



17 β -Estradiol enhances expression of inflammatory cytokines and inducible nitric oxide synthase in mouse contact hypersensitivity

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Abstract

The effects of 17 β -estradiol (E2) on the expression of cytokines, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 in mouse contact hypersensitivity (CHS) were examined. Three week old female mice were ovariectomized, administered 3.2 μ g of E2 subcutaneously, the mice sensitized by application of 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXA) on their backs, and CHS induced by applying OXA to the auricles. E2 significantly increased mRNA expression of interferon (IFN)- γ and interleukin (IL)-10 in the auricle at 6 and 24 h after allergy elicitation in the ear, respectively, when compared to untreated controls. Although there was no effect of E2 on the expression of IL-4 and COX-2 at any time, the expression of iNOS mRNA was increased by E2 treatment at 48 h after elicitation. E2 also enhanced the expression of tumor necrosis factor (TNF)- α and IL-1 β . Histological evaluation revealed that E2 promoted edema of the auricle dermis. The hyperplasia of the epidermis was suppressed by E2 and the cell infiltration observed after elicitation was not altered by E2. These results suggest that E2 enhances the expression of IFN- γ , TNF- α , and IL-1 β to augment the edema of auricle dermis in mouse CHS.

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1. Introduction

Environmental pollutants are believed to be involved in the onset and/or exacerbation of allergies [1]. In particular,

endocrine disrupting chemicals such as 17 β -estradiol (E2) are considered to affect immune responses. E2 has been reported to exert immunological effects in conditions such as menstrual asthma [2], symptom changes in systemic lupus erythematosus [3], and rheumatoid arthritis during pregnancy [4]. However, the mechanisms of the immunological actions of E2 are not fully understood.

While some reports have shown that E2 enhances the Th2-type immune response, other reports have demonstrated that E2 enhances contact hypersensitivity (CHS) as a Th1-type

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immune response, and expression of interferon- γ (IFN- γ) as a Th1-type cytokine [5–8]. Human contact dermatitis, a Th1-type immune reaction, is more severe in the ovulation phase than in the luteal phase [5], while numerous experiments in mice have demonstrated that E2 enhances the production of IFN- γ [6–8]. Hence, it has been suggested that E2 may enhance contact dermatitis by increasing IFN- γ production.

We previously reported that E2 enhances the expression of IFN- γ and augments CHS that is induced by 4-ethoxymethylene-2-phenyl-2-oxazolin-one (OXA) in the mouse auricle [9]. In that study, E2 administration led to an increase in ear swelling at 6 h after allergy elicitation, increased ear swelling of female mice at 24 h. Following CHS in male CD-1 mice, edema of the auricle was evident before 24 h, and cell infiltration was evident from 24 h after elicitation [10]. These data suggest that E2 enhances edema and increase the thickness of the ear.

Edema can be induced by vascular dilation evoked by chemical mediators, including nitric oxide produced by vascular endothelial cells and macrophages, and prostaglandin E2 produced by mast cells [11]. Vascular dilation is also induced by inflammatory cytokines such as IFN- γ , tumor necrosis factor (TNF)- α , and interleukin (IL)-1 β [12,13]. We have previously reported that E2 enhanced inducible nitric oxide synthase (iNOS) in the mouse macrophage cell line J774.1, and that E2 enhanced IFN- γ in mouse CHS [9,14]. These data suggest that E2, both directly and indirectly, enhances iNOS expression to dilate vascular vessels.

To clarify how E2 enhances CHS we assayed expression of various genes including IFN- γ (as a Th1 and inflammatory cytokine), IL-4 and IL-10 (as Th2 cytokines), TNF- α and IL-1 β (as inflammatory cytokines), and iNOS and cyclooxygenase (COX)-2 (as enzymes that produce chemical mediators for vascular dilation).

2. Methods

2.1. Animal gonadectomy and E2 administration

BALB/c mice (Japan SLC Co., Shizuoka, Japan) were treated as previously described [15]. Briefly, all treatments and maintenance of animals were conducted in a specific pathogen-free room at 23 °C and 47%–67% humidity under a 12 h light–dark cycle (lights on, 7:00 a.m.). Three week old mice were gonadectomized under pentobarbital anesthesia, and 3.2 μ g of E2 (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in 100 μ l of olive oil was subcutaneously injected (0.32 mg/kg body weight). As a negative control the same volume of olive oil alone was administered. Permission for this study was obtained from the Animal Experiment Room Administration Committee of Setsunan University, and the experiments were conducted according to the Animal Experiment Guidelines of the Faculty of Pharmaceutical Sciences, Setsunan University.

