Oestrogen inhibits psoriasis-like dermatitis induced by imiquimod in mice in relation to increased IL-10 producing cells despite elevated expression of IL-22, IL-23, IL-17 mRNA

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Abstract

Sex hormones influence the development and natural course of psoriasis. Here, we examined the effects of female sex hormones, particularly oestrogen, on psoriasis-like dermatitis induced using topical imiquimod in mice that underwent either sham operation (Sham) or ovariectomy (OVX), with (hormone replacement treatment: HRT) or without 17β-oestradiol targeting the maximum physiological levels. The number of neutrophils in the skin was higher in the order of OVX-, Sham-, and HRT-treated mice. However, no significant difference was detected in the clinical scores among the three groups due to severe erythema and scale in a few mice out of HRT-treated mice in a set of experiments. OVX- and HRT-treated mice showed increased mRNA levels of interleukin (*IL)-22* and *IL-23* compared with Sham-treated mice; increased *IL-10* mRNA levels were found in HRT-treated mice, possibly due to an increased proportion of forkhead box P3 (Foxp3)- and IL-10 positive large cells (possibly macrophages). Additionally, HRT-treated mice had a more compact stratum corneum with higher expression of loricrin and involucrin than OVX- and Sham-treated mice. This study suggests that oestrogen has a dual potential in the pathogenesis of psoriasis: suppression of inflammation by enhancing IL-10 production and enhancement of inflammation by induction of IL-22 and IL-23 expression.

Keywords: psoriasis, ovariectomy, 17β-oestradiol, IL-10, foxp3 protein, mouse

Background

Psoriasis vulgaris is a skin disease that affects individuals of all ages and has a complex pathology based on the interleukin (IL)-23/IL-17 axis.[1-3] Sex hormones are major factors influencing the development and natural course of psoriasis. Psoriasis vulgaris often begins around puberty, suggesting that sex hormones influence its development.[4] Additionally, menstrual cycle irregularity, and surgical and age-related menopause are risk factors for psoriasis.[5, 6] Pregnancy relieves symptoms in 55% of patients with psoriasis but exacerbates symptoms in 23%.[7] The prevalence of psoriasis is greater in men than in women among Asians [8, 9,10]; however, the same is not true for Caucasians.[1]

Oestrogen, a female sex hormone, includes oestrone (E1), 17β-oestradiol (E2), and estriol (E3). E2 is the most potent oestrogen produced by ovary and the placenta. Many studies on various diseases have indicated that oestrogens have complex immunomodulating effects. Oestrogen not only suppresses immune responses, such as in experimental allergic encephalomyelitis and multiple sclerosis,[11] but also enhances anaphylaxis,[12] contact hypersensitivity,[13] immunoglobulin production (including autoantibodies),[14,15] and experimental autoimmune thyroiditis.[16] E2 has immunosuppressive effects in most inflammatory models, as it induces regulatory T cells and the production of IL-10 and

suppresses Th17 cell development,[17,18] which could all be involved in the pathogenesis of psoriasis. However, E2 has the opposite effect in different and limited situations, such as splenocyte IL-17 production and experimental autoimmune thyroiditis.[18-21] Furthermore, the production of IL-6, which is related to the pathogenesis of psoriasis, is enhanced by oestrogen after injury.[22]

Female sex hormones could affect the pathogenesis of psoriasis; however, evidence supporting the effects and mechanisms of female hormones in mice models of psoriasis is lacking. This study directly assessed the effects of ovariectomy (OVX) and hormone replacement treatment (HRT) to induce the physiologically maximal levels of E2 on the pathogenesis of psoriasis, including IL-10 and forkhead box P3 (Foxp3)-positive cells, using psoriasis-like dermatitis induced by imiquimod (IMQ) in mice.

Questions addressed

This study aimed to address the following questions: 1) Does oestrogen affect the pathogenesis of psoriasis-like dermatitis induced by imiquimod? 2) What is the mechanism through which E2 affects the severity of psoriasis-like dermatitis?

Experimental design

Experiments were performed under the supervision of the Animal Care and Use Committee, Dokkyo University School of Medicine. Female C57/BL6 and BALB/c mice were randomly assigned to either the sham procedure (Sham group) or OVX at 8 weeks of age. HRT was performed immediately after OVX by implantation of subcutaneous slow-releasing E2 tablets (Innovative Research of American, Sarasota, USA) that dispensed 0.025 mg of E2 for 60 days, which maintained the maximum physiological concentration of E2 in mice, as shown in our previous study.[23] Psoriasis-like dermatitis was induced 1 week after OVX and HRT by repeated application of IMQ for 5 days and was evaluated based on clinical scores, including erythema, scale, and induration, as described previously.[24] Skin samples were harvested 24 h after the last application of IMQ. The skin was formalin-fixed and paraffin-embedded for haematoxylin and eosin staining and IL-10, Foxp3, Ki-67, ketatin 6, loricrin, and involucrin immunostaining. The remainder of the skin was stored at -70°C until analysis by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Details of mice, the staining conditions, semi-quantitative immunostaining analysis, and real-time RT-PCR are presented in the Supplementary Materials and Methods and in Table S1. Each group contained at least six mice. All experiments were repeated at least thrice. The results in the figures represent three or more experiments; standard deviations were calculated from a single experiment. When appropriate,

data were analysed using unpaired two-tailed Student's t-tests based on the F-test of equality of variances. A p-value of <0.05 was considered a significant difference.

