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Female sex hormones ameliorate arthritis in SKG mice

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ABSTRACT

The pathology of rheumatoid arthritis (RA) is ameliorated during pregnancy and deteriorated after delivery. Thus, female sex hormones could be involved in the pathogenesis of RA. However, the effects of estrogen and progesterone on the development and progression of RA have been unclear. In this study, we analyzed the effects of female hormones on the pathogenesis of RA by performing ovariectomy (OVX) and hormone implantation in the SKG mouse model of human RA. OVX mice showed severe arthritis and cartilage destruction with increased serum levels of TNF-α and IL-6, when compared with sham-operated mice. In contrast, estrogen-treated mice exhibited remarkable suppression of arthritis, with no bone erosion, little synovial hyperplasia and little infiltration of immune cells. Moreover, serum levels of TNF-α and IL-6 were decreased. In progesterone-treated mice, mild synovial hyperplasia and no immune cell infiltration were observed, with decreased serum levels of IL-6. These results suggest that female hormones, estrogen and progesterone, can play roles in the remission of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that primarily affects multiple joints, occurring in 0.5–1.0% of the world's population. RA is characterized by synovial inflammation, proliferation of synoviocytes, formation of pannus, cartilage destruction, bone erosion, and production of proinflammatory cytokines [1,2]. Among these proinflammatory cytokines, TNF-α and IL-6 have been proven to play critical roles in the development of RA [3,4]. Moreover, biological agents that antagonize TNF-α and IL-6 are highly effective treatments for RA [5,6]. However, about 30% of RA patients fail to respond to anti-TNF-α agents, and these therapeutic strategies (dependent upon the use of biological agents) are expensive and associated with risks for possible side effects including infections and lymphomas [7]. Therefore, we need to develop novel and different types of therapies for RA, especially for patients who fail to respond to biological agents.

Hormonal factors, such as sex hormones, are likely involved in the pathogenesis of RA. Rheumatoid arthritis is more prevalent in women than men, with a ratio of 3:1 [8], and the peak incidence of RA in women is found during the perimenopausal period [9]. A decrease of sex hormone levels during the perimenopausal period may affect the development and progression of RA. On the other hand, the clinical features of RA are ameliorated in approximately 75% of pregnant patients who have high serum levels of sex hormones [10]. Also, a recent study reported that pregnancy can induce complete remission in the SKG mouse model of human RA [11]. This evidence indicates that alterations of serum levels of estrogen and progesterone during the perimenopausal period and pregnancy may affect the pathogenesis of RA. However, it remains unclear whether estrogen and progesterone are involved in amelioration of RA during pregnancy.

To uncover the pathophysiological mechanisms of RA, several animal models of inflammatory arthritis have been developed. The SKG mouse strain, established by Sakaguchi and colleagues [12], provides a convenient animal model of human RA. SKG mice spontaneously develop chronic arthritis under conventional microbial conditions [12], or following injection of β-glucan-containing products under specific-pathogen-free conditions [13]. In contrast to collagen-induced arthritis, SKG mice exhibit not only arthritis but also extra-articular manifestations, such as pneumonitis and rheumatoid nodules. SKG mice also develop high titers of autoantibodies, including rheumatoid factors and type II collagen antibodies. Thus, the characteristics of the SKG mouse model generally resemble the pathogenesis of human RA in its clinical and histological characteristics of joint inflammation and in serological features [12]. However, the pathophysiological roles of female sex hormones in the development and progression of arthritis in SKG mouse model have not been addressed.

To analyze the effects of alterations in estrogen and progesterone levels on the pathogenesis of human RA, we used SKG mice

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to examine whether female hormone depletion or administration alters the development and the progression of arthritis.

2. Materials and methods

2.1. Animals

SKG/Jcl mice were purchased from CLEA Japan, and were housed in a specific-pathogen-free facility under climate-controlled conditions with a 12-h light/dark cycle and were provided with water and standard diet (CE-2, CLEA, Japan) ad libitum. All animals were maintained according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

2.2. Ovariectomy and hormone pellet implantation

Eight-week-old female SKG littermates underwent an ovariectomy or a sham operation under anesthesia two weeks before arthritis induction. Slow releasing pellets of E2 (0.83 mg/day) or P4 (1.25 mg/day: Innovative Research, Sarasota, FL) were implanted subcutaneously in the inguinal region of SKG mice at nine weeks of age, one week before arthritis induction.