2.2. Contact hypersensitivity

After the administration of E2, the backs of the mice were shaved with a clipper under pentobarbital anesthesia, and the mice were sensitized by topical application of 100 μ l of 3% OXA (Sigma-Aldrich Inc., St. Louis, MO) in a 3:1 (v/v) mixture of ethanol and acetone. After 7 days, the mice were challenged by applying 7.5 μ l of 1% OXA in olive oil on both sides of the right auricle, and 7.5 μ l of olive oil on both sides of the left auricle. The allergy elicitations were carried out at 8:00 a.m. after considering the circadian rhythms of mice [16]. Auricle thickness was measured under ether anesthesia using a

digital thickness gauge (Ozaki MFG Co. Ltd., Tokyo, Japan), taking care to avoid denting the edematous skin. The degree of ear swelling was calculated by subtracting the thickness of the left ear from that of the right ear.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) of IFN- γ and IL-4 mRNAs

CHS mice that were either treated with E2, or untreated, were anesthetized with pentobarbital. The uteri were excised and weighed. The right auricles were excised, immersed in Trizol (Gibco BRL Invitrogen Co., Carlsbad, CA), and stored at –80 °C until RNA extraction according to the guanidinium isothiocyanate method. An aliquot (2 μ g) of each total RNA sample was reverse-transcribed using a Superscript First-Strand Synthesis System (GibcoBRL Invitrogen Co.), and 1 μ l of the obtained cDNA solution was used for PCR with Ex Taq polymerase (Takara Bio Inc., Shiga, Japan). Primer sets for IFN- γ , IL-4, IL-10, TNF- α , and IL-1 β were obtained from Takara Bio Inc., while the primer sets for β -actin (an internal control), iNOS, and COX-2 were obtained from GibcoBRL Invitrogen Co. The amplified DNA products were visualized by electrophoresis followed by ethidium bromide staining, and the intensities of the bands on the photographs were analyzed using a charge coupled device (CCD) camera “Light-Capture” (Atto Co., Tokyo, Japan) and software “CS analyzer” (Version 2.06a; Atto Co.). The mRNA levels were calculated as ratios relative to the corresponding β -actin mRNA level.