Results

Representative photographs of psoriasis-like dermatitis in IMQ-treated C57BL/6 mice and the clinical scores are shown in Fig. 1a and 1b, respectively. The total clinical scores were higher in the order of OVX-, Sham-, and HRT-treated mice without statistical significance, except for induration, for which the score was lower in HRT-treated mice than others. High values of erythema and scale scores in several mice in the HRT group seemed to cancel out the difference in total clinical scores from the other groups (Fig. 1a). We observed similar heterogeneity of dermatitis severity using HRT-treated mice in repeated experiments on some occasions. The representative histopathology and immunostaining of psoriasis-like dermatitis in each group are shown in Fig. 1c. The total number of cells and neutrophils in the skin, the thickness of the epidermis, and the number of Ki-67 positive cells in the epidermis were higher in the order of OVX-, Sham-, and HRT-treated mice (Fig. 1c-g). In contrast, the levels of immunostaining with keratin 6 were comparable among three groups of mice (data not shown). These factors indicative of psoriatic dermatitis in HRT-treated mice with clinically severe dermatitis were higher than those with mild dermatitis but less than OVX- and Sham-treated

mice; results were unsuitable for statistical analysis due to sporadic appearance. Immunostaining of loricrin and involucrin showed comparable levels between OVX- and Sham-treated mice, which were lower than those of HRT-treated mice (Fig. 1c, h, i). These data suggest that HRT-treated mice develop less severe psoriasis-like dermatitis than OVX- and Sham-treated mice. Furthermore, OVX induces more severe dermatitis than a sham operation.

To explore the mechanism of the inhibitory effect of HRT and OVX on the development of psoriasis-like dermatitis, we evaluated the mRNA expression of cytokines in the skin using quantitative real-time RT-PCR (Fig. 2a). The *IL-10* levels were significantly increased in HRT-treated mice. *IL-17, IL-22* and *IL-23* levels were significantly elevated in the HRT group compared to the Sham groups. The levels of *IL-22* and *IL-23* in the OVX groups were higher than those in the Sham group and comparable with those in the HRT group. Immunostaining of Foxp3 indicated a comparable number of positive cells in the skin from all groups, while the number of Foxp3-positive large cells was higher in the HRT group than in the others. (Fig. 2b-d). Positive immunofluorescence staining of IL-10 was observed in large cells of the HRT group but not in the Sham and OVX groups (Fig. 2b).

We also examined the effect of OVX and HRT on psoriasis-like dermatitis induced by mild application of imiquimod cream under inhalation anaesthesia with sevoflurane. In this setting,

the imiquimod cream was wiped off the mice immediately after application. Intraperitoneal anaesthesia, through a mixture of medetomidine, midazolam, and butorphanol, was used to keep the mice sedated for an hour to conduct the abovementioned experiments. Mild application of imiquimod induced mild dermatitis with infrequent neutrophilic infiltration even in sham- and OVX-treated mice (Fig. 3c). However, the inhibitory effects of HRT were apparent. Clinical score; number of total cells and Ki-67-positive epidermal cells (Fig.3a-e); and mRNA levels of IL-17, IL-23, and CCL20 were lower in HRT-treated mice than Sham- and OVX-treated mice (Fig. 3h). The number of Ki-67 positive epidermal cells in high power field in the HRT group (3.6 ± 2.6) was significantly lower than in the Sham group (10.4 ± 6.5) and OVX-group 12.4 ± 4.8. Similarly, in cases with complete application of imiquimod, the immunostaining score of loricrin and involucrin was higher in HRT-treated mice than in the others (Fig. 3c, f, g). IL-10 mRNA levels increased in HRT-treated mice (Fig. 3h), and IL-10-positive cells were found only in HRT-treated mice (Fig. 3i). In contrast, the total number of cells in OVX-treated mice was lower than in the Sham-treated mice (Fig. 3c, d). Similar results were obtained for BALB/c mice with a mild application of imiquimod (Supplementary Fig. S1).

