Effect of Sex Hormones on Eosinophilic Inflammation in Nasal Mucosa

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ABSTRACT

We examined the effects of sex hormones on the functions of eosinophils. Treatment of eosinophils with β-estradiol significantly enhanced the eosinophil adhesion to human mucosal microvascular endothelial cells (HMMEC), and eosinophils stimulated by a combination of β-estradiol and progesterone showed significant induced degranulation. On the other hand, testosterone significantly reduced the eosinophil adhesion to HMMEC and eosinophil viability. The experiments from this series of studies might provide a partial explanation for the aggravation of asthma and some forms of rhinitis that occurs during pregnancy. (Allergy and Asthma Proc 19:263–269, 1998)

During pregnancy, aggravation of nasal allergic symptoms is occasionally observed in subjects with nasal allergy.[1] An increase in symptoms of nasal allergy is apparent from the first trimester and tends to become more pronounced as the pregnancy progresses.[2] If the mechanism underlying this "pregnancy effect" could be understood, this understanding could lead to more effective treatments of inflammation in pregnancy.

Estrogen and progesterone are known to be involved in eosinophil infiltration in many organs, including the uterus,[3] spleen,[4] and subdural hematoma membrane.[5] In particular, the uterus during pregnancy is infiltrated by a number of eosinophils that may be induced by estrogen.

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Recent studies have suggested that eosinophil-derived lipid mediators and toxic proteins play an important role in the development of many allergic and inflammatory diseases. In the nasal mucosa, leukotrienes (LT) and platelet-activating factors (PAF) induce nasal swelling by increasing vascular permeability and by dilating the capacitance vessels.[6] LT and PAF, in addition to eosinophilic cationic protein (ECP), induce nasal hyperreactivity to nonspecific stimuli, including histamine.[7,8]

Through the process of chemotaxis to the lamina propria, eosinophils repeat the adhesion and detachment of the endothelial cells in the nasal mucosa. During this time, communication between the cells and mutual influence occurs. To elucidate the influence of female hormones on eosinophil accumulation, it is important to study the effect of female hormones on the adhesion between eosinophils and endothelial cells. Using eosinophils isolated from the peripheral blood of patients with perennial nasal allergy, as well as cultured endothelial cells isolated from human turbinal mucosas, we investigated the effects of sex hormones, first on the adhesion of eosinophils and endothelial cells, and then on the adhesion molecules of eosinophil and endothelial cells. The effects of sex hormones on eosinophil viability and eosinophil degranulation were also examined.

MATERIALS AND METHODS

Antibodies

IgG Murine mAb recognizing lymphocyte function associated antigen-1 (TP1/32) {Upstate Biotechnology Inc. (New York)} is of the IgG_1 subclass. 10 μl/mL of this antibody inhibits binding of T cells to endothelial cells treated with 1 ng/mL TNF-α for 4 hours. Murine mAb recognizing Mac-1 (D12*, IgG2a) was purchased from Becton Dickinson (San Jose, CA), which recognizes the α-chain

of the heterodimeric complex known as Mac-1 or CD11b/CD18(9). Murine mAb recognizing very late antigen-4 (CD49d, 9F10, IgG_1) was purchased from PharMingen (San Diego, CA), which reacts with the integrin $\alpha 4$ chain. Monoclonal mouse anti-human intercellular adhesion molecule-1 (anti-human ICAM-1), vascular cell adhesion molecule-1 (Anti-Human VCAM-1), and endothelial cell-leukocyte adhesion molecule-1 (Anti-Human ELAM-1) were all purchased from Genzyme (Cambridge, MA) and are of the IgG_1 subclass.

Agents

Water soluble β-estradiol, water soluble progesterone and, water soluble testosterone, were purchased from Sigma Chemical Co., (St. Louis, MO).

Eosinophil Purification

Eosinophils were purified from peripheral blood obtained from patients with nasal allergy (mean age 31.2 $\pm$ 7.9 years). The diagnosis of nasal allergy was made on the basis of a typical history of perennial nasal allergy symptoms, eosinophilia in nasal smears, and a positive radioallergosorbent test (RAST) to house dust mites. The leukocyte fraction was obtained by dextran sedimentation. After the removal of mononuclear cells by gradient centrifugation at room temperature using Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden), the eosinophils were isolated by negative immunomagnetic selection using CD16 microbeads and the MACS system (Miltenyi Biotec, Germany).[10] Isolates consisting of > 95% eosinophils, with viability > 97% as judged by trypan blue exclusion, were used in this study.