2.3. Arthritis induction and scoring of clinical signs

Two weeks after ovariectomy, or one week after hormone pellet implantation, arthritis was induced according to a previously published method [14]. Female SKG mice were injected intraperitoneally with three mg curdlan (Wako) to induce arthritis, and then mice were monitored for five weeks. Clinical scores were monitored weekly and scored using a previously published system [12]: 0, no swelling or redness; 0.1, swelling or redness of digit; 0.5, mild swelling and/or redness of wrists or ankle joint; 1, severe swelling of larger joints. Scores of affected joints were totaled for each mouse.

2.4. Microcomputed tomography (μCT) and histological analysis

Five weeks after arthritis induction, mice were euthanized, and legs were removed and fixed with 4% PFA in PBS overnight. μCT scanning of the joints was performed using a SCANCO Medical μCT35 System (SCANCO Medical) with an isotropic voxel size of six μm. After μCT scanning, joints were decalcified in Morse's Solution (10% sodium citrate, 20% formic acid), and embedded in paraffin. Six μm thick tissue sections were stained with hematoxylin and eosin, and imaged (Zeiss).

2.5. Measurement of serum hormones

Serum levels of 17β-estradiol (E2) and progesterone (P4) were measured by ECLIA (SRL, Tokyo, Japan). Serum levels of TNF-α and IL-6 were measured with an ELISA kit according to the manufacturer's instructions (eBioscience).

2.6. Statistical analysis

Clinical score, serum levels of 17β-estradiol, progesterone, TNF-α, and IL-6 were analyzed by a two-tailed Student's *t*-test. Incidence was analyzed with Fisher's exact test. For all graphs, data are represented as means±SEM. Statistical significance is indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. OVX deteriorated arthritis development in SKG mice

We sought to determine the pathophysiological role of female sex hormones in RA. Thus, we assessed arthritis development in SKG mice in which female sex hormone deficiency was achieved by OVX. Two weeks after OVX, arthritis was induced by treating female SKG mice with three mg curdlan i.p., and the development of arthritis was measured weekly. Arthritis incidence and clinical

