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Physiologic Regulation of Postovulatory Neutrophil Migration into Vagina in Mice by a C-X-C Chemokine(s)\textsuperscript{1}


Leukocytes, particularly neutrophils, infiltrate into female genital organs after ovulation in both humans and mice. In mice, a female sexual cycle consists of 5 phases: proestrus, estrus, metestrus-1, metestrus-2, and diestrus. Ovulation occurs at estrus; at metestrus-2, a large number of neutrophils infiltrate into the vaginal epithelium accompanied by an increased neutrophil number in vaginal lavage fluid. Concomitantly, concentrations of a functional IL-8 homologue, murine macrophage inflammatory protein (MIP)-2, were increased significantly in vaginal lavage fluid at metestrus-2 as compared with other phases. On the contrary, MIP-2 was not detected in plasma during the whole course of a sexual cycle. Moreover, immunohistochemical analyses demonstrated that MIP-2 protein expression was prominent at the upper layer of the vaginal epithelium at metestrus-2, in contrast to a marginal staining in the vaginal epithelium at proestrus and estrus. These results suggest that a C-X-C chemokine, MIP-2, was produced physiologically in the vaginal epithelium in a sexual cycle-dependent manner. Furthermore, the administration of neutralizing anti-IL-8R homologue Abs at proestrus abrogated leukocyte infiltration into the vagina at metestrus. However, anti-MIP-2 Abs reduced leukocyte influx at metestrus by \textasciitilde 50\%. Thus, a murine IL-8 homologue, MIP-2, and its related molecules physiologically regulate neutrophil migration into the vagina in a sexual cycle-dependent manner. \textit{The Journal of Immunology}, 1998, 160: 6159–6165.

Female sexual cycles in mice and rats are controlled by various types of hormones, similar to the human sexual cycle, and consist of four stages; each hormone corresponds to a distinct phase: proestrus, estrus, metestrus, and diestrus (1, 2). In mice, metestrus is further subdivided into two phases. Ovulation occurs during the estrus phase after plasma estradiol levels decrease, and plasma-lutenizing hormone levels increase in a surge (1, 2). However, ovulation is not followed by menstruation in mice, in contrast to humans and primates (3). Vaginal smear findings, as well as the appearance of the vagina, change as a sexual cycle proceeds and show distinct features at each cycle in mice (Table I). The vaginal smear test at metestrus-2 shows a marked increase in neutrophil numbers (4). In humans, leukocytes infiltrate into the uterine endometrium, and the number of leukocytes in vaginal smears eventually increase before the start of menstruation (5, 6). Since metestrus ensues from ovulation, these results suggest that leukocytes migrate into genital organs after ovulation in both humans and mice. However, the molecular mechanism of sexual cycle-dependent leukocyte migration remains to be investigated.

Accumulating evidence indicates that a newly identified family of chemokines exhibits various inflammatory activities, including chemotaxis and the activation of a specific type(s) of leukocyte(s), and are involved in various types of inflammatory and immune responses (7, 8). Chemokines are divided into four classes: C-X-C, C-C, C, and C, depending upon whether the first pair of cysteines are separated by one or three amino acids, are adjacent, or are singular. C-X-C chemokines with an “ELR” motif before the first cysteine possess potent neutrophil chemotactic activities (7). Moreover, IL-8 has a pivotal role along with C-X-C chemokines with an ELR motif with regard to neutrophil migration and activation in humans after binding to two closely related but distinct receptors on neutrophils (9). However, in mice, a single type of IL-8 receptor homologue (10–12) has been identified with no exact homologue of the IL-8 gene (8). Hence, it is assumed that in mice, other C-X-C chemokines with an ELR motif, namely macrophage inflammatory protein (MIP)\textsuperscript{3}, KC, and/or growth-related oncogene (GRO)\textsubscript{γ} substitute the functions of IL-8 after binding to an IL-8R homologue (12).

