Estrogen Receptor in the Human Eye: Influence of Gender and Age on Gene Expression

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Purpose. Many epidemiologic studies indicate an increased incidence of certain vision threatening conditions in postmenopausal women. These data suggest that changes in sex steroid homeostasis may affect the physiology of the eye. To provide support to this hypothesis, the expression of estrogen receptor alpha (ERα) in human eye tissues was investigated.

Methods. Complementary studies including RNA analysis by reverse transcription polymerase chain reaction, western blot analysis, and immunocytochemistry were used to provide evidence of ERα expression. Protein detection was carried out using a mouse monoclonal antibody raised against an epitope located in the ligand binding domain of the human receptor. Cellular localization was studied on formalin-fixed paraffin-embedded eye sections using conventional immunohistochemical techniques.

Results. Gender and age differences in ERα mRNA expression were observed in retina. The 65-kDa ERα protein was detected in the retina and retinal pigment epithelium (RPE) of young female eyes but not in eye tissues dissected from men and postmenopausal women. Immunocytochemistry corroborated ERα staining of a young female neurosensory retina and RPE. In addition, ERα could be detected in the ciliary body, in the iris, and in the epithelium of the lens.

Conclusions. The presence of the ERα in the human eye suggests that the sex steroid hormone axis may play a role in the pathogenesis of certain ocular diseases. (*Invest Ophthalmol Vis Sci.* 1999;40: 1906–1911)

Significant gender-based differences in the incidence of many important ocular conditions raise the possibility that estrogens may have direct effects on the eye. Disorders such as age-related macular degeneration (AMD), idiopathic full-thickness macular hole, and cataract have been conclusively associated with gender and age in several epidemiologic studies. These investigations demonstrate an increased prevalence of high morbidity eye diseases in the elderly, and in postmenopausal women in particular.[1–3] Furthermore, an elderly female population under estrogen replacement therapy has shown a reduced prevalence of nuclear cataract, indicating some beneficial effects of estrogens on lens physiology.[4] Although other epidemiologic studies have suggested the prevalence of dry eye and glaucoma among the elderly, especially women, they involved different populations and different clinical end points and as such are inconclusive.[5–7] Thus, the body of epidemiologic data points to an involvement of estrogens in the normal physiology of the human eye. A single study reported previously by Gans et al. failed to find estrogen and progesterone receptors in conjunctival human tissues.[8]

Estrogens are steroid hormones long known for their profound effects on both male and female reproductive systems. Estrogens regulate growth, differentiation, and function of diverse tissues both within and outside the reproductive system. The effects of estrogens are mediated by specific nuclear receptors, the estrogen receptor (ER) α and β types, that act as hormone-inducible transcription factors.[9–11] Relatively recent findings demonstrate important roles of the steroid hormones in the cardiovascular system, in specific brain regions, in the liver, and in the maintenance of bone tissue. Despite the wide range of tissues influenced by estrogens and the broad transcriptional regulatory properties of the ligand-activated ERs, the literature contains limited evidence of these receptors in normal ocular tissues. This, and the large spectrum of physiological alterations occurring in women after the normal hormonal decline of menopause, or in males during andropause, motivated our studies. We report here that the α type of estrogen receptor is normally present in the human eye and that gender and age differences may influence its expression.

Materials and Methods

Tissue Collection

Human eyes were obtained from the Lions Eye Bank, Phoenix, Arizona, and stored at −80°C. Tissues from 4 females, ages 35 (postmortem enucleation time [PET] 3 hours), 49 (PET 3 hours), 74 (PET 1.5 hours), and 77 (PET 3.5 hours) and 3 males, ages 27 (PET 3.5 hours), 45 (PET 5 hours), and 76 (PET 3 hours) were studied. In addition, formalin-fixed paraffin-embedded human eye sections were obtained from the Pathology Laboratory at the UCLA School of Medicine. The majority of the

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samples were harvested during autopsy (females 48, 49, 55, 68, 77, 79, 83, 90, and 93 years of age and males 6, 13, 59, and 65 years of age) except for a unique specimen obtained after enucleation belonging to a female donor (age 17 years).

