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Colony Stimulating Factor-1 exerts direct effects on the proliferation and invasiveness of endometrial epithelial cells

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Abstract

Although macrophage colony stimulating factor (CSF-1) has been suggested to play a role in part in maintaining the chronic inflammatory response in endometriosis, our previous data suggest that CSF-1 may also play a role in early endometriosis lesion formation. In the current studies, we have examined this possibility and have shown that CSF-1, in an autocrine fashion, has a direct effect on endometrial epithelial cell proliferation and attachment to peritoneal mesothelial cells, early steps in endometriosis lesion formation on the peritoneum.

Endometriosis is a benign gynecologic disorder causing infertility in approximately 5%–10% in reproductive women and in about 30% of infertile women. Implantation of menstrual endometrial tissue onto the peritoneum has been suggested as a mechanism leading to ectopic growth of endometrial cells in the peritoneum (1–5). The presence of endometrial lesions leads to chronic inflammatory response and increase in cytokine level profile that may contribute to progression of endometriosis (6, 7).

Macrophage colony stimulating factor (CSF-1) is an important hematopoietic growth factor responsible for differentiation, proliferation and activation of macrophage and is expressed in non-hematopoietic cells (8). CSF-1 acts by binding to its receptor encoded by the *c-fms* gene (9–11). CSF-1 affects cellular survival and proliferation via autocrine and paracrine actions (12). CSF-1 and *c-fms* are expressed by endometrial endometrial epithelial cells (13). Increased levels of CSF-1 have been detected in peritoneal fluid of endometriosis patients, suggesting that it plays a role in the pathology of endometriosis (6).

Our previous studies have shown that CSF-1 is an autocrine growth factor in endometrial epithelial cells (EECs) and is involved in early endometriosis lesion formation (14). In these studies, decreased endometriosis lesion formation was observed by endometrial tissue from donor CSF-1 knockout mice (CSF-1^{op/op}) in the syngeneic transplantation model of endometriosis (14). Although our published studies suggest the involvement of CSF-1 in early endometriosis lesion formation, little is known on the direct effects of CSF-1 on endometrial cells during processes involved in lesion establishment (14–17).

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Our previous study has suggested possible autocrine function of CSF-1 in EECs (12). In this study we have further examined the direct effects of CSF-1 on EEC involvement in endometriosis lesion formation. For this purpose, we have generated CSF-1 knockout clone (EM42KDC) of the EM42 EEC line and examined the effects of diminished CSF-1 expression on attachment of EECs to PMCs, transmesothelial invasion, and cellular proliferation. Our results confirm the role of CSF-1 in early endometriosis and suggest that CSF1 directly affects endometrial epithelial cell involvement in pathological steps of early endometriosis lesion formation.

EM42 EEC line was originally isolated from benign proliferative endometrial tissue resected from a patient undergoing hysterectomy for leiomyomata (18). EM42 cells express estrogen and progesterone receptors (19). EM42 immortalized endometrial cells were cultured in RPMI with glutamine containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) supplemented with glutamine. For EM42KDC, 5 μ g/ml puromycin was also added for culture medium. To eliminate the effects of steroid hormones and growth factors present in FBS in the media, cells were washed with phenol red-free Hanks buffer salt solution (Invitrogen) and incubated in phenol red-free RPMI media containing charcoal-stripped (C-S), heat-inactivated (H-I) FBS (10%) for 24 hrs before treatment.

To generate EM42 clones with CSF-1 knockdown, EM42 cells were transiently infected with Lentivirus (Mission[®] Lentivirus transduction particles, SHLVN, Sigma-Aldrich, St. Louis, MO, USA) containing CSF-1 specific shRNA at multiplicity of infection (MOI) of 10 and supplementing with 300 μ g/ml of polybrene. A total of 6,000 cells were plated per well in a 96 well plate in RPMI media as described above. After 24 hours, medium is changed with 5 μ g/ml puromycin in RPMI (a concentration that was predetermined to effectively kill EM42 cells within 48 hours). Puromycin resistant colonies were picked and grown for analysis of CSF-1 knockdown by RT-PCR and ELISA, as described below. Clone TRCN0000058560 was shown to have the most efficient decrease in CSF-1 mRNA, the target for CSF-1 shRNA, and secreted CSF-1 protein levels (70% reduction) (Fig. 1A).

