Activation of protease-activated receptor 2 stimulates proliferation and interleukin (IL)-6 and IL-8 secretion of endometriotic stromal cells

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BACKGROUND: Inflammation has been proposed to play essential roles in the pathophysiology of endometriosis, in which neutrophils and mast cells have been suggested to be involved. We studied whether the protease-activated receptor 2 (PAR2), which is activated by enzymes from neutrophils and mast cells, in endometriotic stromal cells (ESC) has any implication in the development of the disease. METHODS: Cultured ESC were stimulated with various concentrations of a specific PAR2 agonist peptide. Proliferating activity of the cells was determined using immunostaining of proliferating cell nuclear antigen (a cell proliferation marker), 5-bromo-2′-deoxyuridine incorporation into DNA and cell count. The concentrations of interleukin (IL)-6 and IL-8 were measured using specific enzyme-linked immunosorbent assay kits. The phosphorylation of three mitogen-activated protein kinases (MAPK), i.e. p38 MAPK, p42/44 MAPK and stress-activated protein Kinase/c-jun N terminal Kinase, in ESC was examined with Western blot analysis. RESULTS: Activation of PAR2 stimulated the proliferation of ESC and the secretion of IL-6 and IL-8 from ESC in a dose-dependent manner. Activation of PAR2 stimulated the phosphorylation of all three MAPK, and inhibitors of each MAPK suppressed the PAR2 activation-induced proliferation of ESC. CONCLUSIONS: The activation of PAR2 in ESC may be involved in the pathophysiology of endometriosis by inducing the growth and inflammation of endometriotic lesions.

Key words: endometriosis/interleukin/mitogen-activated protein kinase/proliferation/protease-activated receptor 2

Introduction

Endometriosis is an enigmatic disease that impairs the health of women of reproductive age. Accumulating evidence suggests that peritoneal inflammation plays an important role in the development of the disease, with associated symptoms such as pain and infertility (Harada et al., 2001; Lebovic et al., 2001; Wu and Ho, 2003). Leukocytes in the abdominal cavity have been suggested to contribute to the inflammatory milieu. Macrophages have been studied as a central player in the pathogenesis of endometriosis (Giudice and Kao, 2004). However, relevance of other leukocytes to the disease has been poorly understood.

The concentrations of chemokines, such as interleukin (IL)-8, epithelial neutrophil-activating peptide 78, and growth-regulated α, that induce the activation and chemotaxis of neutrophils, are increased in the peritoneal fluid (PF) in women with endometriosis (Kutteh et al., 1995; Arici et al., 1996; Oral et al., 1996; Szamatowicz et al., 2002; Mueller et al., 2003). Similarly, concentrations of stem cell factor, which stimulates the activation and migration of mast cells, are increased in PF of women with endometriosis (Osuga et al., 2000). In view of the thesis that endometriosis is characterized by recurrent ectopic bleeding (Brosens, 1997), it is conceivable that endometriotic tissues, including ovarian, are repeatedly exposed to neutrophils in the blood. Furthermore, reduced spontaneous apoptosis of neutrophils is suggested to be involved in the development of the disease (Kwak et al., 2002). An increase of mast cells in endometriotic lesions has been demonstrated (Matsuzaki et al., 1998; Uchiide et al., 2002; Fujiwara et al., 2004). Therefore, it is reasonable to speculate that neutrophils and mast cells may play roles in the pathophysiology of the disease.

In recent years, protease-activated receptors (PAR), members of the seven-transmembrane G-protein-coupled receptor family, have been noted to be important mediators of inflammation (Cocks and Moffatt, 2000; Macfarlane et al., 2001). To date, four PAR have been discovered and characterized. PAR2 is unique in that it is activated by various proteases but not by thrombin, while PAR1, PAR3 and PAR4 are all activated by thrombin. Interestingly, proteinase 3, human leukocyte elastase and cathepsin G, which are secreted from neutrophils, are activators for PAR2 (Uehara et al., 2002, 2003). In addition, tryp- tase from mast cells also activates PAR2 (Molino et al., 1997).
In light of the possible implication of neutrophils and mast cells in endometriosis, we speculated that proteases from these cells function via PAR2. In particular, PAR2-mediated proliferative effects reported in certain cells (Fungieri et al., 2002; Gaca et al., 2002) prompted us to investigate whether PAR2 activation may be involved in the development of endometriosis. In the present study, we examined the effects of PAR2 activation on endometriotic cell proliferation, and the possible involvement of mitogen-activated protein kinases (MAPK) therein.

