Full Paper

Protease-Activated Receptor–Stimulated Interleukin-6 Expression in Endometriosis-Like Lesions in an Experimental Mouse Model of Endometriosis

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Abstract. The present study was undertaken to investigate the function of protease-activated receptor (PAR) in endometriotic lesions using a mouse model of endometriosis. Unilateral ovariectomy (uOVX) was performed on female nude mice followed by intraperitoneal transplantation of a suspension mixture of immortalized human endometrial epithelial cells (EM-1) and stromal cells (EtsT-499). Endometriosis-like lesions were observed mostly around the dissection site after transplantation. The expression of interleukin (IL)-6 and cyclooxygenase-2 in the lesions was enhanced in uOVX mice compared to non-uOVX animals. In non-uOVX mice, IL-6 mRNA levels were higher in lesions formed with cells that were pretreated with PAR1/2 agonists (thrombin, 10 U/ml and PAR2-activating peptide, 30 μ M) compared to untreated, intact cells. Peritoneal IL-6 concentrations were also increased in the PAR1/2 agonists–treated group. IL-6 expression induced by PAR activation was blocked by the treatment of cells with serine protease inhibitors. In cultured endometrial cells, simultaneous treatment with PAR1 and PAR2 agonists significantly increased the expression of IL-6. These results suggest that peritoneal bleeding may accelerate IL-6 expression in endometriotic lesions in part through the activation of PAR.

Keywords: endometriosis, endometrial cell, interleukin (IL)-6, protease-activated receptor (PAR), thrombin

Introduction

Endometriosis is a chronic disorder characterized by the presence of functioning ectopic endometrial tissue outside the uterine cavity (1 – 3). The prevalence of endometriosis has been estimated to be approximately 5% – 10% (ca. one hundred and thirty thousand individuals in Japan) among women of reproductive age (4). Endometriosis is typically associated with infertility and pain symptoms. Although the pathophysiology of endometriosis remains unresolved, retrograde menstruation containing endometrial tissue fragments through the fallopian

tubes into the pelvis may be the cause of this disorder (2, 3). Both endometrial glandular and stromal cells appear to be required for the formation of endometriotic lesions. The development of endometriosis is associated with the attachment and the survival of endometrial cells in the peritoneal cavity and the peritoneum. Endometriosis is a multi-factorial disease in which both endometrial and peritoneal factors may be implicated (2, 5). Prominent features of endometriosis include recurrent ectopic bleeding with localized inflammatory reactions and often chocolate cysts in the endometriotic lesions (3). Bleeding seems to accelerate the progression of the disease. Production of prostaglandin (PG) E₂, an inflammatory mediator, is up-regulated in endometriosis, along with increased expression of cyclooxygenase (COX)-2 (5, 6). PGE₂ is a local mediator produced by the endometrium and is associated with endometrial proliferation and im-

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plantation (7). PG content in the peritoneal cavity is high in patients with endometriosis and abnormally elevated PG has been shown to cause decreased immune system function (8, 9). Serum levels of interleukin (IL)-6 have also been shown to be higher in patients with endometriosis compared to healthy women (10). The menstrual effusion contains various types of endometrial cells, including glands, stromal cells, endothelial cells, and blood cells. These cells are not capable of surviving outside the uterine cavity under normal conditions and are removed via the innate immune system. However, the properties of these cells in patients who develop endometriosis may be altered to allow survival through as-yet undefined mechanisms. Infusion of endometrial tissue fragments (7, 11 – 17) or endometriosis-derived cells (18) into the peritoneal space of nude mice results in the development of endometriosis-like lesions, providing a useful model system for studying the mechanisms underlying endometriosis.