Collection of Inferior Nasal Mucosa

Nine perennial allergy patients (5 males and 4 females, mean age 37.8 $\pm$ 6.7 years) were selected. These patients all had eosinophilia in nasal smears and were sensitized to house dust mites. Additionally, they were all positive in the nasal provocation test, skin test, and RAST. They had typical clinical histories of sneezing, rhinorrhea, and perennial nasal obstruction. All patients were confirmed not to have typical symptoms of muco-purulent nasal discharge, and all had radiographic evidence of normal sinuses on recently taken X-ray films. Inferior nasal mucosa samples were obtained at the time of surgery for submucosal resection.

Purification of Endothelial Cells

Human mucosal microvascular endothelial cells (HMMEC) were isolated from human nasal mucosa using the method of Fukuda et al.[11] HMMEC were cultured in a MCDB107 medium (GIBCO) containing 5% fetal calf serum (FCS) and 75 mg/mL endothelial cell growth supplement (Collaborative Research Inc., Bedford, MA). The identity of endothelial cells was verified by expression of factor VIII antigen and inverted phase contrast microscopy. After the two to three passages, the cells were seeded ($10^4/cm^2$) in collagen-type-I-coated culture plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan).

Eosinophil Viability Assay

To determine the effects of female and male hormones on eosinophil viability, eosinophils (1×10^4/well) suspended in RPMI1640 with penicillin and streptomycin were incubated with estradiol, progesterone, or testosterone for 18 to 96 hours. They were then stained with trypan blue, and counted blindly. The viability was calculated using the following formula: (living cells/living cells + dead cells) $\times$ 100(%).

Eosinophil-HMMEC Adhesion Assays

The HMMEC monolayers were observed by phase-contrast microscopy. At confluence, HMMEC were washed three times with phosphate buffered saline (PBS) containing 10 mg/mL gentamicin (Sigma). Eosinophils (2.5×10^5/well) were preincubated in the presence or absence, respectively, of β-estradiol, progesterone, or testosterone at concentrations of 5 or 50 ng/mL for 6 or 18 hours at 37°C. The eosinophils were labeled with ^{35}S by incubation with the [^{35}S] Protein Labeling Mix (Du Pont, Biotechnology Systems, DE) in RPMI without methionin and cysteine (Gibco RBL) for 3 hours at 37°C. Labeled eosinophils were then added and were allowed to adhere to the monolayers for 60 minutes at 37°C. Monolayers with eosinophils were rinsed by PBS, and then lysed by the addition of 2% Triton X-100 (Sigma). A liquid scintillator (SuperMix™, Wallac, Finland) was then added. ^{35}S radioactivity was determined by Micro Beta™ (Pharmacia Biotech, Sweden). Previous studies have shown that eosinophils take up ^{35}S at different rates, depending on the stimulation. Therefore, the number of adherent eosinophils was calculated with each standard curve.

For the anti-adhesion molecule monoclonal antibody (mAb) blocking experiments, 50 ng/mL β-estradiol-stimulated eosinophils were preincubated at 37°C in the presence or absence of saturating concentration of IgG Murine mAb recognizing LFA-1 and Murine mAb recognizing Mac-1 for 15 minutes before their addition to HMMEC.

Flow Cytometric Analysis

For the detection of surface adhesion molecules on eosinophils, the cells that were preincubated in the present or absence of the indicated concentration of β-estradiol, progesterone, or testosterone for 18 hours at 37°C were washed with FACS buffer (PBS containing 0.1% of NaN3 and 1.0% of BSA. The eosinophils were then suspended in IgG Murine mAb-recognizing LFA-1, Mac-1, and VLA-4, respectively. The cells were incubated for 30 minutes at 4°C, washed free of unbound antibodies, and incubated with the 1:50 dilution of FITC-conjugated anti mouse IgG

(Southern Biotechnology Associates, Inc., AL) in PBS for 30 minutes at 4°C. After washing, the cells were suspended in 0.5 mL of buffer. The mean fluorescence of each cell population was then determined by flow cytometry (FACScan, Becton-Dickinson), with quantitative determination of peak fluorescence intensity.

