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A potential role for colony-stimulating factor 1 in the genesis of the early endometriotic lesion

Jani R. Jensen, M.D.^{a,b}, Craig A. Witz, M.D.^{a,c}, Robert S. Schenken, M.D.^a, and Rajeshwar R. Tekmal, Ph.D.^a

^a Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, the University of Texas Health Science Center at San Antonio, San Antonio, Texas

^b Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Mayo Clinic, Rochester, Minnesota

^c Fertility Specialists of Houston, Houston, Texas

Abstract

Objective—To investigate the role(s) of colony-stimulating factor 1 (CSF-1) on the development of early endometriosis in a murine model by comparing rate of lesion formation in mice [1] homozygous for a CSF-1 mutation versus syngeneic controls and [2] after treatment with imatinib, a commercially available tyrosine kinase inhibitor that alters interaction(s) between CSF-1 and its receptor, *c-fms*.

Design—Prospective, placebo-controlled animal study.

Setting—Academic medical center.

Animals—Six- to 8-week old female FVB, wild-type C57BL/6, and CSF-1 op/op mice.

Intervention(s)—Endometrial tissue from donor mice was used to induce endometriosis in murine recipients. In some experiments, mice homozygous for a CSF-1 mutation (CSF-1 op/op) were donors or recipients. In other experiments, donor and/or recipient mice received imatinib.

Main Outcome Measure(s)—Histologic confirmation of endometriosis, rate of lesion formation.

Result(s)—By 40 hours, recipient mice developed a mean of 7.2 ± 0.9 endometriotic lesions that had invaded host surfaces, and mesothelial cells had proliferated over the entire surface of the implants. The CSF-1 op/op mice developed significantly fewer (mean 0.9 ± 0.3) endometriotic lesions versus syngeneic controls. Imatinib treatment resulted in significantly fewer lesions when compared with sham-treated controls.

Conclusion(s)—Colony-stimulating factor 1 has a role in establishing early endometriotic lesions. Agents targeting CSF-1 or its actions have therapeutic potential for treating endometriosis.

Keywords

Endometriosis; animal model; colony-stimulating factor 1; CSF-1; imatinib

Endometriosis is a common gynecologic disease affecting up to 10% of reproductive-age women in the general population and up to 30% of infertile women (1). Despite this high

Reprint requests: Jani R. Jensen, M.D., Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Mayo Clinic, 200 First St. S.W., Rochester, MN 55905 (FAX: 507-284-1774; Jensen.jani@mayo.edu).

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prevalence and the severe symptoms associated with the disease, little is known about the histogenesis of the early endometriotic lesion. Sampson's theory proposes that fragments of menstrual endometrium pass retrograde through the fallopian tubes into the peritoneal cavity where they attach and grow on peritoneal surfaces (2). There is mounting evidence that retrograde menstruation and implantation of endometrial fragments are the primary factors responsible for the development of most endometriotic lesions in the pelvis (3–9).

We have developed novel in vitro models of the early endometriotic lesion. Our models demonstrate that endometrial fragments rapidly adhere (within 1 hour) to intact peritoneal mesothelium and within 24 hours begin to invade the submesothelial extracellular matrix (10–12). Using human peritoneal explants, we demonstrated that fragments of proliferative, secretory, and menstrual phase endometrium, as well as individual endometrial stromal cells and endometrial epithelial cells, disrupt peritoneal mesothelial cells and invade into the extracellular matrix (13–15).

Many important questions about the initial interaction of endometrial cells with the peritoneum remain unanswered. Recently a role for colony-stimulating factor 1 (CSF-1) has been postulated (16). Colony-stimulating factor 1, initially described as a hematopoietic growth factor, has been shown to have important functions in nonhematopoietic cells, including the endometrium. Colony-stimulating factor 1 interaction with its receptor, *c-fms*, has been implicated in the growth, invasion, and metastasis of several types of cancer, including breast and endometrial cancers (17). Colony-stimulating factor 1 and *c-fms* are expressed by endometrial stromal cells, endometrial epithelial cells, and peritoneal mesothelial cells (18). Using immunohistochemistry, Mettler et al. (16) showed increased expression of *c-fms* in endometriotic implants, in both epithelium and stroma, compared with eutopic endometrium. We have demonstrated previously that [1] coculture of endometrial cells and peritoneal mesothelial cells increases expression of CSF-1 and *c-fms* by endometrial cells and peritoneal mesothelial cells (19), [2] CSF-1 interaction with *c-fms* increases endometrial cell proliferation and migration, and [3] decreased production of CSF-1 by endometrial cells leads to decreased cell proliferation and migration, as well as altered transcription of genes implicated in invasion, metastasis, and cell signaling (20).

