Identification of Estrogen and Progesterone Receptor mRNA Expression in the Conjunctiva of Premenopausal Women

Gabriele Fuchsjäger-Mayrl, Johannes Nepp, Christian Schneeberger, Michael Sator, Wolf Dietrich, Andreas Wedrich, Johannes Huber, and Walter Tschugguel

PURPOSE. The purpose of the present study was to identify the expression of estrogen and progesterone receptor mRNA and of estrogen and progesterone receptor protein in the conjunctiva of healthy women.

METHODS. Specimens of conjunctival tissue of 10 premenopausal women (age range, 13–38 years) were obtained during ophthalmic surgery in patients under general anesthesia. Specimens of approximately 4 mm² were taken from superior, nasal, or temporal bulbar conjunctiva adjacent to the bulbus and were immediately deep frozen with liquid nitrogen. Four women underwent strabismus surgery, two had phacoemulsification, and four had vitrectomy. Only three women were taking oral contraceptives. The expression of estrogen receptor (ER)-α, ERβ, and progesterone receptor (PR) mRNA was analyzed by RT-PCR. Western blot analysis on nuclear extracts was performed with the anti-ERα mouse monoclonal antibody AB-15, the anti-ERβ mouse monoclonal antibody 6B12, and the anti-PR mouse monoclonal antibody PgR 656.

RESULTS. In two samples, ERα, ERβ, and PR mRNAs were not accessible because of highly degraded RNA. In the remaining eight samples, an appearance rate of 100% was obtained for all three mRNAs. Similarly, an appearance rate of 100% was obtained for ERα, ERβ, and PR protein in nine tissue samples accessible for analysis; one sample could not be analyzed due to a low amount of tissue.

CONCLUSIONS. This study confirms the existence of estrogen and progesterone receptors in the human conjunctiva of premenopausal females. Because the proteins of estrogen and progesterone were also found in this study’s specimens, the data indicate that the conjunctiva is a target site for sex steroids. Future studies are needed to elucidate the role of these receptors in ocular diseases involving the conjunctiva. (Invest Ophthalmol Vis Sci. 2002;43:2841–2844)

There is evidence that sex steroids, including estrogens, play a role in structural and functional characteristics of ocular tissues. These effects are mediated by two specific estrogen receptors, estrogen receptor (ER)-α and -β, both of which are members of the superfamily of steroid hormone receptors.

The incidence of increasing intraocular pressure (IOP) and/or glaucoma among females increases after menopause, because of a deficiency of sexual steroid hormones. It is caused by an imbalance between secretion of aqueous humor by the ciliary process and reabsorption or outflow of aqueous humor through the trabecular meshwork. Hormone replacement therapy significantly lowers IOP, suggesting a role for estrogen in the control of IOP.

Estrogen is also assumed to play a role in keratoconjunctivitis sicca, a common complication among postmenopausal women. Symptoms of this condition range from mild foreign-body sensation to pain and visual reduction due to ocular surface abnormalities. Topical estrogen has been implicated in the therapy of conjunctivitis sicca. Previous clinical trials are contradictory, however, and both amelioration of symptoms and negative actions on tear films have been reported. Recently, large-scale studies have been published that indicate that hormone replacement therapy (HRT) is associated with a significantly increased prevalence of severe dry eye symptoms.

It has long been recognized that sex steroids, such as androgen, estrogen, and progesterone, play a role in the functional and structural characteristics of the eye. Accordingly, the presence of sex steroid receptors has been investigated in various ocular tissues by different methods. Although these have been described in the cornea of animals and humans, attempts to determine the presence of estrogen receptors in the human conjunctiva by means of immunohistochemistry have shown a negative result. Because of the limited sensitivity of this technique, it is possible that these previous immunohistochemical experiments missed the presence of the receptor. This hypothesis is supported by a recent study in which molecular biological procedures were used to identify estrogen receptors in a small number of human conjunctival samples. We therefore intended to identify expression of estrogen and progesterone receptor mRNA and of estrogen and progesterone receptor protein in the conjunctiva of healthy premenopausal women.

METHODS

The protocol of the study complied with the tenets of the Declaration of Helsinki for research in human subjects. Specimens of conjunctival tissue of 10 premenopausal women (age range, 13–38 years) were obtained during ophthalmic surgery under general anesthesia. All patients who were undergoing surgery gave written informed consent. All biopsy specimens were obtained under identical conditions and before instillation of any topical medication. Tissue specimens (4 mm²) were taken from superior, nasal, or temporal bulbar conjunctiva adjacent to the bulbus and were immediately deep frozen with liquid nitrogen. Four women underwent strabismus surgery, two had phacoemulsification, and four had vitrectomy. Three women in the group were taking oral contraceptives, and the other seven subjects were not taking any hormonal agents.

