Progesterone Enhances Macrophage Colony-Stimulating Factor (M-CSF) Production in Human Endometrial Stromal Cells in vitro
(子宮内膜間質細胞に於けるM-CSF産生とその卵巣性ステロイドホルモンによる調節)
Progesterone Enhances Macrophage Colony-Stimulating Factor Production in Human Endometrial Stromal Cells in Vitro*

HIROSHI HATAYAMA, HIDEHARU KANZAKI, MASAZUMI IWAI, MASATOSHI KARIYA, MARIKO FUJIMOTO, TOSHIHIRO HIGUCHI, KENJI KOJIMA, HIROKI NAKAYAMA, TAKAHIDE MORI, AND JUN FUJITA

Departments of Gynecology and Obstetrics, and Clinical Molecular Biology (H.N., J.F.), Faculty of Medicine, Kyoto University, Kyoto, Japan

ABSTRACT

Increasing evidence suggests that macrophage colony-stimulating factor (M-CSF) is produced in the uterine endometrium and that it plays an important role in the reproductive process. In the present study, using an in vitro decidualization model and human endometrium, we investigated M-CSF messenger RNA (mRNA) expression in human endometrial stromal cells (ESC) by Northern blotting and in situ hybridization. The secreted M-CSF in the culture medium of ESC was measured by enzyme-linked immunosorbent assay. ESC were cultured in the presence of progesterone (P) or estrogen. After a 9-day culture with P, when in vitro decidualization was confirmed by the production of PRL, M-CSF mRNA and protein levels were 3.1 ± 0.5- and 3.2 ± 0.8-fold (mean ± SEM) higher, respectively, than those in cultures without P (P < 0.01). The P-induced increase was dose dependent. On the other hand, estrogen did not increase M-CSF mRNA expression. M-CSF mRNA expression in the first trimester deciduae that expressed PRL mRNA was higher than that in the endometria. By in situ hybridization, ESC as well as epithelial cells were shown to express M-CSF both in vitro and in vivo. These findings indicate that human ESC (decidual cells) express M-CSF mRNA and suggest that they secrete M-CSF in a P-dependent manner during the process of decidualization. (Endocrinology 135: 1921–1927, 1994)

THE ENDOMETRIUM has a biological role in achieving successful implantation through secretory changes in the glandular epithelium and decidual changes in the stromal cells. These changes are primarily controlled by ovarian steroid hormones, but in addition, the endometrial stromal cells (ESC) may be regulated by some cytokines (1–3). ESC also reportedly secrete some cytokines in response to ovarian steroids (4, 5). Macrophage colony-stimulating factor (M-CSF; also referred to as CSF-1) is a growth factor that is produced by macrophages, fibroblasts, and endothelial cells. This cytokine was originally identified as a regulating factor of mononuclear phagocytic cells (6, 7). M-CSF has been identified in human (8–12) and mouse (13–18) endometrial tissues and placenta. The action of M-CSF is mediated by a specific membrane receptor encoded by the c-fms protooncogene (19). This M-CSF receptor, c-fms, is also present in human (10, 20, 21) and mouse (14, 15, 18) endometrium and placenta. These reports suggest that M-CSF plays some role in endometrial and placental function. However, in the human uterus, M-CSF is reportedly localized in uterine epithelial cells (11, 12), not in stromal cells. In this study, by Northern blotting and in situ hybridization for gene expression and enzyme-linked immunosorbent assay (ELISA) for production, we showed that human ESC (decidual cells) also expressed M-CSF messenger RNAs (mRNAs) and produced M-CSF in a progesterone (P)-dependent manner during the process of decidualization.

Materials and Methods

Specimens

Human endometria were obtained from 19 patients, aged 38–48 yr, who had undergone hysterectomy for the treatment of uterine myoma. A small portion of the endometrial tissue from each specimen was examined histologically and dated according to the criteria of Noyes et al. (22). Fourteen specimens were late proliferative, and 5 were secretory. First trimester human deciduae (n = 5) were obtained from patients who had undergone legal abortions. Human placental tissue (n = 1) was obtained from a patient who had undergone elective cesarean section. Informed consent was obtained from all patients.

Cell cultures

The ESC from the proliferative endometrial tissues were isolated as previously described (2). Briefly, specimens were washed, finely minced, and enzymatically digested. After subsequent pipetting, the cell suspension, diluted twice with RPMI-1640, was placed in the centrifugation tube (Corning Glass Works, Corning, NY) and left upright for 10 min at unit gravity. The supernatant, excluding the lowest 2 ml, was transferred into a new tube to collect suspended single cells. After repeating this procedure several times, the cell suspension was washed three times and used as the ESC. The viability, determined by dye exclusion, was at least 90%. Two million viable stromal cells were cultured in 25-ml flasks in RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Dainippon Pharmaceutical Co., Osaka, Japan), 100 IU/ml penicillin, and 100 μg/ml streptomycin for 3–12 days at 37 C in a humidified atmosphere of 5% CO₂ in air. To determine the effects of ovarian steroids, P (10⁻⁸ M) or 17β-estradiol (E; 10⁻⁸ M) dissolved in ethanol or vehicle alone was added to the medium from the beginning...
Fig. 1. The time course of PRL concentration in the medium of cultured human ESC. Human ESC were cultured with or without P (10^{-8} M), or vehicle alone (Control) for the indicated number of days. The PRL concentration was measured by RIA. Each value represents the mean ± SEM of three separate cultures for each P treatment.

