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－C SF 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

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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 lowermost 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.
of the culture. The concentration of ethanol did not exceed 0.1% of the total volume. Culture media were changed every 3 days and stored at -20°C until the ELISA and PRL assay. At the completion of culture, the cells were collected for the isolation of total RNA. To analyze the time-dependent effects of culture, ESC were cultured with E (10^{-8} M) or P (10^{-8} M), and total RNA was extracted every 3 days until day 12.

1. **Flow cytometry analysis**

Flow cytometry was performed as described previously (23). The antibodies were LeuMD (Becton Dickinson, Mountain View, CA) for macrophages, anti-von Willebrand factor for endothelial cells, and CD10 antibodies (Nippon Co., Tokyo, Japan) for stromal cells. CD10 antigens are reportedly expressed on human ESC (23). The negative controls were cells stained with fluorescein isothiocyanate-labeled secondary antibody alone. At least 10,000 cells were analyzed for each specimen.

**PRL assay by RIA and M-CSF assay by ELISA**

The PRL concentration in the culture medium was measured by RIA using a commercial kit (Daichi Radioisotope Laboratory, Tokyo, Japan). The detection limit was 1.0 ng/ml, and the intra- and interassay coefficients of variation were between 1.9-7.5% and 1.6-3.0%, respectively. The concentrations of secreted M-CSF in the culture medium were measured by ELISA established by Humanmu et al. (24). The detection limit was 10 pg/ml, and the coefficient of variation was 7.5%. Each assay was performed in duplicates. These data were analyzed by paired t test.

**In situ hybridization**

In situ hybridization was performed as described previously by Kanetke 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. Digoxigen-labeled single strand RNA probes were transcribed with T3 and T7 RNA polymerases using a DIG RNA Labeling Kit (Boehringer Mannheim GmbH, Biochemical, 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 diluted in PBS for 15 min. They were successively exposed to protease-K, 0.2 M HCl, and 0.25% acetic anhydride in 0.1 M barbituric acid. Thereafter, they were dehydrated with a series of graded ethanol and air dried. Hybridization was carried out overnight at 50°C with about 0.5 ng/ml digoxigen-labeled RNA probes in 50% formamide, 10% deuterium oxide, 10 mM Tris-HCl, pH 7.5, 500 ng/ml polyvinylpyrrolidone, 50 ng/ml BSA, and 1:50 of mouse anti-Dig antibody (1:500 dilution, Boehringer, Mannhein, Germany), and then visualized using the alkaline phosphatase-conjugated anti-Dig probe (Boehringer, Mannhein, Germany) as described. Nuclei were stained with 0.5% Giemsa solution (5 g/liter, 3 g polyvinylpyrrolidone, 3 g BSA, and 150 to 500 ml 0.01 M NaCl and 250 g polyethylene glycol) transfer RNA after hybridization, the slides were digested with ribonuclease A (3 mg/ml) at 37°C for 30 min, then washed twice with 2 x SSC (1 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 digoxigen-labeled labeled probe were detected using a 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 proliferative and 5 secretory), first trimester human decidua (n = 5), and cultured ESC was isolated by centrifugation through a 4 g sodium iodide barbiturate and 5 M LiCl buffer (26). The probe for M-CSF was an 1100-basepair Smal-EcoR1 fragment (nucotides 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 PCR with primer-PCR primers (sense primer, 5' - GCCCGGCGTGGTGTCAA - 3' ; antisense primer, 5' - GAAATGGATGGCGCTTATG - 3') and purified by a QIAquick column (Qiagen, Hilden, Germany). Each PCR product was radiolabeled with [35S]dATP and purified by a QIAquick column. The purified PCR products were subjected to Northern blotting. Twenty micrograms of total RNA were electrophoresed on a 1.5% agarose gel and transferred to nylon membranes, which were then incubated with prehybridization solution (Quick Hyb, Stratagene, La Jolla, CA) for 15 min at 68°C and hybridized with the labeled probe. 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/pg 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 × SSC plus 0.1% sodium deoxydextrall sulfate, followed by 0.1 × SSC and 0.1% sodium dodeyl sulfate. The membranes were washed and used again for Northern blotting, with luciferase ribonucleic acid (5 S) as internal control, because luciferase 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 autoradiographs.

