Platelet-activating factor stimulates cytokine production by human endometrial stromal cells

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Although preimplantation embryo and decidual cells secrete significant amounts of platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF); its precise function in early pregnancy has yet to be established. To investigate the effect of PAF on cytokine synthesis, we measured the cytokine concentration in the culture media of two human cell lines: normal endometrial stromal cells (ESC) and endometrial stromal sarcoma cells (MaMi), following stimulation with a non-metabolized PAF analogue, carbamyl-PAF (C-PAF). Enzyme-linked immunosorbent assays were used to measure five cytokines: interleukin (IL)-6, IL-8, macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein-1α (MIP-1α) and tumour necrosis factor-α (TNF-α). We also evaluated the mRNA expression for IL-6 and IL-8 in ESC after C-PAF stimulation using Northern blot analysis. Non-stimulated ESC and MaMi cells both secreted IL-6, IL-8, and M-CSF, but not MIP-1α or TNF-α. The concentrations of IL-6, IL-8, M-CSF, MIP-1α, and TNF-α in the culture media of both cell lines increased in parallel with increasing amounts of C-PAF. C-PAF stimulated IL-6 and IL-8 transcription in ESC. These results suggest that PAF secretion by decidual tissues and developing embryos may induce cytokine synthesis by the ESC, as part of the cytokine network in the feto–maternal unit. An increase in the local cytokine concentration may be an important factor in the maintenance of early stages of gestation.

Key words: endometrial stromal cell/platelet-activating factor/interleukins/macrophage colony-stimulating factor/macrophage inflammatory protein-1α

Introduction

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) has been shown to be involved in human reproduction, including ovulation, implantation, maturation of the fetal lung, and parturition (Narahara et al., 1996). PAF has been detected in the uterus of humans (Alecozay et al., 1989), rats (Yasuda et al., 1986) and rabbits (Angle et al., 1988). The concentration of PAF in the rabbit endometrium has been shown to be significantly greater than that in the myometrium (Angle et al., 1988). It has been suggested that the capacity to synthesize PAF is relatively high in human endometrial tissue, as indicated by its lyso-PAF-acetyltransferase activity (Nonogaki et al., 1989). Inactivation of PAF by PAF acetylhydrolase present in decidual macrophages may be one of the mechanisms by which PAF concentration is tightly regulated (Narahara et al., 1993).

The secretion of PAF by the human preimplantation embryo has been demonstrated (O’Neill 1989), which appeared to be a requisite for viability. The concentration of PAF in the embryo culture media is related to the age and the stage of development, increasing with the duration of pregnancy (Punjabi et al., 1990; Nakatsuka et al., 1992).

PAF appears to induce a dose-related, decidual-like reaction in the pseudo-pregnant rat (Acker et al., 1989). However, the effects of PAF on endometrial stromal cells (ESC) are largely unknown. We recently established a novel cell line, MaMi, from an endometrial stromal sarcoma, which resembles normal ESC (Nasu et al., 1998a). MaMi cells synthesize various cytokines and are a useful model for analysing the function of the endometrial stroma (Nasu et al., 1998b). We investigated the effect of PAF on the production of interleukin (IL)-6, IL-8, macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein-1α (MIP-1α), and tumour necrosis factor-α (TNF-α) by normal ESC and MaMi cells, since these cytokines act on a variety of immune effector cells in the endometrium.

Materials and methods

Cell culture

All culture reagents were obtained from Gibco-BRL (Gaithersburg, MD, USA). Samples of normal human endometrium were obtained from seven premenopausal Japanese women, each of whom had undergone a hysterectomy for an intramural leiomyoma. All of the samples were diagnosed as late proliferative stage (days 11–13 day of the menstrual cycle) on the basis of standard histological criteria.

Normal ESC were separated from the epithelial glands by digesting the tissue fragments with collagenase, as described previously (Arici et al., 1993; Nasu et al., 1998a,b). Briefly, the tissues were cut into 2–3 mm pieces and incubated with collagenase (200 IU/ml) in Roswell Park Memorial Institute (RPMI) 1640 medium with stirring for 2 h at 37°C. The suspension was then filtered through a 150 μm wire sieve to remove mucus and undigested tissue, followed by
passage through a 80 μm wire sieve to remove intact glands. The resulting stromal cells were washed three times with serum-free RPMI 1640, and cultured at a density of 105 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 IU/ml), and penicillin (100 IU/ml). The medium was replaced every 4 days. The cells used in these experiments were obtained after three passages (15–20 days after isolation), by standard methods of trypsinization.

A line of endometrial stromal sarcoma cells (designated MaMi) had been established previously by the authors (Nasu et al., 1998a). These cells were maintained in the culture medium described above. All cultures were incubated at 37°C in an atmosphere of 5% CO2 in air at 100% humidity.

