Do Genetic Alterations in Sex Steroid Receptors Contribute to Lacrimal Gland Disease in Sjögren's Syndrome?

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Abstract: *Background*: Defects in sex steroid receptors have been linked to the onset, progression and severity, as well as the sex-related prevalence, of a variety of autoimmune disorders, including lupus, rheumatoid arthritis, multiple sclerosis and diabetes. We hypothesize that defects in estrogen receptor α (ESR1), estrogen receptor β (ESR2) and/or the androgen receptor (AR) may also contribute to the development of lacrimal gland autoimmune sequelae in Sjögren's syndrome. To begin to test this hypothesis, we examined whether mutations exist in the coding regions of ESR1, ESR2 and AR transcripts in lacrimal tissues of mouse models of Sjögren's syndrome.

Methods: Lacrimal and submandibular glands were collected from adult MRL/MpJ-Tnfrsf6^{lpr}, nonobese diabetic and/or BALB/c mice. Tissues were pooled according to sex and experiment and processed for cDNA generation. PCR primers were designed to amplify 566-875 base pair segments of the entire open reading frame of each receptor. Segments were amplified, purified and then sequenced. Receptor sequences were assembled and compared to each other and to known NCBI sequences.

Results: Our results show that almost all ESR1, ESR2 and AR sequences in exocrine tissues of male and female autoimmune and non-autoimmune mice were identical to those of NCBI standards. There was a G→A shift at position 998 of the ESR2 complete coding sequence in all tissue samples when compared to NCBI reference sequence U81451.1, but this polymorphism was not found in other ESR2 reference sequences.

Conclusions: Our findings indicate that defects in the coding region of sex steroid receptors do not contribute to the pathogenesis of lacrimal gland disease in mouse models of Sjögren's syndrome.

Keywords: Sex steroid receptors, lacrimal gland, autoimmune disease, androgen, estrogen.

INTRODUCTION

Defects in sex steroid receptors have been linked to the onset, progression and severity, as well as the sex-related prevalence, of a variety of autoimmune disorders, including lupus, rheumatoid arthritis, multiple sclerosis and diabetes [1-9]. These defects, which are often due to gene polymorphisms or alternative splicing, may lead to significant changes in the affinity or specificity of ligand binding, nuclear translocation, receptor dimerization, DNA association and transcriptional activation [10-13].

We hypothesize that defects in estrogen receptor α (ESR1), estrogen receptor β (ESR2) and/or the androgen receptor (AR) may also contribute to the development of lacrimal gland disease in Sjögren's syndrome. This syndrome is an insidious and currently incurable autoimmune disorder, that occurs primarily in women, and is associated with an extensive lymphocyte accumulation in the lacrimal gland, an immune-mediated destruction and/or dysfunction of acinar and ductal epithelial cells, a precipitous decrease in tear secretion and severe dry eye [14, 15]. The precise etiology of Sjögren's syndrome is unknown, but the progression of this disease has been linked to sex steroid effects [14, 16], and to alterations in the structure or function of sex steroid receptors [17]. It is possible that these receptor changes may predispose to the development of autoimmune sequelae, and specifically the lacrimal gland immunopathology and dysfunction, decreased tear secretion and consequent dry eye [14].

To begin to test our hypothesis, we examined whether mutations exist in the coding regions of ESR1, ESR2 and AR transcripts in lacrimal tissues of the MRL/MpJ-Tnfrsf6^{lpr} (MRL/lpr) [18] and nonobese diabetic (NOD/LtJ) [19] mouse models of Sjögren's syndrome. For comparative purposes in these experiments, we also evaluated receptor sequences in lacrimal and submandibular glands of non-autoimmune BALB/c mice.

