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Effects of a tissue-selective estrogen complex on B lymphopoiesis and B cell function

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ABSTRACT

Background/objective: 17β-estradiol (E2) has major effects on the immune system. It induces thymic atrophy, inhibits both T and B lymphopoiesis and stimulates antibody production treatment with E2 has protective effects on the skeleton but is associated with negative side effects in reproductive organs. A tissue-selective estrogen complex (TSEC) comprise of estrogens combined with a selective estrogen receptor modulator (SERM). TSEC therapy displays the bone-protective effects of estrogen, while the negative side effects on reproductive organs are blocked by the SERM. In a recent publication we showed that treatment with the TSEC E2 + bazedoxifene (bza) potently inhibits experimental arthritis and associated osteoporosis. In order to elucidate immunological mechanisms involved in those effects, the aim of this study was to investigate how E2 + bza treatment affects the healthy immune system.

Methods: Ovariectomized C57BL/6N mice were treated with vehicle, E2, bza or E2 + bza. Weights of uterus and thymus were determined and fluorescence-activated cell sorting was used to analyze B cell populations in bone marrow and spleen. Immunoglobulin production from B cells in bone marrow and spleen were determined using ELISPOT.

Results: Addition of bza to E2-treatment totally antagonized the E2-mediated proliferative effect on uterus. On the contrary, addition of bza to E2-treatment did not block the E2-induced thymic atrophy or inhibition of B lymphopoiesis, and did not block the E2-induced increase in immunoglobulin secretion from bone marrow B cells.

Conclusions: Addition of bza to E2-treatment blocks E2-induced uteroproliferation but does not alter E2-mediated effects on thymus, B lymphopoiesis or B cell function.

1. Introduction

The immunomodulative role of estrogens has been described as a paradox since estrogen can act both as an anti-inflammatory and a pro-inflammatory substance (Straub, 2007). It is known that women have a higher inflammatory response to infections compared to men, and also have an increased incidence of autoimmune diseases. It has therefore been suggested that female sex hormones constitute a significant part of the explanation for the skewed sex ratio in chronic inflammatory diseases.

Loss of estrogens during menopause can give rise to menopausal vasomotor symptoms (VMS), such as hot flashes, night sweats, and sleep disruptions (Nelson, 2008). VMS significantly decrease quality of life for the patients (Gartoulla et al., 2015) and untreated VMS correlate with work productivity loss and higher health care costs (Sarrel et al., 2015). In addition, postmenopausal estrogen deficiency disrupts the balance of osteoblast and osteoclast activity by promoting osteoclast activity, skewing the bone turnover towards bone resorption, and may result in development of osteoporosis (Das and Crockett, 2013).

Menopausal symptoms can be treated with hormone replacement therapy (HRT), where estrogen is given in combination with progestin to counteract negative side effects such as endometrial cancer. HRT effectively reduces VMS and protects against osteoporosis, but increases the risk for breast cancer (Ellis et al., 2014). During the years an alternative approach to HRT in postmenopausal women has emerged: treatment with selective estrogen receptor modulators (SERM). A SERM binds to estrogen receptors and can act simultaneously as an agonist or an antagonist depending on the tissue. SERMs have been continuously developed in generations starting with tamoxifene that is used as preventative breast cancer treatment in high-risk patients. Further, the second generation SERM raloxifene is used as preventative breast cancer treatment as well as in treatment of postmenopausal osteoporo-

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sis. Recently also the third generation SERM bazedoxifene (bza) was approved for treatment of postmenopausal osteoporosis. The use of SERMs in clinical practice was reviewed by Ellis et al. (2015). Although SERMs have improved beneficial effects and safety profile compared to regular HRT, they cannot counteract VMS. Thus, there is still a need for an effective treatment that can encounter both VMS and postmenopausal osteoporosis with a safe profile regarding endometrial thickening and breast cancer.

SERM can be combined with E2 to form a tissue-selective estrogen complex (TSEC). The purpose of a TSEC is to treat menopausal symptoms and protect against osteoporosis while minimizing negative effects of estrogen on the endometrium (Lindsay et al., 2009). In the TSEC, bza acts as both an agonist and an antagonist in estrogen responsive tissues and is able to block the stimulatory effect of estrogen on uterus (Lewiecki, 2007).

Effects of estrogen on B and T cells have been thoroughly investigated. Estrogen inhibits B lymphopoiesis in bone marrow at the differentiation step from pro-B cells to pre-B cells (Medina et al., 1993; Medina et al., 2000). Furthermore estrogen is known to alter the distribution of splenic B cell populations, where estrogen-treated mice displays an increase in marginal zone (MZ) B cells, a reduced number of transitional B cells and a slight increase in follicular cells (Grimaldi et al., 2001). Bza has been shown to inhibit B lymphopoiesis in the bone marrow at a later stage in the B cell differentiation process compared to E2 (Bernardi et al., 2014). E2 also stimulates antibody production by B cells in both bone marrow and spleen (Erlandsson et al., 2002), while SERMs lack this effect (Bernardi et al., 2014). In the thymus, E2-treatment reduces the number of T cells and alters the proportions of different T cell subpopulations during T lymphopoiesis (Okasha et al., 2001; Zoller and Kersh, 2006). However, bza does not have any effects on T cell development in the thymus (Bernardi et al., 2015).

