Menstrual cycle-specific inhibition of endometrial stromal cell proliferation by oncostatin M

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We have investigated the possible roles of oncostatin M (OSM), which is a member of the interleukin-6 family of cytokines, in endometrial and endometriotic stromal cell growth. Endometrial and endometriotic stromal cells were collected from the uterus or ovarian chocolate cysts. We observed the expression of mRNA transcripts for OSM, OSM receptor subunit β, leukaemia inhibitory factor receptor subunit (LIFR), and glycoprotein 130 in endometrial and endometriotic stromal cells. We also examined the effects of OSM (0–50 ng/ml) and LIF (0–10 ng/ml) on endometrial and endometriotic stromal cell proliferation and evaluated the effects of OSM on endometrial stromal cell differentiation. The presence of 10–50 ng/ml OSM significantly suppressed endometrial stromal cell growth in secretory phase tissue but not in proliferative phase tissue. In contrast, stromal cells in endometriotic tissues were resistant to the inhibitory effects of OSM. Addition of LIF did not influence the growth of endometrial stromal cells. We also showed that 10 ng/ml OSM stimulated markers of differentiation causing increased prolactin secretion and cyclooxygenase-2 gene expression in endometrial stromal cells from the secretory phase. These results suggest that OSM may play a pivotal role in regulating the growth and differentiation of endometrial cells. Endometriotic cells may behave differently from normal endometrial cells in terms of the inhibitory response to OSM.

Key words: cell proliferation/differentiation/endometriosis/endometrium/oncostatin M

Introduction

Oncostatin M (OSM), originally identified as a growth inhibitor of A375 human melanoma cells, was cloned from the U937 human hepatopoietic cell line (Zarling et al., 1986; Linsley et al., 1990). OSM, which is produced by macrophages, monocytes, and activated T lymphocytes, is a member of the interleukin-6 (IL-6) family of cytokines that includes IL-6, IL-11, OSM, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and ciliary neurotrophic factor (CNTF), and ciliary neurotrophic factor (CNTF), and ciliary neurotrophic factor (CNTF), and cardiothrin-1 (CT-1) (Bazan et al., 1991; Rose and Bruce, 1991; Davis et al., 1993; Du and Williams, 1994; Penica et al., 1996). These cytokines exhibit very similar biological activities when they act on the same cells. This functional similarity is now explained by glycoprotein 130 (gp130), the common signalling subunit of the receptors for these cytokines (Kishimoto et al., 1995). The OSM receptor (OSMR) consists of gp130 and the OSM-specific subunit (OSMRγ) that is structurally similar to gp130. In the human, the LIF receptor, which is the LIF-binding protein (LIFR) in association with gp130, also serves as a receptor for OSM (Gearing et al., 1992; Thoma et al., 1994). Thus, OSM and LIF exhibit equivalent effects through the LIFR, and OSM induces its unique activity through the OSM-specific receptor.

As a pleiotrophic cytokine, OSM elicits many different biological functions in different cell types. Among these functions, its ability to regulate cell growth and differentiation is most notable. OSM stimulates the growth of normal fibroblasts (Horn et al., 1990), normal rabbit vascular smooth muscle cells (Grove et al., 1993), myeloma cells (Zhang et al., 1994), and AIDS-related Kaposi sarcoma cells (Miles et al., 1992; Nair et al., 1992). OSM has also been shown to inhibit the proliferation of a number of cell lines derived from human tumours, including melanoma, breast carcinoma, and lung carcinoma (Zarling et al., 1986; Horn et al., 1990).

We have previously shown that IL-6 inhibits proliferation of endometrial stromal cells derived from the secretory phase of the menstrual cycle (Yoshioka et al., 1999). In the present study, we extended our investigation to the possible roles of other IL-6 family cytokines, and have examined the effects of OSM and LIF on the proliferation and differentiation of endometrial and endometriotic stromal cells.