**Materials and methods**

**Reagents and materials**

Type I collagenase, antibiotics (mixture of penicillin, streptomycin, amphotericin B) and a serine protease inhibitor, aprotinin, were purchased from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (DMEM/F-12) and 0.25% trypsin–EDTA were from Life Technologies (Rockville, MD, USA). PAR2 agonist peptide (PAR2AP) SLIGKV, which stimulates PAR2 specifically, was from BACHEM (Bubendorf, Switzerland). MAPK inhibitors SB202190, PD98059 and SP600125 (inhibitors for p38 MAPK, p42/44 MAPK and SAPK/JNK respectively) were from Calbiochem (La Jolla, CA, USA). Anti-rabbit antibodies of p38 MAPK, phospho-p38 MAPK, p42/44 MAPK, phospho-p42/44 MAPK, SAPK/JNK and phospho-SAPK/JNK were from New England BioLabs (Beverly, MA, USA). Ficol-Paque Plus, Dextran 500 and anti-rabbit horseradish peroxidase secondary antibody was from Amersham Biosciences (Little Chalfont, UK). Goat anti-actin antibody and anti-body and anti-goat horseradish secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Non-immune murine immunoglobulin (IgG)2a and antibodies of proliferating cell nuclear antigen (PCNA), CD45, vimentin and cytokertatin were from Dako (Kyoto, Japan). Mouse monoclonal anti-CD10 antibody was from Novocastra, (Newcastle upon Tyne, UK). Charcoal/dextran-treated fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Deoxyribonuclease I was from Takara (Tokyo, Japan).

**Collection of endometriotic tissues**

Endometriotic tissues were obtained from patients with ovarian endometriomas (n = 24) undergoing laparoscopy or laparotomy before surgery. Endometriotic tissue samples were obtained from the cyst wall of ovarian endometrioma under sterile conditions and transplanted into 60 mm dishes and allowed to adhere at 37 °C for 30 min, after which non-adhering epithelial cells and blood cells were removed with PBS rinses. ESC were cultured in DMEM/F-12 containing 10% FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. When the cells became confluent in 2 or 3 days, they were dissociated with 0.25% trypsin–EDTA, harvested by centrifugation at 200 g for 5 min, replated in 6-well plates at 2 × 10^5 cells/well, 48-well plates at 1 × 10^4 cells/well or 96-well plates at 0.5 × 10^3 cells/well, and incubated at 37 °C in a humidified 5% CO2/ 95% air environment for 24 h. The complete media were then removed and replaced with fresh serum-free media containing antibiotics, and the cells were cultured for an additional 12–24 h. Purity of the stromal cell population was determined by immunocytochemical staining for the following: vimentin (stromal cells), cytokertatin (epithelial cells) and CD45 (monocytes and other leukocytes). The purity of the stromal cells was >98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokertatin and CD45. More specifically, immunostaining with anti-CD10 antibody identified >95% of the cells as endometriotic stromal cells.

**Isolation, purification, and culture of neutrophils**

Human neutrophils (polymorphonuclear leukocytes) were isolated from freshly drawn venous blood samples from healthy volunteers. The methods of neutrophil isolation were divided by three steps, i.e. dextran sedimentation, Ficoll-Paque centrifugation and lysis of contaminated red blood cells.

All manipulations were performed in sterile conditions. Twenty millilitre of venous blood drawn into heparin-coated syringes was mixed with 10 ml of 0.9% saline with 3% Dextran 500, and the mixture was left standing for 30 min at room temperature to allow sedimentation of red cells. After the sedimentation, leukocyte-rich supernatant was recovered and centrifuged at 250 g for 10 min. The pellet was then diluted in 8 ml PBS and carefully layered over 4 ml Ficoll-Paque Plus. After centrifugation at 400 g for 15 min, the supernatant, which contains mononuclear cell layer, was discarded. To lyse contaminated red blood cells, the remaining pellet was resuspended with 0.2% sodium chloride for 30 s, and subsequently was mixed with an equal volume of 1.6% sodium chloride. The purified neutrophils were washed, pelleted and resuspended at 1 × 10^6 cells/ml in DMEM/F-12 containing 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. The cells were then plated in 6-well plates at 2 × 10^5 cells/well and incubated at 37 °C in a humidified 5% CO2/95% air environment for 12 h. After the incubation, the conditioned media were collected and used for the treatment of ESC.