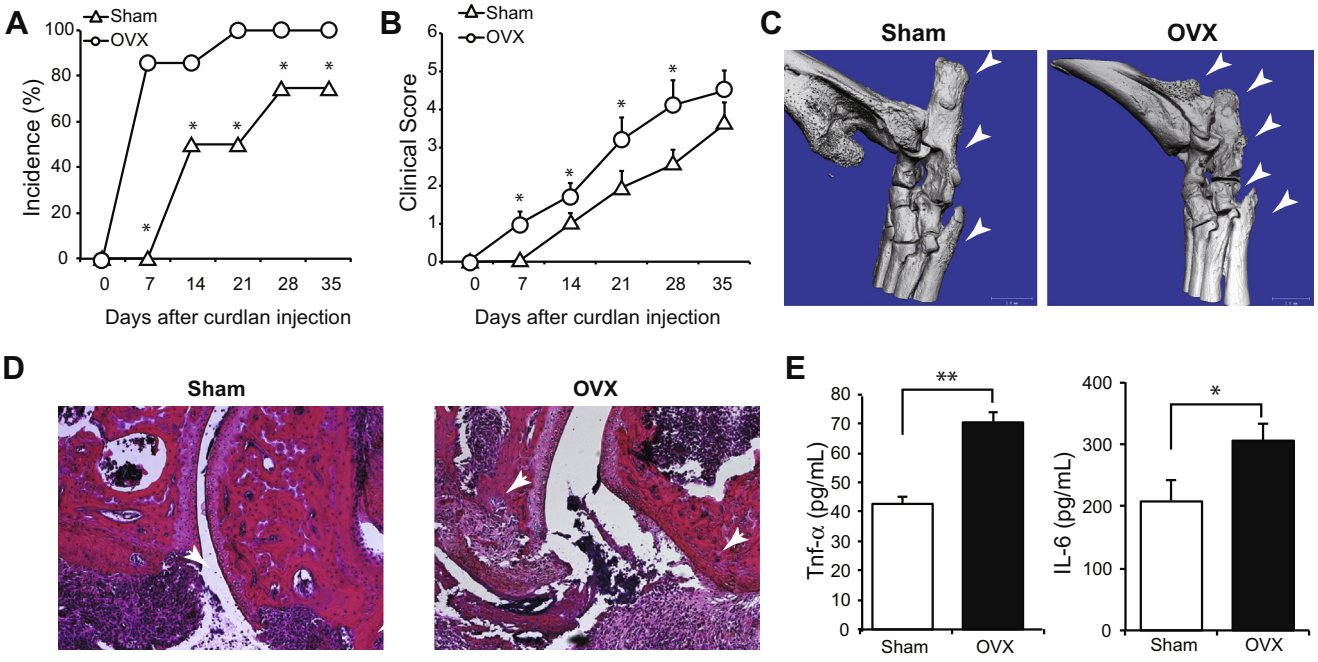

Fig. 1. Arthritis development was deteriorated in ovariectomized mice. Female SKG mice (eight weeks old) underwent OVX ($n = 6$) or a sham operation ($n = 6$) two weeks before arthritis induction. (A) Arthritis incidence and (B) clinical score were recorded for 35 days. (C) Representative μCT scans of left hind paw of SKG mice 35 days after arthritis induction. (D) Representative sections of the talocrural joints stained with H&E. (E) Serum levels of TNF-α and IL-6 were determined by ELISA. Data are presented as means±SEM. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

