TITLE:
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CITATION:

ISSUE DATE:
1995-03-23

URL:
https://doi.org/10.11501/3099671

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 Hawai, J. H. (编集), "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", 畑山 博
Progestrone Enhances Macrophage Colony-Stimulating Factor Production in Human Endometrial Stromal Cells 
in Vitro*

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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.

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 found 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 17ß-estradiol (E; 10^-8 M) dissolved in ethanol or vehicle alone was added to the medium from the beginning.

Received April 22, 1994.

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* This work was supported in part by Grants-in-Aid for Scientific Research 05054448, 05072052, 050671365, and 04054402 from the Ministry of Education and the Takeda Science Foundation.
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 the culture, the cells were collected for the isolation of total RNA. To analyze the time-dependent effects of culture, ESC were cultured with either E (10^{-8} M) or P (10^{-7} M), and total RNA was extracted every 3 days until day 12. The dose-dependent effects of P or E were examined after 9 days of culture with various doses of P (10^{-10}-10^{-6} M) or E (10^{-10}-10^{-6} M).

### Flow cytometric analysis

Flow cytometry was performed as described previously (23). The antibodies were LeuM1 (Becton Dickinson, Mountain View, CA) for macrophages, anticytokinin for epithelial cells, anti- 

### Results

The PRL concentration in the culture medium was measured by RIA corresponding commercial kit (Daiichi Radiotope Laboratory, Tokyo, Japan). The detection limit was 1.0 pg/ml, and the intra- and interassay coefficients of variation were between 1.9-7.1% and 1.6-3.6%, respectively. The concentrations of secreted M-CSF in the culture medium measured by ELISA. Each value represents the mean ± SEM of four separate culture samples. P increased M-CSF mRNA levels in a dose-dependent manner. The concentrations of secreted M-CSF in the culture medium were measured by ELISA. Each value represents the mean ± SEM of three culture samples. P increased M-CSF production in a dose-dependent manner (P < 0.05), by analysis of variance; **P < 0.01**, **P < 0.01** for control, by Fisher's protected least significant difference test.

### Statistics

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.

### Purification of PRL

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 by cultured ESC

In the presence of P, PRL was first immunologically detected in culture medium after a lag of 5-6 days. Subsequently, the level of PRL increased sharply to reach 10-40
Production of M-CSF in Human ESC

Fig. 5. In situ hybridization of cultured ESC with the antisense RNA probe for M-CSF mRNA (B and D) and the control sense probe (A and C). The antisense RNA probe revealed that the transcript for M-CSF mRNA was abundant in the stromal cells cultured in the presence of P for 9 days. Original magnification: A and B, x120; C and D, x154.

Fig. 6. The concentrations of secreted M-CSF in the culture medium. Human ESC were cultured with or without P (10⁻⁷ M) for 9 days. Nine paired culture media with or without P were collected, and the M-CSF concentration was measured by ELISA. P increased M-CSF secretion 3.20 ± 0.6-fold compared with that in its absence (P < 0.01, by paired t test).

Fig. 7. M-CSF and PRL mRNA expression in human endometrial and decidual tissues. Total RNA was extracted from 10 human endometrial tissues (5 proliferative and 5 secretory) and 5 first trimester human decidua. The mRNA levels in secretory phase endometrium and first trimester decidua were higher than that in proliferative phase endometrium (P < 0.05, by analysis of variance; *, P < 0.05; **, P < 0.01, by Fisher's PLSD). There was no significant difference in M-CSF mRNA levels between secretory phase endometrium and decidua. Decidual PRL mRNA was expressed only in the first trimester decidua as a single hybridization band of about 1.1 kb.

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 the in vitro 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, P 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 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 show that the ESC from the ESC also works as a growth factor in the fetal-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 variabilities have been observed among the samples. The same variabilities have been detected in the decidua in vivo. There may exist 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. The correlation between 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 (13). 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 decreased, whereas gene expression and secretion of M-CSF increased in the presence of P. Another possibility is that contaminating macrophages expressed M-CSF in ESC. However, judging from the result 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 mediator, this study revealed that the human ESC (decidual cells) play an important role in the female reproductive tract, and suggested that the expression of M-CSF in ESC was under the control of P in the process of decidualization. The roles that increase in M-CSF, the in vitro and early pregnancy in vivo and the biological actions of M-CSF within the female reproductive tract should be studied further.

Acknowledgment

The authors wish to thank Mr. Takasi Hanazuma of the Grown Cross Corp. for his technical assistance with the ELISA.

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