Materials and methods

Isolation and culture of endometrial and endometriotic stromal cells

Endometrial tissues were obtained from the uteri of cycling premenopausal women who underwent hysterectomy for uterine...
leiomyoma (n = 17) during the proliferative (n = 6) or secretory phase (n = 11). The chocolate cyst lining of the ovaries in patients with endometriosis (n = 9) was the source of endometriotic tissue. Informed consent was obtained from all patients. The menstrual cycle phase was determined by measuring serum oestradiol and progesterone concentrations and by histological examination of the endometrium.

Stromal cells were collected from endometrial and endometriotic tissue according to a published method (Osteen et al., 1989). The procedure has been described in detail previously (Iwabe et al., 1998). The tissues were minced and digested with 0.5% collagenase in Dulbecco’s-modified Eagle’s medium (DMEM) and Ham’s F-12 (1:1, vol/vol) at 37°C for 60 min. The dispersed cells were filtered through a 70 µm nylon mesh to remove the undigested tissue pieces containing the glandular epithelium. The filtered fraction was separated further from epithelial cell clumps by differential sedimentation without centrifugation, i.e., 1 g. The medium containing stromal cells was filtered through 40 µm nylon mesh. Final purification was achieved by allowing stromal cells, which attach rapidly to plates, to adhere selectively to culture dishes for 30 min at 37°C in 5% carbon dioxide in air. Non-adherent epithelial cells were removed.

Stromal cells were cultured in DMEM and F-12. Stromal cells in a 24-well dish were plated at a concentration of 3-5 x 10⁵ cells per dish to adhere selectively to culture dishes for 30 min at 37°C in 5% carbon dioxide in air. Non-adherent epithelial cells were removed. The cells were then incubated for 72 h. Total RNA was then extracted from these incubated cells and subjected to RT–PCR as described above but involving 32 amplification cycles at 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and 15 min at 72°C after each cycle. The following specific PCR primers for cyclo-oenzyme-2 (COX-2) were used: sense 5’-GAGCCGACATGCTGTA-3’, antisense 5’-GAATCTATAGGTTTCAC-3’, LIFR 5’-GAAA-TGAATCCTGGTT-3’, and gp130 5’-CCAGATCCTCGGAA-3’. The membranes were treated with streptavidin conjugated with alkaline phosphatase, and this was followed by chemiluminescence detection. Radioactive film was exposed to the membrane for 15 min at room temperature (Iwabe et al., 1998).

Endometrial stromal cells from the secretory phase were plated in 100 µm culture dishes at a concentration of 5-10 x 10⁵ cells per dish and were allowed to proliferate until confluence (3-5 days). After the cells were preincubated in medium without serum for 24 h at 37°C, medium containing either 0.1% bovine serum albumin (BSA) alone or with 10 ng/ml of OSM (recombinant human OSM; R&D Systems, Minneapolis, MN, USA) was added and the cells were incubated for 72 h. Total RNA was then extracted from these incubated cells and subjected to RT–PCR as described above but involving 32 amplification cycles at 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and 15 min at 72°C after each cycle. The following specific PCR primers for cyclo-oenzyme-2 (COX-2) were used: sense 5’-GCCCCACTACCTTTGGACA-3’, antisense 5’-TTCTTGAAGCCTTGAGGATA-3’ (Kim et al., 1999). The internal probe of COX-2 (5’-ATGCGACACTCG-3’) was used for Southern blot analysis. Amplification of the COX-2 gene could be semiquantified as previously described (Kim et al., 1999). Densitometric analysis of the PCR products was performed using NIH image program software. The densitometric values of COX-2 mRNA were normalized with the corresponding ethidium bromide-stained G3PDH mRNA.