MCF-7 breast adenocarcinoma cells (American Type Culture Collection, ATCC# HTB-22) were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium, 1:1 (vol/vol; D-MEM/F12; GIBCO–BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (GIBCO–BRL), 1 mM sodium pyruvate (Sigma, St. Louis, MO), and antibiotic antimycotic solution (Sigma).

Detection of ERα Transcripts in Retinas

Total RNA was obtained from retinas and MCF-7 cells using TRIzol (GIBCO–BRL). All the RNA pellets were treated with 10 U/μl of RNase–free DNaseI (Boehringer Mannheim, Indianapolis, IN) for 60 minutes at 37°C to eliminate DNA contamination. A pair of primers previously designed by Enmark et al.[12] was used to amplify the ERα open reading frame. Polymerase chain reactions (PCR) were standardized by amplifying a 300–bp fragment of the α–tubulin mRNA with the primers 5′GCCACTTATGCCCCTGTCAT3′ and 5′TCTCTGCACCTTGGCCAGGT3′. First-strand cDNAs were synthesized using 1 μg of total RNA, 50 U of murine leukemia virus reverse transcriptase (MuLVRTase; Perkin–Elmer, Branchburg, NJ). The cDNA products were subjected to PCR in a Robocycler (Stratagene, La Jolla, CA) using 2.5 U AmpliTaq DNA polymerase (Perkin–Elmer) and the following program: 94°C for 2 minutes, 35 cycles (94°C for 60 seconds, 58°C for 80 seconds, 72°C for 90 seconds), 72°C for 10 minutes. The PCR products were separated on a 2.5% agarose gel and blotted onto a Hybond N+ membrane (Amersham Life Science, Arlington Heights, IL), according to the manufacturer's recommendations. The blots were then probed with an internal oligonucleotide that recognized the ERα sequence and that had been radiolabeled with [γ-^{32}P]-dATP using a polynucleotide kinase (Pharmacia Biotech, Piscataway, NJ). Blots were hybridized overnight at 65°C and washed at a final stringency of 0.2% SSC plus 0.2% sodium dodecyl sulfate (SDS) at 60°C.

Detection of ERα by Western Blot Analysis

Retina, retinal pigment epithelium (RPE)/choroid, sclera, and a pool of anterior eye tissues, including cornea, iris, lens, and ciliary body, were dissected from frozen eyes. Sclera and cornea–containing samples were ground in a mortar before extraction. Nuclear extracts from retina and the MCF-7 cell line were prepared according to the method described by Fei et al.[13] with some modifications. Briefly, retinas or cell pellets were homogenized in buffer containing 10 mM Tris–HCl, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 1 mM dithiothreitol (DTT), 0.2% Nonidet P-40, and 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) using a glass homogenizer and 3 to 4 strokes of a Teflon pestle. The samples were centrifuged at 38,000 rpm for 30 minutes at 4°C to sediment the nuclear fraction. Nuclear pellets were resuspended in buffer A (10 mM Tris–HCl, pH 7.6, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, and 0.5 mM PMSF), and the nuclei were broken in an all-glass Dounce homogenizer. Homogenates from RPE/choroid, minced sclera, and anterior eye tissues were obtained after treatment with buffer A under the same experimental conditions. Cellular debris was removed from each sample by centrifugation at 14,000 rpm for 30 minutes in an Eppendorf centrifuge. Protein concentration was determined by the Peterson technique.[14]

Samples (20 μg of nuclear retinal extracts, 40 μg of MCF-7 cells nuclear extracts, 100 μg protein from RPE/choroid, sclera, and anterior eye fractions or 200 ng of the recombinant ERα protein) were electrophoresed on polyacrylamide gels (10%T/2%C) using a Tris/tricine buffer system[15] for 30 hours at 35 mA constant current, and then transferred to nitrocellulose by the method of Towbin et al.[16] at 4 V/cm, 10°C, overnight. Nitrocellulose membranes were blocked in 3% bovine serum albumin (BSA), 0.1% Tween-20 in Tris-buffered saline (TBS: 500 mM NaCl, 20 mM Tris, pH 7.6) for 2 hours at 37°C. Blots were then incubated overnight with primary mouse monoclonal antiserum (1:500 dilution) produced against the human ERα (NeoMarkers, Fremont, CA) and the products were visualized with the Amplified-Alkaline Phosphatase kit (Bio–Rad Laboratories, Hercules, CA) using biotin–streptavidin.

