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IMMUNOHISTOCHEMICAL DETECTION OF ESTROGEN AND PROGESTERONE RECEPTORS IN THE HUMAN LACRIMAL GLAND

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Abstract - The nature and extent of estrogen and progesterone action on the lacrimal gland is not known, and neither are the targets for their action. Immunohistochemical detection of estrogen and progesterone receptors in the human lacrimal glands in both sexes in different age groups was performed in this study. Twenty human lacrimal glands from autopsies were analyzed by the immunohistochemical method of cell counting and the χ^2 test. Estrogen and progesterone receptors were detected in the lacrimal glands of both sexes with significantly higher total and average cell counts in females ($p<0,001$). Estrogen and progesterone receptors are present in human lacrimal glands with age and gender dependent expression.

Key words: Estrogen, progesterone, receptor, lacrimal gland, immunohistochemistry.

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INTRODUCTION

Sex steroid hormones influence many physiological processes in mammals, including reproduction, cardiovascular health, bone integrity, cognition and behavior. Given the widespread role of estrogen in human physiology, it is not surprising that it is also implicated in numerous diseases. Until recently, researchers assumed that the targets for sex hormones are reproductive organs. Investigators have proposed that estrogens play an important role in the anatomy, physiology and sexual dimorphism of the lacrimal gland (Azzarolo et al. 2003, Sullivan et al. 1998). Supporting this proposition are the reports that antiestrogen treatment leads to acinar cell disruption and necrosis, DNA degradation, inflammation, glandular tissue loss and dry eye, as well as the correction of this changes after estrogen administration (Suzuki et al. 2006).

Other researchers have found that neither estrogen insufficiency nor estrogen treatment have any effect on the morphology, total protein content, specific enzyme activity or secretion of the lacrimal gland (Kuscu et al., 2003, Pelit et al., 2003); yet there are reports on negative estrogen influences on the lacrimal tissue, such as glandular regression, the suppression of protein production and reduced tear secretion (Suzuki et al. 2006, Sato et al. 1994, Rocha et al. 1993). The conflicting results may be the consequence of differences in experimental design or the effects of progesterone action that may significantly modify the effects of estrogen. However, the overriding difficulty in clarifying this is because the nature and extent of estrogen and progesterone action on the lacrimal gland is not known, and neither are the targets for their action. Indeed, it has not yet been established whether these hormones have functional receptors on the lacrimal gland (Suzuki et al.

2006). In many diseases estrogen mediates its effects through the estrogen receptor (ER), which may serve as the basis for therapeutic intervention.

Estrogen, androgen and progesterone receptors have been detected in the human lacrimal (Sullivan et al. 1996, Rocha et al. 2000) and meibomian glands (Mircheff et al. 1996) and have been implicated in the dry eye syndrome (Mathers et al. 1998, Suzuki et al. 1996). They were also found in the nuclei of human corneal epithelial, stromal and endothelial cells (Vecsei et al. 2000, Sullivan et al. 2009, Spelsberg et al. 2004).

The signal of estrogens is transmitted either via a specific estrogen receptor or in a receptor- independent way (Fuller, 1991). ERs are ligand-dependent transcriptional activators regulating a gene via complex mechanisms. Two ER isoforms, α and ß, have been isolated, (Pavao et al. 2001) and mapped on different chromosomes (ERα on chromosome 6 and ERß on chromosome 14). Based on mRNA analyses, ERα is predominantly expressed in female tissues, whereas ERß is expressed in different tissues, for example the vascular tissue and central nervous system tissues. The affinity of estradiol is comparable for both receptor subtypes, but ERß seems to be predominant when both receptor subtypes are present. Based on their different structure and distribution, these ER isoforms are thought to have different biological activity. However, the function of ERß is not yet fully understood (Spelsberg et al. 2004).

Owing to insufficient commercial availability, unsatisfactory characterization and limited application on ERß antibodies, only one immunohistochemical study exists regarding this receptor type (Munant et al. 2001) and there are very few studies dealing with the immunohistochemical detection of ERs in tear-producing tissues using monoclonal antibodies (Pavao et al. 2001).

Our belief is that age and gender-related differences in the expression of ER and PR in human ocular tissues do exist. We also believe that it is important to determine the basis of those differences.

Therefore, the purpose of this study was to begin this exploration by immunohistochemical detection of receptors in human lacrimal glands in both genders and different ages.

