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Involvement of estrogen receptor α in pro-pruritic and pro-inflammatory responses in a mouse model of allergic dermatitis

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ABSTRACT

It has been reported that endogenous or exogenous estrogens can affect the immune system, resulting in immune disorders; however, their direct involvement in such conditions remains to be demonstrated. The purpose of this study was to investigate whether estrogen receptors (ER) are directly implicated in pro-pruritic and pro-inflammatory reactions in cutaneous allergy. Initially, enhancement of the pro-inflammatory response by several ER agonists [methoxychlor (MXC), β-estradiol (E2), propylpyrazoletriol (PPT; an ERα agonist), and diarylpropionitrile (DPN; an ERβ agonist)] was examined *in vivo* using a male BALB/c mouse model of allergic dermatitis induced by toluene-2,4-diisocyanate administration. The ear swelling response, itch response, and local cytokine secretion were measured. Subsequently, the mechanism underlying the development of such allergic reactions was analyzed *in vitro* using human epidermal keratinocytes, murine bone marrow-derived dendritic cells (mBMDCs), and the mixed leucocyte reaction assay. Activated cells were exposed to each ER agonist for 24 h, and cytokine secretion and cell proliferation were measured. Our *in vivo* experiments indicated significant up-regulation of pro-inflammatory and pro-pruritic responses in the E2-, MXC-, and PPT-treated groups compared to the control group; however, no change was observed in the DPN-treated group. Levels of cytokines expressed by keratinocytes, such as TSLP and IL-33, were particularly increased by exposure to E2, MXC, or PPT. These *in vivo* results were confirmed *in vitro* in keratinocytes, but not mBMDCs or T cells. Our findings imply that ERα is involved in pro-inflammatory and pro-pruritic responses in cutaneous allergy through activation of keratinocytes.

1. Introduction

The effect of endogenous or exogenous estrogens on the mammalian immune system, particularly in immunosuppression involving thymus atrophy, is well documented (Sakazaki et al. 2007; Fukuyama et al. 2010, 2011a; Bernardi et al. 2015). Interestingly, some reports have also indicated that transient immunosuppression induced by estrogens early in life may exacerbate immune disorders, including atopic dermatitis, asthma, rheumatoid arthritis, and food allergy, in later life stages. In order to examine this phenomenon, our group recently conducted several studies using mouse models of atopic dermatitis and allergic airway inflammation involving oral exposure to the endocrine disruptors methoxychlor (MXC) and parathion during immaturity (4 weeks of age) (Fukuyama et al., 2011a, b, 2012; Nishino et al. 2013). Analysis of allergic responses in maturity (4 weeks after exposure) revealed skin and airway allergic reactions to be significantly increased by pre-exposure to MXC or parathion, suggesting that endocrine disruptors aggravate such reactions in mice. Sobel et al. (2005) presented similar findings with respect to the progression of systemic lupus erythematosus due to administration of an estrogenic substance. Moreover, Sakazaki et al. (2006) demonstrated that 17β-estradiol (E2) enhances inflamed skin contact hypersensitivity in BALB/c mice. However, previous studies have only described the indirect effects of endocrine disruptors on inflammatory diseases, and the events between administration and outcome remain quite uncertain. Indeed, Petzold et al. (2014) have implied that the impact of the endocrine disruptor bisphenol A on asthma risk is strongly age-dependent, and demonstration of a direct relationship between estrogen exposure and the

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development of allergic symptoms is necessary for a proper understanding of the situation. Therefore, the primary aim of this study was to elucidate whether estrogen exposure directly exacerbates allergic responses using a mouse model of allergic dermatitis.

Endogenous or exogenous estrogens exert their effects by binding to estrogen receptors (ERs). Of such receptors, ERα and ERβ are generally of greatest importance and have a considerable impact on the immune system. From a clinical perspective, ERβ-selective agonists might be therapeutically useful for some types of cancer and several other inflammatory diseases. Since most of the undesirable proliferative effects of estrogens involve activation of ERα, fewer efforts have been devoted to the development of pharmaceuticals that ERα-selective agonists than for ERβ-selective agonists (Paterni et al. 2014). However, only very limited data concerning their effects on allergic diseases are currently available. Therefore, the secondary objective of this study was to identify the roles of these ERs in enhancing or diminishing allergenicity in our mouse model of allergic dermatitis using several types of ER agonists selective to ERα and/or ERβ. In addition, the detailed mode of action was confirmed *in vitro*. In particular, type 2 helper T (Th2) cells, dendritic cells and keratinocytes play a central role to develop and promote the cutaneous inflammation and pruritus *via* cytokine release including IL-4, IL-5, IL-8, IL-12, IL-13, TNFα and thymic stromal lymphopoietin (TSLP) (Danso et al. 2014; Mu et al. 2014). Previous reports have shown that Th2 cytokines such as IL-4 and IL-13 promotes T cell activation and differentiation into the Th2 subtype and can switch the antibody isotype from IgM to IgE (Kaplan et al. 1996; Oettgen 2000). TSLP is also a possible candidate protein involved in the initiation, development and progression of atopy and atopic diseases both in mice and in humans (Indra 2013). We particularly focused on the cytokine release to elucidate the functions of ERs.