Immunocytochemical Localization of ER

Formalin-fixed paraffin-embedded sections (10 μm) were deparaffinized in xylene and hydrated through graded alcohols. Immunolocalization of the sex steroid receptor was performed using conventional immunohistologic techniques. Incubation with the primary antibody (1.5 μg IgG/ml) was for 20 hours at 4°C in a moist, air-tight chamber. Slides were exposed for 1 hour to biotinylated goat anti-mouse immunoglobulin (1:1500 dilution). Antibody dilution was prepared in PBS containing 0.5% normal goat serum, 2% Triton X-100, and 5% BSA, and all buffer washes were performed with PBS. As controls, some sections in each experiment were processed replacing the primary antibody with normal mouse serum at the same working dilution. Reactions were visualized using two alternative enzymatic detection systems, an avidin–biotin–peroxidase/3,3′-diaminobenzidine (DAB) kit or the alkaline phosphatase kit coupled with the Vector Blue substrate reagent (Vector Laboratories, Burlingame, CA). Eosin-Y was used as counterstaining, and sections were permanently mounted in the recommended mounting solutions according to the manufacturer.

RESULTS

ERα mRNA Expression in Human Retina

Reverse–transcription PCR (RT–PCR) was used to amplify the ERα mRNA from different retinal tissues (Fig. 1). The primers chosen (5′AATTCAGATAATCGACGCCAG3′ and 5′GTGTTTCAACATTCTCCCTCCTC3′) amplified a 344–bp fragment from the ERα mRNAs (Fig. 1A); a 300–bp fragment amplified from the α-tubulin mRNA served as internal standard for each reaction (Fig. 1B). An ER-positive cell line, the mammary adenocarcinoma cell line (MCF-7), was chosen as control (lane 7), because it had been reported that MCF-7 cells produce high levels of ERα as well as significant but lower amounts of the beta type.[12,17–19] Different ERα mRNA expression levels were detected in RNA isolated from retinas of various donors. Qualitatively, ERα transcripts were found in the retinas of 2 females still having estrus cycles (35 and 49 years of age; lanes 1 and 2) in contrast with a dim band seen in an older female retina (74 years of age; lane 3). A retinal mRNA isolated from a 77-year-old