Imatinib (Gleevec, formerly STI-571; Novartis, Basel, Switzerland), a commercially available orally active agent, is a tyrosine kinase inhibitor used in the treatment of chronic myeloid leukemia and some gastrointestinal tumors. It is thought to target either the Bcr-Abl tyrosine kinase or the KIT- and/or platelet-derived growth factor receptor tyrosine kinases (21–23). Imatinib also inhibits the growth of some nonmalignant cells, including monocytes and macrophages, through pathways independent of these receptor kinases (21). Recently imatinib was demonstrated to target the CSF-1 receptor, *c-fms*. Phosphorylation of *c-fms* was inhibited by therapeutic concentrations of imatinib, and this was not due to down-regulation in *c-fms* expression (21). Imatinib was also found to inhibit CSF-1–induced proliferation of a cytokine-dependent cell line (21). These findings suggest that imatinib may be useful in the treatment of diseases where *c-fms* is implicated, including breast and ovarian cancer and inflammatory conditions such as rheumatoid arthritis (21,23).

On the basis of our in vitro observations, we hypothesized that CSF-1 may have a role in establishing early endometriotic lesions. We also hypothesized that imatinib may disrupt the interaction between CSF-1 and *c-fms* and further affect lesion formation. Here we report the development of an in vivo model of the early endometriotic lesion and use it to demonstrate a potential role of CSF-1 in the pathogenesis of endometriosis. We also show that imatinib treatment results in a significantly decreased rate of endometriotic lesion formation in our in vivo model.

MATERIALS AND METHODS

Establishment of Model

All procedures involving experimental animals were approved by the Institutional Animal Care Program at the University of Texas Health Science Center at San Antonio. Animals were housed according to institutional guidelines and were allowed free access to food and water. Female wild-type (WT) 6- to 8-week-old FVB mice (Jackson Laboratory, Bar Harbor, ME) received 100 $\mu\text{g}/\text{kg}$ of E_2 valerate (Sigma, St. Louis, MO) in corn oil SC for 1 week before induction of treatment and weekly thereafter. Induction of endometriosis was performed with use of a modification of methods previously described (24,25). Donor mice (used in a ratio of one donor to two recipients) were killed via halothane inhalation and cervical dislocation. Uteri were removed and placed into a dish of sterile phosphate-buffered saline solution (PBS). The serosal and myometrial layers were dissected under microscopy. The remaining endometrial fragments were placed in sterile PBS, finely minced with iris scissors, and homogenized by serial passage through 14- to 18-gauge adjuvant-mixing needles (Popper and Sons, Inc., New Hyde Park, NY). Endometrial fragments were pooled, pelleted by centrifugation, resuspended in 1 mL PBS, labeled at 37°C with 20 $\mu\text{mol}/\text{L}$ of CellTracker Green (Invitrogen, Eugene, OR) for 30 minutes, washed twice in PBS, and resuspended in PBS at a volume of 100 μL per recipient mouse. Each recipient mouse received an equal quantity of endometrial fragments (equivalent to one uterine horn per recipient, approximately 35 mg) via intraperitoneal (IP) injection. Recipient mice were killed by halothane inhalation and cervical dislocation 40 hours later, and 3 mL of cold 3% formaldehyde was injected IP. Parietal peritoneum, liver, spleen, uterus and adnexae, and intestines were removed 30 minutes later. Tissues were agitated in formaldehyde overnight and then examined under a fluorescence microscope. The number and location of experimental endometriotic lesions were identified by fluorescence stereomicroscopy (Leica MZ-16FA; Leica Microsystems, Wetzlar, Germany) and recorded. Lesions were dissected from surrounding tissue, paraffin embedded, serially sectioned, and stained with hematoxylin and eosin. The histologic diagnosis of endometriosis was made by observing endometrial glands and stroma within tissue sections. In all experiments, examiners were blinded to the treatment group given to each animal.

