FULL PAPER *Immunology*

Effect of ovarian hormones on maturation of dendritic cells from peripheral blood monocytes in dogs

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ABSTRACT. Previously, we reported that ovarian hormones affect the immune response against *E. coli* isolated from the dogs affected with pyometra. In order to investigate mechanisms underlying the immune modulation, we examined the effects of ovarian hormones on the generation of dendritic cells (DCs), the most potent antigen presenting cell. DCs were differentiated from peripheral blood monocytes (PBMOs) using a cytokine cocktail. Both estrogen receptor and progesterone receptors were expressed by the PBMOs and immature DCs. When various ovarian hormones were added to the culture for the DC differentiation, progesterone significantly decreased the expression of DC maturation markers, such as CD1a, CD80 and CD86, on mature DCs. Conversely, the addition of estrogen to the cultures increased the expression of CD86, but not other maturation makers. Furthermore, DCs differentiated in the presence of progesterone did not stimulate allogeneic mononuclear cells in PB. Taken together, these results indicate that progesterone diminishes the maturation of DCs, leading to decreased immune responses against invading pathogens.

KEY WORDS: dendritic cell, immune response, maturation, ovarian hormone

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Pyometra is a common bacterial disease that is seen in the reproductive system of the female dogs. The majority of disease incidence is observed in the first half of diestrus [1], during which the blood concentration of progesterone is highest, but the level of estradiol-17β is lowest [3]. Pyometra is often observed after treatment with progesterone, used to block estrus [18]. These findings suggest ovarian hormones significantly affect the incidence of pyometra. Previously, we found that response of peripheral blood (PB) mononuclear cells to *E. coli* isolated from pyometra significantly decreased in the first half of distress, but increased in proestrus/estrus [16]. In addition, the response of PB mononuclear cells was significantly suppressed by the addition of progesterone into the culture, but significantly enhanced in the presence of estradiol-17β [16]. This suggests a systemic immune control by ovarian hormone markedly effect on the pathogenesis of pyometra as well as local immune control reported previously [7, 8]. However, it is unknown how these PB cell responses are regulated by ovarian hormones.

Dendritic cells (DCs) are the most potent antigen presenting cells and significantly stimulate not only primary immune response but also secondary responses. Using a cocktail of cytokine produced by activated T cells (T cell-conditioned medium: TCCM), we have succeeded to induce differentiation and maturation of DCs from PB monocytes (PBMOs) of dogs [19]. DCs exist in both immature and mature stages. We also found a significant increase in expression of immune-stimulatory molecules during maturation of the dog DCs [17, 20]. In contrast to mature DCs, immature DCs were reported to induce tolerance [10]. Therefore, maturation of DCs is thought to be very important for immunity against infectious disease.

In the present study, we show that ovarian hormones regulate the maturation stage of DCs in the dog, which ultimately affects immune responses against pyometra in the uterus. Our data expand understanding of the pathogenesis of pyometra.

MATERIALS AND METHODS

Animals: Laboratory-bred female beagle dogs of 2 to 8 years of age were housed and studied in accordance with NIH guidelines, with the regulations of the local Institutional Animal Care and Use, and with accepted veterinary medical practice. The study protocol was also approved by the animal experiment committee of Osaka Prefecture University. The dogs were fed a commercial dog food once daily and given water *ad libitum*. The estrous cycle of the dogs was determined with vaginal smear as described previously [6].

Ovarian hormones: Progesterone, 17α-hydroxy progesterone (17-OHP; a metabolite of progesterone) and estradiol-17β (estrogen) were purchased from Sigma Co. (St. Louis, MO, U.S.A.) and used in this study. All of the hormones were dissolved in ethanol at a thousand times of

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the concentration used in experiments.

Preparation of cells: Collection of PBMOs and induction of DCs were performed as described previously [19]. In brief, PB was collected from dogs in anestrus. $CD14^+$ PBMOs were isolated from PB mononuclear cells using anti-human CD14 microbeads (Miltenyi Biotec GmbH, Begisch-Gladbach, Germany). To induce differentiation to DCs, the isolated PBMOs (1×10^6/m*l*) were cultured in wells of 24 well-culture plates (Asahi Techno Glass, Shizuoka, Japan) with 2 m*l* culture medium which was composed of 1.5 m*l* PRMI1640 supplemented with fetal bovine serum (FBS) (10%) and 0.5 m*l* TCCM. The cultures were incubated at 37°C with various concentrations of ovarian hormones. As the untreated control, vehicle (ethanol) was added to the culture. Cells were harvested on day 6 or day 12 to investigate either immature or mature DCs. Thus, the early 6 days in the culture were considered as the differentiation period, and the late 6 days, as the maturation period.