**Treatment of ESC**

To evaluate the dose effects of PAR2AP on PCNA expression and cytokine secretion of ESC, the wells were replenished with serum-free media with different concentrations of PAR2AP and the cells were incubated for 24 h. The doses used in this study was chosen based on our previous findings about PAR2AP on eutopic endometrial cells (Hirota et al., 2005a). To assess the effects of PAR2AP on DNA synthesis and number of ESC, the cells were incubated with serum-free media with PAR2AP at 30 μmol/l for 24 or 48 h. To evaluate the effects of PAR2AP on MAPK phosphorylation of ESC, the cells were incubated with serum-free media with PAR2AP at 30 μmol/l for different durations. To evaluate the effects of three MAPK inhibitors on PAR2-induced proliferation of ESC, the cells were preincubated with SB202190 (p38 MAPK inhibitor, 10 μmol/l), PD98059 (p42/44 MAPK inhibitor, 25 μmol/l) or SP600125 (SAPK/JNK inhibitor, 10 μmol/l) for 1 h before PAR2AP treatment (30 μmol/l). To evaluate the proliferative effect of neutrophil-derived serine proteases on ESC, the cells were incubated with either serum-free media or conditioned media of neutrophil culture with or without different concentrations of serine protease inhibitor aprotinin for 24 h.
**Immunocytochemical staining for PCNA**

PCNA was chosen as a proliferation marker in view of our previous report that used PCNA to show thrombin-stimulated proliferation of ESC (Hirota et al., 2005b). The cells were fixed with cold methanol: acetone (1:1) at −20°C for 20 min, and washed twice with PBS. The fixed cells were treated with 3% hydrogen peroxide for 5 min to eliminate endogenous peroxidase. After blocking with 1.5% horse serum for 20 min, the cells were incubated with a mouse monoclonal antibody to PCNA (1:200) for 20 min at room temperature. Control cells were incubated with non-immune murine IgG2a, the concentration of which was adjusted to that of the primary antibody. The cells were then incubated with biotinylated horse anti-mouse IgG, followed by avidin peroxidase using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The chromogenic reaction was performed with diaminobenzidine (Vector Laboratories).

Immunostained cells were analysed in a blinded fashion by the same person without knowledge of the treatment group. The PCNA-positive rate (PCNA-positive cells/total cells) was determined by observing >500 nuclei for each sample, and was used for evaluating the proliferating activity of the cells.

Control and PAR2AP-treated cells were also stained with antibodies to vimentin and cytokeratin to evaluate the effect of PAR2AP on ESC purity.

**Western blot analysis**

Western blot analysis was performed as we have reported previously (Yoshino et al., 2003; Hirota et al., 2005a). Cultured cells were homogenized in a lysis buffer containing 50 mmol/l Tris/HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 50 mmol/l dithiothreitol and 0.1% bromophenol blue, and diluted to 1 mg total protein/ml. Concentrations of total protein in the homogenized cells was measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples were resolved by 10% SDS–polyacrylamide gel electrophoresis. Proteins were blotted onto a Hybond-ECL nitrocellulose membrane (Amersham Bioscience, Little Chalfont, UK) and incubated with rabbit antibodies to total p38 MAPK (1:1000), to phospho-specific p38 MAPK (1:1000), to total p42/44 MAPK (1:1000), to phospho-specific p42/44 MAPK (1:1000), to total SAPK/JNK (1:1000), or to phospho-specific SAPK/JNK (1:1000) as primary antibodies, and anti-rabbit horseradish peroxidase antibody (1:1000) as a secondary antibody. The blotted membranes were also developed with a goat antibody to actin (1:1000) as a primary antibody and anti-goat horseradish peroxidase antibody (1:1000) as a secondary antibody. Immunocomplexes were visualized by an enhanced chemiluminescence western blotting system (Amersham Biosciences). Densitometric analysis of bands on developed X-ray films was performed using National Institutes of Health image software.

**The 5-bromo-2′-deoxyuridine (BrdU) proliferation assay**

The BrdU proliferation assay was performed as we have reported previously (Tang et al., 2002; Hirota et al., 2005a,e) using the Biotrak cell proliferation enzyme-linked immunosorbent assay (ELISA) system (Amersham Biosciences) according to the manufacturer’s instructions. Briefly, ESC were treated with serum-free medium with PAR2AP for 24 h, and 100 μl BrdU solutions were added and incubated at 37°C for an additional 2 h. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of 200 μl/well fixative. The peroxidase-labelled anti-BrdU bound to the BrdU incorporated in the newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant colour was read at 450 nm in the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria).

**Cell counting of ESC**

ESC were treated with serum-free medium with or without PAR2AP for 48 h, dissociated with 0.25% trypsin–EDTA, and collected by centrifugation at 200 g for 5 min. The number of ESC, resuspended in 10 ml PBS, was determined using a Coulter Counter Z1 (Beckman Coulter, Fullerton, CA, USA).