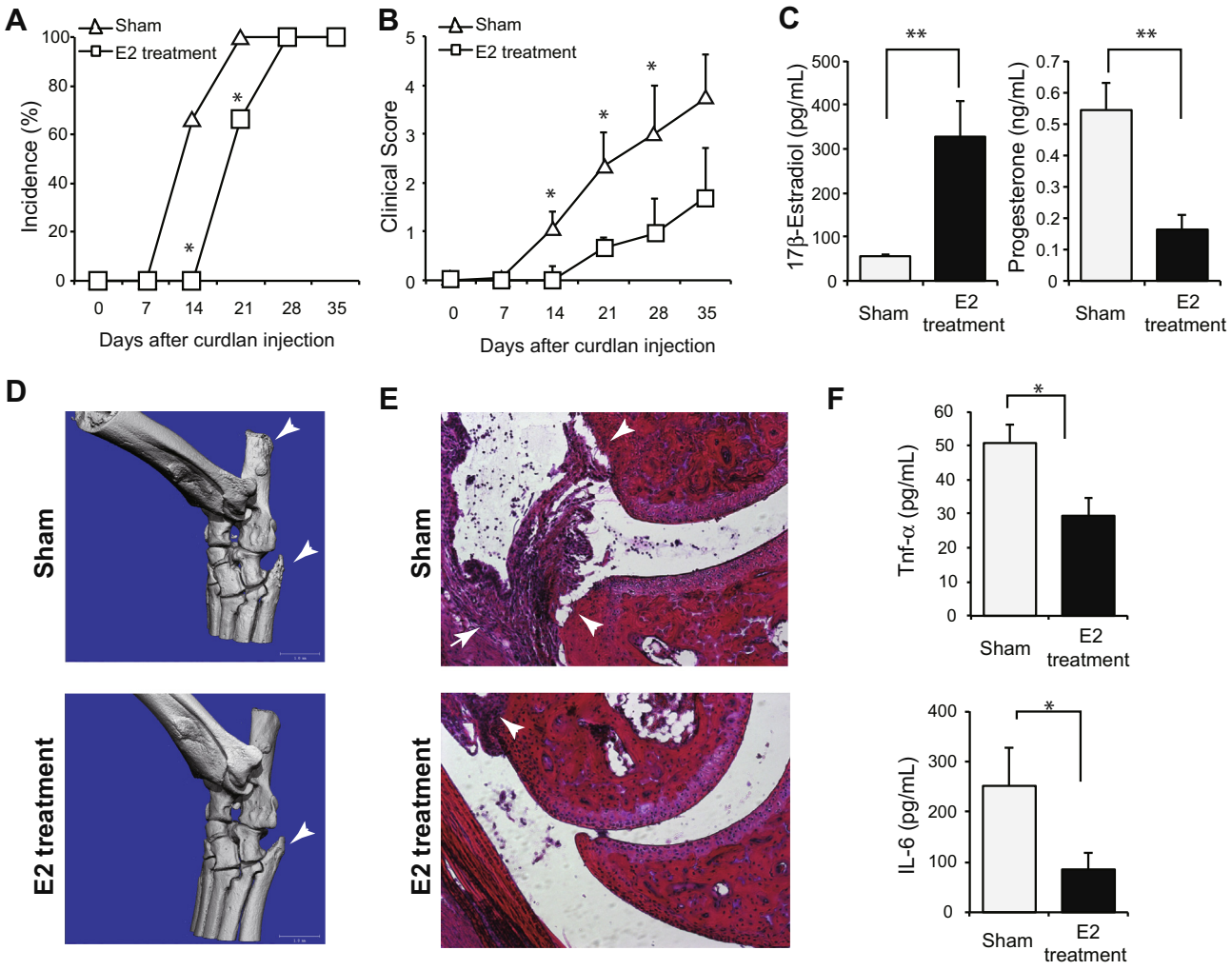

Fig. 2. E2 treatment suppressed arthritis development in SKG female mice. E2 pellets were implanted subcutaneously in the inguinal region of SKG mice one week before arthritis induction (sham: $n = 6$; E2 treatment: $n = 6$). Serum was collected from each mouse 35 days after arthritis induction. (A) Arthritis incidence and (B) clinical score were recorded for 35 days. (C) Serum levels of E2 and P4 measured by ECLIA. (D) Representative μCT scans of left hind paw of SKG mice 35 days after arthritis induction. (E) Representative sections of joints stained with H&E. (F) Serum levels of TNF-α and IL-6 were determined by ELISA. Data are presented as means±SEM. ∗ and ∗∗ indicate $p < 0.05$ and $p < 0.01$, respectively.

scores significantly progressed in OVX-mice from a week after arthritis induction (Fig. 1A and B). To evaluate the changes in joint structure between sham and OVX mice, we performed μCT analysis 35 days after arthritis induction. The fifth metatarsal bone and calcaneus were moderately destroyed in sham mice (Fig. 1C, left panel). In contrast, more severe destruction of the fifth metatarsal bone, calcaneus and cuboid was observed in OVX mice (Fig. 1C, right panel). OVX mice also exhibited periosteal heterotopic bone formation at the distal tibia, which we did not observe in sham-operated mice (Fig. 1C). Moreover, we performed histological evaluation of the talocrural joint using the same sample used for μCT analysis. Talocrural joints of sham-treated mice exhibited moderate subchondral bone erosion with infiltration of inflammatory cells and pannus formation (Fig. 1D, left panel), whereas the joints of OVX mice showed more severe cartilage and bone destruction, inflammatory cell infiltration and pannus formation (Fig. 1D, right panel). To evaluate the effects of ovariectomy on proinflammatory cytokine production, we measured serum TNF-α and IL-6 levels. As the result, serum levels of TNF-α and IL-6 in OVX mice were significantly increased compared with those in sham-operated mice (Fig. 1E). These results indicate that OVX-induced deficiency of female sex hormones deteriorated the development of arthritis in the SKG mouse model via up-regulating the production of TNF-α and IL-6.

3.2. E2 treatment suppressed arthritis development in SKG mice

Based on the observed deterioration of RA in OVX mice, we determined whether female hormones, E2 or P4, were required for the suppression of RA. Thus, we examined the effect of E2 and P4 on arthritis development in SKG female mice. First, we evaluated the pathophysiological role of E2 in arthritis development. Either E2 pellet implantation or a sham-operation was performed one week before arthritis induction. Weekly clinical observations revealed that the onset of arthritis was significantly delayed (Fig. 2A) and clinical scores were markedly suppressed in E2-treated mice compared with sham-operated mice (Fig. 2B). In E2-treated mice, the serum level of E2 was elevated compared with sham-operated mice (Fig. 2C). In addition, μCT analysis revealed that the fifth metatarsal bone and calcaneus were destroyed in sham-operated mice (Fig. 2D, left panel), whereas only the fifth metatarsal bone was damaged in E2-treated mice (Fig. 2D, right panel). In histological evaluation, the joints of sham-operated mice showed moderate subchondral bone erosion with infiltration of