MATERIALS AND METHODS

After the approval of the local ethical committee, twenty lacrimal glands were taken during autopsy. Sixteen glands were taken from people who died in accidents and 4 from stillbirths. There were 10 males and 10 females, the oldest man was 73 years old, while the mean age was 36.5 years (Table 1).

Table 1. Age and gender characteristics for tested lacrimal glands

	Age	Sex	ER	PR	Post mortem time (hours)
1.	premature	m	-	-	12
2.	premature	m	-	-	12
3.	stillbirth	f	+	-	not known
4.	stillbirth	m	-	-	not known
5.	45	f	+	+	12
6.	28	f	+	+	20
7.	37	m	+	-	24
8.	56	m	-	-	22
9.	58	m	-	-	18
10.	73	f	+	-	24
11.	40	f	+	-	8
12.	39	f	+	-	15
13.	58	f	+	-	15
14.	65	f	+	-	20
15.	20	f	+	-	12
16.	21	m	+	-	22
17.	46	f	+	-	18
18.	27	m	-	-	24
19.	68	m	-	-	20
20.	72	m	-	-	12

Following fixation in 10% buffered formalin over 24 h, the lacrimal glands were routinely prepared in an automatic tissue processor and were embedded in paraffin blocks. Samples of normal breast tissue were used as a positive control. The negative control were samples of breast tissue. The paraffin-embedded tissues were cut to 5 μ and were treated as follows: deparaffination in xylene (4 x 3 min) rehydration

Table 2. Distribution of receptor positive cells counts

Case N°	cell count	total cell count
3.	1; 0; 0	1
5.	24;12;8	44
6.	6; 8; 5	19
7.	4; 1; 0	5
10.	5; 7; 4	16
11.	4; 3; 3	10
12.	5; 4; 7	16
13.	2; 1; 0	3
14.	5; 2; 4	11
15.	8; 1; 2	11
16.	4; 6; 3	13
17.	1; 2; 1	4

Table 3. ER and PR receptor positive acinar cells of the lacrimal gland by gender

	Gender	
	Male	Female
Total number of cases	10	10
Cases with ERs and PRs	2 (20,00%)	10 (100%)
Total count of positive cells	18	135
% of positive/ analysed cells	0,12%	0.90%
N° positive cells per case	1,80±4,24	13,50±12,27

in increasing concentrations of alcohol (3 x 3 min). These steps were followed by rapid washing in distilled water. The sections were than treated in 0.001 M citrate puffer under 120°C in an autoclave for 10 min and then washed in PBS (two rapid changes). Endogenous peroxidase blocking with 3% H_2O_2 in mannitol (20 min) was followed by washing in PBS (x2). The sections were then treated by a primary antibody for 24 h at 4°C. We used the ready-to-use Anti-Human Estrogen Receptor (ER) *Clone 1D5* and Anti-Human Progesterone Receptor *Clone PGR-1A6* ("Histofine" Nichirei Biosciences Inc., Tokyo, Japan) as recommended by the manufacturer. Dark brown granules of DAB were treated as positive staining.

The counting of positive reactions was done on 3 fields with 500 cells. Statistical analyses were done using the χ^2 test.

RESULTS

To examine whether estrogen and progesterone receptors occur in the acinar cells of lacrimal glands, we used an immunohistochemical method with commercially prepared anti-ER and anti-PR antibodies. It was possible to detect dark brown DAB deposits in the nuclei of very few cells in the 12 lacrimal glands and progesterone in only 2 cases (Fig. 1). There was cytoplasmic staining in some cases (Fig. 1D).

Results of the presence and distribution of ER and PR positive acinar cells in lacrimal glands

Estrogen and progesterone receptors were detected in the lacrimal glands in both sexes, in all female and 20% male lacrimal glands, with a statistical significance up to $p<0.001$, by χ^2 test analysis. We analyzed the total and average count of acinar cells in

Table 4. ER and PR positive acinar cells of the lacrimal gland by age group

	Age groups (in years)				
	<20	21-30	31-40	41-50	>50
N° cases	5	3	3	2	7
ERs and PRs positive	2 (40,00%)	2 (66,67%)	3 (100,00%)	2 (100%)	3(42,86%)
Total count of positive cells	12	32	31	48	30
% of positive/analyzed cells	0,16%	0,71%	0,69%	1,60%	0,33%
N° positive cells/case	2,40±4,83	10,67±9,71	10,33±5,51	24,00±28,28	4,29±6,35