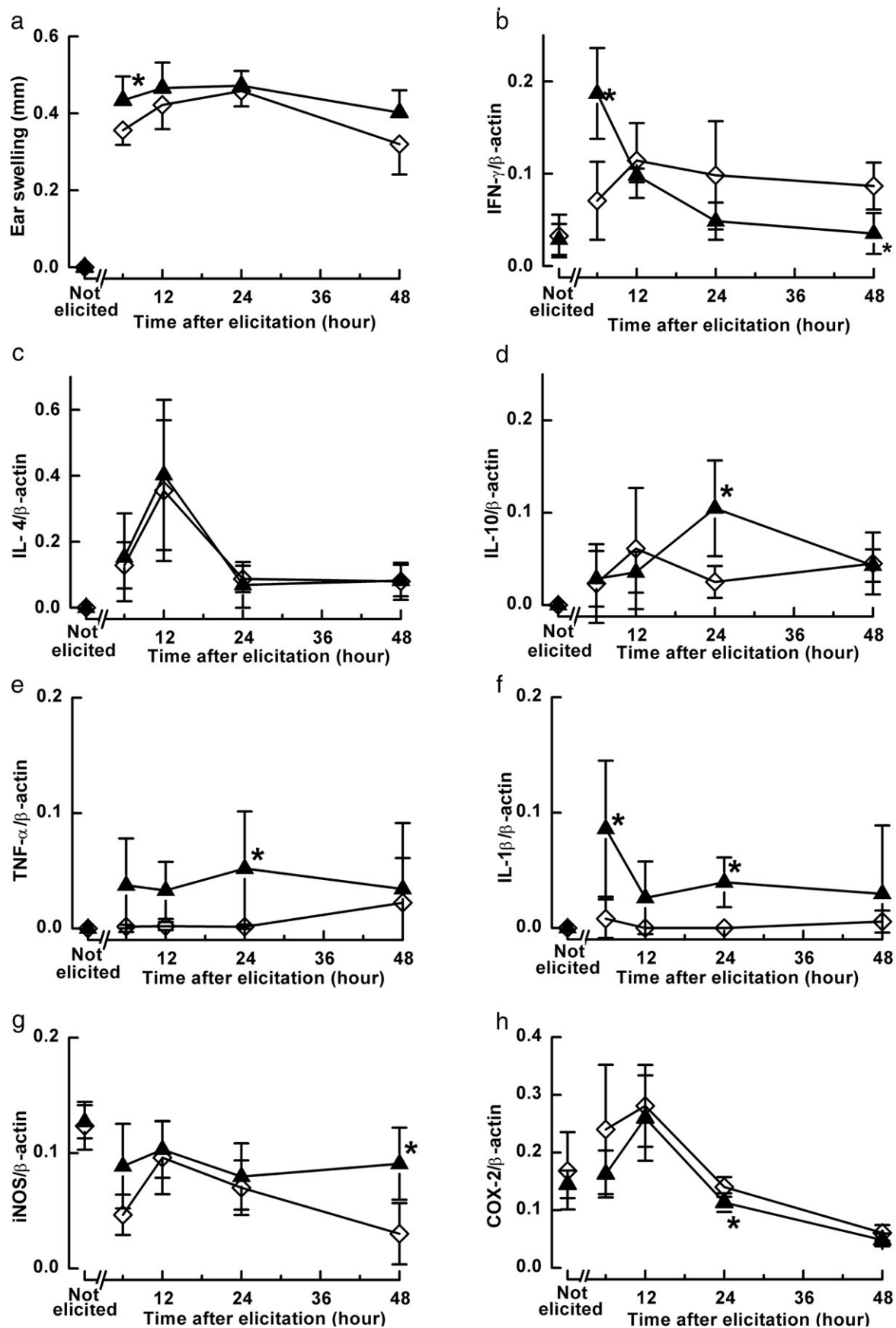
2.4. Statistical analysis

The values obtained from 4 to 5 mice were calculated as the mean $\pm$ standard deviation. Differences among the standard deviations were evaluated by Fisher's *F* tests, while the differences of the means between the vehicle- and E2-treated mice were analyzed by two sample Student's *t*-tests. A *p* value less than 0.05 was considered statistically significant.

3. Results

Auricle swelling in vehicle-treated mice was observed from 6 h after allergy elicitation (Fig. 1a). The swelling was maximum at 24 h, and declined at 48 h. Auricle swelling in E2-treated mice was significantly greater than that in vehicle-treated mice at 6 h, and showed a non-significant trend to increase further at 48 h. These auricle swelling results were similar to our previous report [9].

mRNA expression of IFN- γ (a Th1 type cytokine and an inflammatory cytokine) at the inflamed auricles of vehicle-treated mice was observed from 6 to 48 h after allergy elicitation. E2 significantly increased IFN- γ expression at 6 h when compared to the vehicle-treated mice, and significantly decreased IFN- γ expression at 48 h (Fig. 1b). The mRNA expression of two Th2 type cytokines, IL-4 and IL-10, was determined. IL-4 expression was maximum at 12 h after allergy elicitation in the vehicle-treated group, the levels of which were not altered with E2 (Fig. 1c). IL-10 expression was significantly increased by E2 at 24 h after allergy elicitation when compared to the vehicle-treated mice (Fig. 1d). The mRNA expression of iNOS was significantly increased by E2 at 48 h (Fig. 1e). COX-2 mRNA expression was maximum at 12 h after elicitation, then subsequently decreased at 24 and 48 h in the vehicle-treated group; there was no effect of E2 (Fig. 1f). The mRNA expression of two inflammatory cytokines, TNF- α and IL-1 β , was also determined. The individual differences in the expression levels of the cytokines in E2-treated mice