Eosinophil Degranulation Test

To determine the effects of female and male hormones on eosinophil degranulation, eosinophils (2.5×10^5/well) suspended in RPMI1640 with penicillin and streptomycin were incubated with β-estradiol, progesterone, a combination of β-estradiol and progesterone (Combination), or testosterone for 18 hours. As a positive control, calcium ionophore (Ca I: A23187, Sigma) was used. Supernatants of eosinophils were harvested from the wells and stored at −80°C until assayed. Eosinophil protein X (EPX) was measured using commercially available RIA kits for EPX (Pharmacia EPX RIA, Pharmacia, Sweden). The minimum detectable concentration was 3 ng/mL. The changes in EPX concentration were expressed as a percentage of those observed with PBS.

Statistical Analysis

Results are presented as means ± SED. The statistical significance of differences between groups was determined using Wilcoxon's test.

RESULTS

The Effects of Sex Hormones on Eosinophil Viability

Eosinophil viability was observed at 24, 48, 72, and 96 hours after eosinophil separation. Resting eosinophil viability was 73 ± 8% at 24 hours, 53 ± 3% at 48 hours, 35 ± 8% at 72 hours, and 24 ± 9% at 96 hours. At 24 hours sex hormones did not affect the eosinophil viability. Figure 1 shows the eosinophil 48-hour-viability after incubation with IL-5 and sex hormones. 100 pg/mL IL-5 significantly increased the eosinophil viability to 81 ± 3% at 48 hours. Female hormones and 5 ng/mL testosterone had no effect on the eosinophil viability; however, 50 ng/mL testosterone significantly shortened the eosinophil viability to 35 ± 6% ($p < 0.05$).

Eosinophil-HMMEC Adhesion Experiment

The rate of resting eosinophil adhesion to HMMEC was 17.3 ± 1.3%. The effects of female and male hormones on eosinophil adhesion to HMMEC are shown in Figure 2. 50 ng/mL β-estradiol significantly increased adherence to HMMEC as well as did IL-5, the positive control. Eosinophils stimulated by 50 ng/mL β-estradiol increased adhesion to HMMEC to 22.4 ± 2.2% ($p < 0.05$). On the other hand, 50 ng/mL testosterone significantly decreased the percentage of adherent eosinophils to HMMEC to 13.2 ± 2.5% ($p < 0.05$). We investigated the adhesion of eosinophils incubating with sex hormones for only 6 hours; however, the rates of the adhesion did not change from the control.

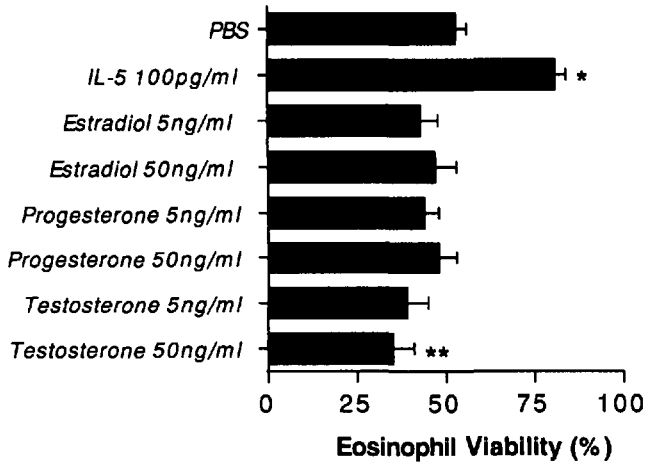

*Figure 1. Effects of sex hormones on eosinophil survival rate at 48 hours after eosinophil incubation. β-estradiol, progesterone, and 5 ng/mL testosterone had no effect on the eosinophil viability; however, 50 ng/mL testosterone significantly shortened the eosinophil viability to 35 ± 6% ($p < 0.05$). Each bar represents mean ± SED, n = 9. *$p < 0.05$ versus control.*

Treatment of 50 ng/mL β-estradiol-stimulated eosinophils with anti-LFA-1 mAb before their addition to HMMEC reduced the number of eosinophils adhering to endothelial cells to 73.3 ± 12%. However, treatment of 50 ng/mL β-estradiol-stimulated eosinophils with the anti-Mac-1 mAb had no effect on the eosinophil adherence to endothelial cells, as shown in Figure 3.