Reverse transcription (RT) -PCR: Total RNA was from PBMOs (1×10^6), immature DCs (1×10^6) or ovarian cells as a positive control. The RT reaction and the PCR for estrogen receptor (ER) α, ERβ and a housekeeping gene 18s rRNA was performed as previously described [6]. To perform the PCR for progesterone receptor (PR), using Primer3 software [13], we designed primers as follows: sense, 5′-CAGGTGTACCAGCCGTACCT-3′; antisense, 5′-ACAGGTTGTGGGAGAGCAAC-3′.

Flow cytometry: Flow cytometry (FCM) was performed as described previously [17, 20]. Cells harvested from the cultures were stained with the following monoclonal antibodies (mAbs): FITC-conjugated mouse anti-human CD1a mAb (clone NA1/34-HLK, Serotec, Oxford, U.K.), rat anti-canine MHC class II mAb (clone YKIX.334.2, Serotec), PE-Cy5-conjugated mouse anti-human CD14 mAb (clone TÜK4, Serotec), biotin conjugated rat anti-mouse CD80 mAb (clone 1G10, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and mouse anti-human CD86 mAb (clone FUN-1, Becton Dickinson). Staining with biotin conjugated mAb was followed by incubation with streptavidin-PerCP-Cy5.5 (Becton Dickinson). Before staining, the anti-CD86 mAb was labeled by PE conjugated goat anti-mouse IgG (Molecular Probe) for 5 min at room temperature. The excess of PE-goat anti-mouse IgG was neutralized by excess purified mouse IgG (Sigma). After the staining, the cells were fixed and analyzed using a flow cytometer (FACSCalibur™: Becton Dickinson). Background of the fluorescence was adjusted using following isotype-control: FITC-conjugated mouse IgG2a (eBioscience, San Diego, CA, U.S.A.), FITC-conjugated rat IgG2a (eBioscience) and biotin-conjugated rat IgG2a (eBioscience). To determine the expression intensity of the surface antigens specified above, the mean peak channel of the fluorescence intensity was calculated by software (CellQuest™: Becton Dickinson) after the FCM analysis and expressed as mean expression intensity (MEI).

Mixed leukocyte reactions (MLR): MLR was carried out as described previously [19]. In outline, PBMOs were incubated in the cultures for DC-induction with progesterone, 17-OHP or estrogen for 12 days. After the incubation, cells in graded doses were cultured with allogeneic PB mononuclear cells (10^5) in 0.2 m*l* RPMI 1640 supplemented with 10% FBS for 4 days. Cell proliferation was quantified by incubation of the cells with [^{3}H]-thymidine (PerkinElmer® Life and Analytical Sciences, Boston, MA, U.S.A.).

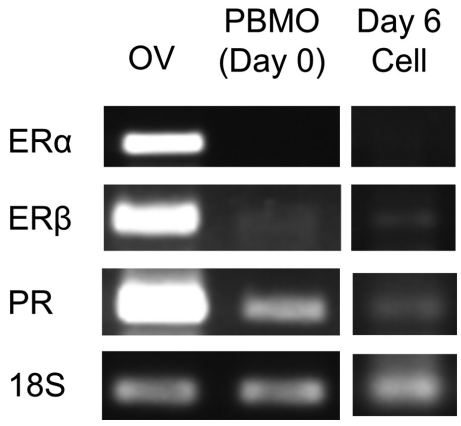

Fig. 1. Expression of receptor for ovarian hormones. The expression of receptor for ovarian hormones by PBMOs and the cells collected from the DC-inducing culture (Day 6 cell) was examined by RT-PCR and compared with that by ovary (OV) used as a positive control.