MATERIALS AND METHODOLGY

Animals

Age-matched BALB/c mice were purchased from Taconic Laboratories (Germantown, NY). Young adult MRL/lpr and NOD/LtJ (NOD) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals (n = 5 mice/sex/strain/experiment; n = 3 separate experiments) were housed in constant temperature rooms with fixed light/dark periods of 12 hours length. When indicated

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(BALB/c = 9 weeks old; MRL/lpr = 4.6 months old; NOD = 5 months old), animals were sacrificed by CO_2 inhalation and salivary and/or lacrimal glands were obtained, pooled according to sex and experiment, frozen in liquid nitrogen and then processed for molecular biological procedures. All studies with mice were approved by The Schepens Eye Research Institute Animal Care and Use Committee and adhered to The Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

RNA Isolation and cDNA Synthesis

Glandular total RNA was isolated by using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and RNAqueous spin columns (Ambion, Austin, Tx). The RNA samples were analyzed spectrophotometrically at 260 and 280 nm to determine concentration and then evaluated on a RNA 6000 Nano LabChip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), to verify RNA integrity. Aliquots of RNA were exposed to DNase I, then converted to cDNA by using oligo (dT)15 primer (Promega, Madison, WI) and SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was precipitated with alcohol and resuspended in distilled water.

Primer Design and PCR Amplification

Primers were designed to amplify 566-875 base pair segments of the entire open reading frame of the AR, ESR1 and ESR2 (Table **1**). These designs were accomplished by using Primer3 freeware [20] and reference sequences from the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/). The primers were also designed to ensure that: [a] the 5' coding regions were amplified beyond the start/stop codon, which prompted our use of two NCBI sequences for AR and ESR2; and [b] there was a 90-200 base pair overlap on both the 3' and 5' regions of receptor segments (Fig. **1**).

Segments were amplified from cDNA with appropriate primers (200 nM), a Platinum Taq DNA Polymerase High Fidelity Kit (Invitrogen) and the following thermocycler program: 1 cycle (94° C for 120 seconds), 35 cycles (94° C for 45 seconds, 55° C for 60 seconds, 72° C for 90 seconds) and 1 cycle (72° C for 300 seconds). Certain amplifications required the addition of 5% dimethyl sulfoxide (DMSO) or 1 M betaine (Table **1**). An aliquot of each PCR reaction was examined by using a DNA 500 or 1000 LabChip (Agilent) with the 2100 Bioanalyzer, or by utilizing a 1.5% agarose tris-borate-EDTA gel. The agarose gels were visualized by employing SYBR Safe DNA Gel Stain (Invitrogen) and UV illumination. All amplification reactions produced bands at the expected molecular weight size.

Sequencing and Data Analyses

Amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega) and their concentrations were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Amplicon samples, as well as sense and anti-sense primers, were sent to the DNA Sequencing Center for Vision Research at the Massachusetts Eye and Ear Infirmary (Boston, MA). Sequencing reactions were performed on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) with a BigDye Terminator Cycle Sequencing Kit Version 3.0 (Applied Biosystems).

Table 1. Primers and Enhancers Used to Amplify Segments of Androgen and Estrogen Receptor mRNAs

Receptor	Sense	Anti-Sense	Enhancer
Androgen			
AR(NTD1)	GCTAGCTGCAGATACTACATCATC	GCTGCCTTCGGAGATTACCT	DMSO
AR(NTD2)	CTCCGCCGACATTAAAGACA	GCCGTAGTCCAATGGGTTCT	DMSO
AR(DBD)	GACACTTGAGATCCCGTCCT	TCGTTTCTGCTGGCACATAG	None
AR(LBD1)	GGACATGCGTTTGGACAGTA	TACTGAATGACCGCCATCTG	None
AR(LBD2)	TGTGCATGTGGTCAAGTGG	AACAAAGGCAGAGCCACAAT	None
AR(LBD3)	TTAACGTCCTGGAAGCCATT	GGAGCTTGGTGAGCTGGTAG	None
Estrogen 1			
ESR1(NTD1b)	GGGAGCCAGTCTGTAACTCG	AGGCATAGTCATTGCACACG	Betaine
ESR1(NTD2)	CTTTCCCCCAGCTCAACAG	AAGGACAAGGCAGGGCTATT	DMSO
ESR1(DBD)	ATGACTTGGAAGGCCGAAAT	GGTGGATGTGGTCCTTCTCTT	DMSO
ER1(LBD1)	TGGAGATCTTTGACATGTTGCT	GCTAGTCATACATGACATGGGTAAA	DMSO
Estrogen 2			
ESR2(NTD1)	CGCAAGACATGGAGATCAAA	TTTTACGCCGGTTCTTGTCT	DMSO
ESR2(NTD2)	ACTAGTCCAAGCGCCAAGAG	AGAACGAGGTCTGGAGCAAA	DMSO
ESR2(DBD)	GAAGCTGGCTGACAAGGAAC	CTCTGTCGAGCAGCACTCAG	DMSO
ESR2(LBD1)	CCAACCTCCTGATGCTTCTT	GGAATGCGAAACGAGTTGAT	DMSO