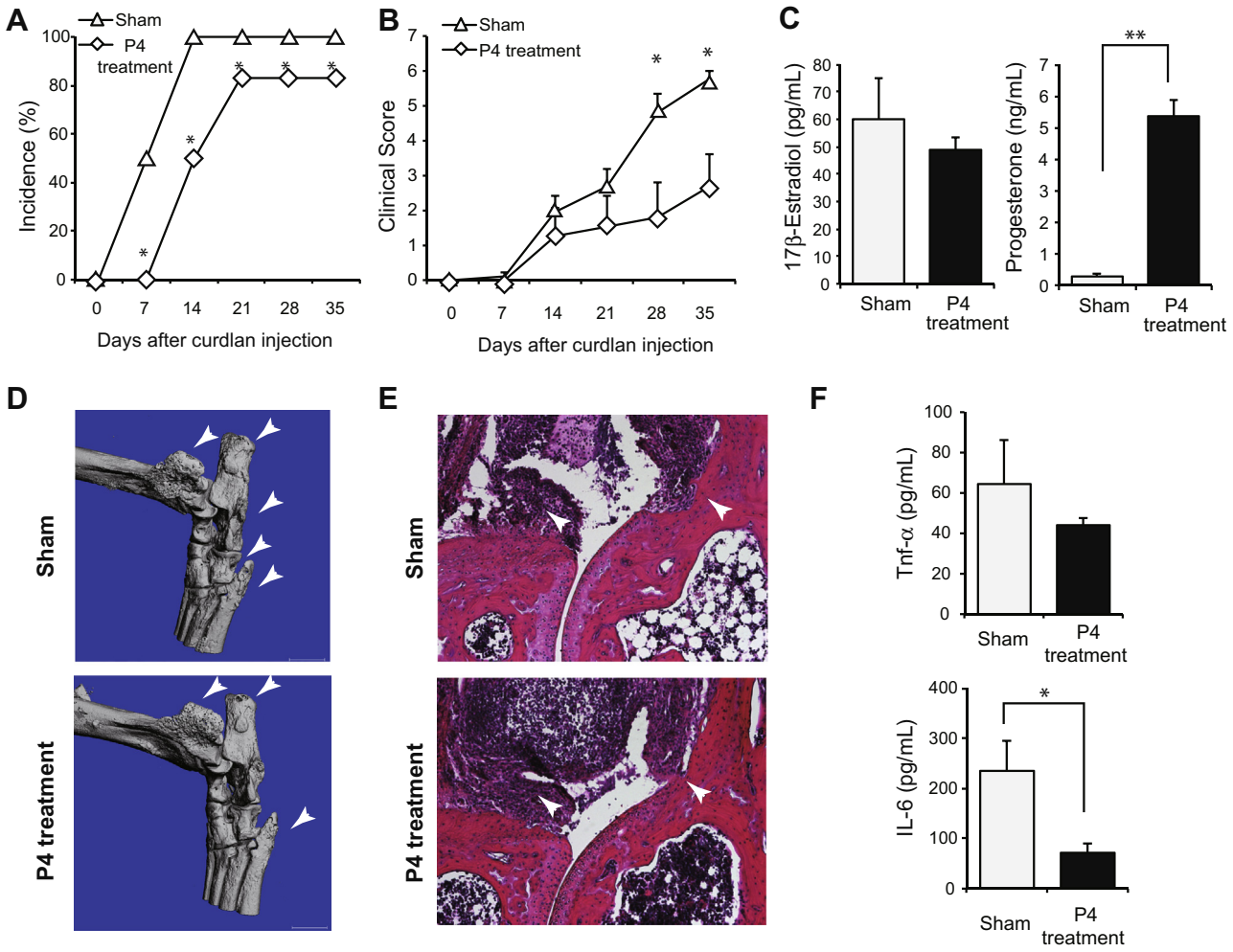

Fig. 3. P4 treatment ameliorated arthritis development in SKG female mice. P4 pellets were implanted in the same way as E2 pellets (sham: $n = 6$; P4 treatment: $n = 6$). Serum was collected from each mouse 35 days after arthritis induction. (A) Arthritis incidence and (B) clinical scores were recorded for 35 days. (C) Serum levels of E2 and P4 were measured by ECLIA. (D) Representative μCT scans of the left hind paw of SKG mice 35 days after arthritis induction. (E) Representative sections of joints stained with H&E. (F) Serum levels of TNF-α and IL-6 determined by ELISA. Data are presented as means±SEM. $*$ and $**$ indicate $p < 0.05$ and $p < 0.01$, respectively.