Detection of Surface Adhesion Molecules on Eosinophils and HMMEC by Flow Cytometric Analysis

Eosinophil adhension molecules LFA-1, Mac-1 (CD11b/18), and VLA-4, were detected on resting eosinophils, at rates of 96.2 ± 0.5%, 94.5 ± 1.0%, and 83.9 ± 1.9%, respectively (Table I). Any significant effect on the expression of these adhesion molecules was not observed following 18-hour incubations with sex hormones. Furthermore, no change in the adhesion molecule detection of IL-5-activated eosinophils by sex hormones was observed.

Effects of Sex Hormones on Eosinophil Degranulation

The effects of 50 ng/mL β-estradiol, progesterone, Combination, testosterone, respectively, and A23187 on eosinophil degranulation were examined in six cases. The changes in the concentration of EPX in the supernatant were evaluated by RIA. The concentration of EPX in the unstimulated eosinophils after 18 hours at 37°C was 33.3 ± 10.7 ng/mL. The EPX concentration from eosinophils stimulated by A23187 was significantly increased to 236.8 ± 64.0 ng/mL. The concentration of EPX in the supernatant obtained after the incubation of 50 ng/mL combination was

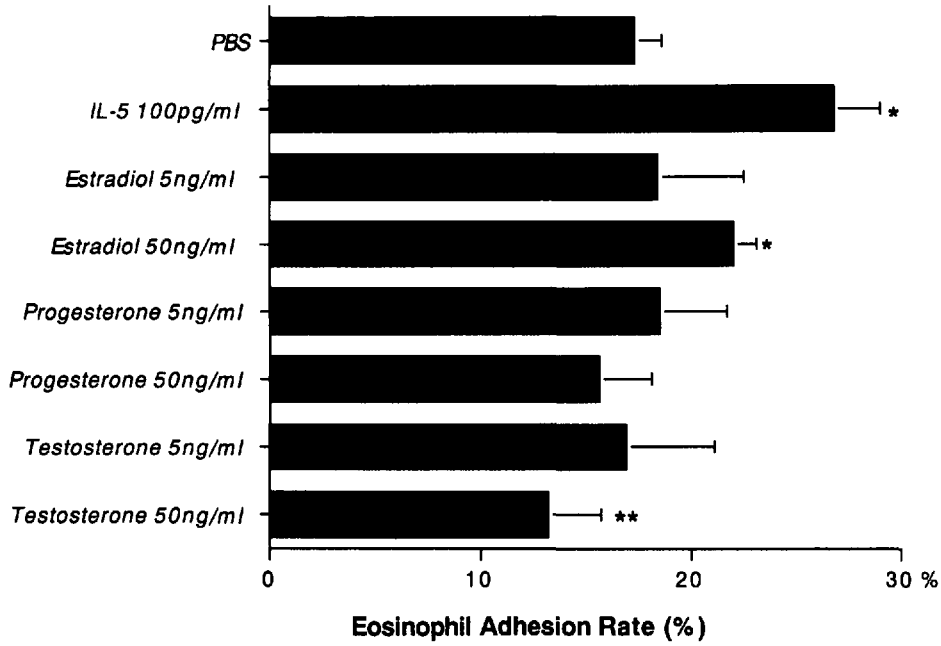

*Figure 2. Effects of sex hormones on eosinophils adhesion. Eosinophils were stimulated with β-estradiol, progesterone, and testosterone for 18 hours. (n = 8) Eosinophils stimulated by 50 ng/mL β-estradiol significantly increased adhesion to HMMEC to 22.4 ± 2.2% ($p < 0.05$). On the other hand, 50 ng/mL testosterone significantly decreased the percentage of adherent eosinophils to HMMEC to 13.2 ± 2.5% ($p < 0.05$). Each bar represents mean ± SED, *$p < 0.05$ versus control.*