Herein, we evaluate the physiologic role of MIP-2 in leukocyte migration during metestrus in mice. We observed that MIP-2 was produced locally at metestrus, and that the administration of Abs to the IL-8R homologue abrogated neutrophil infiltration into the vagina at metestrus. However, anti-MIP-2 Abs reduced leukocyte influx into vaginal lavage fluid (VLF) at metestrus by 50%. These results provide the first evidence that MIP-2 and its related chemokines are produced in the vagina in a cycle and regulate physiologic neutrophil migration.

\textsuperscript{1}Abbreviations used in this paper: MIP, macrophage inflammatory protein; GRO, growth-related oncogene; VLF, vaginal lavage fluid; H&E, hematoxylin and eosin.

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Materials and Methods

Mice

Pathogen-free female BALB/c mice were obtained from CLEA (Toyama, Japan) and bred ad libitum in the Animal Research Facility at Kanazawa University (Kanazawa, Ishikawa, Japan). The light-dark cycle was continually maintained at a 12-h interval. We used 7- to 10-wk-old mice for the experiments and complied with the standards outlined in the “Guideline for the Care and Use of Laboratory Animals” on the Takara-machi campus of Kanazawa University.

Antibodies

IgG fractions of rabbit anti-mouse IL-8R homologue and rabbit anti-glutathione S-transferase Abs were prepared as described previously (13). Sera were obtained from rabbits that had been immunized four times with mouse MIP-2 expressed in Escherichia coli (13). IgG fractions were obtained using a protein G-Sepharose column (Pharmacia-Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.

Collection of VLF and genital organs

At total of 20 μl of PBS was instilled into the vagina of an unanesthetized mouse. VLF were collected by gentle suction and instillation that was repeated several times. Some mice were sacrificed to obtain genital organs. The organs were fixed with 10% neutral buffered formaldehyde solution for hematoxylin and eosin (H&E) staining. In some experiments, mice at proestrus were injected i.v. with 200 μg of either rabbit anti-mouse IL-8R homologue, anti-MIP-2, or anti-glutathione S-transferase IgG as a control in 100 μl of PBS.

Determination of a sexual cycle and its leukocyte count

A total of 4 μl of VLF was placed on a slide glass, air-dried, fixed with methanol, stained with Giemsa solution, and examined microscopically. The sexual cycle of an individual mouse was determined based on the criteria shown in Table I. The number of leukocytes was counted with a hemocytometer on a small amount of VLF that had been stained with Türk solution.

Measurement of VLF chemokine levels

After centrifugation at 12,000 revolutions per min for 5 min at 4°C, VLF was diluted with PBS and stored at −20°C until measurement. MIP-2 concentrations were determined by a sandwich ELISA. Briefly, a 96-well plate was coated overnight at 4°C with 100 μl of 1 μg/ml of rabbit anti-mouse MIP-2 IgG in 0.05 M carbonate buffer (pH 9.6). The wells were then postcoated with 150 μl of 1% BSA/PBS for 1 h at 37°C. A total of 50 μl of either sample or standard rMIP-2 was subsequently added to each well and incubated for 2 h at 37°C. Then 50 μl of biotinylated rabbit anti-mouse MIP-2 IgG (1 μg/ml in 1% BSA/PBS) was added to each well and incubated for 2 h at 37°C. Subsequently, 50 μl of streptavidin-conjugated alkaline phosphatase (Life Technologies, Gaithersburg, MD) (diluted to 1:1000 with 1% BSA/PBS) was added to each well and incubated for 2 h at 37°C. Finally, 50 μl of p-nitrophenylphosphate (1 mg/ml in 1 M diethanolamine, pH 9.8) was added to each well and incubated for 60 min at room temperature. The resulting absorbance at 405 nm was read with a microplate reader (MPR-A4i; Tosoh, Osaka, Japan). The detection limit of this assay system was consistently ~50 pg/ml. KC levels in VLF were determined using a specific ELISA against mouse KC (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Immunohistochemical analyses