**Statistical differences**

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 ± SEM, 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 3 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.
PRODUCTION OF M-CSF IN HUMAN ESC

Effects of P or E on M-CSF mRNA levels in cultured ESC

To examine the effects of ovarian steroids on the M-CSF mRNA level, human ESC were cultured with P (10^-8 M) or E (10^-8 M) for 3-12 days. M-CSF mRNA in ESC was evident as a single hybridization band of about 4.0 kilobases (kb). In the presence of P, the M-CSF mRNA level increased from day 3 and appeared to reach a plateau after 9 days (Fig. 2). The M-CSF mRNA level in the control group was not affected by the length of culture. After 12 days in culture, the M-CSF mRNA level was significantly higher in the presence of P than in its absence (P < 0.05-0.01). In contrast, E did not affect the M-CSF mRNA level (Fig. 3). To analyze the dose dependence of the increase in M-CSF mRNA and production levels, ESC were incubated with various doses of P or E for 9 days. P increased the M-CSF mRNA levels in a dose-dependent manner, with 10^(-8), 10^(-7), and 10^(-6) M resulting in 1.7 ± 0.7, 2.9 ± 1.1, and 3.4 ± 1.1-fold increases, respectively, compared to the level without P (Fig. 4A). P also increased the production level in a dose-dependent manner (Fig. 4B). E did not affect M-CSF mRNA expression at any examined doses (data not shown).

Detection of M-CSF-producing cells in vitro

In situ hybridization of P-treated ESC with the antisense RNA probe revealed that the transcript for M-CSF mRNA was abundant in more than 90% of the cultured cells (Fig. 5).

Discussion

Using the in vitro decidualization model of human ESC, we examined M-CSF mRNA expression and its hormonal regulation. The separated ESC, when cultured with P, underwent the morphological changes and produced PRL, indicating that the in vitro transformation of ESC by P mimics in vivo decidualization. Using this in vitro system, we demonstrated that P increased M-CSF gene expression and production in a dose-dependent manner. M-CSF secretion in the culture medium increased as well as mRNA expression. The effect of P was evident within the range of its physiological concentrations that circulate during the luteal phase and in early pregnancy, that is at the time of endometrial decidualization. The finding that M-CSF mRNA expression in secretory phase-endometrium and first trimester decidua, which is affected by ovarian P, was higher than that in the proliferative phase-endometrium, was consistent with the in vitro results. On the contrary, E did not affect the expression of M-CSF mRNA. This is the first evidence to show that human ESC (decidual cells) produce M-CSF in a P-dependent manner.

The importance of M-CSF in the reproductive process has been suggested by studies of the osteopetrotic (op/op) mutant mouse, which lacks M-CSF entirely and shows infertility in a homozygous (op/op) X (op/op) mutant cross and reduced implantation numbers in a heterozygous cross (16). The exact reason for the infertility in the op/op mouse is not clear, but the disturbance of implantation due to the lack of M-CSF in the uterus may be one of the causes. Oocytes express c-fms, the M-CSF receptor mRNA, during their maturation (18), and supplementation of the embryo culture medium (M6 medium) with M-CSF significantly enhances the development of two-cell embryos to blastocysts (17). In situ hybridization, we demonstrated that the stromal cells in...
placental 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 corpus luteum affects the uterus. As for the response to M-CSF, it is thought to be determined by the PRL production, considerable variabilities have been observed among the samples. The same variabilities have been detected in the deciduae in vivo. There may exist some other factors in addition to P that affect M-CSF production in human ENS and deciducal cells.

In this study, we 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. Thus, the increase in 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 immunohistochemistry (12) or PCR (11). However, in these reports, M-CSF was detected only in the epithelial cells, not in the stromal cells. In this study, the cultured ESC was shown to express M-CSF mRNA. The contaminating epithelial cells in the in vitro culture may have produced the M-CSF. However, this is unlikely, because the ratio of epithelial cells was very low, and it decreased, whereas gene expression and secretion of M-CSF increased in the presence of P. Another possibility is that contaminating macrophages expressed M-CSF mRNA. However, judging from the results of in situ hybridization, in which most of the cultured cells expressed M-CSF, the stromal cells (decidual cells) produced M-CSF under our conditions in vitro. In fact, by in situ hybridization of the endometrium, the ESC was shown to express M-CSF mRNA as well as epithelial cells. The expression of M-CSF in ESC may have been lower than the sensitivity of the immunohistochemical methods previously employed.

In conclusion, although M-CSF is thought to play important roles in various reproductive processes as a local media, this study revealed that the human ESC (decidual cells) are also an important source of this cytokine and suggested that the expression of M-CSF in ESC was under the control of P in the process of decidualization. The roles of M-CSF in ESC production and early pregnancy in vitro and the biological actions of M-CSF within the female reproductive tract should be studied further.

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References