Protease-activated receptors (PARs) have been identified as critical mediators of inflammation and immune responses (19 – 21). PARs belong to a unique four-member family of G protein-coupled receptors (PAR1 – PAR4). Cleavage of PARs at a specific site in the extracellular N-terminal domain by serine proteases exposes an N-terminal domain of the receptor that acts as a tethered ligand. The tethered ligand binds to the second extracellular loop of the receptor to induce a variety of intracellular signaling pathways. PAR1, PAR3, and PAR4 function as thrombin receptors, whereas PAR2 is insensitive to thrombin but can be activated by trypsin, tryptase, and coagulation factors VIIa/Xa. PAR1, PAR2, and PAR4 are also selectively activated by short synthetic peptides (termed PAR-activating peptides) based on the tethered ligand sequence. PARs can trigger a variety of inflammatory responses, including pain and leukocyte infiltration (21). Administration of PAR1-activating peptide causes edema and enhancement of vascular permeability, mediated in part through the alteration of mast cell function (22). Interestingly, PAR1 (23) and PAR2 (24) are expressed in uterine endometrial stromal cells, and PAR1 activation increases the expression of IL-8 and monocyte chemoattractant protein-1 (MCP)-1 and increases mitogenic activity in cultured stromal cells (23). Furthermore, activation of PAR2 has been shown to stimulate proliferation and IL-8 secretion in endometrial cells (24). Based upon these observations, Osuga et al. (25) suggested that PAR may play a role in endometriosis, and PAR activation has been proposed to contribute to the pathogenesis and pathophysiology of endometriosis. We recently observed that human endometrial cells that were transplanted into the peritoneal cavity of nude mice formed neovascularized lesions several days after

implantation. Preliminary data showed that the formation of these endometriosis-like lesions was enhanced by ovariectomy (OVX) and that most of the lesions were formed around the site of excision, suggesting that bleeding and/or tissue injury associated with OVX may stimulate the development or inflammation of lesions, possibly through the activation of PAR1/2. This system therefore could serve as an *in vivo* whole animal model for evaluating the involvement of PARs in the pathophysiology of endometriosis. In the current study, we examined the role of PAR1 and PAR2 activation in the formation of endometriosis-like lesions and the expression of proinflammatory mediators in this experimental mouse model of endometriosis.

Materials and Methods

Chemicals

The PAR1 agonist thrombin and PAR2-activating peptide (PAR2 AP) (Ser-Leu-Ile-Gly-Arg-Leu-amide, SLIGRL-NH₂, HPLC Purified Synthetic Peptide) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and Abgent (San Diego, CA, USA), respectively. Indomethacin and 17 β -estradiol (E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and PGE₂ was from Cayman (Ann Arbor, MI, USA). Matrigel (high concentration; BD Matrigel Basement Membrane Matrix) for preparing the cell suspensions was from BD Biosciences (Bedford, MA, USA). Serine protease inhibitor cocktail (Calbiochem 565000) was purchased from Merck (Darmstadt, Germany). The protease cocktail contained AEBSF, aprotinin, elastatinal, and Glu-Gly-Arg-chloromethyl ketone (GGACK). Staurosporine was obtained from Alomone Labs Ltd. (Jerusalem, Israel).

Endometrial cell culture

The immortalized human endometrial glandular epithelial cell line (EM-1) (26) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako) containing 50 U/ml penicillin, 50 μ g/ml streptomycin, 0.25 mg/ml Fungizone (Life Technologies, Tokyo) and 10% (vol/vol) fetal bovine serum (FBS; HyClone, South Logan, UT, USA). The immortalized endometrial stromal cell line EtsT-499 (EtsT) (27) was grown in basal medium (DMEM/F12) without phenol red (Life Technologies) containing the above antibiotics as well as antimycotics and 10% (vol/vol) FBS. Cells were grown in culture dishes, detached using phosphate-buffered saline (PBS) containing 5 mM ethylenediamine-tetraacetic acid (EDTA), and then resuspended at a concentration of 3.4×10^6 EM-1 cells/125 μ l or 1.6×10^6 EtsT cells/125 μ l.

Animals and induction of endometriosis

Female nude mice (8 weeks of age, BALBc/nu nu) were purchased from Japan SLC, Inc. (Shizuoka). Animals were maintained in pathogen-free conditions in an air-conditioned room (temperature of $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and humidity of $55\% \pm 5\%$) under controlled lighting (12 h light/dark cycle) with free access to food and water. All animal handling protocols and surgical procedures were approved by the Institutional Animal Care Committees at Tokyo University of Pharmacy and Life Sciences in compliance with institutional guidelines for experimental animal care. For unilateral (right side) ovariectomy (uOVX), a 1-cm longitudinal incision of the musculature of the body wall of the back was made under anesthesia with ether. Human endometrial EM-1 epithelial cells (3.4×10^6) and EtsT stromal cells (1.6×10^6) were mixed and finally resuspended in 50% Matrigel and then the suspension (5×10^6 cells/500 μl per mouse) was administered from and directly adjacent to the dissection site into the peritoneal cavity with a micropipette to induce the formation of endometriosis-like lesions. Where indicated, both types of cells were treated with 10^{-7} M E2 just before implantation and the animals were administered E2 (10 μg , s.c.) once a day until autopsy to determine the effects of excess E2 on the characteristics of the lesions [morphology, weight, and expression of CD31, vascular endothelial growth factor (VEGF), and PARs]. Mice were euthanized by cervical dislocation 5 days after the transplantation. The lesions were removed, homogenized in RNA extraction buffer and then subjected to mRNA analysis.

