAP-6. Representative data from three different experiments (n=3) are shown. Data are the mean ± SD of triplicate samples.

Figure 3

A: The detection of both ERK-1 and ERK-2 phosphorylation by the treatment of DSCs with TRAP-6. The bands of ERK-1, ERK-2, and GAPDH are detected at molecular weights 42, 44, and 37 kD, respectively. Neither ERK-1 nor ERK-2 was activated under 0 min. The activity of ERK1/2 in decidual stromal cells was increased by treatment with TRAP-6 at 5 min, but the increase was attenuated 4 hr later. Representative data from three different experiments (n=3) are shown.

B: The detection of p70 S6 kinase phosphorylation by the treatment of DSCs with TRAP-6. The bands of p70 S6 kinase and GAPDH are detected at molecular weights 70, 85 and 37 kD, respectively. p70 S6 kinase was not activated under 0 min. The activity of p70 S6 kinase in DSCs was increased at 1 hr, but the increase was attenuated 8 hr later. Representative data from three different experiments (n=3) are shown.

Figure 4

The detection of *in vitro* wound repair by the treatment of ESCs and DSCs with/without TRAP-6. ESCs/DSCs grown to confluence in a 6-cm dish were challenged with/without TRAP-6 (100 μM) for 48 hr. Areas with lesions were photographed. ESC control (**A-a**), ESC treated with TRAP-6 (**A-b**), DSC control (**A-c**), DSC treated with TRAP-6 (**A-d**). After 48 hr, 64.2 ± 14.1% of the wounded area was repaired by DSCs treated with TRAP-6, whereas 22.6 ± 16.5% of the wounded area was repaired by ESCs treated with TRAP-6. The wound repair of the DSCs was significantly enhanced compared to that of the ESCs (B). Representative data from three different experiments (n=3) are shown.

a : **P＜0.01,　b, c : *P＜0.05

Figure 5

The expression levels of p21 mRNA and protein secretion over 16 days. **A:** ESCs were incubated with/without db-cAMP and MPA for the indicated times. Total RNA was extracted from the cells and subjected to a real-time PCR to determine the p21 levels. **B:** The protein was assayed by a western blot analysis. The bands of p21 and GAPDH are detected at the molecular weights 21 and 37 kD, respectively. Data were normalized to GAPDH mRNA/protein levels. Representative data from three different experiments (n=3) are shown as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. mRNA: **p<0.01 vs. day 0. Protein: **p<0.01 vs. day 8. Open bars are control and closed bars mean being added db-cAMP (0.5mM) and MPA(100nM).

Conflict of interest:

There are no conflicts of interest to declare.

Author's roles:

Y.K.: study design, sample processing, manuscript writing; K.G., Y.K.: cell culture, ELISA assay, western immunoblot analysis; RT-PCR; T.U.: data interpretation; H.N.: manuscript correction.