Abbreviations: AR – androgen receptor; ESR1 – estrogen receptor 1; ESR2 – estrogen receptor 2; NTD – N-terminal domain; DBD – DNA binding domain; LBD – ligand binding domain; DMSO – dimethyl sulfoxide.

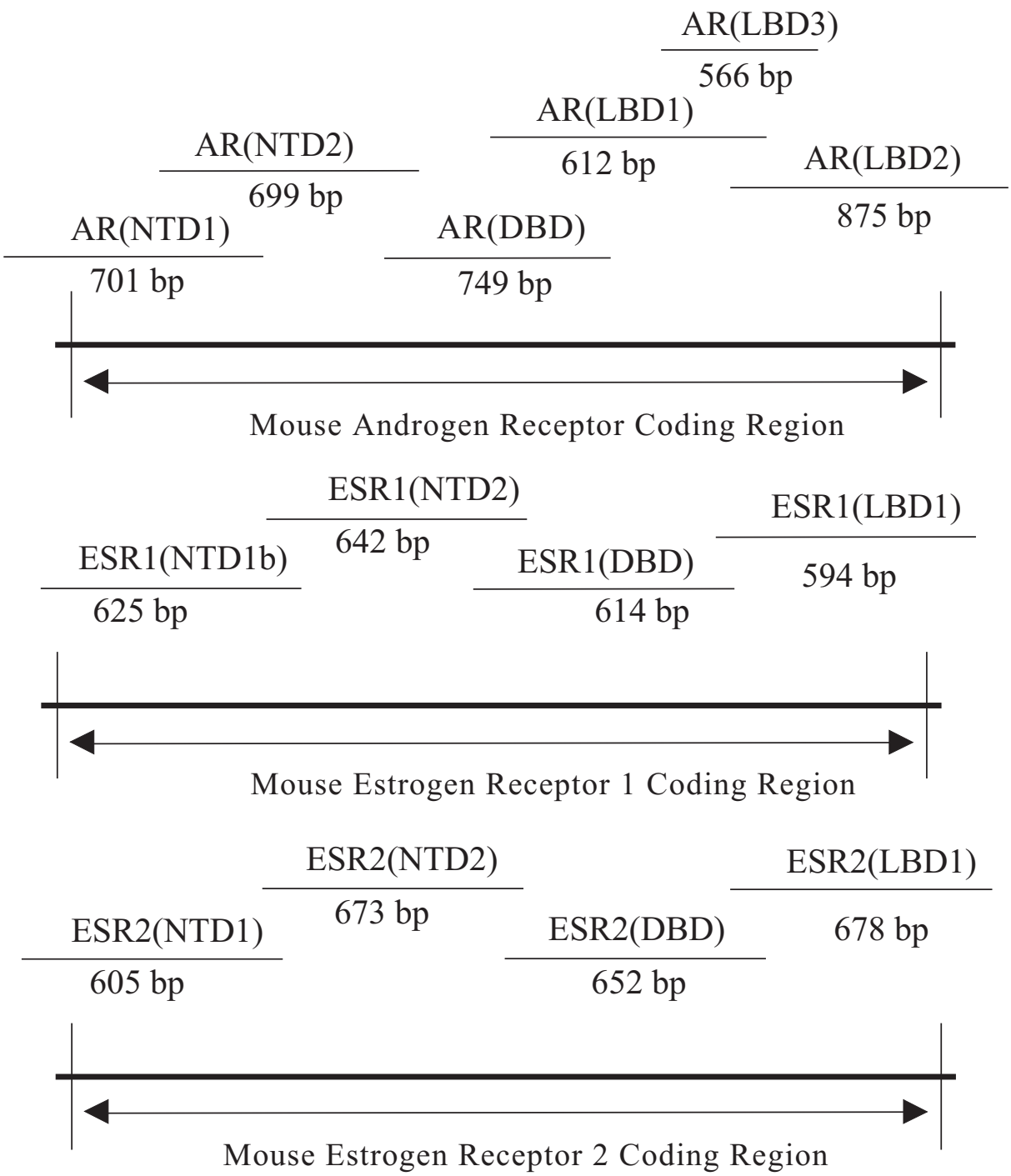

Fig. (1). Amplicon sizes for each of the sex hormone receptors. Abbreviations are defined in the legend to Table **1**.

Tracing data were collected with 3100 Data Collection Software Version 1.1 (Applied Biosystems). Amplicons from all cDNA preparations were sequenced at least once by using the sense primers. Additional sequencing was conducted on selected amplicons by utilizing the anti-sense primers. Amplicon sequences were assembled and compared to each other and to known sequences from NCBI with CLC Gene WorkBench (CLC bio, Cambridge, MA). The reference sequences used were NM_013476.2 and X59592.1 for the AR, M38651.1 and NM_007956.2 for ESR1, and AK054413.1, U81451.1 and NM_010157.3 for ESR 2.

RESULTS

To determine whether defects in sex steroid receptors may contribute to the development of lacrimal gland disease in Sjögren's syndrome, we examined whether mutations exist in the coding regions of ESR1, ESR2 and AR transcripts in lacrimal tissues of male and female MRL/lpr and NOD mice. For comparison, we also evaluated receptor sequences in lacrimal and submandibular glands of non-autoimmune male and female BALB/c mice.

Our results show that almost all ESR1, ESR2 and AR sequences in exocrine tissues of male and female autoimmune and non-autoimmune mice were identical to those of NCBI standards (Fig. **2**). More specifically, for the AR all experimental samples produced an assembled sequence identical to NM_013476.2.