Statistical analysis: Experimental groups were compared using analysis of variance followed by Fisher's protected least significant difference (PLSD), implemented using StatView® software (Hulinks Inc., Tokyo, Japan). To compare two parameters against each other, Student's *t* test was used. The significance level was set at $P<0.05$.

RESULTS

Expression of ovarian hormonal receptors: As the first step for elucidating the effect of ovarian hormones on the maturation of DCs, we examined expression of the receptors for ovarian hormones in PBMOs and DCs generated *in vitro*. As shown in Fig. 1, the PBMOs expressed PR. They slightly expressed ERβ, but not ERα. Similarly, cells harvested on day 6 of the culture expressed ERβ and PR. However, cells harvested on day 12 did not express any receptor (data not shown).

Effect of ovarian hormones on the maturation of DCs: Since we have found that an antigen presenting molecule, CD1a and co-stimulatory molecules, CD80 and CD86, significantly relate to DC maturation [17, 20], we investigate effect of ovarian hormones on the expression of these molecules. It is confirmed that expression of all surface markers investigated in this study significantly increased on the day 6 cultured cells, compared with monocytes as reported previously [17, 20] (data not shown). As shown in Fig. 2, the intensity of the expression was expressed as MFI calculated by the software mentioned. Moreover, since we have found that 17-OHP does not affect the immune responses of the dog PB mononuclear cells, we used that as a control for progesterone. As shown in Fig. 3, when the expression of CD80 or CD86

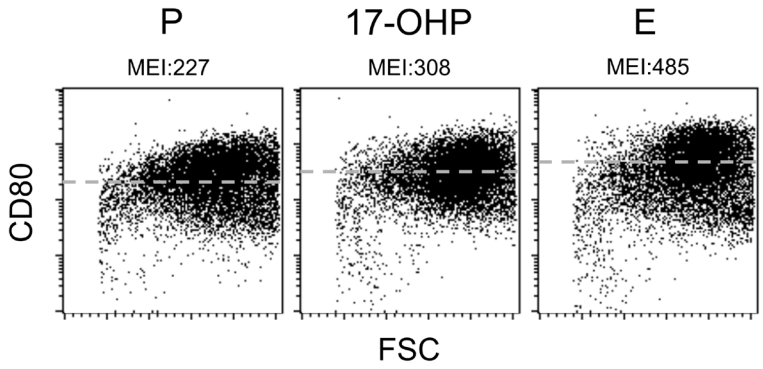

Fig. 2. CD80 vs. FSC profile of cells incubated with various ovarian hormones. PBMOs (10^6/m*l*) were incubated with progesterone (P) (1 μM), 17α-hydroxy progesterone (17-OHP; a metabolite of progesterone) (1 μM) or estrogen (E) (2 nM) in the presence of 25%TCCM for 12 days. After the incubation, expression of CD80 and forward scatter (FSC; size) of cells was examined in FCM. Dotted lines indicate the mean expression intensity (MEI).

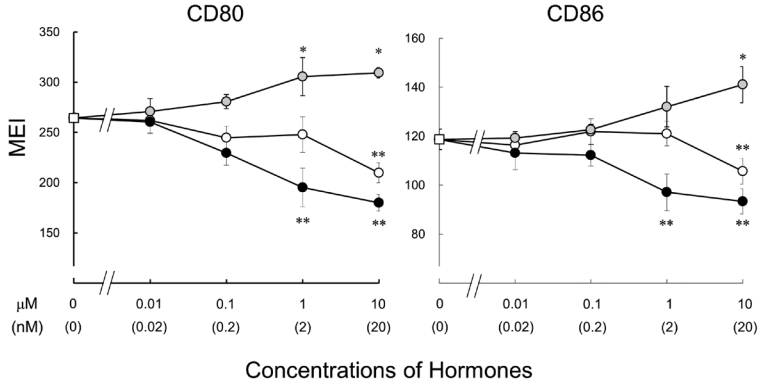

Fig. 3. Effect of ovarian hormones on expression of surface antigens characteristically expressed by DCs. PBMCs were incubated with various concentrations of progesterone (black circles), 17-OHP (white circles) or estrogen (gray circles) in the presence of TCCM for 12 days. As the untreated control, vehicle (ethanol) was added to the culture (a white square). After the incubation, the MEI of CD80 or CD86 was evaluated in the FCM. Four experiments were independently performed using nine dogs. Results were expressed as mean ± SE. *significantly increased and **significantly decreased, compared with the untreated control, $P<0.05$ by Fisher's PLSD