Conclusions & perspectives

For the first time, we report the inhibitory effect of administering E2 targeting the maximum physiological levels on psoriasis-like dermatitis in relation to increased IL-10 mRNA levels and IL-10-positive cells in BALB/c and C57BL/6 mice. The C57BL/6 mice provide a better background than other strains, including BALB/c mice, for modelling psoriasis disease mechanism[25]. However, the effects of E2 differed between full induction and incomplete induction of dermatitis. Upon induction of psoriasis-like dermatitis in association with dominant neutrophilic infiltration, E2 inhibited the neutrophilic infiltration but enhanced erythema score and mRNA expression of IL-22 and IL-23, which might induce or exacerbate psoriasis-like dermatitis. In contrast, E2 inhibited dermatitis and mRNA expression of IL-17, IL-23, and CCL20 in incomplete dermatitis induction with infrequent neutrophilic infiltration. OVX also qualitatively affected the pathology. In the full dermatitis induction setting, OVX enhanced neutrophilic infiltration and mRNA expression of IL-22 and IL-23. In contrast, inflammation in the skin and mRNA level of IL-17 in OVX-treated mice were weakened compared with Sham-treated mice in the incomplete induction model.

The inhibitory effect of E2 could be associated with increased levels of *IL-10* mRNA and IL-10-positive cells, suggesting induction of regulatory immune responses. In terms of the

similarity of morphology and distribution, IL-10-producing cells appeared to be Foxp3-positive macrophages in HRT-treated mice; however, we could not identify the cell type of IL-10-producing cells. IL-10 is secreted by T and B cells, macrophages, dendritic cells, and keratinocytes. Its relevance to psoriasis has been repeatedly reported in successfully treated patients as well as mouse models of psoriasis.[26 27 28 29,30 31] IL-10-deficient mice develop severe psoriasis-like dermatitis,[29] and loss of IL-10 in regulatory B cells enhances psoriasis-like dermatitis in CD19-deficient mice.[32] Conversely, IL-10 therapeutic potency is limited in patients with psoriasis; systemic administration of recombinant IL-10 only partially improves psoriasis in patients.[33 34] The partial suppression of psoriasis-like dermatitis by E2 in this study, as well as its association with increased *IL-10* and an increased proportion of Foxp3-positive large cells, was consistent with the results of previous clinical and laboratory findings regarding the importance of IL-10 in this process.[17,35]

Interestingly, some HRT-treated mice exhibited robust dermatitis even though they showed steady induction of IL-10 mRNA and IL-10-producing cells. This phenomenon could be associated with increased mRNA levels of IL-17, IL-22 and IL-23 in HRT-treated mice compared with OVX- and/or Sham-mice. In contrast, HRT repressed mRNA levels of IL-17 and IL-23 in experiments with incomplete induction of dermatitis. Considered together, the

capability of IL-10 induced by HRT targeting at the maximum physiological levels of E2 seems to be close to the threshold to inhibit the pathogenesis of psoriasis-like dermatitis. Alternatively, E2 could potentiate to induce or exacerbate psoriasis-like dermatitis via induction of IL-23 and IL-22 when the inhibitory effects of E2 via IL-10 would be impaired. Iwano et al. showed that oestrogen and oestrogen receptor α agonists accelerate dermatitis in psoriasis-like dermatitis induced by imiquimod.[36] However, they used 50 times more E2 than our study and an amount of oestrogen receptor α agonist estimated to be suitably effective. Adachi et al. recently reported inhibitory effects of E2 in psoriasis-like dermatitis, possibly via suppression of IL-1β from neutrophils[37]. They also used an E2 dosage six times more than our study. These findings suggest that oestrogen may have dual effects on inflammation in psoriasis depending on the doses and association with other factors, such as stimulants and modifiers, including another female ovarian hormone, progesterone, and androgen. This potential of E2 is consistent with the clinical observation that high oestrogen levels observed during pregnancy have an inhibitory effect on psoriasis, wherein 55% of patients reported improvement, 21% reported no change, and 23% reported worsening of symptoms during pregnancy,[7] and also consistent with development of psoriasis at puberty [4].

The effect of oestrogen on epidermal differentiation as a key pathology of psoriasis must be

discussed. We previously showed that hairless Hos:HR-1 mice that received OVX showed decreased expression of the epidermal differentiation molecules loricrin and involucrin,[23] while HRT enhanced the expression of them. Similar findings of epidermal differentiation molecules were observed in psoriasis-like dermatitis in this study, probably leading to the protection of dermatitis.

Thus, female hormones could influence the pathogenesis of psoriasis-like dermatitis through immunological and epidermal modification. Although these effects may not be directly pertinent for human psoriasis, these findings, with abundant supportive data on the protective effect of oestrogen on psoriasis, suggest that similar mechanisms could contribute to the pathogenesis of psoriasis and should be considered for the treatment of psoriasis.