For ESR1, the sequences for the male and female BALB/c and MRL/lpr submandibular and/or lacrimal glands were identical to those of reference sequence M38651.1. In contrast, the sequences generated from NOD lacrimal glands showed different nucleotides at positions 1299 and 1338 in the ligand binding domain coding region, when compared to the sequence for M38651.1 (Fig. **3**). However, the NOD sequence showed complete homology to the ESR1 sequence for NM_007956.2. Given that translation of the M38651.1 and NM_007956.2 sequences produce identical proteins, the nucleotide variations found in NOD lacrimal glands should not result in any functional differences.

For ESR2 there was a G→A substitution at position 998 of the complete coding sequence in all tissue samples when compared to NCBI reference sequence U81451.1 (Fig. **4**), but this polymorphism was not found in other ESR2 reference sequences. The U81451.1 was used as a primary reference sequence for ESR2 because it appears to be the only full length, non-splice variant listed in the NCBI databank.

DISCUSSION

Polymorphisms in ESR1, ESR2 and AR have been linked to variety of conditions, including breast, endometrial and prostate cancer, cardiovascular disease, diabetes, metabolic syndrome, male infertility, premenstrual dysphoric disorders, depression, cholelithiasis, osteoporosis, preeclampsia, Grave's and Kennedy's diseases, macular degeneration, meibomian gland dysfunction, lupus, rheumatoid arthritis and multiple sclerosis [1-13,21-37]. Indeed, over 300 different mutations have been identified in androgen receptors in non-ocular tissues, which may result in partial or complete insensitivity to androgens [13]. These genetic alterations may be intronic or exonic [38].