inflammatory cells and pannus formation. On the other hand, the joints of E2-treated mice exhibited no cartilage erosion, little synovial hyperplasia and small infiltration of inflammatory cells (Fig. 2E). Serum TNF-α and IL-6 levels were measured to confirm the effects of E2 treatment on proinflammatory cytokine production. We detected reduced serum TNF-α and IL-6 levels in E2-treated mice compared with those in sham-operated mice (Fig. 2F). These results suggested that E2 treatment could suppress the development of arthritis and inhibit production of TNF-α and IL-6.

3.3. P4 treatment ameliorated arthritis development in SKG mice

We next assessed the effects of P4 on arthritis development in SKG female mice. P4 pellets were implanted in the same way as the E2 treatment. The onset of arthritis in P4-treated mice was significantly delayed and suppressed (Fig. 3A). There was not a significant difference in the clinical scores between P4-treated mice and sham-operated mice until day 21 of arthritis induction. However, the clinical scores of P4-treated mice were significantly suppressed from day 28 to day 35 compared with those of sham-operated mice (Fig. 3 B). Although the serum E2 level was not altered in P4-treated mice, a significant increase of the serum P4 level was confirmed in P4-treated mice, compared with that in sham-operated mice (Fig. 3C). In μCT analysis, the fifth metatarsal bone, calcaneus and cuboid were moderately damaged, and periosteal heterotopic bone formation was observed in sham-operated mice (Fig. 3D, left panel). In contrast, the destruction of the fifth metatarsal bone and calcaneus was milder in P4-treated mice compared with that of sham-operated mice, although periosteal heterotopic bone formation was also observed (Fig. 3D, right panel). In histological evaluation, the joints of sham-operated mice showed moderate subchondral bone erosion with infiltration of inflammatory cells and synovial hyperplasia. On the other hand, synovial hyperplasia was also observed in the joints of P4-treated mice; however, neither subchondral bone erosion nor infiltration of inflammatory cells was confirmed (Fig. 3E). To determine the effects of P4 treatment on proinflammatory cytokine production, we analyzed serum TNF-α and IL-6 levels. There was no significant difference in serum TNF-α levels between P4-treated mice and sham-operated mice, while serum IL-6 levels of P4-treated mice were increased compared with those of sham-operated mice (Fig. 3F). These results suggested that P4 treatment might inhibit the development of arthritis through repression of IL-6 production.

4. Discussion

In this study, we showed that female sex hormone deficiency permitted progression of arthritis in the SKG mouse model of RA

with increasing serum levels of proinflamatory cytokines, such as TNF-α and IL-6. This result recapitulated the high incidence of RA observed in female patients during the perimenopausal period and deterioration of RA after delivery. Therefore, our results indicated that decreased serum levels of female sex hormones might permit the onset and progression of RA in female patients.

We also demonstrated that arthritis development was markedly repressed in E2-treated mice and P4-treated mice. E2-treated mice showed little synovial hyperplasia and small infiltration of inflammatory cells with decreased serum levels of TNF-α and IL-6, whereas P4-treated mice showed synovial hyperplasia and no infiltration of immune cells, with inhibition of IL-6, but not TNF-α, production. These results indicated that transcription of TNF-α gene might be regulated by E2/estrogen receptor signaling, but not P4/progesterone receptor signaling.