Fig. 2. The effect of P on M-CSF mRNA expression in cultured human ESC. Human ESC were cultured with or without P (10^{-8} M) for the indicated number of days. Twenty micrograms of total RNA extracted from these tissues were hybridized with the probes for M-CSF and S26 ribosomal protein. The densitometric analysis of M-CSF mRNA was normalized by the S26 protein and expressed relative to E (control) on day 3. Each value represents the mean ± SEM of four separate cultures (bottom). The M-CSF mRNA level with E was not different from that of the vehicle alone at each time point.

12. The dose-dependent effects of P or E were examined after 9 days of culture with various doses of P (10^{-9} - 10^{-6} M) or E (10^{-10} - 10^{-6} M).

Flow cytometric analysis

Flow cytometry was performed as described previously [23]. The antibodies were LeuM3 (Becton Dickinson, Mountain View, CA) for macrophages, anticytokeratin for endothelial cells, and CD10 antibodies (Nihon News Co., Tokyo, Japan) for stromal cells. CD10 antigens are reportedly expressed on human ESC [23]. The negative controls were stained with fluorescein isothiocyanate-conjugated second antibody alone. At least 10,000 cultures were analyzed for each specimen.

RIA assay for M-CSF and PRL by ELISA

The PRL concentration in the culture medium was measured by RIA using a commercial kit (DaiChem Radioisotopes Laboratory, Tokyo, Japan). The detection limit was 1.0 ng/ml, and the intras- and interassay coefficients of variation were between 1.9-7.1% and 1.6-3.6%, respectively. The membranes were washed and used again for Northern blotting, and the coefficient of variation was 7.5%. Each assay was performed in triplicate. The data were analyzed by paired t test.

In situ hybridization

In situ hybridization was performed as described previously by Kaneko et al. [25]. Briefly, a plasmid containing the human M-CSF complementary DNA (cDNA) was linearized with the appropriate enzymes to generate sense and antisense templates. Digoxigenin-labeled single strand RNA probes were transcribed with T3 and T7 RNA polymerases using a DIG RNA Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany). Human nonpregnant endometrial tissues were fixed with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h and embedded in paraffin after dehydration by the conventional method. Human ESC were cultured for 9 days in Lab-Tek chamber slides (Nunc, Naperville, IL) with P (10^{-8} M). Before hybridization, the culture supernatant was removed, and the cells were washed with PBS, then fixed with 4% paraformaldehyde in PBS for 15 min. They were successively exposed to protease-K 0.2 μg/ml and 0.25% acetic anhydride in 0.1 M triethanolamine. Then, they were dehydrated with a series of graded ethanol and air dried. Hybridization was carried out overnight at 50°C with about 0.5 μg/ml digoxigenin-labeled RNA probes in 50% formamide, 1% deuterium oxide, 10 mM Tris-HCl, 50 mM sodium citrate, and 0.02% sodium dodecyl sulfate. After hybridization, the slides were digested with RNase-A (3 μg/ml) at 37°C for 30 min, then washed twice with 2 x SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.2 x SSC for 20 min each at 50°C. The hybridized digoxigenin-labeled probes were detected using an Nucleic Acid Detection Kit (Boehringer Mannheim, Indianapolis, IN). Hybridization with the sense probe at the same time under identical conditions served as the negative control.