Ethics statement:

Experiments were performed under the supervision of the Animal Care and Use Committee, Dokkyo University School of Medicine.

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Authors' Contributions

K.Kobayashi and **S.Chikazawa** contributed equally to this work.

K. Kobayashi: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Validation, Visualization, and Writing - original draft; **S. Chikazawa:** Data curation, Formal analysis, Methodology, and Resources**; YC:** Conceptualization, Data curation, Investigation, Methodology, and Resources; **NI:**

Methodology and Resources**;** **SS:** Methodology and Resources**;** **K. Katagiri:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing-review & editing, Supervision, and Validation. All authors have read and approved the final manuscript.

Conflicts of Interest: None to disclose.

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Figure legends

Figure 1. Hormone replacement treatment inhibits the full induction of imiquimod-induced psoriasis-like dermatitis. Mice received a sham operation (Sham), ovariectomy (OVX), or OVX with hormone replacement treatment with 17β-oestradiol (HRT) 1 week before initiating daily application of imiquimod on the back skin for 5 consecutive days. These panels of mice were applied imiquimod under intraperitoneal anesthesia with mixture of medetomidine, midazolam, and butorphanol that can keep mice sedated for an hour, which induces robust dermatitis like psoriasis with neutrophilic infiltration, so this model is referred to as full induction. The skin with psoriasis-like dermatitis was harvested 24 h after the last application of imiquimod. The first day of topical application of IMQ was defined as day 1. (a) Representative pictures of the psoriasis-like dermatitis in the three groups. (b) Clinical scores of each group. The total clinical score was calculated by summing the scores for erythema, scaling, and induration. (c) Haematoxylin and eosin staining (x200 and x400, Scale bar = 50 μm and 25 μm, respectively) and immunostaining for Ki-67, loricrin and involucrin of the skin from the three groups. Number of total cells (d) and neutrophils (e) in the dermis at high power field (×400). (f) Epidermal thickness. (g) Number of Ki-67 positive cells in the epidermis at high power field (x400). Scores of immunostaining for involucrin (h) and loricrin (i). Immunostaining

intensity was scored on a four-point scale by 10 blinded individuals. The highest intensity staining was scored as four points, no staining was scored as zero points, and intermediate intensities were scored as one, two, or three points, according to the intensity as shown in our previous study.[23] Results are expressed as mean ± standard deviation. N = 6 or 7 for each group. *p < 0.05, ** P <0.01, *** P <0.001, **** P <0.0001

Fig. 2 Cytokine expression and immunostaining for Foxp3 and IL-10 in a psoriasis-like dermatitis model. Skin was harvested from the sham, ovariectomy (OVX), and OVX with hormone replacement treatment with 17β-oestradiol (HRT) groups 24 h after the last application of imiquimod. (a) Cytokine and Fox3p mRNA expression in the skin were evaluated by quantitative reverse transcriptase-polymerase chain reaction. (b) Representative immunostaining of Foxp3- and IL-10 positive cells in the skin. White arrows indicate Foxp3-positive large cells (×400). Scale bar = 25 μm for Foxp-3 and scale bar =100 μm for IL-10. (c) Number of Foxp3-positive cells in the skin in high power filed (HPF, ×400). (d) Number of Foxp3-positive large cells in HPF. Results are expressed as the mean ± standard deviation. N = 6 or 7 for each group. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001

Foxp3: forkhead box P3

Figure 3

Hormone replacement treatment inhibits the incomplete induction of imiquimod-induced psoriasis-like dermatitis. Mice received a sham operation (Sham), ovariectomy (OVX), or OVX with hormone replacement treatment with 17β-oestradiol (HRT) 1 week before initiating daily application of imiquimod under inhalation anesthesia with sevoflurane. In this setting, mice wiped the imiquimod cream immediately after application, which results in induction of mild inflammation with infrequent infiltration of neutrophils even in Sham-mice, , so this model is referred to as incomplete induction. (a) Representative pictures of the psoriasis-like dermatitis in the three groups. (b) Clinical scores of each group. (c) Haematoxylin and eosin staining and immunostaining for loricrin and involucrin of the skin from the three groups. (d) Number of infiltrating cells in the skin (×200). Scale bar = 50 μm. (e) Epidermal thickness. (f, g) Scores of immunostaining for loricrin and involucrin of the skin from three groups as described before. (h) Cytokine expression in the skin were evaluated by quantitative reverse transcriptase-polymerase chain reaction. (i, j) Representative of immunostaining for IL-10 and Foxp3. (k) Percentage of Foxp3-positive cells among all infiltrating cells. Results are expressed as mean ± standard deviation. N = 6 or 7 for each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p <$

0.001

Foxp3: forkhead box P3