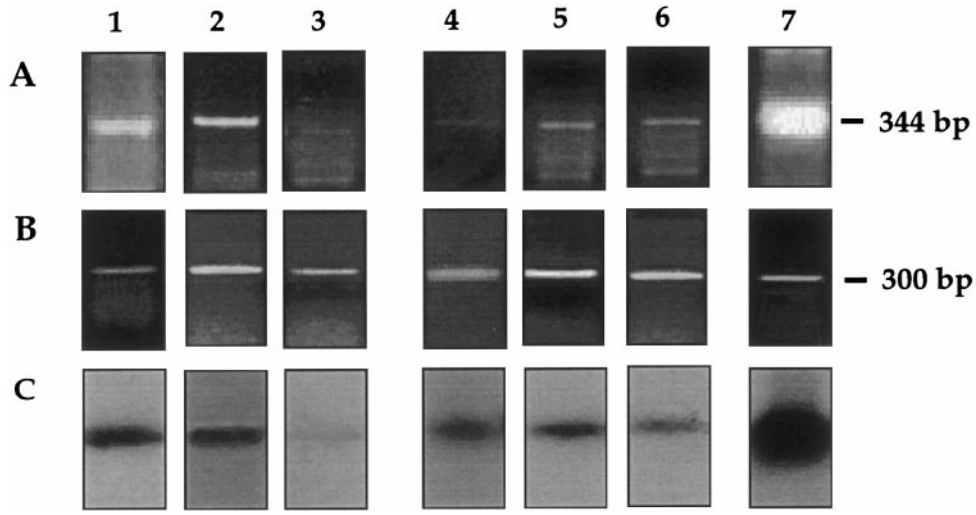

FIGURE 1. Gender and age differences of estrogen receptor gene expression in human retinas. (**A**) Total RNA from retinas of 3 females, 35 (*lane 1*), 49 (*lane 2*), and 74 (*lane 3*) years of age; of 3 males, 27 (*lane 4*), 45 (*lane 5*), and 76 (*lane 6*) years of age, and from MCF-7 cells (*lane 7*) was obtained. PCR detection of ERα transcripts was performed using 1 μg of total RNA and a primer pair corresponding to nucleotides 457 through 477 and 801 through 779 of the human ERα cDNA. (**B**) Another set of reactions using 1 μg of total RNA and a pair of primers designed from the sequence of the mouse α-tubulin cDNA amplified a 300-bp fragment (internal control). (**C**) A 23-mer fragment of the ERα gene was radiolabeled and used as a probe, confirming the identity of the RT–PCR products in (**A**). Bars indicate the expected size of the PCR products.

female did not exhibit ERα RT–PCR product (data not shown). Two young men (27 and 45 years of age; lanes 4 and 5) and an old man (76 years old; lane 6) all showed ERα transcripts in their retinal RNAs. Male retinas seemed to have mRNA levels intermediate between young and old female retinas; in addition, the amount of transcript did not change with increasing age. An internal ERα specific oligonucleotide probe (5′CCAATGACAAGGGAAGTATGGCT3′) confirmed the identity of the PCR products (Fig. 1C).

Immunodetection of ERα by Western Blot Analysis

Western blot analysis using a mouse monoclonal antibody raised against the human ERα (Fig. 2) showed an intensely labeled 65–kDa protein corresponding to the ERα recombinant protein (lane 1). Two lower-molecular-weight bands were also labeled, but they may correspond to degradation products of the recombinant protein. Nuclear extracts obtained from the positive control cell line MCF-7 similarly exhibited the 65–kDa ERα protein (lane 10). ERα was present in the nuclear retinal extract and in the total protein extract from RPE/choroid isolated from the eyes of a young female donor (lanes 2 and 4, respectively). ERα was not observed in the retina (lane 3) or RPE/choroid (lane 5) samples from the 77-year-old donor eye; no positive reaction could be detected in tissues from 2 other older females or 3 men examined under the same experimental conditions (data not shown). ERα seems to be absent from cornea (lanes 6 and 7) and sclera (lanes 8 and 9) extracts in all the samples analyzed (only shown are samples from the 35- and 77-year-old female donors). The additional higher-molecular-weight proteins seen on the western blot analysis are not specific, because they are also present in blots analyzed in parallel where the primary antibody was replaced by normal mouse serum (data not shown).

Immunolocalization of ERα in Human Eye Sections

Expression of ERα was studied on paraffin-embedded sections from a 17-year-old female donor eye. In this particular case, the eye was fixed immediately after enucleation, eliminating any possibility of rapid ERα degradation. Immunohistochemical analysis of sections from diverse donors (see the Materials and Methods section) corroborated the results obtained with the 17-year-old donor eye and were in agreement with the data generated by western blot analysis. The sections from older eyes showed the predictable cellular death caused by the aging process.

Immunolocalization of ERα-expressing cells was carried out with the same mouse monoclonal antibody used on the western blot (Figs. 3, 4, and 5). No positive-labeled nuclei were observed when the primary antibody was replaced with normal mouse serum (Fig. 3A). ERα immunostaining was observed across the neuroretina, in most of the nuclei of the outer nuclear layer and inner nuclear layer, in a few cells in the outer plexiform layer, presumably horizontal or bipolar cells, and in some nuclei of the ganglion cell layer (Fig. 3B). Also evident was a strong reaction in the axons of the ganglion cells. In general, the presence of positive staining in other subcellular compartments except for nuclei appears as a common problem of the immunocytological techniques.[20]

A blue chromogen was used to analyze ERα expression in RPE. This allowed us to distinguish our end product from the pigments normally present in RPE cells. No positive staining could be observed in the absence of the primary antibody (Fig. 3C) in contrast to a strong reaction in some of the RPE nuclei in its presence (Fig. 3D).