**Purity of endometrial cells cultures**

Cells were cultured immunochemically with antibodies to vimentin, keratin factor VIII and leukocyte common antigen (Dako, Glostrup, Denmark). The purity was >98%; the contaminants being epithelial cells, endothelial cells and leukocytes.

**PAF stimulation of cell cultures**

Cells (5×10^5) were plated on 6-well culture plates (Corning, New York, NY, USA) in 1 ml of culture medium with 10% of FBS, and cultured until they were fully confluent. The media were replaced with fresh medium containing differing amounts of carbamyl-PAF (1-O-alkyl-2-N-methylcarbamyl-sn-glycero-3-phosphorylcholine, C-PAF) (Calbiochem, La Jolla, CA, USA) (10^-12 to 10^-8 M), a non-metabolizable analogue of PAF. The media were collected 0–24 h after the addition of C-PAF, and stored at –70°C until assay. Experiments were performed in triplicate and repeated four times.

**Measurement of IL-6, IL-8, M-CSF, MIP-1α, and TNF-α**

Concentrations of IL-6, IL-8, M-CSF, MIP-1α, and TNF-α were determined in the culture media, using commercially available enzyme-linked immunoassorbent assays (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Samples were diluted as necessary. The inter-assay coefficients of variation, intra-assay coefficients of variation, and sensitivities are shown in Table I.

**Northern blot analysis of IL-6 and IL-8 mRNA expression**

Cells (1×10^6) were plated in 75 cm² culture flasks (Corning) in 15 ml of culture medium with 10% FBS and cultured until fully confluent. The media were replaced with fresh medium containing 10^-9 M C-PAF. Total RNA was extracted from the cultures cells, as described previously (Nasu et al., 1997). Briefly, the cells were disrupted with 2 ml of Isogen solution (Nippon Gene, Tokyo, Japan), maintained at room temperature for 5 min, and shaken vigorously for 15 s after the addition of 0.2 ml of chloroform. The homogenates were centrifuged at 12 000 g at 4°C for 15 min, and 0.5 ml of isopropanol was added to the aqueous phase. This was then maintained at room temperature for 10 min and centrifuged at 12 000 g at 4°C for 10 min. A volume of 75% ethanol (Wako, Osaka, Japan) was added to the precipitate. The mixture was shaken vigorously and centrifuged at 12 000 g at 4°C for 15 min. The precipitate was then dried briefly, re-dissolved in water, and Northern blotting was performed as described previously (Sambrook et al., 1989). Human IL-6 and IL-8 cDNA were labelled with [α-32P]dCTP using a random-primed DNA labelling kit (Amersham Life Science, Bucks, UK). The labelled probe was purified on a Sepharose-G50 column (Pharmacia, Piscataway, NJ, USA). Total RNA (20 µg) was subjected to electrophoresis in agarose/formaldehyde gels and transferred to nylon membranes (Hybond N; Amersham Life Science). The membranes were hybridized to a 32P-radio labelled probe for IL-6 or IL-8. Following hybridization, the membranes were washed and exposed at –70°C to Kodak XRP-5 film (Eastman Kodak Company, Rochester, NY, USA) with intensifying screens (Eastman Kodak Company). The expression of mRNA for β-actin was used as an internal control.

**Statistical analysis**

Results are presented as mean ± SD and were analysed by Bonferroni–Dunn test with StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). P < 0.05 was considered to be statistically significant.

**Results**

When control, non-stimulated ESC were incubated for 24 h, small amounts of IL-6, IL-8, and M-CSF were detected in the supernatant; the concentrations of MIP-1α and of TNF-α were both below the level of detection. The concentrations of IL-6, IL-8, M-CSF increased in a dose-related response to the addition of C-PAF. Relatively high concentrations of C-PAF also induced the production of MIP-1α and TNF-α (Figure 1). Similar results were obtained with cultures MaMi cells (Figure 2).

When the ESC were stimulated with C-PAF at 10^-9 M, cytokine concentrations were found to increase with time. The concentrations of IL-6 were 156 ± 15, 345 ± 19, and 748 ± 56 pg/ml respectively after 4, 12, and 24 h incubation with C-PAF. Similarly, the concentrations of IL-8 were 5760 ± 500, 36 100 ± 1700, and 78 500 ± 18 000 pg/ml after 4, 12, and 24 h incubation. M-CSF concentrations were 55.7 ± 2.5, 99.0 ± 22.9, and 276 ± 53 pg/ml respectively after 4, 12, and 24 h incubation and TNF-α concentrations were 200 ± 19, 271 ± 26 and 380 ± 27 pg/ml respectively after 4, 12, and 24 h incubation. MIP-1α was detected only after 24 h stimulation (9.4 ± 1.4 pg/ml).

