Heat Shock Cognate-70 Gene Expression Declines during Normal Aging of the Primate Retina

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PURPOSE. Despite documented age-related changes in retinal function and histology, little is known about the pattern of gene expression during normal aging of the vertebrate retina. This study was undertaken to definitively characterize gene expression in the primate retina during aging.

METHODS. Human retina cDNA library clones were arrayed at high density on nylon membranes and screened with mixed cDNA probes generated from young (4-year-old) and old (80-year-old) human retinas. Clones showing a more than twofold difference in intensity were rescreened by dot blot analysis with the same probes and with mixed cDNA probes generated from young (2–3 years) and old (27–35 years) rhesus monkeys. One clone identified by its differential (age-putative) signal, and age-related differential expression was used for analysis of Northern blot analysis of total retinal RNA from human donors (35 weeks to 94 years of age) and two rhesus monkeys (2 and 27 years of age). The identified clone was sequenced and compared with entries in the GenBank/EMBL databases. Western blot analysis was performed on protein isolated from the retina of human donors aged 4 to 64 years and rhesus monkeys aged 18 months and 35 years.

RESULTS. Approximately 1.6% of the 55,368 retina-expressed sequences examined show age-related changes between tissues from young and old donors. The mRNA level one clone, identical with heat shock cognate (HSC)70, was altered during normal retinal aging in primates. Regression analysis of Northern blot analysis signals from 23 human donors suggested that there may be a two- to threefold decrease in HSC70 mRNA levels in the human retina by the eighth decade of life. Western blot analysis also showed lower levels of the 70-kDa HSC protein in older tissues of both primates.

CONCLUSIONS. HSC70 mRNA levels apparently decline during normal aging of the primate retina. Because the heat shock 70 protein family may play important roles in ocular development and protection from various biologic and environmental stresses, decreased HSC70 levels in the retina during aging may contribute to the apparent increased susceptibility of the retina to age-acquired retinal disease. (Invest Ophthalmol Vis Sci. 2000;41:2857–2862)
may be a suitable gene model for studying age-related alternations in retina-expressed genes, because it is relatively easy to obtain good quality tissues and to monitor age-related changes. We recently described a strategy for evaluating gene expression in retinal tissues of young and old human donors and reported the identification of a clone (designated dd112) that showed a significantly lower expression in retina from old versus young donors. The identity of this clone was not determined, and it was not known whether the age-related changes observed in human retina correlate with those in the retina of rhesus monkeys. Clone dd112 has now been identified as heat shock cognate (HSC)70, the constitutively expressed member of the 70-kDa heat shock protein (HSP) family. We screened additional human donor samples and performed multispecies analysis by evaluating the expression of clone dd112 in young and old rhesus monkeys. Because mRNA levels do not always directly correlate with protein levels, we also determined the level of the 70-kDa heat shock cognate protein in human retinal extracts.

HSPs are differentially expressed in response to various biologic and environmental stresses, indicating that they are important in maintenance of cellular function. In particular, HSC70 plays a role in regulation of normal protein folding; preventing damage to proteins, intracellular processing of newly synthesized proteins, facilitation of protein translocation, and enhancement of protein degradation. Studies have demonstrated that there is precise cellular and developmental regulation of HSC70 in ocular tissues, indicating that this chaperone may have specific cellular roles during ocular development and in vertebrate retinal neurogenesis. Because appropriate expression of HSC70 is critical for cellular activity, and HSC70 expression varies during vertebrate retinal development, it is likely that alterations in HSC70 activity could ultimately result in the accumulation of incorrectly folded intracellular proteins, changes in nucleocytoplasmic transport, and/or dysfunction in protein degradation. A decrease in HSC70 intracellular levels could contribute to age-related dysfunction and disease susceptibility, especially in the highly metabolically active retinal environment. The apparent decrease in the level of this gene in older tissues may be associated with the onset and/or progression of age-related diseases of the retina.