Anterior eye tissues such as the lens epithelium (Figs. 3F and control 3E), the nonpigmented cell layer of the ciliary body epithelium (Figs. 3H and control 3G), and cells in the stroma of the iris and around the capillaries (Fig. 3I) also showed immu-

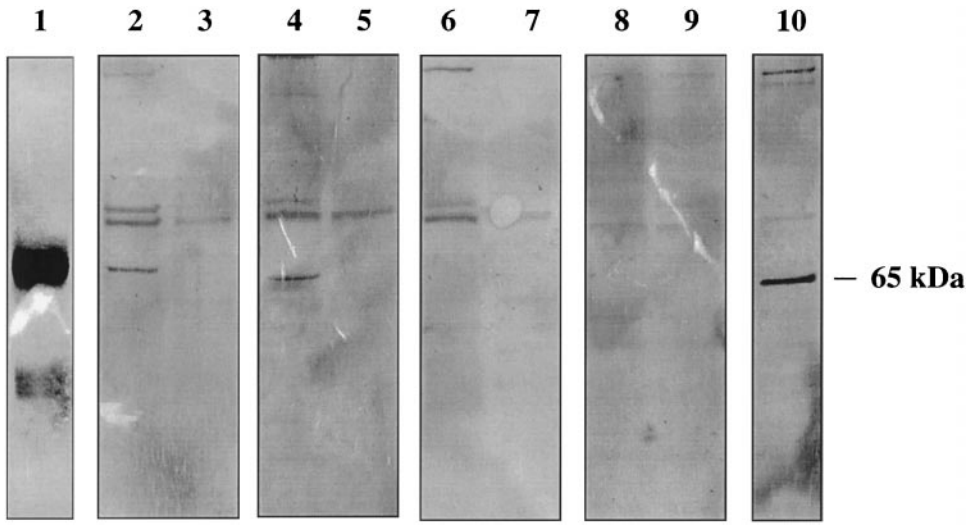

FIGURE 2. Immunodetection of ERα in various eye tissues. Proteins from different samples were separated by SDS–PAGE: ERα recombinant protein (*lane 1*), nuclear fractions from retina (*lanes 2 and 3*), total extracts from RPE/choroid (*lanes 4 and 5*), a pool of anterior eye structures (*lanes 6 and 7*), sclera (*lanes 8 and 9*), and nuclear extracts from the MCF-7 cells (*lane 10*). A mouse monoclonal antibody produced against the human ERα and an alkaline phosphatase coupled system were used to visualize the 65-kDa protein. Tissues were obtained from two female donor eyes, 35 (*lanes 2, 4, 6, and 8*) and 77 (*lanes 3, 5, 7, and 9*) years of age. Molecular weight of the ERα is indicated.

noreactivity, indicating the presence of ERα. The receptor could not be visualized in the corneal epithelium (Fig. 3J).

DISCUSSION

It is well known that age is a risk factor for loss of visual function in many diseases. Several clinical disorders appear during the aging process. Symptoms observed in menopausal women have been considered related to the abrupt decline in hormonal activity after the reproductive years. The aging male also experiences a number of physical alterations associated with a 30% decrease in circulating testosterone and other minor androgens, as well as prolactin, growth hormone, and insulin growth factor-1, although male estrogen levels remain unchanged throughout life.[21]

Several epidemiologic observations suggest a potential participation of estrogens in the homeostasis of the eye, but the mechanisms involved remain unclear. Hormone replacement therapies have been associated with a decreased incidence of ocular diseases, such as glaucoma,[22] AMD,[23] and cataract,[24,25] further suggesting a central role for estrogens in ocular physiology. The classic cellular mechanism by which steroids act is through intracellular receptors, which modulate transcription and protein synthesis on the target cell after becoming activated. Recently, an alternative nongenomic pathway has been proposed to explain the rapid onset short-term effects of sex steroids in the brain.[26] The authors suggested that cellular signaling mechanisms of neurotransmitters and steroids (estrogens being the most studied) exhibit significant similarities and proposed a new model of action through ligand-gated ion channels or G protein–coupled second-messenger systems.

In this study, we have used three complementary techniques to provide evidence for the presence of ER in the human eye. Our results suggest that the clinically observed modulatory properties of estrogens may be mediated by the expression of the ER in the ocular tissues. Although addressing mechanistic issues requires further study, synergistic estrogen effects through a nongenomic pathway cannot be discarded.