Collection of peritoneal fluid and assessment of lesions

One milliliter of saline was infused into the peritoneal cavity and then the infused saline containing diluted peritoneal fluid was collected using a 1-ml syringe for IL-6 and PGE₂ assay. The peritoneum and visceral organs were examined visually and lesions exhibiting the distinctive morphology of a matrigel-containing lesion were removed.

Fluorescence imaging of carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE)-expressing tissues

In the initial experiment, endometrial EM-1 and EtsT cells at subconfluence were fluorescently labeled with CFDA-SE (10 μM), a cell-permeable and non-polar lipophilic fluorescent derivative (28), for 15 min in PBS. Amine-reactive coupling of CFDA to intracellular proteins results in stable long-term intracellular retention, allowing in vivo monitoring of the cells. The labeled endometrial cells were implanted into nude mice, as described above. Five days after transplantation, mice were anesthetized with thiopental and their peritoneal cavity

was illuminated with a Maestro in vivo imaging system (CRi, Woburn, MA, USA) (Fig. 1A).

RNA isolation and semi-quantitative reverse transcriptase (RT)-PCR

Total RNA was extracted from endometriosis-like lesions using Isogen (Nippon gene Co., Ltd., Tokyo). RNA concentration was determined using a NanoVue spectrophotometer (GE Healthcare UK, Ltd., Little Chalfont, Buckinghamshire, UK). RNA (0.25 μg) was reverse-transcribed and amplified using a One-Step RNA PCR Kit (Takara Bio Inc., Otsu). The sense (S) and anti-sense (A) primers used for RT-PCR were as follows: 5'-TGTGTACACCGGAGTGTGTTGTAG-3' (S) and 5'-ACTGTCATGAGCAAGATAGAGGC-3' (A) for human PAR1; 5'-GTTGATGGCACATCCCCACGTC-3' (S) and 5'-GTACAGGGCATAGACATGGC-3' (A) for human PAR2; and 5'-AGCCACATCGCTCAGACA-3' (S) and 5'-GCCAATACGACCAAATCC-3' (A) for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a house-keeping gene which was used as an internal control. The thermal cycle parameters for PCR were as follows: 20 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 2 min. The amplified products were separated on 1.5% agarose gels and analyzed as described earlier (29).

Quantitative real-time RT-PCR

Quantitative changes in the mRNA expression of human IL-6, VEGF₁₂₁, and COX-2 were examined by real-time RT-PCR using the iScript™ One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA, USA). Measurement and data analysis were performed essentially as described (30). Transcript levels of each gene were normalized to G3PDH. The specific primers used are shown in Table 1. Each primer was specific for either a human or mouse gene. PCR was run using the iQ5 Real-Time PCR Detection System (Bio-Rad) under the following thermal PCR conditions: 95°C for 10 s for cleavage and 60°C for 30 s for both annealing and extension for 45 cycles. Fluorescence was detected during the annealing–extension reaction. iQ5 Optical system software (Bio-Rad) was used to collect the data and calculate the threshold cycle (Ct). The expression of each gene was normalized to G3PDH and analyzed by the comparative Ct method.

Measurement of IL-6 and PGE₂

Concentrations of IL-6 were measured using a human IL-6 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). The levels of PGE₂ were determined using a PGE₂ Enzyme Immunoassay (EIA) kit (Enzo Life Sciences, Plymouth Meeting, PA, USA), according to the manufacturer's instructions. The sensitivity of the