Tetrazolium dye assay for measuring cell proliferation

Proliferation of the cells was determined spectrophotometrically by measuring the incorporation of tetrazolium dye (MTT assay). The tetrazolium dye assay used in this study was a previously described system (Iwabe et al., 1998). Endometrial and endometriotic stromal cells were cultured in culture medium with 10% fetal bovine serum to a seeding density of 3 to 5 x 10⁵ cells/well for 96-well culture tissue culture plates (100 µl/well), and incubated at 37°C for 12 h. The medium was changed to a serum-free medium containing 1 mg/ml BSA. Cells were then treated continuously with 100 µl of various concentrations of OSM (0-50 ng/ml) or LIF (0-10 ng/ml, recombinant human LIF; Strathmann Biotech GmbH, Hannover, Germany). An antibody against human OSM (1 µg/ml monoclonal mouse anti-human OSM; R&D Systems) was used to neutralize the specific effects of OSM. An antibody against human IgG1 (1 µg/ml monoclonal mouse anti-human IgG1; Cosmo Bio Ltd, Tokyo, Japan) was used as a control. Each plate had one control row (6 wells) containing cytokine-free medium. After the cells had been incubated for 72 h, 20 µl tetrazolium dye solution (2.5 mg/ml) was added to each well and the plates were incubated for another 4 h. We chose 72 h incubation time as has been previously described (Yoshioka et al., 1999). Dimethylsulfoxide (150 µl) was then added and the plates were vigorously shaken on a plate shaker to render soluble the tetrazolium dye–formazan product. Absorbance was measured at 590 nm with a microplate reader (model 450; Bio-Rad Laboratories Inc, Hercules, CA, USA).

Assay of prolactin secretion

Endometrial stromal cells from the secretory phase and endometriotic stromal cells were plated in 24-well dishes (Costar, Cambridge, MA, USA) at a concentration of 2 x 10⁵ cells per well. The cells were preincubated in medium without serum for 24 h and then received either media containing 0.1% BSA alone or media with various...
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**Figure 1.** Reverse transcription–polymerase chain reaction (PCR) Southern blot analysis of OSM, OSMRβ, LIFR and gp130 gene expression in endometrial stromal cells from proliferative and secretory phases and in ovarian chocolate cysts. Specific PCR products were confirmed by hybridization of oligonucleotide probes. G3PDH was used as a positive control.

concentrations of OSM (0–50 ng/ml) for 3–72 h. The concentration of prolactin in the culture supernatants was determined using prolactin radioimmunoassay kits (SPAC-S Prolactin Kit; Daiichi Radioisotope Laboratories Ltd, Tokyo, Japan). The values were adjusted by the number of cultured cells.

**Statistical analysis**
The SD of the absorbance of tetrazolium dye assay (percentage of control value), and densitometric analysis of COX-2 mRNA prolactin secretion assay were assessed by one-way analysis of variance, followed by the Fisher’s protected least significant difference test. The data are presented as mean ± SE. *P < 0.05 was accepted as indicating statistical significance.

**Results**

**Expression of OSM, OSMRβ, LIFR, and gp130 genes in endometrial and endometriotic cells**
The mRNA expression of OSM, OSMRβ, LIFR and gp130 in endometrial cells from the proliferative and secretory phases and in endometriotic cells from chocolate cysts was examined by RT–PCR. Representative results from each group are shown in Figure 1. RT–PCR demonstrated that the OSM, OSMRβ, LIFR, and gp130 genes are expressed in both the endometrial and endometriotic stromal cells.

**Effects of OSM and LIF on endometrial stromal cell proliferation**
To investigate the effects of OSM on endometrial stromal cell growth, tetrazolium dye assays were performed after the cells were treated with various concentrations of OSM. OSM had no effect on the growth of stromal cells in tissue from the proliferative phase (n = 6) (Figure 2A). On the other hand, the addition of concentrations of 10 and 50 ng/ml OSM induced significant inhibition of cell proliferation from secretory phase tissues (n = 11). The proliferation rate in cultures exposed to 10 ng/ml OSM was 66.6 ± 6.3% of control values. The inhibitory effects of OSM were negated by anti-OSM antibody (Figure 2B).

Since OSM and LIF exhibit similar effects through the LIFR in various cell types, we investigated the effects of LIF on endometrial stromal cell growth. However, growth inhibition was not observed in endometrial cells collected during either the proliferative phase or the secretory phase (Figure 2C,D).

**Effect of OSM on endometriotic stromal cell proliferation**
Endometriotic stromal cells derived from ovarian chocolate cysts were also treated with various concentrations of OSM. No inhibitory effects on the growth of endometriotic stromal cells were observed with the various concentrations of OSM regardless of the menstrual cycle phase (Figure 3).