CSF-1 Op/Op Mice

Female 6- to 8-week-old mice (C57BL/6 background) homozygous for a CSF-1 mutation (CSF-1 op/op) were obtained from Jackson Laboratory. For these experiments, both CSF-1 op/op and syngeneic WT mice served as endometrial fragment donors and recipients. All mice (donors and recipients) received 100 $\mu\text{g}/\text{kg}$ of E_2 valerate in corn oil SC for 1 week before induction of treatment and weekly thereafter. Killing of donors (ratio of one donor to two recipients), tissue processing and labeling, and induction of experimental endometriosis in recipient mice was performed as described above. The number of lesions developed in CSF-1 op/op mice was compared with that in WT mice. For some experiments, CSF-1 op/op mice were used as donors and recipients. For cross experiments, CSF-1 op/op mice were used as donors and WT mice as recipients, and vice versa.

Imatinib Treatment

Wild-type C57BL/6 6- to 8-week-old mice (donors and recipients) were treated with E_2 valerate as described above. Imatinib (Gleevec, formerly STI-571; Novartis) was dissolved in sterile PBS to create a 10 mg/mL stock solution. Both donor and recipient mice were treated with imatinib at 50 mg/kg IP either once or twice daily for 7 days. The doses of imatinib were determined from other in vivo experiments using murine models (26–28). Control WT mice were injected with 100 μL IP of sterile PBS (sham) either once or twice daily. After 7 days of imatinib or sham treatment, donor mice were killed (one donor to two recipients), and endometriotic lesions were induced as described above. Recipient mice continued to be treated

with imatinib or PBS until being killed 40 hours later. Recipients were killed and experimental endometriotic lesions were quantified as described above. The number of endometriotic lesions was compared among control (PBS treated) recipients with those treated with either once or twice per day imatinib.

Cross-treatment experiments were also performed. In these investigations, WT C57BL/6 donors were treated with imatinib 50 mg/kg IP once per day for 7 days before killing. Endometrial fragments were processed, labeled, and subsequently injected IP into naive (untreated) WT C57BL/6 recipients. Recipients were killed after 40 hours and tissues were dissected and examined as above. Labeled endometrial fragments from untreated WT C57BL/6 donors were injected IP into WT C57BL/6 recipients that had been pretreated with imatinib once per day for 7 days. Recipients continued to receive imatinib once per day until being killed 40 hours after endometrial fragment injection. Tissues were dissected, agitated overnight, and then examined as described above.

Statistics

Numbers of lesions observed are reported as the mean $\pm$ SEM. Analysis of variance (ANOVA) was used to compare treatment groups with controls. A significant difference between groups was accepted when alpha was $<.05$.

RESULTS

Establishment of Model

All of the WT FVB recipient mice ($n = 16$) developed endometriotic lesions within 40 hours. The mean number of lesions was 7.2 ± 0.9 (range 3–13). Endometriotic lesions were identified on the peritoneum, liver, intestines, spleen, and uterus-adnexae, with the most frequent site being the peritoneal surface. Lesions were visible under fluorescence microscopy at magnification (Fig. 1). Histology confirmed that ectopic endometrial tissue was attached to the peritoneal surface of the recipient. No attached fragments were seen over an intact mesothelium. Rather, there was disruption of the underlying mesothelium, invasion of ectopic endometrium into the submesothelial stroma, and regrowth of mesothelium over the attached endometrial fragment.

CSF-1 Op/Op Mice

When CSF-1 op/op mice were used as donors and recipients, there were significantly fewer endometriotic lesions formed compared with experiments in which C57BL/6 mice were both donors and recipients (i.e., controls, Table 1). Wild-type C57BL/6 mice receiving CSF-1 op/op tissue developed a similar number of lesions compared with controls. In contrast, CSF-1 op/op mice receiving WT C57BL/6 tissue developed significantly fewer lesions than controls.