The organs were fixed for 4 h at 4°C in periodate-lysine-paraformaldehyde and washed sequentially for 4 h with PBS containing 10, 15, and 20% sucrose. The tissues were subsequently embedded in OCT compound (Miles, Elkhart, IN), frozen in dry ice-acetone, and cut into 6 μm-thick sections using a cryostat (Bright, Huntingdon, U.K.). After the endogenous peroxidase activity had been inhibited according to the method described by Isohe et al. (14), sections were incubated with either rabbit anti-mouse MIP-2 antiserum or preimmune serum that had been diluted at 1:1000 overnight at 4°C. Subsequently, the slides were incubated with horseradish peroxidase activity had been inhibited according to the method described by Isohe et al. (14), sections were incubated with either rabbit anti-mouse MIP-2 antiserum or preimmune serum that had been diluted at 1:1000 overnight at 4°C. Subsequently, the slides were incubated with horseradish

Table I. Characteristic features of VLF smears at each sexual stage of mice

<table>
<thead>
<tr>
<th>Sexual Cycle</th>
<th>Prominent Types of Cells</th>
<th>Leukocyte Number on VLF Smears</th>
</tr>
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<tbody>
<tr>
<td>Proestrus</td>
<td>Oval or polygonal nucleated epithelial cells; few cornified cells</td>
<td>–</td>
</tr>
<tr>
<td>Estrus</td>
<td>Cornified epithelial cells devoid of nuclei; scattered number of nucleated epithelial cells</td>
<td>–</td>
</tr>
<tr>
<td>Metestrus-1</td>
<td>Cornified cells desquamate with conglomeration</td>
<td>++</td>
</tr>
<tr>
<td>Metestrus-2</td>
<td>Exfoliated epithelial cells</td>
<td>+++</td>
</tr>
<tr>
<td>Diestrus</td>
<td>Scattered number of epithelial cells; mucus is present</td>
<td>+</td>
</tr>
</tbody>
</table>

FIGURE 1. A. The leukocyte number in VLF at each sexual cycle. The number of leukocytes in VLF was determined as described in Materials and Methods. The mean and SE were calculated on at least 10 mice for each phase. Error bars indicate one SE. Statistical significance is indicated by an asterisk. B. Morphologic analysis on leukocytes in VLF at metestrus-2. Giemsa staining was performed on a cytopsin preparation of VLF obtained at metestrus-2.
peroxidase-conjugated anti-rabbit Ig F(ab\(^9\)) \(_2\) fragments. After visualization with 3,3' -diaminobenzidine (Dojin Chemical, Kumamoto, Japan), the sections were counterstained with hematoxylin and mounted with resin.

**Statistical analysis**

The mean and SE were calculated, and statistical analyses were performed using paired and unpaired Student's \(t\) tests and an ANOVA test. \(p < 0.01\) was accepted as statistically significant.

**Results**

**Cyclic changes in leukocyte numbers in VLF and the vagina**

We determined the number of leukocytes in VLF during a sexual cycle. Consistent with the previous reports (4), the leukocyte number in VLF changed during the cycle, peaking at metestrus-2 and reaching nadir at diestrus (Fig. 1A). Moreover, a microscopic examination identified most of the leukocytes in VLF at metestrus as neutrophils (Fig. 1B). A histologic examination demonstrated that a few leukocytes were present in the vaginal epithelium at proestrus (data not shown) and estrus (Fig. 2A). However, polymorphonuclear cells were present in the vaginal epithelium at metestrus-1 and -2, and prominent polymorphonuclear cell aggregates were apparent at metestrus-2 (Fig. 2, B and C). Leukocytes were sparse in the vagina at diestrus (Fig. 2D). These results imply that neutrophils infiltrate into the vagina during the postovulatory period, metestrus, similar to a process seen in humans, where neutrophils infiltrate into the uterine endometrium before menstruation (5, 6).