Isolation of total cellular RNA from deep-frozen tissue was performed by using a commercially available system (TRI Reagent; Molecular Research Center Inc., Cincinnati, OH) and quantified by measuring
the optical density at 260 nm. cDNA was synthesized in 25 μL total volume, containing a commercially available reverse transcription reaction mix (Random Primed Reverse Transcription Reaction Mix; ViennaLab, Vienna, Austria), 20 U RNasin, 100 U murine Moloney leukemia virus (MuMLV) reverse transcriptase (ViennaLab), and 1 μg total RNA. Reactions were incubated at room temperature for 10 minutes, followed by incubation for 50 minutes at 37°C and 5 minutes at 95°C.

To control for errors in input of cDNA used in PCR reactions, amplification of the ubiquitous β2-microglobulin cDNA was performed in parallel using β2-microglobulin-specific primers. Polymerase chain reactions (PCRs) were performed on an amplification system (GeneAmp PCR System 2400; PE-Applied Biosystems, Weiterstadt, Germany). PCR was performed in a total volume of 25 μL containing 2 μL cDNA template, 25 pmol of each primer (all primer sequences and mapping positions are listed in Table 1), 250 μM dNTPs, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% (wt/vol) gelatin, 1.5 mM MgCl2, 0.1% Triton X-100, and 0.5 U Taq polymerase (Super Taq DNA Polymerase; Vienna-Lab). All amplification profiles have been optimized with regard to the respective exponential phase of the amplification reaction and were as follows: 94°C for 30 seconds, 48°C (ERα) or 50°C (β2-microglobulin, PR, and ERβ) for 30 seconds and 72°C for 30 seconds for 30 cycles. ERα, ERβ, PR or 22 (β2-microglobulin) cycles. To simplify the performance and to increase the reproducibility of PCR, PCR-master mixes containing primers, dNTPs, and buffer were prepared and used in all amplification reactions. In addition, tube contents including all PCR components and distilled water instead of cDNA served as the negative control to check for the presence of DNA that may have carried over from prior reactions. All PCR reactions were performed at least twice in separate experiments.

To ensure that we amplified correct ERα, ERβ, and PR cDNA-fragments, we sequenced the amplified products on an automatic sequencer (Prism 310 Genetic Analyzer System; PE-Applied Biosystems) and compared it with published sequences (BLAST Similarity Search; provided by the National Center for Biotechnology Information, Bethesda, MD; http://www.ncbi.nlm.nih.gov). We found 100% homology with published ERα, ERβ, and PR sequences.

PCR products (ERα: 123 bp; ERβ: 215 bp; PR: 202 bp; β2-microglobulin: 231 bp) were separated on agarose gels (5–10 μL PCR product; 3% SB Fine Gel Agarose; Severn Biotech Ltd., Worcester, UK) and visualized by performing the electrophoresis on fluorescent dye (1:10,000 dilution; SYBR Green I; Molecular Probes Inc., Eugene, OR)–containing gels.

For the analysis of ERα, ERβ, and PR protein expression, Western blot analysis was performed on recombinant human ERα-protein (Affinity BioReagents, Golden, CO) and ERβ-protein (Alexis Biochemicals, San Diego, CA) and nuclear protein fractions. Nuclear protein extracts were obtained from lysed tissue with the use of a commercially available protein isolation system (NE-PER; Pierce, Rockford, IL). Protein concentrations were measured by spectrophotometry using a protein assay reagent kit (MicroBCA-Protein Assay Reagent; Pierce). One hundred nanograms of the recombinant ERα-protein and 20 μg nuclear protein fraction were electrophoresed by SDS-polyacrylamide-gel electrophoresis on 8% to 18% gradient gels (ExcelGel; AP Biotech, Uppsala, Sweden) and transferred onto nitrocellulose membranes (Hybond-ECL; AP BioTech). Membranes were blocked in a solution consisting of 2.5% nonfat dry milk and 2.5% BSA in PBS (pH 7.2) containing 0.05% Tween-20. Immunoreactions were performed with an anti-ERα mouse monoclonal antibody AB-15 (dilution 1:50; NeoMarkers, Fremont, CA), an anti-ERβ mouse monoclonal antibody 6B12 (dilution 1:1000; Genetex, San Antonio, TX), or an anti-PR mouse monoclonal antibody PgR 656 (dilution 1:1600; Dako, Carpinteria, CA). An appropriately diluted isotype matched IgG1 monoclonal antibody (Coulter Clone, Beckman Coulter, Hialeah, FL) was used as the respective negative control. This was followed by an HRP-conjugated goat anti-mouse (1:50,000; Pierce) IgG. Specific reaction products were detected by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Pierce).