Imatinib Experiments

All WT C57BL/6 recipient mice treated with imatinib once or twice per day developed endometriotic lesions. There was no significant difference ($P>.05$) in number of lesions in recipients treated with imatinib once per day versus twice per day (Table 2). The number of lesions formed in recipients given imatinib at either dose was significantly less than the number of lesions in C57BL/6 control mice treated with sham injection of saline solution. There was no difference in number of lesions formed in control mice after once per day versus twice per day sham injections (data not shown).

For imatinib cross-treatment experiments: when untreated (naive) endometrial fragments from WT C57BL/6 donors were given to recipients that were pretreated for 7 days with imatinib, the mean number of lesions formed was not statistically different from the number of lesions

formed in controls (PBS-treated WT donors and recipients). When endometrial fragments from imatinib-treated donors were given to WT (naive) recipients, the mean number of lesions formed was significantly less than the number of lesions formed in controls (Table 2).

DISCUSSION

In the present study, we describe an in vivo model to investigate the early endometriotic lesion. Our in vivo findings confirm what we have previously described in vitro, namely that attachment and invasion of a new endometriotic lesion is a rapid process. We previously demonstrated in vitro that endometrial fragments adhere to intact peritoneal mesothelium within 1 hour and then become invasive, disrupting peritoneal mesothelial cells within 24 hours. In our current in vivo model, we show that endometrial fragments were able to attach to and invade peritoneal surfaces, and the implants were completely covered with mesothelium within 40 hours. Our findings regarding attachment agree with those of other investigators; however, this is the first report to demonstrate in vivo that endometriotic tissue becomes covered with mesothelial cells within 40 hours after placement into the peritoneal cavity. Using nude mouse models, Aoki et al. (29) and Nisolle et al. (30) separately demonstrated that menstrual endometrium was able to attach to the mesothelium as early as 24 hours after transplantation. Lu et al. (31) showed that human endometrium attached to the peritoneum or peri-intestinal fatty tissues of immunocompetent mice by 2 days after transplantation and that by day 3 after transplantation mesothelial cells wrapped around the transplanted endometrium.

Our findings also suggest a role for CSF-1 in the induction of early endometriotic lesions. This is supported by the observations that [1] mice homozygous for a CSF-1 mutation (CSF-1 op/op) developed significantly fewer experimental endometriosis lesions than WT controls and [2] mice treated with imatinib, a *c-fms* kinase inhibitor that disrupts *c-fms* signaling, had fewer endometriotic lesions when compared with controls. There were no significant differences between the numbers of endometriotic lesions formed in mice treated with imatinib once versus twice daily.

Our cross-treatment experiments using the CSF-1 op/op mice reveal some interesting findings. Wild-type mice developed endometriotic lesions at a similar rate as controls when given transgenic endometrial fragments, suggesting that the transgenic endometrial tissue is capable of establishing endometriotic lesions. The observation that transgenic mice given WT endometrial fragments developed significantly fewer lesions than controls suggests that CSF-1 has an effect at the level of the peritoneum or peritoneal fluid environment. Alternatively, there may be other factors within the endometrial fragment itself or peritoneal factors that affect interactions between the endometrial fragment and the peritoneum, thereby affecting induction of endometriosis. Other mouse models have implicated immune factors such as vascular endothelial growth factor and matrix metalloproteinase-2 as augmenting the formation of early endometriosis (31). In contrast, treating mice with a protein kinase C inhibitor appeared to partially inhibit the development of ectopic endometrial implants (32). Human studies show that cytokines and growth factors within the peritoneum differ in women with and without endometriosis (33). Taken altogether, it appears that the peritoneal environment is an important determinant of whether endometriosis develops.