However, polymorphisms in the coding regions of ESR1, ESR2 or AR transcripts do not appear play a role in lacrimal gland disease in Sjögren's syndrome. Sex steroid receptors

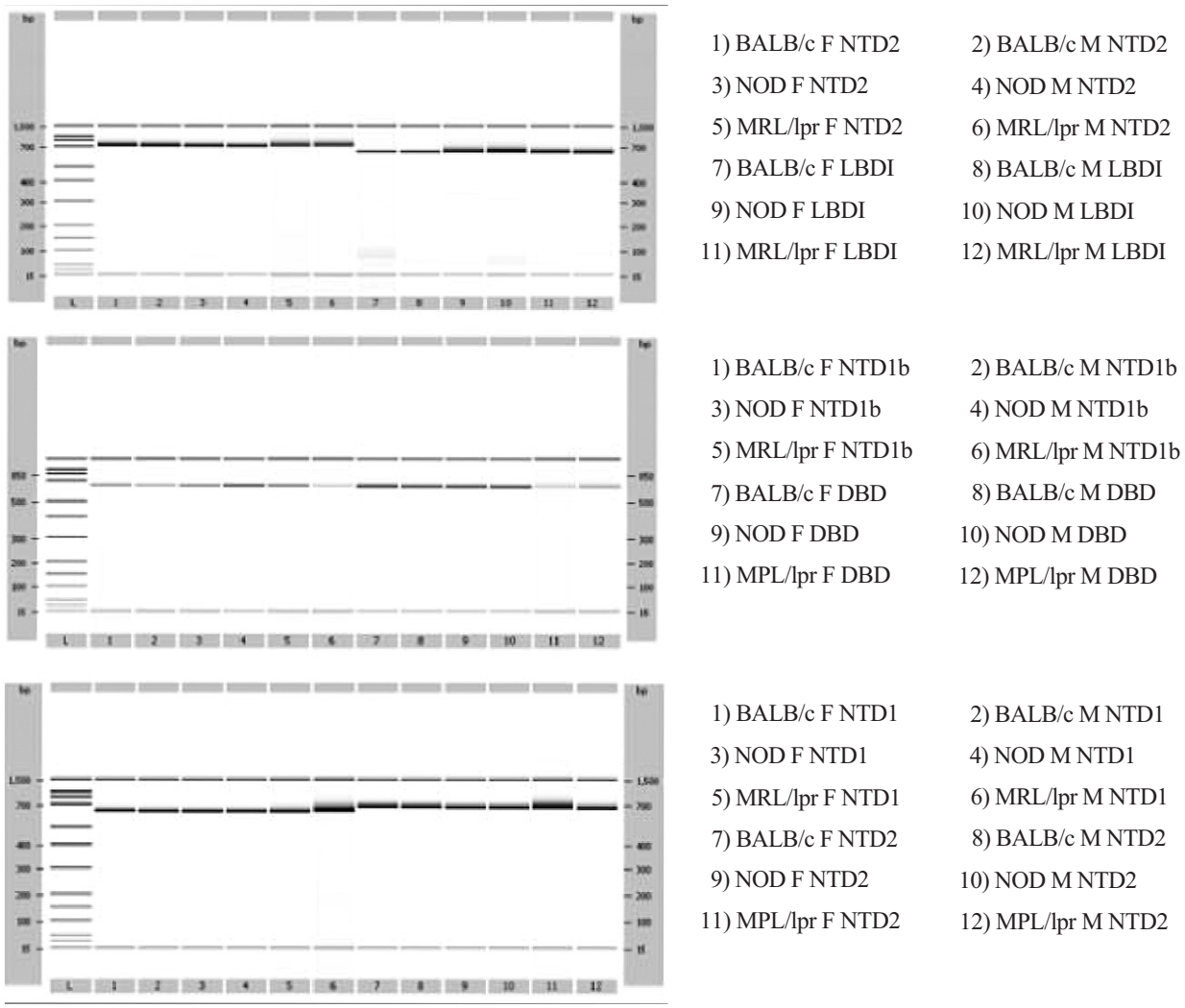

Fig. (2). Examples of AR, ESR1 and ESR2 sequences in lacrimal glands of male (M) and female (F) autoimmune and non-autoimmune mice. Images are from the bioanalyzer and molecular weight standards were run in lane "L." Domain abbreviations are reported in the Table **1** legend, and anticipated band sizes are shown in Fig. (**1**). The sizing accuracy is ± 10%.

Fig. (3). Alignment of NOD ESR1 sequences to an NCBI wild type reference sequence. Each number (i.e. 1, 2, 3) represents a different experimental sample. Each sample originated from the lacrimal glands of 5 mice.

Fig. (4). Alignment of ESR2 sequences to an NCBI wild type reference sequence. All samples were from lacrimal glands (LG), except for those originating from submandibular glands (SMG).

are ligand-activated transcription factors that mediate sex hormone actions and are comprised of N-terminal, DNA-binding and ligand-binding domains. Our analyses of the mRNA sequences of these domains for the ESR1, ESR2 and AR in lacrimal tissues of male and female autoimmune mice revealed that functional polymorphisms are absent. Indeed, our data indicate that sex steroid receptor coding region sequences in autoimmune, as well as non-autoimmune, lacrimal glands are essentially identical to those of normal NCBI standards.

For our studies we evaluated 2 mouse models of Sjögren's syndrome: the MRL/lpr model, which appears to have a disease driven by Th1- and Th2-mediated processes [39], and the NOD model, which seems to have a defect in T cell regulation [40]. Lacrimal glands of MRL/Mp mice contain multifocal and extensive lymphocytic infiltrates, significant tissue disruption and apparent fibrosis [18, 41]. The magnitude of lacrimal gland inflammation in these mice, as with humans, is significantly greater in females as compared to males [42]. This sexual dimorphism has been linked to the effects of sex steroids, with estradiol increasing and testosterone decreasing the extent of inflammation [43].

