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Influence of sex hormones and genetic predisposition in Sjögren's syndrome: A new clue to the immunopathogenesis of dry eye disease

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ABSTRACT

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by lymphocytic infiltration, destruction of lacrimal and salivary glands and the presence of serum autoantibodies. Most women that suffer from SS are post-menopausal however, not all post-menopausal women develop SS, suggesting that other factors, in addition to the decrease in ovarian hormones, are necessary for the development of SS. The purposes of this study were to investigate a) the time course of lymphocytic infiltration and apoptosis in the lacrimal gland after ovariectomy, b) if a predisposed genetic background for SS aggravates the effects of decreasing levels of sex hormones in the lacrimal glands and c) if physiological doses of estrogen or androgen prevent the effects observed after ovariectomy. Six weeks old mice that are genetically predisposed to SS (NOD.B10.H2^b) and control (C57BL/10) mice were either sham operated, ovariectomized (OVX), OVX + 17β estradiol (E_2) or OVX + Dihydrotestosterone (DHT). Lacrimal glands were collected at 3, 7, 21 or 30 days after surgery and processed for immunohistochemistry to measure CD4$^+$, CD8$^+$ T cells, B220$^+$ B cells, nuclear DNA degradation and cleaved caspase-3 activity. Quantification of the staining was done by light microscopy and Image Pro Plus software. The results of our study show that lymphocytic infiltration preceded lacrimal gland apoptosis after ovariectomy. Moreover, removal of ovarian sex hormones accelerated these effects in the genetically predisposed animal and these effects were more severe and persistent compared to control animals. In addition, sex hormone replacement at physiological levels prevented these symptoms. The mechanisms by which decreased levels of sex hormones caused lymphocytic infiltration and apoptosis and the interaction of lack of sex hormones with the genetic elements remain to be elucidated.

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease that targets primarily lacrimal and salivary glands resulting in dry eye and dry mouth disease. One of the main hallmarks of SS is mononuclear cell infiltration of the lacrimal and salivary glands. The lymphocytic infiltrates consist of CD4$^+$ T cells, CD8$^+$ T cells, B220$^+$ B cells and macrophages (Nguyen et al., 2007; Nguyen and Peck, 2009). Although the majority of the infiltrate seems to be composed of T cells (Mitsias et al., 2002), and the increase in CD4$^+$ T lymphocyte population has been recognized as an integral part of the pathogenesis of SS, new evidence suggests that B cell hyperactivity also plays an important role in both the pre-clinical and clinical phases of the disease (Nguyen et al., 2007; Tobon et al., 2010).

Another major characteristic of SS is glandular epithelial cell death. Acinar and ductal epithelial cells in the lacrimal and salivary glands of SS patients have been shown to undergo apoptosis or programmed cell death (PCD). Caspase-3, a prominent apoptosis-associated molecule, plays a major role by cleaving proteins that are essential for cell survival (Masago et al., 2001; Elmore, 2007).

SS primarily affects post-menopausal women, suggesting a role for sex hormones in the prevention of the pathogenesis of this disease. However, the fact that not all post-menopausal women develop SS, suggests that other elements, such as a genetic component are also necessary for the development of this disease.

In the present studies we used genetically predisposed NOD.B10.H2^b and control C57BL/10 mice to investigate the time course of lymphocytic infiltration and apoptosis that occur in the lacrimal gland after ovariectomy, and to determine whether decreased levels of ovarian sex hormones can accelerate and aggravate the characteristic symptoms of this disease in the genetically predisposed mice. In addition, we treated these mice with

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physiological doses of E_2 or DHT to investigate if hormone replacement can prevent the symptoms observed after ovariectomy.

2. Materials and methods

2.1. Animals and treatments

NOD.B10.H2^b breeders were obtained from the animal facilities at the University of Florida, Gainesville, FL., and were bred and maintained under veterinary services at Florida Atlantic University, Boca Raton, FL. Sexually mature female C57BL/10 mice, aged 5 weeks, were purchased from Jackson Laboratory (Bar Harbor, Maine, USA), and housed for one week prior to surgery. All mice were housed in constant temperature rooms with fixed light/dark intervals of 12 h length. All animal experiments were approved by the Florida Atlantic University Animal Care Committee and were in accord with the NIH Guiding Principles for the Care and Use of Animals and the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research.

Six weeks old female mice from each strain were anesthetized with a mixture of xylazine (0.011 mg/g) and ketamine (1.0 mg/g) i.p. and either sham operated, OVX, OVX and treated with E_2 or DHT. A small incision of the skin in the back of the neck was made and a pellet containing sex hormones or a matching placebo (Innovative Research of America, Sarasota, FL) was placed under the skin. The OVX mice treated with E_2 or DHT received a pellet that provided a physiological concentration of the desired hormone previously determined for these mice. Mice treated with E_2 received a 0.005 mg pellet (0.16 μg/day) and mice treated with DHT received a 0.10 mg pellet (3.3 μg/day).