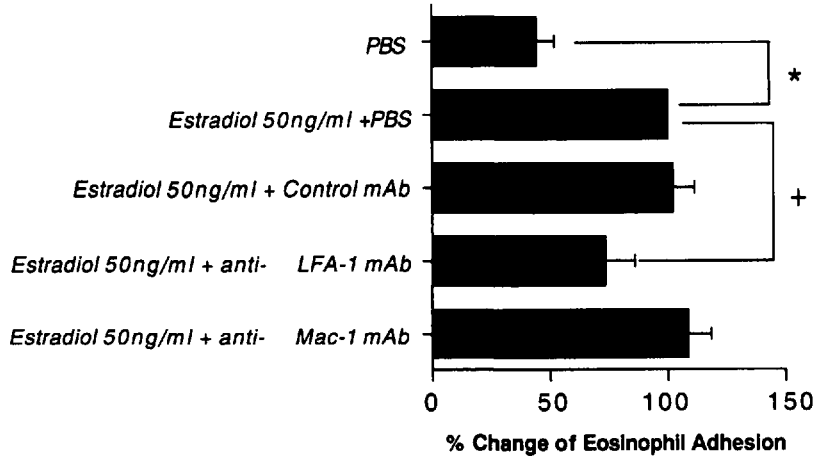

*Figure 3. Effects of anti-Mac-1 and anti-LFA-1 antibodies on estradiol-enhanced eosinophil binding to HMMEC. Results are expressed as percentage increase over the adhesion obtained on HMMEC without each antibody in the presence of IL-5 (100 pg/mL) or estradiol (50 ng/mL). Each bar represents mean ± SED, n = 7. Treatment with anti-LFA-1 mAb reduced the number of eosinophils adhering to endothelial cells to 73.3 ± 12%. However, treatment with the anti-Mac-1 mAb had no effect on the eosinophil adherence to endothelial cells. *$p < 0.05$ versus control, †$p < 0.1$ versus control.*

statistically higher than that of unstimulated eosinophils. However, 50 ng/mL β-estradiol, progesterone, and testosterone had no effect on eosinophil degranulation (Fig. 4).

DISCUSSION

In this study, the effects of female hormones on the functions of eosinophils and endothelial cells have been investigated. The percentage of adherence of eosinophils to endothelial cells significantly ($p < 0.05$) increased after 18-hour stimulation by β-estradiol (50 ng/mL) (Fig. 2). Eosinophils have relatively short half-lives. However, we have observed that 6-hour stimulations of female hormones do not change the adhesion of eosinophils to HMMECs. Therefore, we incubated eosinophils with sex hormones for 18 hours. We also confirmed that sex hormones do not affect the eosinophil viability in this experiment. On the other hand, we were able to confirm that a combination of β-estradiol and progesterone could induce, to a minor degree, eosinophil degranulation.

TABLE I

Effect of Sex Hormones on Expression of Eosinophil Adhesion Molecules

	PBS (%)	Estradiol (50 ng/mL)	Progesterone (50 ng/mL)	E + P (50 ng/mL)
LFA-1	96.2 ± 0.5	94 ± 1.0	95.8 ± 0.7	N.D.
Mac-1	94.5 ± 1.0	92.6 ± 2.8	93.8 ± 1.1	94.9 ± 0.7
VLA-4	83.9 ± 1.9	84.1 ± 2.1	85.9 ± 1.5	84.0 ± 0.89

N.D. not done

Values are mean fluorescence intensities determined by flow cytometry. Each bar represents mean ± SE, n = 11. Any significant change in the expression of LFA-1, Mac-1 (CD11b/18), and VLA-4 was not observed following 18-hour incubations with sex hormones.