Fig. 1. The development of endometriosis-like lesions that express PAR1, PAR2, CD31, and VEGF in a mouse model of endometriosis. A: The right side of the ovary was removed from 8-week-old female nude mice. Immortalized human endometrial EM-1 epithelial cells and EtsT-499 stromal cells were fluorescently labeled with 10 μ M CFDA-SE, mixed, and then administered (5×10^6 cells total) intraperitoneally from and directly adjacent to the dissection site with a micropipette. Five days after transplantation, fluorescent signals were monitored using a Maestro in vivo imaging system. B – D: E2 treatment was performed as described in Materials and Methods. A cell suspension containing E2 (17β -estradiol, 10^{-7} M) was transplanted into the peritoneal cavity. Mice were sacrificed by cervical dislocation 5 or 10 days after transplantation and endometriosis-like lesions were recovered by laparotomy (B, left panel). Macroscopic lesions attached to the peritoneum and intraperitoneal organs that were visible to the eye were collected and weighed (B, right panel). Representative photographs of endometriosis-like lesions formed around the site of ovarian dissection in E2-treated and control groups. C: Real-time quantitative RT-PCR analysis of the expression of mouse CD31 and human VEGF₁₂₁ mRNA levels were normalized to G3PDH. Data are expressed relative to mRNA levels in the absence of E2 treatment after 5 days and represent the means $\pm$ S.E.M. of three independent experiments. D: Semi-quantitative RT-PCR analysis of the expression of human PAR1 and PAR2.

assays was approximately 3 and 40 pg/ml, respectively. Peritoneal fluid derived from uOVX- or PAR agonists-treated animals was collected. For the PGE₂ assay, peritoneal PGs were extracted with ether, as previously de-

scribed (31). For endometrial EM-1 and EtsT cells, culture medium was used directly for measurement.

Table 1. The sequence of primers for real-time RT-PCR

Gene name	Sequence (S: sense, A: Anti-sense)	Product size	Annealing temp. (°C)
IL-6 (human)	S: 5'-CAGGAGCCCAGCTATGAACT-3'	85	60
	A: 5'-AGCAGGCAACACCAGGAG-3'		
COX-2 (human)	S: 5'-CAGCACTTCACGCATCAGTT-3'	127	60
	A: 5'-CGCAGTTTACGCTGTCTAGC-3'		
VEGF ₁₂₁ (human)	S: 5'-GAGCTTCCTACAGCACAACAAA-3'	99	60
	A: 5'-CTCGGCTTGTCACATTTTTC-3'		
CD31 (mice)	S: 5'-CTGGGAGGTCGTCCATGT-3'	59	60
	A: 5'-CACAGGACTCTCGCAATCC-3'		
G3PDH (human)	S: 5'-AGCCACATCGCTCAGACA-3'	66	60
	A: 5'-GCCCAATACGACCAAATCC-3'		
G3PDH (mice)	S: 5'-AAGCTGTGGCGTGATGG-3'	135	60
	A: 5'-TTCAGCTCTGGGATGCCTT-3'		

Cell proliferation assay

To determine the effect of PAR1 and PAR2 agonists on the proliferation of EM-1 and EtsT cells, changes in cell number after thrombin and/or PAR2 AP treatment were monitored by proliferation bioassay. Cells (3×10^3 cells) were seeded in 96-well culture plates in DMEM containing 1% FBS and then treated with thrombin and/or PAR2 AP for 24 h before the evaluation of cell proliferation. For the proliferation bioassay, cells were incubated with WST-8 (Cell counting kit-8; Dojindo, Kumamoto) for 20 min at 37°C. Staining intensity was determined by measuring the absorbance at 450 nm with a microtiter plate reader. The data are expressed as ratios of test to control value.

Assay of caspase-3 and -7 activity

EM-1 and EtsT cells were cultured in 96-well tissue culture plates and then treated with 20 nM staurosporine in the presence or absence of thrombin and/or PAR2 AP. After 6 h, cells were lysed with Caspase-Glo 3/7 reagent (Promega, Madison, WI, USA) and the contents of the wells were gently mixed on a plate shaker, according to the manufacturer's instructions. After incubation at room temperature for 1.5 h, the activity of each sample was determined with a luminometer.

Statistical analyses

Values represent means $\pm$ S.E.M., except for the results of immunoblot and semi-quantitative RT-PCR analyses. The statistical significance of the results was tested using Dunnett's test for multiple comparisons. Differences with a P -value < 0.05 were considered to be significant. All statistical analyses were performed using Stat View (ver. 5; SAS Institute Inc., Cary, NC, USA).