Expression of the ERα gene was detected by RT–PCR using a set of primers located within exons 2 and 4. Differences observed in the ERα mRNA levels of several eyes can be directly associated with gender and age, because the housekeeping α-tubulin gene, used as internal standard in the reaction, exhibited similar expression in all the samples. Apparently, gender and age are factors that may influence the expression of the receptor. This suggests that the reduced amounts of circulating estrogens in women after menopause and at all times in men may lead to reduced transcriptional levels of the ERα mRNA in the target ocular tissues.

The high homology displayed between different members of the superfamily of steroid receptors and the relatively recent finding of a new ER type, the ERβ, have made difficult the identification of a specific antibody for detection of ERα. For our protein analyses, we used a monoclonal antiserum raised against an epitope located in the E domain (amino acids 302–553) of the human ERα, previously characterized by Abbondanza et al.[27] We considered the presence of a protein band identical in molecular size to the recombinant ERα protein an additional confirmation of the receptor identity in our samples. The complete absence of ERα in retinal extracts from three men and three postmenopausal women compared with a significant protein expression in the retina of a young female was in agreement with the mRNA differences observed by RT–PCR. However, production of very low levels of ERα, below the sensitivity of detection of our assays could also explain the absence of the immunoreactive protein in those samples. It is important to mention that sample loading was normalized by measuring the amount of total protein in each extract. Differences observed in the intensity of nonspecific reactive bands after immunostaining (for instance, retinal nuclear extracts from the young and old females) could also be the result of changes in the relative amount of protein occurring during cellular aging.

At this time, the significance of ER in human eye tissues is not clear. However, together with epidemiologic evidence, the results presented here suggest that alterations in ER physiology may be involved in the pathogenic mechanisms underlying AMD, idiopathic full-thickness macular hole, glaucoma, cataract, and dry eye. Interaction of steroid–receptor complexes with responsive genes containing a consensus sequence for ER binding can result in either induction or repression of tran-