RNA was isolated using the Qiagen RNeasy plus (Valencia, CA, USA) with a genomic DNA removal step as per manufacturer's protocol. Real-time PCR and subsequent analyses were carried out with 0.25% SybrGreen in the Cepheid (Kingwood, TX, USA) SmartCycler. Specific primers were used to assess the expression of CSF-1, *c-fms* and the housekeeping gene GAPDH as described previously (11, 12). Melt curve analysis was performed after each real-time PCR to ascertain PCR product specificity. Relative expression was determined using the formula $2^{-\Delta\Delta C_t}$.

ELISA was carried out following protocol by R & D Systems (Minneapolis, MN, USA) Quantikine Human M-CSF after 24 and 48hrs of seeding cells into appropriate cell culture vessel. For proliferation assay, EM42 and EM42KDC cells were seeded at 2,000 cells per well in a 96 well plate as described previously (15). Cells were incubated in phenol red-free RPMI media C-S, H-I FBS (10%) for 24 hrs before treatment. After 48 hrs, cell growth was assayed using the Cell Titer-glo kit (Promega, Madison, WI, USA) as described by the manufacturer's protocol.

Attachment assay of endometrial cells to mesothelial cells was performed as described previously (17, 20). In brief, EM42 cells were labeled with calcein-AM (Molecular Probes; 5 mM) for 20 min at 37°C. The cells were plated at 20,000 cells per well over 96-well plates with confluent LP9 PMCs. After incubation at 37 °C for 1 hr plates were inverted, submerged in a bath of PBS containing calcium and magnesium (Invitrogen) and incubated at 37 °C for 15 min on an orbital mixer (Barnstead/ThermoLyne, Dubuque, IA, USA) set at 20 r.p.m., allowing non-adherent endometrial cells to precipitate under gravity. Fluorescence

readings were taken for each well before and after washing. The percentage of attached endometrial cells was calculated for each well (fluorescence value after washing/fluorescence value before washing).

The 3D invasion assay modeling transmesothelial invasion was described previously (20). Briefly, 20,000 EM42 control and EM42KDC cells were plated after labeling with CellTracker Green[®] (Molecular Probes-Invitrogen) on a confluent layer of LP9 PMCs on top of growth factor-reduced Matrigel[™], coated on 8-mm pore membranes in 24-well invasion chambers (BD Bioscience, San Jose, CA, USA). After 20 hrs, cells that did not invade on the upper surface of the membranes were mechanically removed with cotton tip applicators. Invaded cells on the bottom of the coated membranes were visualized using a fluorescence microscope. Invaded cells were counted using the Image J software (<http://rsbweb.nih.gov/ij/>). Invasion assays were performed in duplicates and repeated at least twice.

Real-time RT-PCR, cellular proliferation, attachment and trans-mesothelial invasion data are presented as means. ANOVA followed by Tukey's post-hoc analysis was used for statistical analysis.

Knock down of CSF-1 expression by specific lentivirus shRNA was determined at the RNA and protein levels by RT-PCR and ELISA, respectively. EM42KDC cells showed decreased CSF-1 expression by 72–80% compared to control in EM42 cells at the RNA and protein levels (Fig 1A). *c-fms* expression was also diminished due to CSF-1 knockdown, suggesting that *c-fms* expression was directly proportional to CSF-1 concentration and that CSF-1 signaling is diminished in EM42KDC (Fig 1A).

EM42KDC showed statistically significant decreased cell growth ($p < 0.05$) compared to control EM42 cells. Treatment of EM42KDC cells with CSF-1 rescued cellular growth (Fig 1B). EM42KDC cells showed significantly decreased ($p < 0.05$) attachment to PMCs compared to EM42 control cells (Fig 1C). Addition of CSF-1 at 10 ng/mL rescued attachment of EM42KDC to PMCs (Fig. 1C). Non-significant decrease in the transmesothelial invasiveness of EM42KDC compared to EM42 parental cells was observed (Fig. 1D).