**Measurement of IL-6 and IL-8**

After the treatments, the conditioned media were collected, centrifuged and stored at −80°C until assay. Concentrations of IL-6 and IL-8 were measured using specific ELISA kits (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Absorbance was read at 450 nm with the DigiScan Microplate Reader. Cultured cells were homogenized, and total protein in the homogenized cells was measured by a protein assay kit. Data were standardized by total protein of cell lysates.

**Statistical analysis**

Data were evaluated using Student’s t-test and analysis of variance with post-hoc analysis (Fisher’s protected least significance). P < 0.05 was accepted as significant.

**Results**

**PAR2AP induced PCNA expression in ESC**

As shown in Figure 1A, the immunocytochemical staining clearly demonstrated PCNA-positive nuclei in ESC, whereas cytoplasm appeared to be virtually PCNA negative. The PCNA-positive rate in the ESC was increased with PAR2AP treatment in a dose-dependent manner (Figure 1B). The increase was significant, from 0.3 to 300 μmol/l. The purity of ESC was >98% both in the control and PAR2AP-treated cells, judged by vimentin-positive and cytokeratin-negative cells (data not shown).

**PAR2AP stimulated phosphorylation of p38 MAPK, p42/44 MAPK and SAPK/JNK**

As demonstrated in Figure 2, PAR2AP at 30 μmol/l stimulated the phosphorylation of all three MAPK (p38 MAPK, p42/44 MAPK and SAPK/JNK) within 5 min. The phosphorylation levels of p38 MAPK significantly increased between 5 and 30 min, followed by a decrease to basal levels at 60 min. The phosphorylation levels of p42/44 MAPK significantly increased between 5 and 15 min and decreased at 60 min as compared with control, followed by recovery to basal levels at 120 min. The phosphorylation levels of SAPK/JNK significantly increased between 5 and 60 min, followed by a decrease to basal levels at 120 min. PAR2AP did not alter control actin protein levels (data not shown).

**MAPK inhibitors suppressed PAR2AP-induced PCNA expression in ESC**

Inhibitors of each MAPK (SB202190, PD98059 and SP600125) significantly suppressed the PAR2AP-induced increase in the PCNA-positive rate in ESC (Figure 3).
PAR2AP stimulated DNA synthesis and increased number of ESC

As shown in Figure 4A, 30 μmol/l PAR2AP significantly increased BrdU incorporation into DNA in ESC (139 % of control). PAR2AP (30 μmol/l) also significantly increased ESC number (124% of control, Figure 4B). Values are presented as the mean ± SEM of three independent experiments using different ESC preparations. *P < 0.0001; **P < 0.01; ***P < 0.05 (all versus control).

Neutrophil-derived serine proteases promoted PCNA expression in ESC

As shown in Figure 5, the conditioned medium of neutrophil cell culture had a proliferative effect on ESC. This effect was abolished by aprotinin, a serine protease inhibitor, at a dose of 1 and 10 μg/ml.

Effects of PAR2AP on the secretion of IL-6 and IL-8 in ESC

PAR2AP induced a dose-dependent increase in IL-6 and IL-8 secretion by ESC (Table I). The minimal effective concentrations for producing significant increases in the secretions of IL-6 and IL-8 were 30 and 300 μmol/l in ESC respectively.

Discussion

In the present study, we demonstrated that the activation of PAR2 stimulates proliferation of ESC. This stimulatory effect was abrogated by inhibitors of p38 MAPK, p42/44 MAPK and SAPK/JNK.

A current paradigm is that local inflammation associated with endometriosis plays important roles in the pathophysiology of the disease; various proinflammatory molecules are suggested to promote the progress of the disease (Osuga et al., 2002; Wu and Ho, 2003). In the present study, we showed that PAR2AP, like serine protease(s) secreted from neutrophils, stimulated the proliferation of ESC, suggesting another possible link between
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Inflammation and the development of the disease. Specifically, apart from cytokines, several proteases secreted from leukocytes recruited and activated in the disease may stimulate proliferation of ESC via PAR2 activation, though a further in vivo study is needed to reinforce the thesis. We have also recently shown that PAR2 activation stimulates the proliferation of stromal cells of the eutopic endometrium (Hirota et al., 2005a). PAR2 thus appears to participate in various physiological and pathological events in different sites.