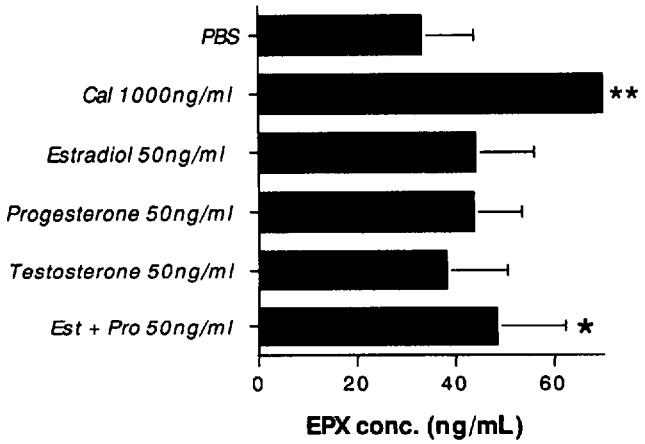

Figure 4. *Effects of sex hormones on eosinophil degranulation. The change in the concentration of EPX in the supernatant was determined by RIA. Each bar represents mean ± SED, n = 6. *p < 0.05 versus control. The concentration of EPX in the supernatant obtained after the incubation of 50 ng/mL Combination was statistically higher than that of unstimulated eosinophils. However, 50 ng/mL β-estradiol, progesterone, and testosterone had no effect on eosinophil degranulation.*

We previously reported that female hormones may influence the autonomic nervous receptors in nasal mucosae, inducing an increase of nasal discharge and nasal mucous swelling.[12] On the other hand, several articles have noted that approximately one-third of pregnant women experience exacerbated asthmatic symptoms, one-third experience reduced symptoms, and one-third experience no change in symptoms during pregnancy. These changes in the severity of maternal asthma may be explained by some of the many physiologic adaptations that occur in pregnancy, such as an increase in free cortisol, the effects of female hormones on smooth muscles of bronchi, and a significant decrease in expiratory reserve volume resulting from the elevation of the diaphragm.[13]

We studied 59 pregnant women with nasal allergy to determine the effect of pregnancy on nasal allergy.[20] About 10% of the women experienced increased symptoms of nasal allergy; however, none of the pregnant women with nasal allergy experienced decreased symptoms. The symptoms of nasal allergy generally increased during the first trimester, and in almost none of the patients who experienced such an aggravation did the symptoms subsequently decrease during the third trimester. Therefore, patients with asthma or nasal allergy are thought to differ in allergic response during pregnancy. One of the reasons is that nasal turbinates, the main allergic reactive spots in the nose, have no smooth muscle. The second reason is that nasal allergy is unlikely to be affected by respiration. An increase in free cortisol could improve the symptoms of nasal allergy during pregnancy; however, none of the patients experienced such an improvement. We therefore suspect that critical exacerbating factors in nasal allergy during pregnancy, such as female hormones, more than offset any benefit from elevated cortisol levels. Female hormones are thought to be particularly critical because their levels increase by factors of 100–1000 times over the levels in nonpregnant women. This is one of the most dramatic physiological changes to occur during pregnancy.

The serum levels of sex hormones is very limited in infants of less than 5 or 6 years old; however, thereafter the level of β-estradiol increases rapidly to the beginning of adolescence. Levels of both β-estradiol and progesterone are highest in young women in their 20s. The change in the serum concentrations of these female hormones closely matches the change in incidence of nasal allergy in females. The levels of β-estradiol and progesterone in pregnant women are 0.1–52 ng/mL and 4–390 ng/mL, respectively. These levels are 100–1,000 times as much as those found in nonpregnant women. The concentrations of the female hormones used in this study (5 and 50 ng/mL) were, therefore, within the typical physiological range.

Inflammatory reactions associated with chronic allergic diseases and experimentally induced late phase allergic reactions are characterized by the preferential influx of eosinophils. The extravascular chemotaxis includes 1) rolling on endothelial cells, 2) adhesion to endothelial cells, 3) transmigration through their intercellular spaces, 4) remaining between endothelial cells and pericytes, and 5) chemotaxis into tissues. Eosinophils attach first to endothelial cells in response to the chemotaxis in inflammatory areas, and are then guided into lamina propria in the nasal mucosa through the gradient of chemotactic factor concentration and the direct contacts between eosinophil-endothelial cell adhesion molecules.