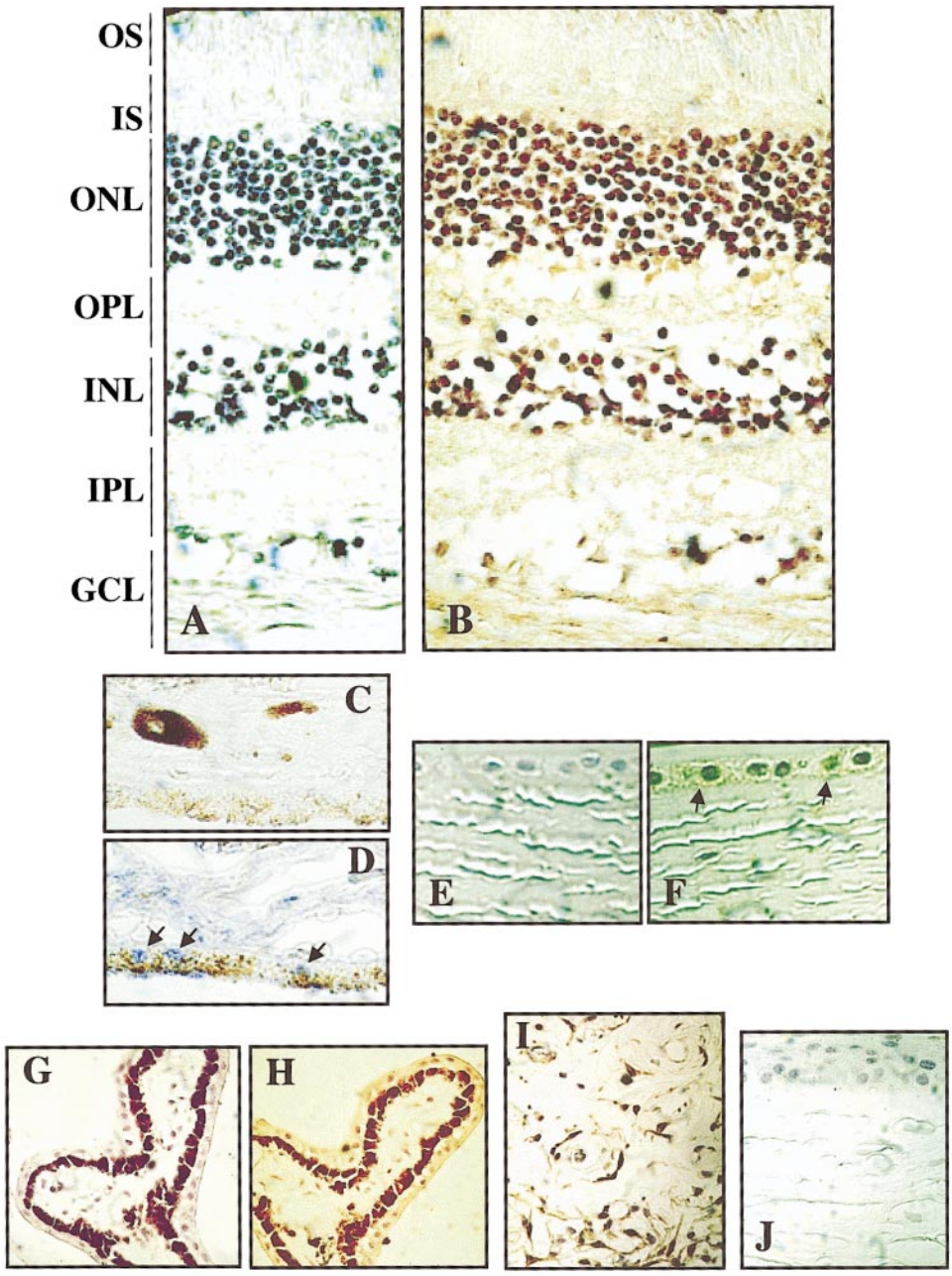

FIGURE 3. Expression of ERα. Immunolocalization of ERα was performed using paraffin-embedded sections from a human donor eye formalin-fixed right after enucleation (female, 17 years of age). (**A**) Retinal nonspecific staining determined by replacing the primary antibody with normal mouse serum. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer. (**B**) Positive-labeled brown nuclei (DAB staining) visualized across the retina after immune reaction with an anti-ERα monoclonal antibody (magnification, ×725). Nonspecific (**C**) and specific (**D**) immunostaining in the RPE, visualized with the alkaline phosphatase detection system and using a blue chromogen in the developing reaction. *Arrows* indicate ERα-expressing nuclei in the monolayer (magnification, ×600). Nonspecific (**E** and **G**) and specific (**F**, **H**, **I**, and **J**) reactions in the lens epithelium, ciliary body epithelium, iris, and cornea, respectively, observed after immunostaining with the monoclonal antibody and developing with the peroxidase/DAB system. *Arrows* in (**F**) indicate positive reaction with the antibody (magnification, ×400). Note the absence of immunoreactivity in the corneal section.

scription, depending on the target gene and tissue. The broad number of estrogen-induced proteins (e.g., progesterone receptor, cathepsin D, α2-macroglobulin, cytochrome P-450 aromatase, c-*fos*, c-*myc*, heat shock protein 27, tumor growth factor α, pS2) identified to date may justify the multifaceted involvement of the steroid hormones in the control of processes such as cell proliferation, differentiation, physiology, and development. Interestingly, most of these proteins have been found in human eye tissues, such as RPE, retina, or ciliary body.[28–33] Moreover, these proteins seem to be coexpressed with the ER, and, thus, their regulation in the eye may be controlled by estrogens.

The presence of ERα in the epithelia of several ocular tissues such as retina, lens, ciliary body (nonpigmented), and, also, in the iris stroma, brings up the possibility that ER could be regulating the transcriptional expression of different target genes in those tissues. Furthermore, the localization of ERα in neuronal cells of the retina and in specific brain areas suggests participation of the ERα in the regulation of neuronal function.

In summary, in an attempt to begin to investigate the molecular mechanisms underlying gender-based predispositions in certain ocular diseases, we have been able to demonstrate for the first time the presence and localization of ERα in human eye tissues.

Note added in proof

After submission of this manuscript, another group described the presence of ER in bovine and rat retinas.[34] As it could be predicted, our findings in the human retina exactly correlate with their description in the homologous mammalian tissue.

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