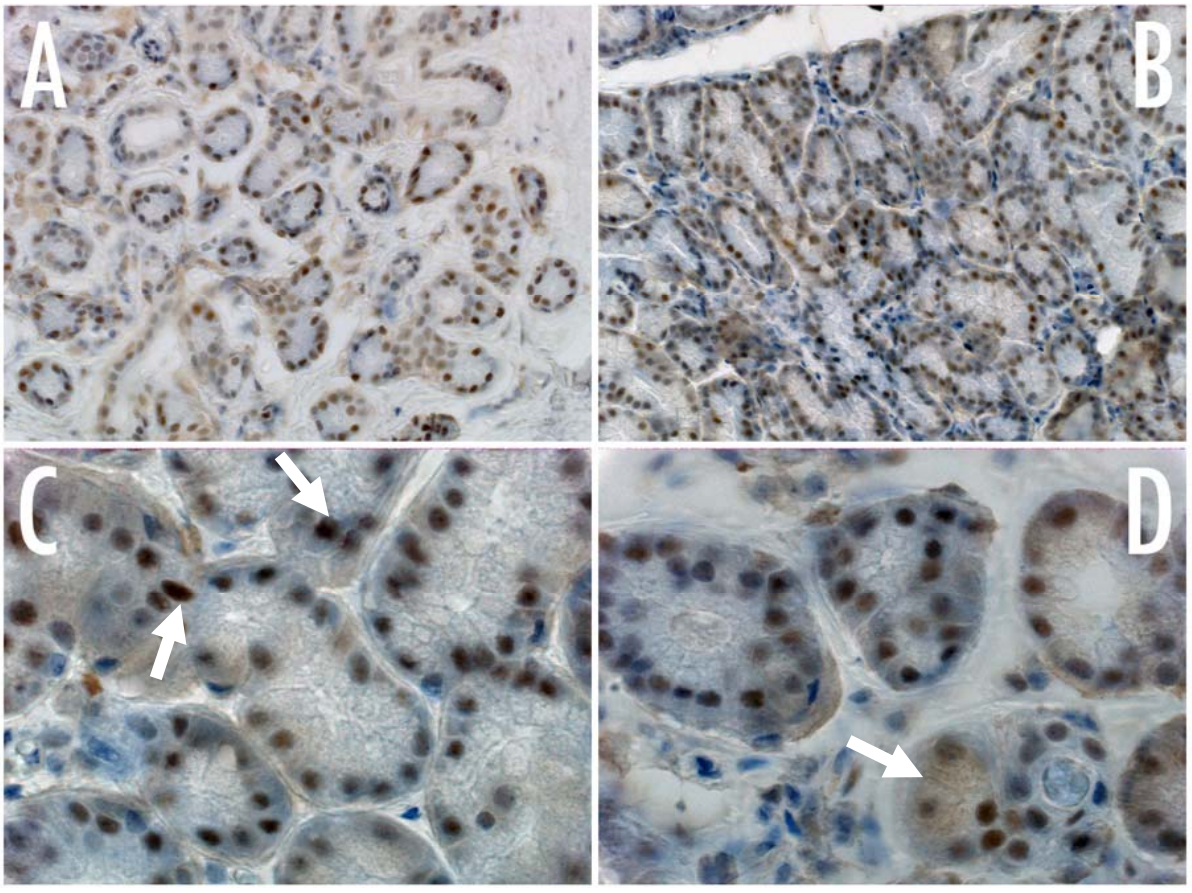

Fig. 1. Estrogen and progesteron receptors in acinar cells of lacrimal glands: expression is of wariable intensity (A and B, En Vision x20); Nuclear expression (C white, En Vision x100); citoplasmic coloration (D, white errow, En Vision x100).

the lacrimal glands with ERs and PRs. The samples without ERs and PRs were regarded to have zero cell count. Having analyzed 1500 cells in each case, we found that the total representative of the count of ER and PR bearing cells was 135 (0.90%) and this is statistically significantly higher in comparison to the male gender where the total cell count was 18 (0.12%), $p < 0.001$. We also found that the average number of positive stained cells was significantly higher in females (13.50±12.27 vs.1.80 ±4.24) $p<0.001$.The results are shown in Table 3. Although we couldn't demonstrate statistical differences between the age groups, it is evident that the incidence of ER- and PR-bearing cells is highest in the age groups of 31-40 and 41-50, where it was 100%. The lowest incidence was in the youngest group (40.00%). The distribution and presence of ER and PR positive cells was found to be significantly different in the group of 41-50 years of age (48, i.e. 1.6%) in comparison to other groups by χ^2 test ($p<0.001$). Similar results were found by testing the middle-aged group with the subjects younger than twenty, and by testing the eldest group. The results of the distribution of ER and PR according to the age are shown in Table 4.