Results

The development of endometriosis-like lesions that express PAR1/2, CD31, and VEGF in a mouse model of endometriosis

Immortalized human endometrial EM-1 epithelial cells and EtsT stromal cells were fluorescently labeled with CFDA-SE and then implanted into the peritoneal cavity of uOVX mice. After 5 days, fluorescent signals corresponding to labeled cells were detected inside the upper abdomen (Fig. 1A). To determine the effect of excess E2 on the size of the endometriosis-like lesions formed by the implanted cells in the peritoneal cavity, cells suspended in matrigel-containing E2 (10^{-7} M) were transplanted into the peritoneal cavity of uOVX mice and then E2 ($10 \mu\text{g}/\text{mouse}$) was administered subcutaneously once a day for 5 or 10 days thereafter. Most of the endometriosis-like lesions (about 80%) were observed around the dissection site in each mouse, as depicted in Fig. 1B. Approximately 20% of the lesions were non-adherent and floating in the peritoneal fluid. Small lesions were obtained in the peritoneal cavities of non-uOVX mice (data not shown). Visible lesions that were attached to the uOVX site and the intraperitoneal organs were collected and weighed (Fig. 1B). There was no significant difference in total lesion weight between control and E2-treated mice. The effect of E2 on angiogenic activity in the lesions was determined by measuring the mRNA levels of mouse CD31, platelet-endothelial cell adhesion molecule 1 (PECAM1), and human VEGF (Fig. 1C). The mRNA levels of CD31 and VEGF₁₂₁ tended to be elevated after E2 treatment compared to control mice, but this difference was not statistically significant. The expression of human PAR1 and PAR2 mRNA was detected in

the lesions on day 5 (Fig. 1D), but there was no apparent effect of E2 on PAR1 and PAR2 mRNA levels.

Effect of PAR agonists on the size and characteristics of endometriosis-like lesions

Subconfluent EM-1 and EtsT cells were treated with the PAR agonists thrombin (a PAR1 agonist) and PAR2 AP (a PAR2 agonist) and then infused into the peritoneal cavity of non-uOVX mice in order to determine whether the formation and characteristics of endometriosis-like lesions was affected by PAR1/2 stimulation. PAR agonist treatment resulted in a 20-fold increase in human IL-6 levels in the peritoneal fluid (Fig. 2B) relative to mice that received untreated control cell, and also significantly enhanced IL-6 and COX-2 mRNA levels in the lesions 5 days after implantation (Fig. 2C). However, there was no effect of PAR agonist treatment on lesion weight (Fig. 2A).

Effect of serine protease inhibitors on PAR agonist- or uOVX-induced IL-6 and COX-2 expression in endometriosis-like lesions

The expression of human IL-6 and COX-2 in endometriosis-like lesions was increased by uOVX to levels

similar to those seen in non-uOVX mice receiving PAR agonist-treated cells (Fig. 3). PGE₂ content in endometriosis-like lesions collected from the peritoneal cavity of uOVX mice was significantly higher than that of non-uOVX animals (Control) (Table 2). The expression of IL-6 and COX-2 was suppressed to control levels in uOVX mice by treatment of the transplanted cells with serine protease inhibitors (uOVX + SPI).

Effect of PAR agonists on IL-6 and COX-2 expression in cultured endometrial cells

To determine whether PAR activation affected the expression of IL-6 and COX-2 in endometrial glandular and stromal cells, cultured EM-1 and EtsT cells were treated with thrombin and/or PAR2 AP (Fig. 4). Co-treatment with thrombin and PAR2 AP stimulated IL-6 mRNA expression in both cell types (Fig. 4: A, D) and up-regulated COX-2 mRNA in EM-1 cells. Furthermore, the levels of IL-6 secreted into the medium were significantly elevated by combined treatment with thrombin and PAR2 AP (Fig. 4: B, E). The mRNA levels of PAR1 and PAR2 in EM-1 (Fig. 4C) and EtsT (Fig. 4F) were not affected by co-treatment with PAR agonists.

Fig. 2. Effect of PAR1 and PAR2 agonists on the expression of IL-6, VEGF, and COX-2 in endometriosis-like lesions. Thrombin (10 U/ml), a PAR1 agonist, and PAR2 AP (30 μM), a PAR2 agonist, were added to the cell suspension mixture (3.4×10^6) of EM-1 and EtsT cells, and then the suspension was administered to non-OVX female nude mice. Animals were sacrificed 5 days later to assess and characterize the formation of endometriosis-like lesions. A: Lesions were collected and weighed. B: Animals were euthanized by cervical dislocation. One milliliter of PBS was injected intraperitoneally and then peritoneal fluid diluted in PBS was collected. The concentration of human IL-6 was determined by ELISA. Data (n = 5) represent the means ± S.E.M. of two independent experiments. * $P < 0.05$ vs. the control (Cont). C: The expression of human IL-6, COX-2, and VEGF₁₂₁ was evaluated by real-time quantitative RT-PCR. Data (n = 6) represent the means ± S.E.M. of three independent experiments. * $P < 0.05$ vs. Cont.