on the day 12 cultured cells was investigated, 17-OHP did not significantly affect the expression up to 1 μM. By addition of progesterone, the expression of CD80 and CD86 of cells decreased in a dose dependent manner and significantly lower than that of the 17-OHP culture at 1 μM, when compared with an untreated control. In contrast, by addition of estrogen, the expression of those molecules increased in a dose dependent manner and significantly higher CD80 expression at 2 and 20 nM when compared with an untreated control. The significantly higher CD86 expression was also observed with 20 nM estrogen. Then, we investigated the expression of those co-stimulatory molecules and antigen-presenting molecules, MHC class II and CD1a on the day 6 and the day 12 cells in the cultures containing 1 μM progesterone, 1 μM 17-OHP or 2 nM estrogen. As shown in Fig. 4, both progesterone and estrogen did not significantly affect the expression of any investigated molecules on the day 6 cells and that of MHC class II on the day 12 cells. The expression of CD80 of the cells in the progesterone culture did not increase from day 6 to day 12. However, the expression of the cells in the 17-OHP and the estrogen culture significantly increased during the 6 days. The expression of CD1a and CD86 on the day 12 cells in culture of any hormones used significantly increased when compared with that on the day 6 cells. Among the day 12 cells, however, the expression of those molecules of the cells in the progesterone culture was significantly lower than that on cells in the 17-OHP or the estrogen culture.

Effect of ovarian hormones on the immune stimulation by DCs: Finally, we examined the immune-stimulating ability of DCs that were exposed to ovarian hormones during differentiation and maturation. In this experiment, we added ovarian hormones into the DC-inducing culture to mimic the maximal

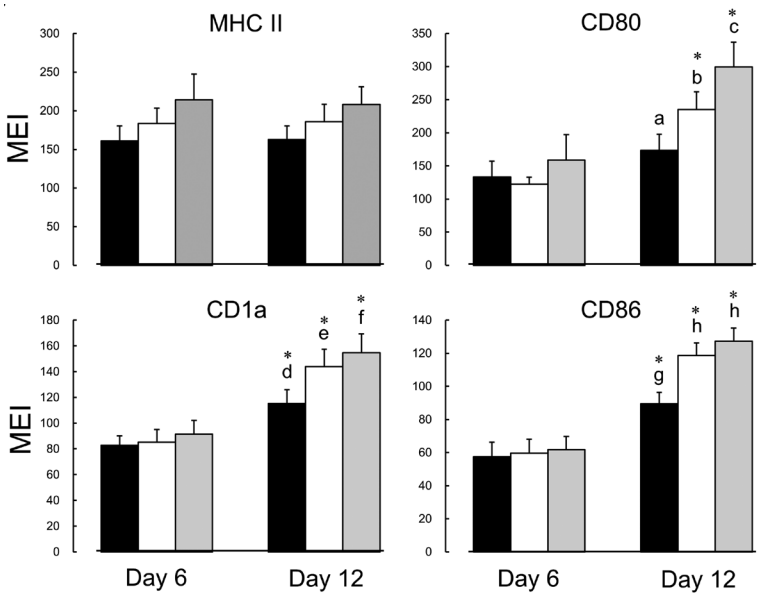

Fig. 4. Effect of ovarian hormones on expression of surface antigens characteristically expressed by DCs. PBMOs were incubated with progesterone (black bars), 17-OHP (white bars) or estrogen (gray bars) in the presence of TCCM for 6 or 12 days. After the incubation, the MEI of indicated molecules was evaluated in the FCM. Nine experiments were independently performed using nine dogs. Results were expressed as mean ± SE. *significantly increased, compared with "Day 6". a vs. b, b vs c, d vs. e, e vs. f and g vs. h, $P<0.05$ by Fisher's PLSD.

concentration in blood during the estrus cycle of the dog, i.e. 0.1 μM progesterone (17-OHP) and 0.2 nM estrogen [3]. As shown in Fig. 5, the proliferation of PB mononuclear cells increased as was with the addition of DCs generated in the culture of estrogen or 17-OHP. However, when DCs generated in the progesterone culture were added to the MLR, the proliferation did not increase with the escalating number of the DC.