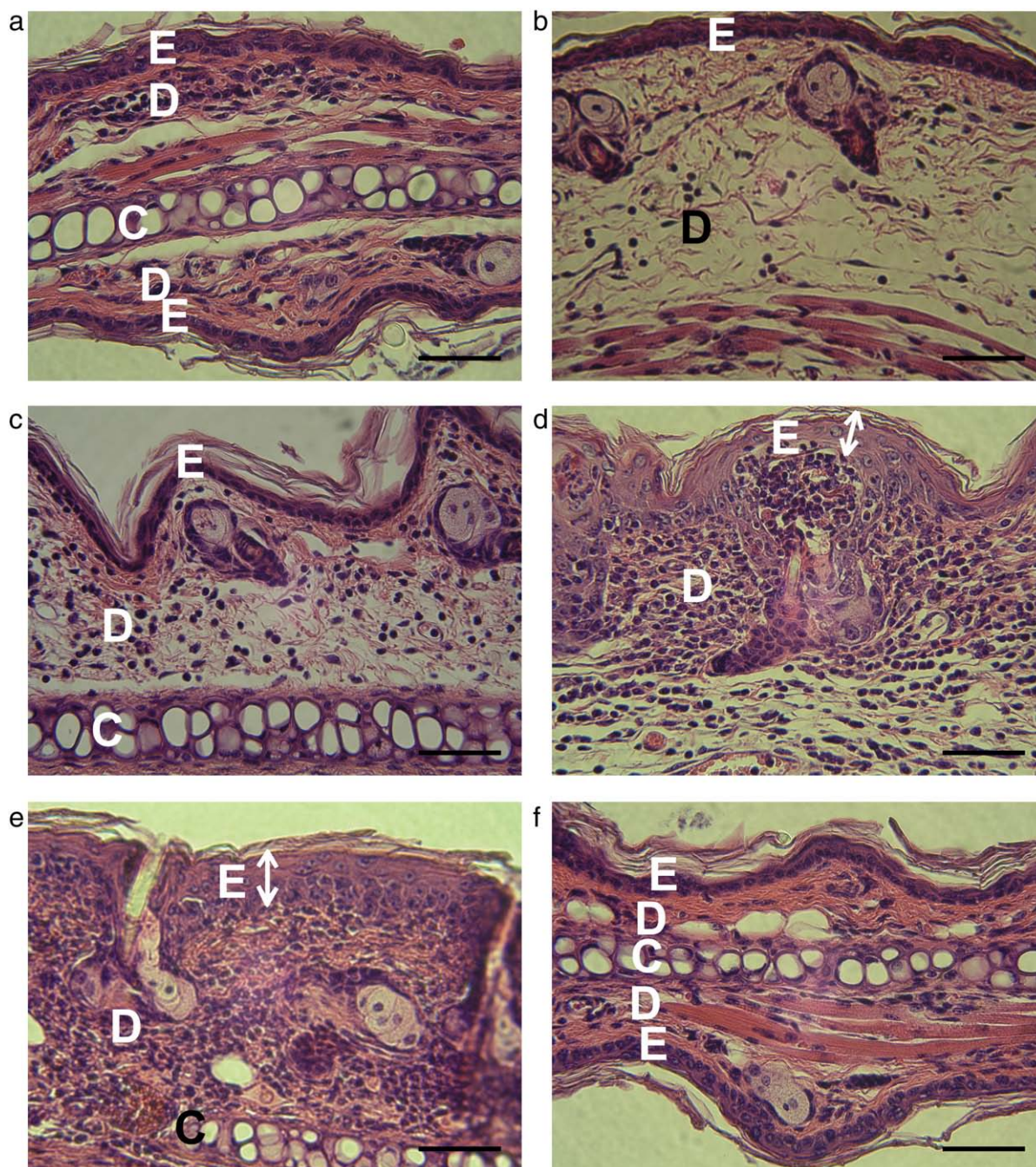


Figure 2 Time-course of histological changes in the auricle of mice following contact hypersensitivity (CHS). Gonadectomized female mice were administered either vehicle or E2. CHS in the ear was elicited by OXA, and the ear was fixed at different time points. (a–e) Images of vehicle-administered mice. (f–j) Images of E2-administered mice. (a and f) Immediately following challenge. (b and g) + 6 h. (c and h) + 12 h. (d and i) + 24 h. (e and j) + 48 h after the elicitation. C, cartilage; D, dermis; E, epidermis. Scale bars, 50 μ m. Representative data are shown.

were large; however, E2 increased TNF- α mRNA expression at 24 h and IL-1 β mRNA expression at 6 and 24 h when compared to the vehicle-treated mice (Fig. 1g,h).

The inflamed auricles of the mice were examined histologically with hematoxylin and eosin staining before elicitation, and at 6, 12, 24, and 48 h after elicitation (Fig. 2). The epidermises

Figure 1 The effects of E2 on ear swelling and mRNA expression levels in inflamed ears. Quantification of mRNA levels was based on the intensities of amplified cDNA product bands relative to that of β -actin. (a) Ear swelling, (b) IFN- γ , (c) IL-4, (d) IL-10, (e) iNOS, (f) COX-2, (g) TNF- α , (h) IL-1 β . Diamonds represent vehicle-administered mice; triangles represent E2-administered mice. Results are presented as mean $\pm$ S.D. ($n=4-5$ /group). * $p<0.05$.