Fig. 3. Effect of serine protease inhibitors on the enhanced expression of IL-6 and COX-2 in endometriosis-like lesions of uOVX mice. Non-uOVX mice administered PAR agonist-treated (PAR agonist) and untreated cells (Cont) were obtained as described for Fig. 2. uOVX was performed as described for Fig. 1. A cell suspension mixture of EM-1 and EtsT cells in matrigel with (uOVX + SPI) or without (uOVX) the addition of serine protease inhibitors was administered intraperitoneally. Five days after transplantation, mice were sacrificed and endometriosis-like lesions were recovered. The expression of IL-6 and COX-2 in the lesions was assessed by real-time quantitative RT-PCR. Data ($n = 6$) represent the means $\pm$ S.E.M. of two independent experiments. * $P < 0.05$ vs. Cont, # $P < 0.05$ vs. uOVX. SPI, serine protease inhibitors.

Table 2. PGE₂ content in peritoneal fluid and endometriosis-like lesions

Group	pg / total volume peritoneal fluid	pg / 10 mg lesion
Control	480 $\pm$ 144.2	79 $\pm$ 10.5
uOVX	1925 $\pm$ 480.2	161 $\pm$ 14.8*

Unilateral ovariectomy (uOVX) was performed as described for Fig. 1. A cell suspension containing a mixture of EM-1 and EtsT cells in matrigel was administered intraperitoneally. Five days after transplantation, mice were sacrificed and the peritoneal fluid and endometriosis-like lesions were recovered. PGE₂ content was measured using a PGE₂ ELISA kit. Data represent the means $\pm$ S.E.M. of four animals. * $P < 0.01$ vs. Control.

Effect of indomethacin and PGE₂ on PAR agonist-induced IL-6 and COX-2 expression in endometrial cells

PAR stimulation has been shown to enhance PGE₂

release by human alveolar epithelial cells (32). Elevated levels of eicosanoids may be associated with increased IL-6 levels in airway epithelial and smooth muscle cells (33). Because COX-2 is highly expressed in glandular cells, we investigated whether secreted PGE₂ might modulate the expression of IL-6 and COX-2 through PAR in cultured cells (Fig. 5). PGE₂ treatment accelerated thrombin / PAR2 AP-induced IL-6 and COX-2 expression, whereas indomethacin had no effect in both EM-1 and EtsT cells. Indomethacin also did not interfere with the ability of PGE₂ to enhance thrombin / PAR AP-stimulated IL-6 and COX-2 expression. Treatment with PGE₂ or indomethacin alone did not affect IL-6 and COX-2 mRNA levels.

Effect of PAR agonists on proliferation and apoptosis induction in cultured EM-1 cells

The effects of PAR stimulation on cell proliferation and apoptosis induced by staurosporine were examined in cultured EM-1 cells (Fig. 6). There was no significant effect of PAR activation on cell number after 24 h (Fig. 6A). Staurosporine induced an approximately 5-fold increase in caspase-3/7 activity, and this was unaffected by thrombin and/or PAR2 AP treatment (Fig. 6B). The similar results were obtained using EtsT cells (data not shown).

Discussion

The development of endometriosis may be influenced by the quantity and quality of retrograde menstruation (2, 3). In particular, inflammation in the peritoneal cavity caused by retrograde menstruation may be crucial for the progression of endometriosis. The exposure of ectopic endometrial cells to local peritoneal factors such as proinflammatory cytokines, growth factors, and PGs derived from endometrial cells and peritoneal cells could alter their properties and contribute to endometriosis. In the present study, normal endometrial gland cells and stromal cells transplanted into the peritoneal cavity of nude mice after uOVX formed endometriosis-like lesions around the uOVX dissection site. Several methods for studying the development and pathology of endometriosis using experimental animal models have been described, most involving the implantation of endometrial cell suspensions derived from patients with endometriosis (12–16). This type of model uses transformed cells that are already in an altered, pathological state. One important aspect of the present model is the use of a suspension of normal endometrial cells, not endometriotic cells, to induce the formation of endometriosis-like lesions. Thus, this new model could serve as a useful tool for addressing how normal endometrial cells transition