In autoimmune diseases, it has been reported that estrogen has both immuno-stimulating and immuno-suppressive functions, while progesterone has an immuno-suppressive function [15,16]. A recent study reported that estrogen treatment exacerbated the symptoms of systemic lupus erythematosus (SLE) in a NZB/NZW F1 mouse model [17]. However, progesterone inhibited lupus development in some situations [18]. In contrast, estrogen administration inhibited disease development of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, by inhibition of Th1 and Th17 cell differentiation and suppression of the production of proinflammatory cytokines, such as TNF-α, IFN-γ and IL-17 [19]. Moreover, estrogen suppressed hepatocellular carcinoma by inhibition of IL-6 production [20]. Progesterone also exerted protective effects on EAE development via suppression of IL-2 and IL-17 production [21]. However, the progesterone-targeted immune cells are still unknown. In addition, progesterone enhanced immuno-modulatory effects of mesenchymal stem cells by up-regulating IL-6 production [22]. In RA, IL-6 is produced by synovial fibroblasts after stimulation by TNF-α [23]. Taken together, these previous reports and our current results suggest that estrogen might ameliorate inflammation of arthritis by regulating Th1 and Th17 cell differentiation and by suppression of TNF-α and IL-6 production, while progesterone might inhibit arthritis development, especially immune cell infiltration via suppression of IL-6 production by synovial cells. Moreover, the results from the histological analyses suggested that bone destruction in arthritis was also ameliorated in E2 and P4 treatment mice (Figs. 2 and 3). In RA, osteoclasts can be differentiated and activated by TNF-α and IL-6, resulting in destruction of bone and articular cartilage [24,25]. From this point of view, reduced bone erosion in E2 and P4 treatment mice might be caused by inhibition of TNF-α and/or IL-6 production. On the other hand, it has been reported that estrogen directly suppresses bone resorption via inducing apoptosis of osteoclasts [26], although effects of progesterone on osteoclast functions are still unknown. Therefore, estrogen and progesterone might directly exert their effects on inhibition of bone erosion in RA through suppression of osteoclast differentiation, longevity and/or activity. Taken together, our results suggest that estrogen and progesterone might play different yet crucial roles in the development of RA. Also, our data indicate that estrogen and progesterone might be involved in pregnancy-induced amelioration of RA.

Although we demonstrated the involvement of estrogen and progesterone in the pathogenesis of RA, the molecular basis of estrogen and progesterone action is still unclear because hormone administration alters systemic endocrine systems. Moreover, we need to determine which cells (synovial fibroblasts, macrophages, T cells or other immune cells located in lesions of arthritis) play critical roles in the estrogen- and progesterone-induced amelioration of RA development. It is well known that the actions of estrogen and progesterone are mediated by estrogen receptors (ERα, ERβ) and progesterone receptors (PR-A, PR-B) [27,28]. Genetic mouse models lacking ERs and PRs can provide insights into the molecular basis of actions of estrogen and progesterone [29–31]. In addition, Cre/loxP technology provides new insights into tissue- and/or cell-type specific molecular functions of ERs and PRs [26,32,33]. Therefore, to determine the cell-type specific functions of ERs and PRs in the pathogenesis of RA, conditional knockout mice of ERs and PRs should be examined.

Our results indicate that estrogen and progesterone treatment might be effective for therapeutic approaches to RA. However, hormone treatment may cause various adverse effects, including increased risks of endometrial and breast cancer, venous thromboembolism and gallbladder disease [34]. Therefore, selective estrogen receptor modulators (SERMs) and selective progesterone receptor modulators (SPRMs) have been developed to reap the benefits of the hormones while preventing their side effects [35,36]. Indeed, it was reported that tamoxifen, one of the SERMs, exerted beneficial effects on SLE in NZB/NZW F1 female mice [37]. Further studies are needed to evaluate the effects of SERMs and SPRMs on the pathogenesis of RA in the SKG mouse model.

There is a limitation in our current study. In our study, the duration until disease onset and the severity of arthritis in sham-operated mice were varied among each series of experiments. Possible reason of this issue can be considered that we did not perform all the experiments simultaneously and we used SKG mice born in different date among each series of experiments. However, statistical significant differences in phenotypes of arthritis development between sham and treatment groups in each experiment can support our claim.

In conclusion, although further studies are needed to reveal the molecular mechanisms of ERs and PRs in the pathogenesis of RA, treatment with SERMs and SPRMs might offer novel therapies for RA patients who fail to respond to treatment with biological agents.

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