Our cross-treatment experiments with imatinib-treated and WT (control) mice revealed different findings from what we observed in our transgenic mouse experiments. When WT recipients received imatinib-treated endometrial fragments, there were significantly fewer lesions than in controls. This finding contrasts with our observation that CSF-1 op/op endometrial fragments given to WT mice resulted in lesion formation at a similar rate as in controls ($P = .07$). It is possible that imatinib affects other signaling pathways in addition to *c-fms* and thus is further able to inhibit endometriotic lesion formation. The observation that

CSF-1 op/op recipients of WT endometrium developed significantly fewer lesions than WT controls suggests that complete inhibition of CSF-1/*c-fms* signal transduction leads to an altered peritoneal environment. This suggests that CSF-1/*c-fms* signal transduction by peritoneal factors (e.g., mesothelium, peritoneal fibroblasts, and immune cells) contributes to the histogenesis of the endometriotic lesion. Alternatively, imatinib may affect other pathways besides CSF-1/*c-fms* signal transduction in the endometrial fragments leading to decreased lesion formation.

Our data suggest that CSF-1 plays an important role in the genesis of the early endometriotic lesion. Although our findings are preliminary, it is attractive to consider imatinib, a commercially available orally active agent with Food and Drug Administration approval to treat some myelogenous and gastrointestinal tumors, as a potential therapeutic agent to treat established endometriotic lesions or prevent formation of new ones. Imatinib has emerged as a first-line agent to treat chronic myelogenous leukemia, largely because of its excellent side effect, safety, and clinical response profiles (34). Most data on reproductive effects of imatinib come from animal studies and suggest that high doses of imatinib increase fetal loss and the rates of certain malformations (exencephaly, encephalocele, and cranial bone hypoplasia) in rats but not rabbits (35). There are now approximately 50 case reports in the literature regarding imatinib in human gestation. Nearly all human cases reported favorable outcomes (36). One human study showed that imatinib appears to cross the placenta poorly but may be detected in high concentrations in breast milk (37). Currently, though, reproductive-age women taking imatinib are advised to avoid pregnancy and breast-feeding.

In conclusion, our data suggest that CSF-1 plays a role in the genesis of the early endometriotic lesion. Further studies on imatinib's effects on endometrial and peritoneal mesothelial cell interactions are warranted to assess the potential of this novel approach to treat or perhaps prevent endometriotic lesion formation.

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FIGURE 1.

Endometrial tissue attached and invaded into peritoneum at 40 hours (A–D), in bright field and fluorescence. *Dashed box (E)* of a separate lesion is magnified in panel F. *Arrowheads* show mesothelial cell growth over invaded endometrial tissue.

TABLE 1

Endometriotic lesion formation in CSF-1 op/op mice versus controls.

EM donor (No.)	Recipient (No.)	Mean No. lesions $\pm$ SEM	Range
WT (7)	WT (14)	8.57 $\pm$ 0.39	6–11
CSF-1 op/op (4)	CSF-1 op/op (7)	0.86 $\pm$ 0.34 ^a	0–2
CSF-1 op/op (3)	WT (6)	6.83 $\pm$ 0.75 ^b	5–10
WT (2)	CSF-1 op/op (4)	2.0 $\pm$ 0.41 ^a	1–3

Note: EM = homogenized endometrial tissue; No. = number of mice.

^a $P < .001$ compared with WT by ANOVA.

^b $P > .05$ compared with WT by ANOVA.

TABLE 2

Endometriotic lesion formation in imatinib-treated groups versus controls.

EM donor (No.)	Recipient (No.)	Mean No. lesions $\pm$ SEM	Range
Ctl (7)	Ctl (14)	8.57 $\pm$ 0.39	6–11
Imat QD (4)	Imat QD (7)	5.43 $\pm$ 0.69 ^a	3–8
Imat BID (4)	Imat BID (7)	4.14 $\pm$ 0.46 ^{a,b}	3–6
Ctl (3)	Imat QD (6)	8.83 $\pm$ 1.01 ^c	5–11
Imat QD (3)	Ctl (6)	5.67 $\pm$ 0.42 ^a	4–7

Note: EM = homogenized endometrial tissue; No. = number of mice; Ctl = saline-treated C57BL/6 mice; Imat QD = C57BL/6 mice treated once daily with imatinib 50 mg/kg IP; Imat BID = C57BL/6 mice treated twice daily with imatinib 50 mg/kg IP.

^a $P < .001$ compared with Ctl by ANOVA.

^b $P > .05$ compared with Imat QD by ANOVA.

^c $P > .05$ compared with Ctl by ANOVA.