Fig. 4. Effect of thrombin and/or PAR2 AP on IL-6 and COX-2 expression in cultured endometrial cells. EM-1 and EtsT cells (4×10^4 cells/well) were incubated for 24 h with thrombin (10 U/ml) and/or PAR2 AP (30 μ M). A, D: Total RNA was analyzed by real-time-quantitative RT-PCR for IL-6 and COX-2 expression levels. B, E: IL-6 levels in culture media were measured by ELISA. C, F: Semi-quantitative RT-PCR analysis of the expression of PAR1 and PAR2 was performed in intact (no treatment) and both thrombin- and PAR2 AP-treated cells. Three independent sets of experiments were performed in triplicate and the data represent the means $\pm$ S.E.M. * $P < 0.05$ vs. intact cells, ** $P < 0.05$ vs. PAR2 AP-treated cells.

Fig. 5. Effect of indomethacin and/or PGE₂ on IL-6 and COX-2 expression induced by co-treatment with thrombin and PAR2 AP in endometrial cells. EM-1 and EtsT cells (4×10^4 cells/well) were pretreated with indomethacin (10^{-7} M) and/or PGE₂ ($0.3 \mu\text{M}$) and then incubated for 24 h with thrombin (10 U/ml) and PAR2 AP ($30 \mu\text{M}$). RNA was isolated and subjected to real-time quantitative RT-PCR for IL-6 and COX-2 expression. Three independent sets of experiments were performed in triplicate and the data represent the means $\pm$ S.E.M. * $P < 0.05$ vs. intact cells, ** $P < 0.05$ vs. thrombin / PAR2 AP-treated cells without indomethacin and/or PGE₂.

into endometriotic cells. The only caveat to this, of course, is that the mechanism of lesion formation in this model is assumed to reflect certain aspects of the pathophysiology of endometriosis.

Estrogen is believed to be involved in the progression of endometriosis. Endometriotic lesions increase in size with repeated rounds of proliferation and bleeding in response to the cyclic secretion of estrogen (2, 3). Furthermore, increased levels of estrogen in the local milieu may be supplied by the lesion itself through aberrant expression of steroidogenic acute regulatory protein (StAR) and aromatase (34). Recently, estradiol-3-*O*-sulfamate (E2MATE), an inhibitor of steroid sulfatase, which is involved in estrogen biosynthesis, was shown to effectively decrease endometriotic lesions without affecting systemic estradiol levels (35). Previously, it was shown that the replacement of estrone increases the growth of implanted endometriotic structures in OVX mice compared to non-treated OVX animals (11). In another mouse model, the infusion of human endometrial

tissue that had been treated with E2 into the peritoneal cavity resulted in the appearance of ectopic lesions, whereas progesterin inhibited the formation of ectopic endometrial lesions (16). In the current study, there was no effect of exogenous estrogen treatment on the size of the endometriotic lesions. This result is consistent with an earlier report showing no effect of estrone on the number and weight of lesions or the levels of estrogen receptor expression in an immunodeficient mouse model (17). Although the reasons for these discrepancies are not clear, one could argue that slight differences in the experimental models could account for the observed differences. In our model, animals that had undergone uOVX were treated with a high dose of E2 immediately after the operation. These conditions could conceal the stimulatory effects of E2 on the lesions. If physiological E2 levels are sufficient to significantly influence lesion formation, it would be difficult to detect the effect of exogenous E2 in our model system.

PAR1, a thrombin receptor, is expressed in various

Fig. 6. Effects of thrombin and/or PAR2 AP on proliferation and chemical-induced apoptosis in EM-1 cells. A: EM-1 cells (3×10^3 cells/well) were incubated for 24 h with thrombin and/or PAR2 AP. The number of cells was evaluated using the WST-8 proliferation assay. Three independent sets of experiments were performed in triplicate and the data represent the means $\pm$ S.E.M. B: Cells (2×10^4 cells/well) were treated for 6 h with staurosporine (20 nM) in the presence or absence of thrombin (10 U/ml) and/or PAR2 AP (30 μ M). The activity of caspase-3 was measured using a luminescent assay (the Caspase-Glo[®] 3/7 Assay). Three independent sets of experiments were performed in triplicate and the data represent the means $\pm$ S.E.M. * $P < 0.05$ vs. Cont.