Each animal received 0.05 mg/kg of the analgesic buprenorphine intramuscularly 12 and 24 h after surgery. Animals were monitored daily for eating, drinking, wound healing and incision status. At each experimental time point, mice were anesthetized as described above, lacrimal glands were removed, placed in OCT, rapidly frozen in liquid nitrogen and processed for immunohistochemistry analyses as described below. Lacrimal glands were collected from the sham group during the diestrus phase when the estrogen levels in serum are the lowest (Nelson et al., 1982). The diestrus phase was chosen so that the least amount of estrogen difference would be compared between sham and OVX groups. Blood was obtained by cardiac puncture which resulted in the death of the animal.

2.2. Vaginal smears

Vaginal smears were examined daily before and after surgery to classify the phases of the estrous cycle and to determine whether ovariectomy was successful. The sexual cycle of female rodents is a well-defined period characterized by distinct phases of hormonal fluctuation and corresponding changes in the vaginal lining: (A) Proestrus stage – mostly nucleated epithelial cells; (B) Estrus stage – significant increase in estradiol, cornified cells are predominant, (C) Metestrus stage – progesterone level rises, many cornified cells which are larger and more clumped than estrus, (D) Diestrus stage – decrease in estradiol, leukocytes are predominant with some epithelial cells. The estrous cycle lasts around 4–5 days, and the stages are not of equal duration. Thus, in a cycle of 4 days, the combined proestrous–estrus stage last only about 24 h (Champlin et al., 1973; DeLeon et al., 1990).

To obtain a vaginal smear, one drop of sterile water was gently expelled into the vagina using a sterile tip, aspirated back into the tip twice, and then transferred to a microscope slide. Dry smears were stained with the Jorvet Dip Quick Stain (Jorgensen Lab. Inc, Loveland, CO) and examined by light microscopy with an Olympus Provis AX70 microscope (Olympus America Inc., Melville, NY) equipped with a digital camera.

2.3. Ovarian hormone levels

Serum levels of androstenedione, testosterone (T), DHT and E_2 were measured in the C57BL/10 and NOD.B10.H2^b mice using an immunoassay test kit (Diagnostic Automation Inc. Calabasas, CA), following the manufacturer's instructions. The lowest levels of detectable hormone using this assay are: E_2 1 pg/ml; *T* 0.05 ng/ml, DHT 6 pg/ml and androstenedione 5 pg/ml.

2.4. Detection of CD4$^+$ T, CD8$^+$ T and B220$^+$ B lymphocytes by immunohistochemistry

To detect CD4$^+$ T, CD8$^+$ T and B220$^+$ B cells, frozen lacrimal glands were serially sectioned at 5 μm on a Cryostat (Leica Microsystem, Inc., IL, USA) and placed on Superfrost-Plus (Fischer Scientific, Pittsburgh, PA, USA) glass slides. Sections were fixed in cold acetone for 10 min, blocked with Background Terminator (Bio Care Medical, CA, USA) for 20 min at room temperature and incubated overnight at 4 °C with primary antibody, either purified monoclonal rat anti-mouse CD4, -CD8 or -B220 (BD Pharmigen, Minneapolis, MA) in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Sections were then quenched in 0.3% H_2O_2, 40% methanol in PBS for 30 min at room temperature. Sections were incubated for 30 min at room temperature with goat anti-rat-biotin secondary antibody (Millipore, MA, USA), followed by incubation with Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature and then with Sigma Fast 3,3:-Diaminobenzidine (DAB Peroxidase Substrate) (Sigma St. Louis, MO). The reaction was stopped by rinsing the sections with PBS followed by distilled water and then counter-stained with methyl green. The sections were then rinsed in distilled water, dehydrated with 95% and 100% alcohol, cleared in xylene and mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Immunohistochemistry negative controls were obtained by omitting the primary antibody.

2.5. Detection of nuclei DNA degradation by the TUNEL assay

Lacrimal gland sections obtained as described under Section 2.4 were stained using the *in situ* apoptosis detection kit ApopTag® (Millipore, MA, USA) as recommended by the manufacturer. For negative controls the TdT enzyme was substituted with distilled water.

2.6. Detection of cleaved caspase-3 by immunohistochemistry

Lacrimal gland sections obtained as described under Section 2.4 were fixed in 1% paraformaldehyde for 10 min at room temperature, followed by post-fixation in precooled ethanol: acetic acid (2:1) at −20 °C for 5 min. Sections were then quenched with 3% H_2O_2 for 5 min at room temperature and blocked for 20 min with Background Terminator (BioCare Medical, CA, USA) at room temperature. After incubation overnight at 4 °C with rabbit anti-cleaved caspase-3 primary antibody (BioCare Medical, CA, USA), sections were incubated for 30 min with MACH-2 goat anti-rabbit HRP polymer secondary antibody (BioCare Medical, CA, USA) at room temperature. The sections were then stained with Cardassian diaminobenzidine chromagen (CDC) (BioCare Medical, CA, USA), and counter-stained with methyl green (Sigma–Aldrich Corporation, St. Louis, MI). Sections were rinsed in distilled water, dehydrated with 95% and 100% alcohol, cleared in xylene and mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Immunohistochemistry negative controls were obtained by omitting the primary antibody.