It has been hypothesized that estradiol through its receptors mediates the progression of autoimmune disease in MRL/lpr mice, and that in this disorder, the estrogen receptor is functionally and/or structurally altered [44]. In support of this hypothesis, the ligand-binding affinity of estrogen receptors is significantly elevated [45], whereas the DNA-binding activity of estrogen receptors is significantly reduced [46], in non-ocular tissues of MRL/lpr mice. It is possible that such changes could occur in lacrimal glands of these mice, but given our mRNA data, it would seem that such alterations would have to be post-translational.

In our experiments we also examined lacrimal tissues of NOD mice. We were particularly curious as to whether these animals had a polymorphism in their lacrimal gland androgen receptor. In the NOD strain lacrimal tissues of male mice are far more inflamed than those of female [42, 47] and this sex difference appears to be due to androgen action [48]. This anomalous hormone effect is mediated through the lacrimal microenvironment [47], and is in striking contrast to the androgen-induced decrease of inflammation in salivary and pancreatic tissues of NOD mice [47, 49], and in exocrine glands of other murine autoimmune strains [14]. However, as shown in this study, the mRNA sequence of the androgen receptor coding region is normal in lacrimal tissues of NOD mice. It may be that the aberrant androgen action in lacrimal glands of male NOD mice may be due to unique effects of this hormone on the local lymphoid microenvironment, in a manner different than that found in other tissues [50].

CONCLUSION

In summary, defects in sex steroid receptors are known to play an important role in the sex-related prevalence, as well as the development and progression, of a number of autoimmune disorders [1-9]. Such defects are commonly due to genetic polymorphisms and lead to functional alterations in receptor activity [10-13]. However, our findings in this study indicate that defects in the coding region of sex steroid receptors do not contribute to the pathogenesis of lacrimal gland disease in mouse models of Sjögren's syndrome.

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