cells, including alveolar epithelial cells, mast cells, macrophages, infiltrating neutrophils, fibroblasts, endothelial cells, and smooth muscle cells (19–21). Activation of PAR1 induces the expression of IL-8 in vascular endothelial cells (36) and induces inflammation (21, 37). PAR1 has also been implicated in cancer cell invasion and tumor progression (36, 38, 39). PAR2 functions to increase PG and cytokine levels in epithelial cells (33, 40). In the current study, the fact that such dramatic lesion formation was observed at the site of ovarian removal where bleeding was present during and after uOVX suggests that tissue damage and bleeding may trigger an inflammatory reaction via PAR activation in this model. PAR is expressed in uterine endometrial stromal cells (23, 24) and PAR activation has been shown to stimulate the production of matrix metalloproteinase (MMP)-1/2, VEGF, and IL-8 (41). One of the potential contributing factors in the progression of endometriosis is neutrophil elastase, which has been implicated in the inflammatory response in endometriotic tissues (42) and can activate PAR1/2 (21). Thus, bleeding may cause PAR1/2 activation through elastase activation. Interestingly, in the current study, the expression of IL-6 and COX-2 in endometriosis-like lesions was significantly

higher when endometrial cells were treated with a PAR agonist before implantation into non-uOVX nude mice. Elevated IL-6 and COX-2 expression was observed in the lesions of uOVX mice and this expression was reduced to control levels by treatment with serine protease inhibitors. The inhibitors were added to the cell suspension just before cell infusion to achieve sufficient inhibition of PAR activation in the implanted cells. These results suggest that PAR signaling may be involved in IL-6 and COX-2 expression in endometriotic lesions. Increased levels of IL-6 in the peritoneal fluid were also observed in mice implanted with PAR agonist-treated cells. In addition, PAR agonists enhanced IL-6 secretion in cultured endometrial gland and stromal cells. IL-6 has been shown to be a potential nonsurgical serum marker for predicting the development of endometriosis (10), and the concentrations of IL-6 in peritoneal fluid were higher in patients with endometriosis and correlated positively with dysmenorrheal and pelvic pain (43, 44). Similar mechanisms might be at work in the current experimental model, wherein PAR signaling pathways activated by bleeding mediate the increased expression of IL-6. However, PAR probably does not contribute to the enlargement of endometriotic lesions, as we observed no

effect of PAR treatment on the weight and size of the lesions. Moreover, PAR agonists did not alter EM-1/EtsT proliferation or chemically-induced apoptosis. In contrast, PAR stimulation enhanced the proliferation and migration of endometrial stromal cells derived from endometrial lesions (23, 24). This discrepancy might reflect differences between freshly isolated cells and the cell lines used in the present study.

IL-6 and COX-2 expression in cultured EM-1 and EtsT cells was significantly increased by combined treatment with thrombin and PAR2 AP, similar to that seen in vivo. Activation of both PAR1 and PAR2 could be necessary to trigger a strong inflammatory reaction, since either agonist alone was less effective than both in stimulating IL-6 and COX-2 expression. In human alveolar epithelial cells, treatment with a PAR2 agonist promoted PGE₂ release, partially through the activation of the PI3-kinase / Akt / NF- κ B pathway (32). The mitogen activated protein (MAP) kinase pathway transduces signals leading to COX-2 upregulation. Furthermore, results from a recent study showed that thrombin promotes chemokine production via the MAP kinase pathway in human endometrial stromal cells (41). Additional studies are needed to determine the signaling pathways involved in the induction of IL-6 and COX-2 expression by PAR1 and PAR2, as well as the underlying mechanism of acceleration of PAR1/2-induced expression of these genes by PGE₂. In the current study, PGE₂ treatment enhanced PAR agonist-stimulated IL-6 and COX-2 expression in cultured glands and stromal cells but indomethacin did not, suggesting that endogenous PGE₂ secreted from endometrial cells is not involved in enhanced IL-6 and COX-2 expression, at least over the short term. Our results suggest that paracrine PGE₂ from cells such as blood cells, endothelial cells, or infiltrating cells in the lesion may stimulate gene expression. The increased attachment of endometrial cells to abdominal tissue, proliferation, or resistance to apoptosis may be closely related to the formation of endometriosis-like lesions (45). However, there was no effect of PAR stimulation on EM-1 cell viability or apoptosis. These results are consistent with results in vivo showing no change in the weight or size of the lesions formed in the peritoneal cavity in animals receiving PAR-treated cells or intact cells.

In conclusion, the results of the current study suggest that peritoneal bleeding may accelerate the expression of proinflammatory mediators in endometriosis-like lesions partly through PAR activation in a uOVX-induced experimental model of endometriosis.

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