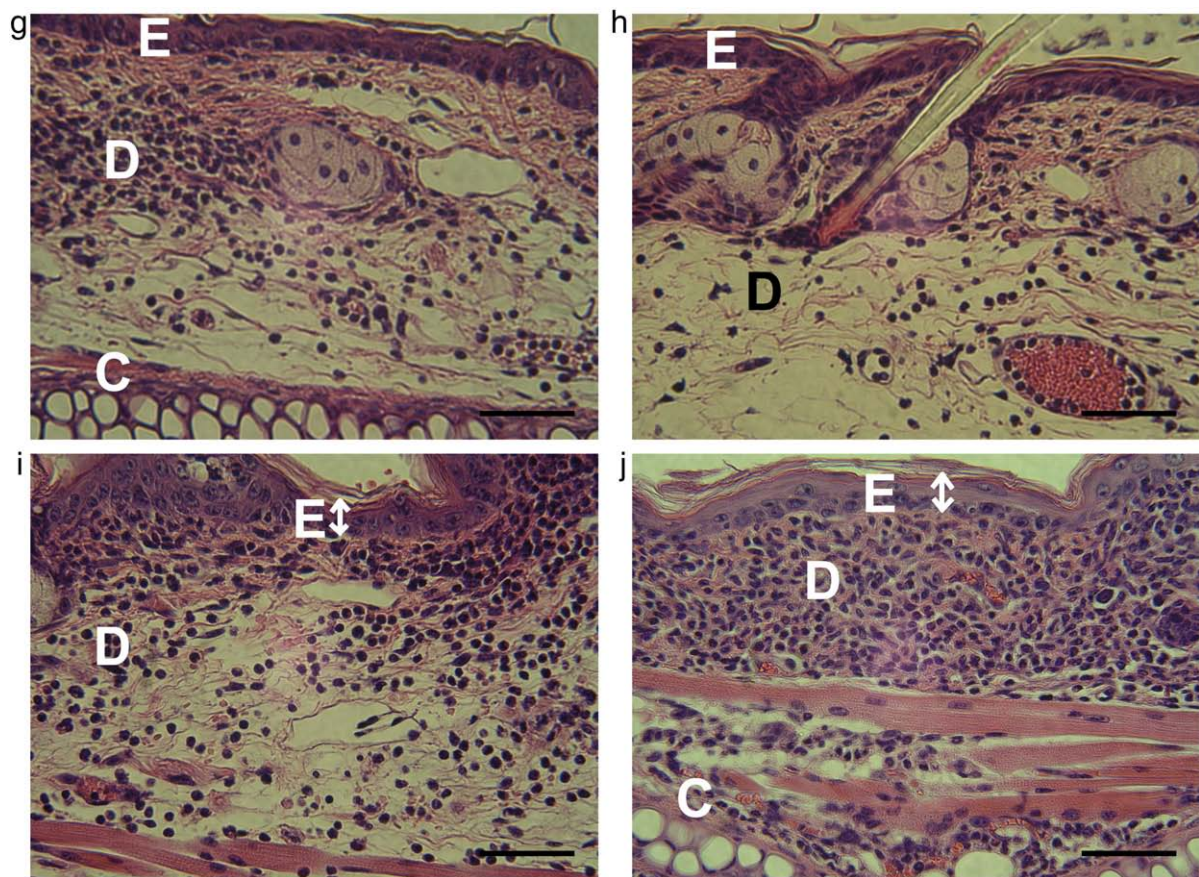


Figure 2 (continued).

of the vehicle-administered mice showed hyperplasia at 24 and 48 h (Fig. 2a–e). The dermis showed significant edema at 6 h after allergy elicitation (Fig. 2b,g), and infiltration of mononuclear cells from 12 h to 48 h (Fig. 2c,d,h,i). At 48 h, the edema of the dermis had almost disappeared, and the dermis was completely infiltrated by mononuclear cells (Fig. 2e,j). E2 looks like increasing cell infiltration in dermis (Fig. 2b,g) but the cell concentration is uneven and in the whole section there was no change in cell infiltration in the dermis of E2-administered mice when compared to vehicle-administered mice. The thickness of epidermis at 48 h of the control mice was $29.9 \pm 1.9 \mu\text{m}$ and that of the E2-administered mice was $16.0 \pm 3.0 \mu\text{m}$. The thickness of dermis at 6 h of the control mice was $126 \pm 28 \mu\text{m}$ and that of the E2-administered mice was $181 \pm 11 \mu\text{m}$. There was less hyperplasia of the epidermis of E2-administered mice than that of vehicle-administered mice at 24 and 48 h after allergy elicitation (Fig. 2d,e,i,j), while the edema was greater in the dermis of E2-administered mice until 24 h (Fig. 2g–j).