Cytokine production by MaMi cells showed a similar time course after stimulation with C-PAF (10^-9 M). The concentrations of IL-6 were 23.3 ± 2.4, 87.6 ± 4.7, and 243 ± 26 pg/ml respectively after 4, 12, 24 h incubation with C-PAF at 10^-9 M. Similarly, IL-8 concentrations were 175 ± 14, 451 ± 22, and 1550 ± 110 pg/ml after 4, 12, and 24 h incubation respectively. M-CSF concentrations were 32.7 ± 2.0 pg/ml after 4 h, 53.2 ± 4.6 pg/ml after 12 h, and 128 ± 1 pg/ml after 24 h incubation. MIP-1α was detected only after

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sensitivity (pg/ml)</th>
<th>Coefficient of variation (%)</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>0.70</td>
<td>1.7–4.4</td>
</tr>
<tr>
<td>IL-8</td>
<td>4.4</td>
<td>5.4–9.2</td>
</tr>
<tr>
<td>M-CSF</td>
<td>8</td>
<td>1.6–3.0</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>6.0</td>
<td>1.3–2.8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.4</td>
<td>4.4–5.3</td>
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IL-6 = interleukin-6; IL-8 = interleukin-8; M-CSF = macrophage colony-stimulating factor; MIP-1α = macrophage inflammatory protein-1α; TNF-α = tumour necrosis factor-α.
Figure 1. Concentrations of (A) interleukin (IL)-6, (B) IL-8, (C) macrophage colony-stimulating factor (M-CSF), (D) macrophage inflammatory protein-1α (MIP-1α), and (E) tumour necrosis factor-α (TNF-α) in the culture media of endometrial stromal cells (ESC) after stimulation for 24 h with different concentrations of carbamyl-(1-O-alkyl-2-N-methylcarbamyl-sn-glycero-3-phosphorylcholine) (C-PAF). Results are expressed as mean ± SD (n = 4). *P < 0.005; **P < 0.0005; ***P < 0.0001, compared with non-stimulated control. N.B. scale in each panel is different.

24 h stimulation (9.4 ± 1.4 pg/ml). TNF-α was not detected at this level of stimulation.

Only weak expression of IL-6 and IL-8 mRNA was detected in non-stimulated ESC, which was markedly induced by C-PAF at 10⁻⁹ M (Figure 3).

Discussion
The uterus undergoes morphological and physiological changes during gestation so as to accommodate the conceptus. The most obvious changes occur in the endometrium, where a variety of cells actively proliferate and differentiate, a process that is referred to as decidualization. This process is normally initiated at the end of the secretory phase of the menstrual cycle. PAF has been reported to induce a dose-dependent decidua-like reaction in pseudopregnant rats (Acker et al., 1989), and to be secreted by the preimplantation embryo (O’Neill, 1989). The production of PAF by the embryo may, therefore, play a role in implantation.

The mode of regulation of PAF concentration at the implantation site has not yet been established. Embryonic production of PAF may activate the uterine endometrium to produce PAF.
Effects of PAF on endometrial stroma

Figure 2. Concentrations of (A) interleukin (IL)-6, (B) IL-8, (C) macrophage colony-stimulating factor (M-CSF), (D) macrophage inflammatory protein-1α (MIP-1α), and (E) tumour necrosis factor-α (TNF-α) in the culture media of MaMi cells after stimulation for 24 h with different concentrations of carbamyl-(1-O-alkyl-2-N-methylcarbamyl-sn-glycero-3-phosphorylcholine) (C-PAF). Results are expressed as mean ± SD (n = 4). *P < 0.005; **P < 0.0005; ***P < 0.0001 compared with non-stimulated control. N.B. scale in each panel is different.

thereby further amplifying the signal (Narahara et al., 1996). The presence of PAF receptors in a purified endometrial membrane preparation has been reported (Kudolo and Harper, 1990). We have previously demonstrated the production of the PAF inactivating enzyme, PAF-acetylhydrolase, by decidual macrophages and suggested that paracrine regulation of PAF concentration may take place in the decidua (Narahara et al., 1993). Because of the mode of regulation of PAF concentration, PAF may exert its effect over a limited area in the decidua, including the site of implantation.

ESC can produce and secrete a variety of cytokines, including IL-6 (Tabibzadeh et al., 1989; Montes et al., 1995; Nasu et al., 1998a,b), IL-8 (Arici et al., 1993; Nasu et al., 1998a,b), M-CSF (Saito et al., 1993; Hatayama et al., 1994; Kanzaki et al., 1995; Nasu et al., 1998b), MIP-1α (Dudley et al., 1995), and TNF-α (Jaattela et al., 1988; Casey et al., 1989). Cytokine expression is important during implantation and early pregnancy, since cytokines play key roles in the attraction and activation of the immune effector cells. Such inflammation-associated cytokines as IL-1β, TNF-α, and IFN-γ have been found to regulate cytokine secretion by ESC (Nasu et al., 1998a,b).
expression of IL-6 and IL-8 was stimulated. PAF produced by the decidual tissue and developing embryo induces the production of these cytokines by ESC, which then act to attract and activate various inflammatory cells into the decidua. PAF may be involved in implantation and increased cytokine production may play a role in the maintenance of early pregnancy.

**References**


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