The activation of PAR2 has been shown to increase the expression of IL-6 and IL-8 in respiratory endothelial cells and neutrophils (Asokananthan et al., 2002; Shpacovitch et al., 2004). These molecules are also proinflammatory and have been suggested to be involved in the pathophysiology of endometriosis (Ota et al., 2001; Chishima et al., 2002; Berkkanoglu and Arici, 2003). We observed that PAR2 activation induced, besides proliferation of ESC, the secretion of IL-6 and IL-8 from ESC. These findings imply that PAR2 activation may also play roles in the disease by stimulating the secretion of proinflammatory molecules. It is interesting to speculate that PAR2-induced IL-8 secretion by ESC may promote the migration of neutrophils, consequently causing self-perpetuating inflammation at endometriotic lesions.

In the pathogenesis of endometriosis, another protease system, such as the plasminogen-activating system, is suggested to be involved (Bruse et al., 2004, 2005). Together with our

Figure 3. Effects of MAPK inhibitors on PAR2AP-induced PCNA staining in ESC. (A) Immunocytochemical staining using an anti-PCNA antibody in ESC cultured in the absence or presence of MAPK inhibitors for 24 h. PCNA-positive nuclei were more abundant in the cultured ESC treated with PAR2AP (30 μmol/l) relative to those in the control cultures. The effects of PAR2AP were inhibited by three MAPK inhibitors. 1, control; 2, PAR2AP; 3, SB202190 (p38 MAPK inhibitor, 10 μmol/l); 4, PAR2AP + SB202190; 5, PD98059 (p42/44 MAPK inhibitor, 25 μmol/l); 6, PAR2AP + PD98059; 7, SP600125 (SAPK/JNK inhibitor, 10 μmol/l); 8, PAR2AP + SP600125; 9, negative control (non-immune murine immunoglobulin 2a). Magnification: ×200. (B) PCNA-positive rate (PCNA-positive cells/total cells) in ESC cultured in the absence or presence of MAPK inhibitors for 24 h. Values are presented as the mean ± SEM of four independent experiments using different ESC preparations. *P < 0.0001 versus control; **P < 0.0005; ***P < 0.0001 (both versus PAR2AP without MAPK inhibitors).

Figure 4. PAR2AP induced DNA synthesis and increased number of ESC. (A) The effect of PAR2AP on the proliferation of ESC was examined by measuring 5-bromo-2′-deoxyuridine incorporation into DNA by using a cell proliferation enzyme-linked immunosorbent assay. ESC were treated with 30 μmol/l PAR2AP for 24 h. Values are the mean ± SEM of the combined data from four independent experiments using different ESC preparations. *P < 0.05 versus control. (B) The effect of PAR2AP on the number of ESC was examined by using an automatic cell counter. ESC were treated with 30 μmol/l PAR2AP for 48 h. Values are the mean ± SEM of the combined data from four independent experiments using different ESC preparations. *P < 0.05 versus control.
Secretion of interleukin (IL)-6 and IL-8 from endometriotic stromal cells (ESC) contributes to the development of endometriosis. The findings suggest that various proteases contribute to the pathophysiology of the disease. The present finding that the stimulation of PAR2 activated all three MAPK in ESC may imply pleiotropic functions of PAR2 in endometriotic tissues, given that the activation of each MAPK can lead to the expression of various sets of molecules functioning in proliferation, differentiation, apoptosis and inflammation (Pearson et al., 2001). Our previous study showed that IL-1β-induced inflammatory responses were mediated by p38 MAPK in endometrial stromal cells (Yoshino et al., 2003). PAR2-induced activation of MAPK may not only stimulate proliferation of ESC, but also induce various responses of the cells that contribute to the development of endometriosis.

Table 1. Secretion of interleukin (IL)-6 and IL-8 from endometriotic stromal cells (ESC) incubated with different concentrations of protease-activated receptor-2 agonist peptide (PAR2AP) for 24 h

<table>
<thead>
<tr>
<th>PAR2AP (μmol/l)</th>
<th>0 (= control)</th>
<th>3</th>
<th>30</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (fold of control)</td>
<td>1 ± 0.29</td>
<td>1.69 ± 0.37</td>
<td>4.73 ± 1.21^a</td>
<td>8.44 ± 1.94^b</td>
</tr>
<tr>
<td>IL-8 (fold of control)</td>
<td>1 ± 0.19</td>
<td>1.07 ± 0.12</td>
<td>1.30 ± 0.12</td>
<td>2.31 ± 0.41^c</td>
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Values are the mean ± SEM of combined data from at least four independent experiments using different ESC preparations. The values represent relative ratios of the concentrations, compared with those in the control. Data were standardized by total protein of cell lysates.

Acknowledgements

The authors thank Ms Emi Nose for technical assistance.

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Submitted on September 10, 2004; resubmitted on May 12, 2005; accepted on July 6, 2005

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