Female hormones, especially β-estradiol, have been suggested to enhance the eosinophil chemotaxis at the first two stages: rolling and adhesion. Treatment of 50 ng/mL β-estradiol-stimulated eosinophils with anti-LFA-1 mAb before their addition to HMMEC reduced the number of eosinophils adhering to endothelial cells. However, treatment of 50 ng/mL β-estradiol-stimulated eosinophils with

the anti-Mac-1 mAb had no effect on the eosinophil adherence to endothelial cells (Fig. 3).

The expressions of LFA-1, Mac-1 (CD11b/18), and VLA-4, the adhesion molecules of eosinophil, were not changed in our study (Table I). Regulated upon activation normal T expressed and presumably secreted (RANTES) is known to enhance the eosinophilic adhesion without an increase in the eosinophil adhesion molecule expressions.[14] In the same way, female hormones may enhance the adherence by the increase of affinity of eosinophil adhesion molecules, particularly in LFA-1, without significantly increasing their expressions. On the other hand, a male hormone testosterone at a concentration of 50 ng/mL reduced the eosinophil adherence as shown in Figure 2. Testosterone may inhibit eosinophilic chemotaxis by reducing the eosinophil adhesion.

Some previous studies have reported that female hormones may be a chemotactic factor of eosinophils. Lee et al.[15] reported that estradiol, via its nuclear receptor, stimulated the production of a chemotactic factor that induces the migration of eosinophils into rat uteri when 1 mg estradiol was given 24 hours before death. They hypothesized that estradiol might stimulate the production of a uterine eosinophil chemotactic factor. Luque et al.[16] proposed that progesterone promoted a massive infiltration of the rat uterine cervix by eosinophils. The eosinophil infiltration was concerned to loosen the collagenous framework by collagenase. This infiltration could result in opening the cervix at the time of delivery. Female hormones are likely to act as a chemotactic factor and promote the adhesion of eosinophils to endothelial cells and to further the subsequent chemotaxis into extravascular tissues. However, it will be necessary to confirm by the Boyden Chamber method whether female hormones have the role of the eosinophil chemotactic factor as is shown by PAF, LTB4, and IL-5.

Female hormone receptors on eosinophils have been identified by an autoradiographic technique.[17] The receptors on endothelial cells were recognized on human large vessels and human umbilical vein endothelial cells.[18]

Tchernitchin et al.[19] reported that the treatment of adult ovarectomized rats with 30 mg estradiol/100 g body weight increased eosinophil degranulation in vivo and in vitro. We further elucidated the effects of sex hormones on eosinophil degranulation. The EPX content in supernatants of cultures significantly increased when a combination of β-estradiol and progesterone was added as a stimulus (Fig. 4). At a similar dosage, the survival rates of sex hormone-treated and nontreated eosinophils did not differ significantly, indicating that the increase in EPX was not due to eosinophil cell death but to eosinophil degranulation by combination of β-estradiol and progesterone. Considering that eosinophil derived LT and PAF induce nasal swelling[6] and that LT, PAF, and ECP induce nasal hyperreactivity to nonspecific stimuli, including histamine,[7,8] female hormones may accelerate eosinophilic inflammation and increase nasal allergy symptoms.

In summary, we were able to confirm that β-estradiol significantly ($p < 0.05$) enhanced the adherence of eosinophils to endothelial cells. On the other hand, the expressions of eosinophil adhesion molecules did not show a significant change after stimulation by female hormones β-estradiol and progesterone. A combination of β-estradiol and progesterone could induce, to a minor degree, eosinophil degranulation, however. It was therefore theorized that female hormones might enhance the eosinophil adhesion to endothelial cells via increasing the affinity of eosinophil adhesion molecules. This can induce further eosinophil chemotaxis into extravessel and lamina propria in the nasal mucosa. As activated eosinophils secrete the specific granules by eosinophil-degranulating agents, female hormones can function as eosinophil-degranulating agents. Female hormones can be thought to enhance eosinophil function and accelerate allergic inflammation. On the other hand, testosterone may regulate allergic inflammation by reducing eosinophil adhesion to endothelial cells and also eosinophil viability. By these reactions, testosterone can control the eosinophil recruitment.

The experiments from this series of studies provided data consistent with an effect of estrogen upregulating aspects of eosinophil adherence. If these findings can be extrapolated to humans, they might provide a partial explanation for the aggravation of asthma or some forms of rhinitis during pregnancy.

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