**Effect of OSM on the differentiation of endometrial stromal cells**
It is well known that endometrial stromal cell differentiation is associated with increased prolactin secretion (Maslar and Riddich, 1979) and COX-2 gene expression (Han et al., 1996). To determine whether OSM can induce differentiation of endometrial stromal cells of the secretory phase, we investigated the effects of OSM on the secretion of prolactin and COX-2 gene expression in endometrial cells from the secretory
The addition of 1–10 ng/ml of OSM significantly stimulated prolactin secretion by endometrial stromal cells of the secretory phase (Figure 4A). The levels of prolactin produced by endometrial stromal cells were increased after 24 h culture (Figure 4B). In endometriotic stromal cells, OSM did not stimulate prolactin secretion (Figure 4C). We also showed that 10 ng/ml OSM could enhance the expression of the COX-2 gene in endometrial stromal cells of the secretory phase (Figure 5).

Discussion

We have demonstrated for the first time that gene expression of OSM and its receptor subunits are detected in both endometrial and endometriotic stromal cells. OSM could inhibit the growth of endometrial stromal cells derived from the secretory phase, but not the proliferative phase. OSM also enhanced prolactin production and COX-2 gene expression in endometrial stromal cells. Based upon these findings, we suggest that OSM functions as a differentiation factor causing a reduced rate of endometrial cell growth.

OSM was initially described in 1986 as a growth regulatory molecule that could inhibit the growth of human melanoma cells (Zarling et al., 1986) and stimulate the growth of several normal fibroblast cells (Horn et al., 1990). Recently it was reported that OSM inhibits the growth of primary normal mammary epithelial cells in a manner similar to its effect in breast cancer cells (Liu et al., 1997, 1998). The growth inhibitory activity of OSM on primary normal mammary epithelial cells was associated with morphological changes (including cytoplasm enlargement and the appearance of

Figure 3. Effect of oncostatin M (OSM) on the growth of endometriotic stromal cells of ovarian chocolate cysts. Endometriotic stromal cells were obtained from the proliferative (n = 2) and secretory (n = 7) phases. Cell proliferation was determined spectrophotometrically by incorporation of tetrazolium dye. Results are expressed as the percentage of control values (in absence of OSM).

Figure 4. Effect of oncostatin M (OSM) on the production of prolactin in secretory endometrial stromal cells and endometriotic stromal cells. Secretory endometrial cells were incubated in medium containing OSM (0–50 ng/ml) for 24 h (A) or OSM (1 ng/ml) for 3–72 h (B). Endometriotic stromal cells were incubated in medium containing OSM (0 and 1 ng/ml) for 24 h (C). The culture supernatants were used to measure prolactin in duplicate using prolactin radioimmunoassay kits. The values were adjusted by the number of cultured cells. *P < 0.05; **P < 0.01, versus control value.

Figure 5. Reverse transcription–polymerase chain reaction (PCR) Southern blot analysis of COX-2 gene expression in endometrial stromal cells from the secretory phase (n = 3) and incubated with media alone or 10 ng/ml oncostatin M (OSM). Specificities of PCR products were confirmed by hybridization of oligonucleotide probe. Relative densities of COX-2 mRNA signals were normalized with the corresponding ethidium bromide-stained G3PDH signals. The ratio of the COX-2 gene to G3PDH in control samples (without OSM) was arbitrarily defined as 1. Each bar represents the mean ± SEM.
intracellular vacuoles) which are attributed to a cellular differ-
entiation process (Zhang et al., 1995). Similarly, we found
that the inhibition in proliferation of endometrial stromal cells
from the secretory phase was associated with the decidualiza-
tion of stromal cells. Although we did not find apparent
morphological changes in cultured stromal cells, differentiation
was suggested by the increased secretion of prolactin and

Recent evidence shows that growth factors, such as epi-
dermal growth factor, mediate the growth-promoting action of
oestrogen in uterine tissue (Nelson et al., 1991). Alternatively,
oestrogen may enhance epithelial cell proliferation by blocking
the synthesis of cell proliferation inhibitors secreted by stromal
cells. Current results, in conjunction with several recent obser-
vations, support the contention that IL-6 and OSM may be
among the inhibitory factors that control homeostasis of the
endometrium (Tabibzadeh et al., 1989; Zarmakoupis et al.,
1995; Yoshioka et al., 1999).