**Murine MIP-2 levels in VLF throughout a sexual cycle**

A potential role of MIP-2 in neutrophil migration in the mouse prompted us to determine MIP-2 levels in VLF by a specific ELISA. MIP-2 was detected in VLF during the whole course of a sexual cycle with a peak at metestrus-2 (Fig. 3A). In contrast, we did not observe any significant increase in VLF KC levels in any phase (Fig. 3B). Moreover, during the whole course of a sexual cycle, MIP-2 was not detected in plasma (data not shown), suggesting that the MIP-2 was produced locally. Vaginal epithelial cell layers at proestrus (Fig. 4A) and estrus (data not shown) were marginally stained positively with anti-MIP-2 but were not stained positively with preimmune sera (data not shown). At metestrus-2, keratinocytes in the upper layer of the vaginal epithelium showed marked immunoreactivities against anti-MIP-2 Abs, while infiltrated neutrophils were weakly stained with the Abs (Fig. 4, B and C). These results suggest that MIP-2 was produced locally during the cycle, particularly by keratinocytes in the vagina and coincidentally with neutrophil infiltration into the vagina.

**Effects of rabbit anti-mouse IL-8R homologue and anti-mouse MIP-2 Abs on leukocyte influx into the vagina**

Mice possess two additional MIP-2-related but distinct chemokines, KC and GRO\(\gamma\) (8). However, these three chemokines bind exclusively to a single type of mouse IL-8R homologue (12). Hence, we administered neutralizing polyclonal Abs to the mouse IL-8R homologue at proestrus to evaluate the roles of these chemokines in the histologic changes seen at metestrus. At 48 h post-treatment, which corresponds to metestrus, no significant difference was observed between untreated or control Ab-treated mice in terms of leukocyte number in VLF. However, the administration of anti-IL-8R homologue Abs abrogated the increase at this time point (Fig. 5A). No difference was observed between control and
anti-IL-8R homologue Ab-treated animals with regard to the keratinization of epithelial cells in vaginal smears (data not shown). A histologic examination demonstrated that treatment with anti-mouse IL-8R homologue Abs markedly reduced the number of infiltrated leukocytes and leukocyte aggregates in vaginal epithelial layers at metestrus as compared with control Ab-treated mice (Fig. 5, B–E). To evaluate the contribution of MIP-2 to neutrophil influx into the vagina, we administered anti-MIP-2 Abs at proestrus. Anti-MIP-2 but not control Abs reduced the number of leukocytes in VLF by ~50% (Fig. 6). These results suggest that MIP-2 and its related chemokines are involved in physiologic neutrophil migration at metestrus.

Discussion
A previous immunohistochemical analysis detected the presence of an immunoreactive IL-8 protein in the perivascular regions of the human uterine endometrium throughout the stages of the human menstrual cycle (15). No sexual cycle-dependent changes in immunoreactivities was observed (15), probably because the method employed was a qualitative one. In contrast, our present quantitative analysis demonstrates a sexual cycle-dependent increase in mouse VLF MIP-2 levels at metestrus that is coincident with neutrophil infiltration into the vaginal epithelium. A similar cycle-dependent neutrophil infiltration into the uterus has been
documented in humans after ovulation (5, 6). Since mouse MIP-2 presumably substitutes some functions of IL-8 in mice (12), these results suggest that IL-8 and/or IL-8-related chemokines are produced locally in a sexual cycle-dependent manner and induce leukocyte infiltration into genital organs after ovulation in humans as well as mice.

IL-8 is a chemokine that is predominantly active on neutrophils in humans (16); at this point, no exact homologue of IL-8 has been identified in mice (8). Murine homologues of GROs, MIP-2, KC, and GROγ possess a so-called ELR motif before the first cysteine residue, a characteristic which is similar to other chemokines with neutrophil chemotactic activities, including human IL-8 (8, 17). Actually, these homologues exhibit potent chemotactic activity for mouse neutrophils. However, a single type of IL-8Rs homologue exists in mice (10–12) and can bind MIP-2, KC, and GROγ (12) in contrast to the presence of two types of IL-8Rs in humans (9). Although we did not observe any sexual cycle-dependent increase in KC levels in VLF, the inhibition of neutrophil migration at metestrus by the anti-IL-8R homologue cannot exclude the possibility that GROγ is also involved in neutrophil infiltration at metestrus. This possibility was further supported by a partial reduction of the neutrophil number in VLF by anti-MIP-2 Abs. However, these results also could not exclude the possibility that these chemokines primed the action of other endogenous chemoattractants such as C5a and leukotriene B4, which induced leukocyte migration cooperatively.