**RESULTS**

In two samples ERα, ERβ, and PR mRNA was not accessible because of highly degraded RNA. In another sample ERα, ERβ, and PR protein analysis could not be performed because of the low amount of tissue. In the remaining samples an appearance rate of 100% was obtained for ERα, ERβ, and PR protein expression, Western blot analysis was performed on recombinant human ERα-protein (Affinity BioReagents, Golden, CO) and ERβ-protein (Alexis Biochemicals, San Diego, CA) and nuclear protein fractions (Table 2). RT-PCR analysis and Western blots of the conjunctival tissues are shown in Figures 1 and 2, respectively. Similarly, an appearance rate of 100% was also obtained for ERα, ERβ, and PR protein in the tissue samples accessible for analysis.

**Table 1. DNA Amplification: Primer Sequences and Mapping Positions**

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<th>Primer</th>
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<th>5′</th>
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**Table 2. Expression of ERα, ERβ, and PR mRNA and ERα and ERβ Protein in Conjunctival Tissues by RT-PCR**

<table>
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<tr>
<th>Sample</th>
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<th>ERβ mRNA</th>
<th>PR mRNA</th>
<th>ERα Protein</th>
<th>ERβ Protein</th>
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ND, Not determined, because of degraded RNA (samples 9, 10) or too little tissue (sample 8).
investigate the specificity of the antibodies used, 100 ng recombinant human ERα and 100 ng recombinant human ERβ proteins were immunostained with either AB-15 monoclonal ERα (Fig. 3a) or 6B12 monoclonal ERβ antibody (Fig. 3b).

**DISCUSSION**

The present data demonstrate the presence of ERα, ERβ, and PR mRNA, as well as ERα, ERβ, and PR protein in the conjunctiva of premenopausal women. This finding is in contrast to previous immunohistochemical studies, most likely because of the higher sensitivity of RT-PCR used in the current study. Our results are in keeping, however, with a recently published study, in which estrogen and progesterone receptors were identified in a small number of human conjunctival samples. Estrogen receptors have been identified in a variety of ocular tissues in animals and humans. Esmali et al. detected estrogen receptors in the meibomian glands of the upper eyelid by immunohistochemical staining. In mouse corneas, as well as in human corneas, estrogen receptors have been verified recently by means of RT-PCR. By contrast, Vecsei et al. did not detect estrogen receptors in human corneas by using immunohistochemistry.

The presence of ERα, ERβ, and PR mRNAs in human conjunctiva does not prove that mRNAs are translated in this tissue. We detected ERα, ERβ, and PR protein in our human conjunctival samples, which strongly indicates that the sex steroid receptor mRNAs are indeed translated. Previous immunohistochemical studies failed to detect receptor proteins in the human conjunctiva, possibly because of the limited sensitivity of the methods used.

Evidence that the conjunctiva is an estrogen-sensitive epithelium arises from a variety of studies. Cyclic variations in conjunctival smears paralleling the menstrual cycle were observed in premenopausal, but not in postmenopausal, women. Moreover, maturation of conjunctival smears is paralleled with hormonal changes in women with normal menstrual cycles, but not in postmenopausal women. The presence of estrogen and progesterone receptors in the conjunctiva and the meibomian gland may also be related to the sex differences in the incidence of dry eye syndrome, which has recently been confirmed in a large-scale study in a managed-care population. The presence of sex steroid receptors and their proteins in the human conjunctiva also represents an explanation for sex steroid-induced changes in allergic responses, goblet cell maturation, and maturation of the conjunctival epithelium.

**FIGURE 1.** ERα, ERβ, PR, and β2-microglobulin (βMGL)-specific RT-PCR analysis in six conjunctival tissue samples (M: Molecular weight marker; top to bottom: 1000, 700, 500, 400, 300, 200, 100, and 50 bp).

**FIGURE 2.** Western blot analysis performed on four nuclear extracts (one in each of the four lanes) from conjunctival tissue. Twenty micrograms of nuclear protein extracts were immunostained with AB-15 monoclonal ERα, 6B12 monoclonal ERβ-antibody, or PgR 636 monoclonal PR antibody.
Conjunctiva, as reported previously, obviously, our results may also have implications for future therapeutic approaches in diseases with conjunctival involvement, such as allergic conjunctivitis and dry eye syndrome.

In conclusion, this study reports on the presence of both estrogen receptor subtypes, α and β, and the progesterone receptor as well as their respective sex steroid proteins in the human conjunctiva of premenopausal females. This clearly indicates that these sex steroids may play a role in the structural and functional features of the human conjunctiva.

References


15. Matsumoto K, Minamitani T. Sequence definition: Homo sapiens mRNA for beta-2-microglobulin, complete cDNA; submitted to the DDB/EMBL/GenBank databases; accession number AB021288.