2. Material and methods

2.1. Materials

Dinitrochlorbenzene (DNCB), MXC, RPMI-1640 medium, and β-Estradiol (E2) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide (LPS), Pefabloc® SC, polyinosinic–polycytidylic acid sodium salt [poly(I:C)], propylpyrazoletriol (PPT; a selective ERα agonist), diarylpropionitrile (DPN; a selective ERβ agonist), hydroxychlor (HPTE; a major metabolite of MXC), toluene-2,4-diisocyanate (TDI), and TMB Liquid substrate system for membranes were obtained from Sigma-Aldrich Co., LLC. (Tokyo, Japan). Methylpiperidinopyrazole dihydrochloride (MPP; a selective ERα antagonist) was supplied by Tocris Bioscience (Minneapolis, MN). Methyl-^{3}H-thymidine was provided by PerkinElmer Japan Co., Ltd. (Kanagawa, Japan). A DC protein assay kit, TGX precast gels (10%) was purchased from Bio-Rad Laboratories, Inc. (Tokyo, Japan). Dynabeads Mouse T-Activator CD3/CD28 reagents were obtained from Thermo Fisher Scientific, Inc. (Kanagawa, Japan). EpiVita basal medium with hydrocortisone was supplied by Cell Applications, Inc. (San Diego, CA). Recombinant mouse GM-CSF was provided by PeproTech, Inc. (Rocky Hill, NJ). ELISAs for mouse and/or human IL-4, -5, -8, -12, -13 and -33, TNFα and TSLP; antibodies for human GAPDH, ERα/NR3A1 and ERβ/NR3A2; and mouse IgG HRP conjugated antibody were purchased from R&D Systems (Minneapolis, MN). Mouse BD Fc Block PE-conjugated anti-mouse CD3, PE-Cy7-conjugated anti-mouse CD4, and FITC-conjugated anti-mouse CD40 antibodies and a Mouse T Lymphocyte Enrichment Set-DM were obtained from BD Pharmingen (Tokyo, Japan). An APC-conjugated anti-mouse CD11c antibody was supplied by Miltenyi Biotec K.K. (Tokyo, Japan). NucleoSpin RNA II, SYBR® Premix Ex Taq™ GC, primers and Western BLoT Blocking Buffer were provided by TaKaRa Bio (Tokyo, Japan). Pro-Prep Protein extraction solution was ordered from iNtRON Biotechnology, Inc. (Seongnam, South Korea). Human ERα and ERβ Reporter Assay Systems were purchased from Indigo Biosciences, Inc. (State College, PA).

2.2. Mice

Seven-week-old male, female and ovariectomized BALB/c mice and female C57BL/6 mice were purchased from Charles River Japan Laboratories (Kanagawa, Japan). C57BL/6 mice were only used for isolating the T cells for mix leucocyte reaction assay. The animals were kept in a controlled environment with 12 h of light per day at 22 ± 2 °C and 50 ± 20% humidity. The mice received a certified pellet diet and water *ad libitum*. The study protocol was approved by the Institute of Environmental Toxicology Animal Care and Use Committee, which is fully accredited by AAALAC International (IACUC Protocol No. AC17030).

2.3. Responses to oral or topical administration of E2 or MXC in a TDI-induced male mouse model of allergic dermatitis

The mouse model of allergic dermatitis was induced by repeated topical sensitization with Th2 hapten TDI according to the method described by Fukuyama et al. (2015). To exclude the effects of the estrus cycle and endogenous estrogen, male mice were used to generate the model in this study. In the first experiment, TDI-sensitized mice were orally or topically administered E2 (0.01% for topical application and 0.1 mg/kg for oral administration) or MXC (1% for topical application and 10 mg/kg for oral administration) 48, 24, and 4 h before the final TDI treatment. In this study, E2 and MXC are used as representative estrogens which can bind to the estrogen receptors. E2 or MXC was topically applied onto both rostral neck skin and ear auricle (30 μl for rostral neck skin and and 25 μl for ear auricle). Both substances were diluted in corn oil and acetone for oral administration and topical application, respectively. E2 and MXC concentrations used in the topical and oral dosing were decided according to the previous studies as well as preliminary dose-finding test (data not shown), which did not induce the systemic toxicity and immunosuppression (Fukuyama et al. 2010; Fukuyama et al. 2011a; Fukuyama et al. 2011b; Fukuyama et al. 2011c; Fukuyama et al. 2012). Immediately after TDI challenge, scratching behavior was monitored by video for 60 min. The ear swelling response was determined by subtracting ear thickness before TDI administration from that 24 h afterwards. Animals were euthanized 24 h after TDI challenge under isoflurane inhalation anesthesia. The ear auricle and auricular lymph node (LN) were isolated for FACS analysis, cytokine measurements and histological evaluation.

2.4. Responses to oral administration of selective agonists of ERα (PPT) and ERβ (DPN) in a TDI-induced mouse model of allergic dermatitis

In the second experiment, a similar protocol with 2.3. was used, with the addition of oral administration of selective agonists of ERα (PPT; 2.5 and 5 mg/kg) and ERβ (DPN; 2.5 and 5 mg/kg). Since males can convert testosterone to estradiol *via* aromatase; similar experiment with ovariectomized female mice instead of male mice was also performed with the oral administration of 5 mg/kg of each PPT and DPN. Both substances were suspended in olive oil by ultrasonication for 5 min just before each administration. At the doses for PPT and DPN used, none of the test substances employed in this study resulted in any signs of systemic toxicity or immunosuppression, according to our preliminary experiments (data not shown).

2.5. Responses to oral administration of PPT and DPN in a DNCB-induced male mouse model of allergic dermatitis

Th1 hapten DNCB-induced mouse model of allergic dermatitis was generated according to the method described by Yu et al. (2015) with slight modifications. In short, the abdominal skin of the BALB/c mice was depilated with a commercially available depilating cream. On the day after depilation, the abdominal skin was stripped 10 times with adhesive tapes. Just after the tape stripping, a 50 μl of 1% DNCB in