DISCUSSION

In this study, ERs were identified by an immunohistochemical method in most of the samples of lacrimal glands with variable expression, and PRs were detected in two cases. Our resuts show a sta-

tistical significant association of ER expression according to gender, being the highest in the acinar cells of the lacrimal glands in the group of middle-aged women (30-50 years). These results are among the few in the literature dealing with the problem (Rocha et al. 2000, Sullivan et al. 1996, Spelsberg et al. 2004). The slight immunohistochemical staining of the PRs in the acinar cells of the lacrimal gland may be explained by tissue-specific receptor expression.

Since the scores for the evaluation of ER and PR expression for ocular tissues do not exist, we used the ratio of stained cells per 1500 cells analyzed in the lacrimal gland per case. Negative immunohistochemical results in detection of ER and PR, which are in contrast to the proof of ERs on transcriptional level, were previously attributed to the fixation, embedding, rehydration and specificity of the antibody itself (Spelsberg et al. 2004). We postulate that our results are the consequence of the affinity of antibodies used in the immunohistochemical procedure. We also postulate that investigators were discouraged to investigate ER and PR in lacrimal gland acinar cells after so many unsuccessful attempts to prove their presence published in the literature. Although small amounts of detectable receptor sites for estrogen and progesterone were found in experimental animals, it was postulated that the amount of mRNA is insufficient to produce detectable, high affinity and steroid-specific estrogen and progesterone binding sites in the lacrimal gland (Sullivan et al. 1998). It was assumed that if a similar translation defect occurs in a human lacrimal gland, it would indicate that estrogen receptor protein may not mediate the controversial effects in dry eye syndrome (Wickham et al. 2000). Dry eye syndrome mainly affects peri- and post-menopausal females and it was thought that the decreased levels of estrogen and progesterone, as well as androgens, influenced this state (Mathers et al. 1998). In climacteric women, presumably the lower estrogen level is responsible for the higher incidence of dry eye syndromes such as keratoconjuctivitis sicca (KCS) (Kramer et al. 1990). Attenuation of androgen synthesis during the menopause contributes to KCS by leading to meibomian gland dysfunction (Sullivan et al. (2009), Krenzer et al. (2000)). Since estrogen-dependent cycle-associated changes of the conjunctiva have been observed, systemic hormone replacement therapy (HRT) was suggested for patients with KCS (Kramer et al. (1990)). On the contrary, data from the Women's Health Study, a large cohort study involving more than 25,000 post-menopausal women, indicated an increased risk of KCS in women who used systemic HRT, particularly estrogen alone (Schaumberg et al. 2001). Topical application of both estrogen and progesterone has shown better results (Sator et al. 1998). As it is the main organ in producing aqueous part of the tear film, the lacrimal gland ER and PR receptors were investigated in this study.

Our results confirm the presence of ER and PRs receptors in the acinar cells of the lacrimal glands in both sexes, with variable, but significantly higher, expression in females. These results are in correlation with and support the observed better results after topical application of estrogen for the treatment of keratoconjuctivitis sicca. Although we could not demonstrate the statistical significance of receptor expression between the age groups because of the small number of cases, it is obvious that receptor expression was highest in middle-aged women. This can be the up-regulating process in capturing the lower amounts of circulating hormones.

In conclusion age-related ER and PR expressions and lacrimal gland functions related to estrogen receptors could further help in understanding the pathophysiological aspect of ocular diseases.

The identification of mRNAs for sex steroid receptors in the ocular tissues in animals and humans (Smith et al. 1999, Fuchsjager-Mayrl et al. 2002, Tachibana et al. 2000), Wichkam et al. 2004) and proof of mRNA translocation in them, as well as, proof of estrogen and progesterone receptor proteins in the lacrimal gland from our study, offer a new therapeutic approach to some of ocular disorders.

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