Previous studies have implicated CSF-1 in endometriosis (9, 10, 12). Increased levels of CSF-1 in the peritoneal cavity suggests altered macrophage function in women with endometriosis (6). The chronic inflammatory response in endometriosis has been attributed in part to elevated CSF-1/macrophage population (6, 7). Our data suggest that CSF-1 is also involved in early establishment of endometriosis lesions by direct effect on EECs. Our studies have shown that CSF-1 is involved in early endometriosis lesion formation in a syngeneic mouse model of endometriosis, using CSF-1^{op/op} knockout mice (14). Diminished early endometriosis lesion formation was detected using these mice as recipients and donors in syngeneic intraperitoneal implantation of endometrial tissue (14).

Our data suggest that CSF-1 may exert a direct role in attachment of endometrial tissue to the peritoneum, an essential step in early establishment of endometriosis lesion. Elevated CSF-1 levels in the peritoneum could lead to increased growth of lesions by increasing cellular proliferation. Less prominent effects were noted on invasion by EM42KDC, suggesting that CSF-1 may not have a strong, direct effect on invasiveness by EECs. Our data does not exclude the possibility of indirect effects by CSF-1 on other cell types within the invading tissue (such as macrophages) that may influence the microenvironment such as altering the composition of extracellular matrix facilitating invasion (21). Thus, autocrine/paracrine signaling involving CSF-1 between the endometriosis lesion and macrophages,

leading to increased macrophage infiltration, could result in enhanced invasiveness and may contribute to disease progression from early to deep endometriosis (21–23).

Our current studies dissect the direct effect of CSF-1 on EEC attachment to PMCs, invasion and proliferation, early processes of endometriosis lesion formation. CSF-1 signaling may be a critical factor affecting endometrial epithelial cell behavior during early endometriosis lesion formation, a finding that is consistent with our previous results showing decreased early endometriosis lesion formation in the endometriosis syngeneic mouse model (14).

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Figure 1.

CSF-1 knockdown in EM42KDC cells affects EEC proliferation and attachment to PMCs. Graphs and error bars represent mean and standard error of mean, respectively. **A**, Real Time RT-PCR was used to detect CSF-1 mRNA (left graph) in EM42 and EM42KDC (n=3) as described in Materials and Methods (*p<0.05 compared to EM42). ELISA (n=2) was used to detect secreted CSF-1 levels (right graph) using the R&D quantakine kit as directed by the manufacturer's protocol (*p<0.05 compared to EM42, 24 hr; **p<0.05 compared to EM42, 48 hr). Expression of the CSF-1 receptor (c-fms) was determined (n=5) by real-time RT-PCR (left graph) in Em42 and EM42KDC (*p<0.05 compared to EM42). **B**, CSF-1 knockdown leads to decreased proliferation. Cellular proliferation in EM42KDC cells growth was compared to the growth of EM42 control cells. CSF-1 was supplemented to EM42KDC at indicated concentrations (n=6 for each cell/treatment). Relative to the control, decreased proliferation was observed in the EM42KDC cells, which was increased by supplementing 10 ng/ml of CSF-1. (*p<0.05 compared to control). **C**, CSF-1 knockdown decreased EM42 attachment to PMCs. Attachment assay was performed as described in Material and Methods. EM42 cells were used as control cells. CSF-1 was supplemented to EM42KDC at indicated concentrations (n=8 for each cell/treatment). Decrease in attachment was observed in the EM42KDC cells compared to control. (*p<0.05 compared to EM42 cells). **D**, CSF-1 knockdown leads to decreased trans-mesothelial invasion. Invasion of EM42 cells through a PMC layer was assayed in vitro. A decrease in invasiveness of EM42KDC cells compared to control was observed (p=0.2).