DISCUSSION

In this study, we examined the effects of ovarian hormones on the generation of DCs to investigate a mechanism underlying the immune modulation by ovarian hormones, which is intimately connected to the incidence of pyometra in the dog [16]. It has been reported that progesterone directly inhibits the development of Th1 cells [12] and that estradiol-17β enhances Th1 cell responsiveness via estrogen receptor-α and -β [11]. However, it is expected the differentiation and maturation of DCs should be affected by the hormones because DC is the sole initiator of primary immune responses and the most potent activator of secondary responses [15]. According to the previous study [19], we induced DCs from PBMOs and estimated the cells collected from the DC-inducing culture on day 6 as the immature DC and cells collected on day 12 as the mature DC.

When the receptors for ovarian hormones were examined, the expression of PR and ERβ was found on PBMOs, precursors and immature DCs. Expression of receptors for ovarian hormones in the DCs has been reported in other species [2, 4, 5]. These results suggest that PBMOs and the immature DCs are affected by progesterone and estrogen.

In the examination of surface markers, progesterone significantly decreased expression of CD1a, CD80 and CD86 on the mature DC, but not on the immature DCs. We have found that the expression of these markers significantly relates to DC maturation [17, 20]. Moreover, although the significant decrease of the expression was shown at ten times more concentration of that physically observed, the allogeneic MLRs significantly decreased using the DCs derived from the culture containing progesterone at the concentration physically observed in blood during diestrus [3]. These results suggest that progesterone significantly inhibits the DC-maturation, but not the DC-differentiation. The inhibitory effect of progesterone on the maturation was also observed in mouse DC [9]. The immature DCs not only show weak in immune stimulation, but also induce immune tolerance [10]. In contrast to our results, Segere *et al.* reported that progesterone and estrogen did not effect on the maturation of dendritic cells derived from human monocytes [14]. However, in their study, hormones were added to the cultures where monocytes had differentiated into immature DCs (on day 4) and incubated with the immature DCs for only 24 hr. In our system, the hormones were added at the beginning of the culture and existed for 12 days. Thus, the discrepancy between their result and ours may be due to the difference of the experimental system. In the diestrus of the dog, the maximal level of progesterone on blood is maintained for 20–30 days, while the level of estrogen is kept very low [3]. Therefore, our system more reflects the hormone condition in the diestrus of the dog.

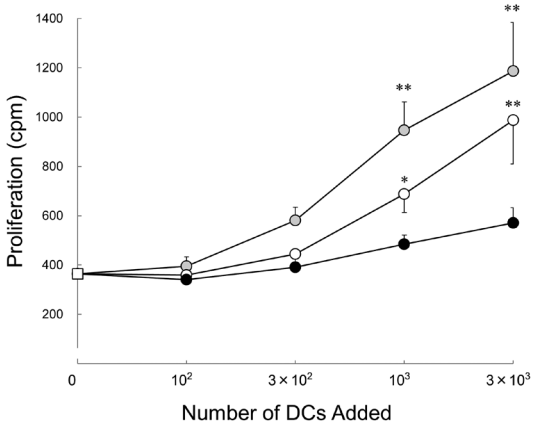

Fig. 5. Effect of ovarian hormones on immune stimulatory activity of DC. Allogeneic PB mononuclear cells were incubated for 4 days with various numbers of the cells cultured with progesterone (black circles), 17-OHP (white circle) or estrogen (gray cycles) in the presence of TCCM. After the incubation, proliferation of the PB mononuclear cells was evaluated by measuring radioactivity of [^{3}H]-thymidine incorporated. The white square indicates the background proliferation of PB mononuclear cells (no DCs added). Four experiments were independently performed using eight dogs. Results were expressed as mean ± SE. Significantly increased $P<0.05$ (*) and $P<0.001$ (**), compared with the background by Fisher's PLSD.

Taken all evidences together, it is concluded that the inhibition of the DC-maturation by progesterone is a mechanism of the immune suppression in the diestrus, which indirectly induces the increased incidence of pyometra in the dog.

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