4. Discussion

CHS is a type IV allergy which involves Th1 cell responses [17]. An antigen that induces type IV hypersensitivity can also induce type I hypersensitivity when it is repeatedly applied [18]. Thus, CHS may also involve a Th2 type immune response. Numerous reports demonstrate that E2 enhances the Th2 response. Alternatively, E2 enhances the production of IFN- γ ,

a Th1 cytokine [6–8]. In the present study, E2 enhanced IL-10 expression at 24 h. Further, E2 enhanced IFN- γ expression before IL-10 expression. These data seem to contradict the Th1/Th2 balance paradigm, which suggests that Th1 type and Th2 type immune responses suppress each other [19], but it is not. Similar phenomena have been observed in human systemic lupus erythematosus (SLE) and a MRL/lpr mouse model of SLE [20,21]. In human SLE, the type-II response is dominant; however, some patients have a higher IFN- γ level [20]. The present study revealed that IFN- γ and IL-10 are produced sequentially, and not simultaneously. Askenase et al. have reported that B1, NK, and CD8 $^{+}$ T cells are active at 6 h after elicitation, and helper T cells active at 24 h [22]. Estradiol benzoate increases CD5 $^{+}$ B cells in the thymus of rats [23]. E2 promotes IFN- γ production by invariant natural killer T cells of C57BL/6J mice [24]. In our experience, E2 did not enhance IFN- γ expression of Jurkat cells stimulated by concanavalin A (data not shown), which may indicate that E2 did not induce Th1 cells to produce IFN- γ . Further investigation about whether E2 activates B1 or NK cells to produce IFN- γ should be needed.

In the present study, the expression of IFN- γ corresponded with the presence of auricle swelling. E2 enhanced auricle edema at 6 h after elicitation, and showed a tendency to enhance edema at 24 h when compared to vehicle-treated mice. The increase in IFN- γ expression at 6 h coincided with the increase in auricle swelling at 6 h. E2 failed to promote swelling at 24 h, enhanced IL-10 expression at 24 h, and suppressed the IFN- γ effect at 48 h.

We have reported that E2 enhances iNOS expression in the mouse macrophage J774.1 cell line [14]. In the present study, it was expected that macrophage activity would be augmented directly by E2, or indirectly via the E2-induced increase in IFN- γ . Further, we hypothesized that IL-10 and IL-4 would repress the macrophage activity. The present study demonstrated that iNOS expression was increased by E2 at 48 h after allergy elicitation, after IL-10 and IL-4 expression declined (Fig. 1e). Generated NO would dilate blood vessels and enhance edema or cell infiltration at 48 h after elicitation [25].

In the present study E2 did not enhanced COX-2 expression. There was also no facilitation of degranulation by E2 in RBL-2H3 cells, the rat basophilic leukemia cell line that has characteristics identical to those of mast cell (data not shown). These data indicate that E2 did not enhance mast cell activity, which corresponds with the absence of effects of E2 on immediate swelling.

The histological results confirmed that edema, especially in a lower part of dermis, was evident at 12 h after elicitation, and cell infiltration was evident from 24 h, as previously reported in male CD-1 mice [10]. E2 suppressed the edema of the epidermis; however, E2 promoted the edema of the dermis. A previous study demonstrated that E2 repressed the expression of inflammatory cytokines *in vitro* [26]. However, in the present study, E2 enhanced the expression of three inflammatory cytokines, IFN- γ (at 6 h), TNF- α (at 24 h), and IL-1 β (at 6 and 24 h) in the entire auricle (Fig. 1b,g,h). Thus, while E2 may decrease the expression of inflammatory cytokines in keratinocytes in the epidermis, E2 may increase inflammatory cytokines in certain cells in the dermis, and exacerbate the edema of the dermis.

In summary, E2 had no effect on the expression of IL-4, IL-10, COX-2, or iNOS at 6 h after allergy elicitation when the auricle swelling was augmented. However, E2 enhanced the expression of inflammatory cytokines, IFN- γ and IL-1 β at 6 h. E2 also increased the expression of IL-10, TNF- α , and IL-1 β at 24 h. Further investigation between these cytokines and skin edema in CHS should be needed.

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