The biological functions of OSM and LIF have been shown
to overlap in several cell types in which either the LIF receptor
only or both the LIF and OSM-specific receptors are expressed.
On the other hand, OSM, but not LIF, inhibits the growth of
A375 human histiocytic melanoma cells (Zarling et al., 1986;
Brown et al., 1987; Malik et al., 1989) as well as a variety of
tumour cell lines of lung, breast, ovary, and stomach origin
(Horn et al., 1990). In addition, LIF has no effect on normal
mammary epithelial cell growth in contrast to the inhibitory
activity of OSM (Liu et al., 1998). Similarly, growth inhibition
by OSM, but not LIF, was observed in the endometrial cells
collected during the secretory phase. LIF binds only to LIFR,
whereas OSM binds to the OSM-specific receptor with a
higher affinity than it binds LIFR (Mosley et al., 1996). Based
on these data, growth inhibition of endometrial stromal cells
by OSM may be mediated by the OSM-specific receptor but
not by LIFR.

In a previous study, we found that IL-6 inhibits proliferation
of endometrial stromal cells derived from the secretory
phase but not from the proliferative phase of the menstrual cycle
(Yoshioka et al., 1999). The exposure of the endometrial
stromal cells in the proliferative phase to oestradiol and
progesterone for 10 days actually induced the inhibitory
response to IL-6. In the current study, we also found a similar
phenomenon, that only secretory phase endometrium responds
to OSM. Although we performed similar experiments to those
for IL-6, OSM did not significantly inhibit proliferation of
endometrial cells derived from the proliferative phases after
treatment with oestrogen and progesterone.

We currently do not understand how sex steroids can make
endometrial cells more responsive to IL-6 and OSM in the
secretory phase. The activation of tyrosine kinases (JAK) and
transcription factors (STAT) is necessary for the signalling of
IL-6 type cytokines. One study has suggested that STAT
activation by epidermal growth factor or interferon-γ is correl-
ated with suppression of cell growth and that the signalling
pathway appears to negatively regulate cell growth in response
to cytokines (Chin et al., 1996). The differential response of
stromal cells of different menstrual phases may be regulated
by signal transducers and activators of transcription or by
related substances. Recent studies have suggested that a cross-
talk between steroid hormones and cytokines signalling path-
ways is important for understanding cytokine actions. Further
studies are needed to resolve these issues.

It is also important to note that endometrial cells derived
from different phases of the menstrual cycle, but cultured under
uniform culture conditions, may not resemble the condition of
either the proliferative or secretory phase. Therefore, data
appearing to show that cytokines such as OSM affect endomet-
rial tissue differently depending on the menstrual cycle should
be evaluated with caution.

Growth factors and cytokines in peritoneal fluid have been
postulated to play a role in the pathogenesis of endometriosis
by facilitating the growth of endometrial cells at ectopic sites
(Halme et al., 1987). We have recently demonstrated that
IL-8 levels in peritoneal fluid are elevated in patients with
endometriosis and that IL-8 stimulates proliferation of the
cultured endometrial and endometriotic stromal cells (Iwabe
et al., 2000). In contrast, IL-6 had inhibitory effects on the
growth of endometrial cells, but endometriotic cells were
resistant to IL-6 growth inhibition (Yoshioka et al., 1999).
Similarly, inhibition of proliferation by OSM was not observed
in the stromal cells of ovarian chocolate cysts. The results
suggest that endometriotic cells have different biological char-
acteristics, particularly in their response to the IL-6 family of
cytokines, as compared with eutopic endometrial cells. An
increasing body of evidence points to biochemical differences
between the endometriotic tissues and endometrium. Further
studies directed toward the regulatory mechanism of IL-6 and
OSM response in endometrial and endometriotic tissues are
necessary to resolve these issues.

In conclusion, OSM may induce a differentiation process
of the endometrium, resulting in a reduced rate of growth and
induction of differentiation.

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