A sexual cycle is regulated by the coordinate actions of various sexual hormones, particularly estradiol and progesterone (1–3). The metestrus phase in mice is characterized endocrinologically by decreased estradiol and increased progesterone levels (2). Progesterone at the level observed at metestrus could inhibit IL-8 production by human endometrial cells in vitro (18, 19). These in vitro data do not support an increased MIP-2 concentration in murine VLF at metestrus. The discrepancy may be explained by a difference in the modulation of human IL-8 and mouse MIP-2 production by progesterone or by a different sensitivity to progesterone between human uterine endometrial and murine vaginal epithelial cells. However, IL-1α and TNF-α, which are potent inducers of IL-8 and MIP-2 production by a wide variety of cells (7, 8), were produced in murine uterus at estrus and before metestrus (20). Similarly, IL-1α expression was enhanced in the uterine endometrium in humans during the secretory phase (21), which corresponds to the mouse metestrus phase. Thus, locally produced IL-1α and TNF-α may overcome the inhibitory effects of progesterone and eventually induce local MIP-2 production.

We did not observe any marked morphologic changes in vaginal epithelial cells upon the administration of anti-IL-8R homologue Abs. At parturition in humans, IL-8 levels in the uterine cervix increase and are accompanied by neutrophil infiltration and the
FIGURE 5. A. Effects of anti-mouse IL-8R homologue Abs on VLF leukocyte number. Mice were either untreated, treated with the anti-mouse IL-8R homologue, or treated with control Abs at proestrus as described in Materials and Methods. Leukocyte numbers in VLF were determined at 48 h posttreatment. Each group consisted of 10 mice. The mean and one SE were calculated, and error bars indicate one SE. Statistical significance is indicated by an asterisk. B–E. Effects of anti-mouse IL-8R homologue Abs on morphology in vaginal tissues. H&E staining was performed on those vaginal tissues that were obtained from mice at 48 h postinjection with anti-mouse IL-8R homologue (D and E) or control Abs (B and C) at proestrus. Three vaginal organs were obtained from each group; representative results are shown. Vaginal spaces are indicated with asterisks. Original magnification: B and D, ×50; C and E, ×100.
increased activities of neutrophil-derived collagenases (22). Activated collagenases presumably loosen tightly bound collagen fibrils in the cervix, thereby causing cervical ripening, a prerequisite for labor (23). This assumption was supported by the observation that a local application of IL-8 could induce cervical ripening in rabbits and guinea pigs (24, 25). Thus, MIP-2, produced locally at metestru, might induce the exfoliation of vaginal epithelial cells by destroying the extracellular matrix with neutrophil-derived collagenases.

C-C chemokines with an ELR motif possess potent angiogenic activities in addition to neutrophil chemotactic activities (26, 27). In the cascade of reproduction, angiogenesis is seen as coincident with ovulation, endometrial proliferation, corpus luteal growth, decidualization, and placental formation (28). The crucial role of angiogenesis in sexual cycles was suggested by the observation that the chronic administration of an angiogenesis inhibitor, AGM-1470, into mice inhibited endometrial maturation and corpus luteal formation and eventually impaired implantation (28). Hence, a pulsatile increase in MIP-2 may cause endometrial proliferation and corpus luteal formation at metestru by inducing angiogenesis. However, treatment with anti-IL-8R Abs did not reduce the frequency of implantation and delivery as compared with untreated or control Ab-treated mice (our unpublished observations). Since a single injection of AGM-1470 has a marginal effect on implantation (28), repetitive injections of anti-IL-8R homologue Abs may also be required. No information has been available on the fertility of female mice that are homoyzygous deficient in the IL-8R homologue gene (29). A more detailed analysis of these mice might unravel the roles of the IL-8-related chemokines, such as MIP-2, KC, and/or GROγ, in the physiologic female reproduction system.

References