Northern blotting

Total RNA from human endometrium (n = 10-5 preoperative and 3 secretory), first trimester human decidua (n = 5), and cultured ESC was isolated by centrifugation through a 4° saturated ammonium sulfate and 5°C CsCl (26). The probes for M-CSF was an 810-bp-long SalI-EcoRI fragment (nucleotides 1010-1820) of human M-CSF cDNA (27). The probe for human PRL was prepared as follows. Two micrograms of placenta total RNA were reverse transcribed with a random primer (Pharmacia LKB, Tokyo, Japan). The resulting cDNA mixture was amplified by 40 cycles of PCR with human PRL-specific primers (sense primer, 5'-TCCCCCGTGTGTCCTGTTA-3'; antisense primer, 5'-GAA-ATGGATGTCGCTTGA-3'). The PCR product was cloned into the pBluescript SKI -I plasmid (28), and the insert was verified by sequencing (29). The PCR/RFLP probe used in Northern blotting was isolated by centrifugation through a 1.5% agarose gel and transferred to nylon membranes, which were then incubated with prehybridization solution (Quick Hy, Stratagene, La Jolla, CA) for 15 min at 68°C and hybridized with the labeled probes. The probes for M-CSF and PRL were labeled by random primer labeling (30) to a specific radioactivity of 0.8-1.0 x 10^{6} cpm/μg DNA. Hybridization with the labeled probe was performed for 1 h at 68°C. After hybridization, the membranes were washed at room temperature in 0.2 x SSC plus 0.1% sodium dodecyl sulfate, followed by 0.1 x SSC and 0.1% sodium dodecyl sulfate. The membranes were washed and used again for Northern blotting, with human placental protein S26 as an internal control, because its mRNA expression level is virtually constant in many tissues (31). The mRNA level was calculated on the basis of the hybridized signal measured by densitometric scanning of the autoradiograph.

Statistical differences between sample means were calculated by analysis of variance, followed by Fisher's protected least significant differences test. The results are expressed as the mean ± S.E., and P < 0.05 was considered significant.

Results

Purity of ESC

We measured the purity of the ESC fraction by flow cytometry. The fraction contained about 2-3% epithelial cells, 1-2% macrophages, and 95% stromal cells, and the contamination by endothelial cells was negligible after days of culture. The ratio of epithelial cells decreased to less than 2% after 9 days of culture. The ratio of contaminating macrophages in the control group without steroids and the E and P groups was 0.9-1.6%, 0.7-0.8%, and 0.7-0.9%, respectively, on day 9 of culture. The ratio of macrophages was very low and not affected by the culture with either E or P.
Detection of M-CSF-producing cells in vitro

Detection of M-CSF-producing cells in vitro was undetectable in the presence of E. In contrast, E did not affect the expression of M-CSF mRNA as well as the secretion of M-CSF mRNA in the presence of P. The control sense probe did not significantly stain the cells (Fig. 8, B and D). The control sense probe did not significantly stain the cells (Fig. 8, B and D).

Discussion

Using the in vitro technique, we examined M-CSF mRNA expression in the human endometrium, in situ hybridization was carried out. The antisense RNA probe revealed that the stromal cells expressed M-CSF mRNA as well as the epithelial cells (Fig. 8, B and D). The control sense probe did not significantly stain the cells (Fig. 8, A and C).

**Production of M-CSF in Human ESC**

All of the examined samples from the human endometria and human decidua expressed M-CSF mRNA (Fig. 7). M-CSF mRNA levels in secretory phase endometrium and first trimester decidua were higher than that in the proliferative phase endometrium (P < 0.05 and P < 0.01, respectively). There was no significant difference in the M-CSF mRNA levels between the secretory phase endometrium and the decidua. Decidual PRL mRNA expression was detected only in the first trimester decidua as a single hybridization band of about 1.1 kb (Fig. 7).

**Detection of M-CSF-producing cells in the nonpregnant human endometrium**

To identify the cells expressing M-CSF mRNA in the human endometrium, in situ hybridization was carried out. The antisense RNA probe revealed that the stromal cells expressed M-CSF mRNA as well as the epithelial cells (Fig. 8, B and D). The control sense probe did not significantly stain the cells (Fig. 8, A and C).

**Production of M-CSF in Human ESC**

M-CSF and PRL expression in the human endometrium and decidua

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placentall villi express M-CSF mRNA and suggested a role for M-CSF as a growth factor for the tissue macrophages and trophoblasts that express c-fms (10). In the present study, we showed that the ESC produce M-CSF. This suggests that the M-CSF from the ESC also works as a growth factor in the feto-maternal interface, because the implanted embryo expressing c-fms is surrounded by the stromal cells (decidual cells). Moreover, the P-dependent increase in M-CSF mRNA in the stromal cells suggests the importance of M-CSF during the luteal phase and early in pregnancy, when the P from the corpus luteum affects the uterus. As for the response to P in M-CSF production, considerable variations have been observed among the samples. The same variations have been detected in the decidua in vivo. There may exist some other factors in addition to P that affect M-CSF production in human ESC and decidual cells.

In this study, we also examined the relationship between decidualization and M-CSF gene expression. In the in vitro system, PRL production and morphological changes in the ESC occurred on day 9, whereas the level of M-CSF mRNA increased on day 3. Therefore, M-CSF mRNA expression in the secretory phase endometrium that did not yet express the PRL mRNA was also higher than that in the proliferative endometrium. These findings indicate that the increase in M-CSF production precedes the decidualization changes both in vivo and in vitro. Whether M-CSF induction by P is a prerequisite for PRL production in the ESC remains to be determined.

In the human endometrium, epithelial cells have been reported to produce a large amounts of M-CSF by immuno- 

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