Combined treatment of estrogen and bza is used in the clinic today for treatment of VMS and prevention of postmenopausal osteoporosis. The effect is well studied in these treatment targets, but the effects in other settings, such as effects on the immune system, remains to be investigated. E2 and SERMs have both stimulatory as well as inhibitory effects on different parts of the immune system. Conversely, the aim of this study was to elucidate effects of treatment with the TSEC E2 + bza on B cell development and B cell function in healthy mice.

2. Materials and methods

2.1. Mice

The study was approved by the regional ethical committee for animal experiments. Female C57BL/6N mice were purchased from Scanbur (Germany) and kept in groups of 6–8 mice per cage under standard environmental conditions. They were fed soy-free chow and tap water *ad libitum*.

2.2. Ovariectomy

Mice were ovariectomized (ovx) at the age of 7 weeks under Isoflurane (Baxter Healthcare Corporation, Chicago, IL, USA) inhalation. Ovaries were removed through a midline incision of the skin followed by flank incisions of the peritoneum and the skin was closed with metallic clips. Carprofen (Rimadyl, Orion Pharma Animal Health, Sollentuna, Sweden) was used as pre-operative analgesia. Successful ovx was confirmed by weighing of uteri at termination of the experiment. After surgery, mice were allowed to rest for 13 days before start of treatments.

2.3. Hormones and treatment

The mice were treated with subcutaneous injections of 17β-estradiol-3-benzoate (E2; 0.1 μg/mouse/day, Sigma Aldrich, St Louis, MO, USA), bazedoxifene (bza; 24 μg/mouse/day, a gift from Pfizer, Inc., NY, USA), or the combination of E2 (0.1 μg/mouse/day) and bza (24 μg/mouse/day) (E2 + bza) dissolved in 100 μL miglyol oil (Vendico Chemical AB, Malmo, Sweden), for 15 consecutive days. Control group received miglyol oil alone (veh; 100 μL/mouse/day). Treatment doses were based on previous titration studies where the addition of bza to E2 was able to antagonize E2-induced uterine proliferation (Andersson et al., 2016a). The dose of bza was comparable to doses used in clinical settings ensured by body surface area calculations (Reagan-Shaw et al., 2008).

2.4. Tissue collection and single cell preparation

At the endpoint of experiments mice were anesthetized with ketamine (Ketalar®, Pfizer) and medetomidine (Domitor®, Orion Pharma), blood was collected, and mice were euthanized by cervical dislocation and cardiac puncture. One femur was dissected and bone marrow cells were collected for flow cytometry analysis. Uterus, thymus, and spleen were dissected and the weights recorded. Single cell suspensions of spleen and thymus were obtained by mashing organs through 70 μm cell strainers. Bone marrow cells were flushed from the femur using a syringe and PBS. Cells from spleen and bone marrow were treated with Tris-buffered 0.83% NH_4Cl solution (pH 7.4) for lysing erythrocytes and then re-suspended in FACS buffer (PBS supplemented with 1% FCS and 0.1% NaN_3). Cells were counted using an automated cell counter (Sysmex Europe GmbH, Nordenstedt, Germany).

2.5. Flow cytometry analysis

Bone marrow cells (0.5×10^6) were stained for B220-FITC (Biolegend, San Diego, CA, USA), c-kit (CD117)-APC (Biolegend), CD19-PE (Becton Dickinson (BD) Pharmingen, Franklin Lakes, NJ, USA), CD25-APC (BD), IgM-PE (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Splenocytes (0.5×10^6) were stained for IgM-PE (Southern Biotech), B220-FITC (Biolegend), CD21-APC-Cy7 (Biolegend), CD23-PE-Cy7 (eBioscience, Vienna, Austria), and CD93-APC (eBioscience). A graphic overview of the markers used to identify the different B lymphocyte differentiation and maturation stages in bone marrow and spleen is shown in Fig. 1. The cells were analyzed using a FACSVerse (BD) and the data analysis was performed in FlowJo version 10.1 (Three Star, Ashland, OR, USA). Gating strategies for each cell population are indicated in the figures.

2.6. ELISPOT

The number of immunoglobulin (Ig) secreting cells in bone marrow and spleen were determined using ELISPOT (Tarkowski et al., 1984). In short, nitrocellulose plates (Millipore, Billerica, MA, USA) were coated with goat anti-mouse IgM, IgG, and IgA $F(ab)_2$ fragments (Southern Biotech). The plates were blocked with FCS in PBS and then 5×10^4 and 1×10^4 cells in RPMI medium without phenol red (Lonza, Basel, Switzerland) were added in duplicates. Plates were incubated for 3.5 h in 37 °C, followed by an overnight incubation with addition of alkaline phosphatase-conjugated goat anti-mouse IgM, IgG and IgA secondary antibodies (Southern Biotech). Spots were revealed using 5-bromo-4-chloro-3-indolyl phosphate (Sigma*fast*™, BCIP/NBT, Sigma-Aldrich). The numbers of Ig-secreting cells were counted, the mean was calculated, and expressed as spot forming cells (SFC) per 10^3 $B220^+$ B cells, which were determined by FACS analysis.

2.7. ELISA

Levels of B cell activation factor (BAFF) in serum were measured by Quantikine ELISA (MBLYS0, R & D Systems, MN, USA), according to the protocol provided by the manufacturer.