**Materials and Methods**

**Tissue Collection**

Human donor eyes were obtained from the Maryland Eye Bank and the National Disease Research Interchange (NDRI, Philadelphia, PA). The time between human donor death to tissue dissection and preservation varied from 12 to 48 hours. Donor eyes were enucleated within 8 hours of death, chilled on wet ice, and transported to the laboratory. All eyes were grossly examined before dissection and were rejected for the study if there were any signs of ocular disease, sepsis, or intraocular disease. A list of samples used in the study, hours to tissue dissection, and medical history are shown (Table 1). The donor samples listed were chosen to give a fair representation of different decades of life. Tissue was dissected if the donor had any ocular or chronic wasting disease. Adolescent monkey eyes (2–3 years of age) were obtained from John Cogan (Department of Biologics, Bethesda, MD), and eyes from old monkeys (27–35 years of age) were obtained through the Obesity and Diabetes Research Center, Department of Physiology, University of Maryland. Tissues were dissected within 1 hour of the animal’s death. All animals were treated and euthanized humanely, according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinas were dissected on ice, and frozen at −70°C until use. Total RNA was extracted from retinal tissues using RNAzol B (Tel-Test; Friendswood, TX), reconstituted in diethyl pyrocarbonate (DEPC)-treated water and stored at −70°C until needed.

**cDNA Array Screening**

Putative gene sequences changing expression during aging were identified by cDNA array screening using mixed age-specific retinal cDNA probes, generated from total poly(A+) mRNA from retinas of young (4-year-old) and old (80-year-old) human donors, using methods previously described. Briefly, retinal cDNA was prepared using reverse transcriptase (Superscript II-RT; Gibco, Rockville, MD) primed with oligo(dT) linked to a unique amplification primer (3′rapid amplification of cDNA ends [RACE] primer; Gibco). Purified first-strand cDNA was then tailed with dCTP using terminal deoxynucleotidyl transferase (Gibco) and reacted to probe from young (18 months) and old (35 years) rhesus monkeys. Probes are prereacted with human repetitive DNA (cot-1; Gibco) and reacted to prehybridized, duplicate nylon membranes (22 × 22 cm) containing 27,684 cDNA clones per membrane at 65°C for 17 hours in Hybrisol II (Oncor, Gaithersburg, MD). After stringent washing (0.1× SSC-0.1% sodium dodecyl sulfate [SDS] at 65°C), membranes were exposed to autoradiographic film (BMR; Eastman Kodak, Rochester, NY) at −70°C, or to a radiosensitive phosphoscreen (Storm Imager; Molecular Dynamics, Sunnyvale, CA) at −20°C. Clones exhibiting age-apparent differences were then rescreened using mixed retina cDNA probes from young (18 months) and old (35 years) rhesus monkeys. Clones exhibiting significant differences in signal intensity between young and old cDNA probes of both species were isolated and sequenced to determine identity.

**Northern Blot Analysis**

Putative age-related candidate clone cDNA was reacted against total RNA from human donors (55 weeks to 94 years) and rhesus monkeys (18 months and 35 years), as previously described. Electrophoresis and Northern blot analysis, RNA from the rhesus monkeys was prepared from young (18–24 months) and old (27–35 years) monkeys. Total RNA loading of each sample was normalized by spectrophotometry (GeneQuant; Amersham–Pharmacia, Piscataway, NJ) and by densitometric comparison on an imaging workstation (NucleoVision; Nucleo-Tech, San Mateo, CA) of the ethidium bromide–stained 18S rRNA band for each sample. Reacted blots were exposed to film (X-AR; Kodak) at −70°C, and developed films were densitometrically scanned and signal intensity normalized using 18S rRNA loading. Because HSC70 shares considerable homology with HSP70 in the 3′ region of its mRNA, we also performed Northern blot analysis using a [γ-32P] adenosine...
triphosphate (ATP)-kinased HSC70-specific oligonucleotide probe to confirm HSC70-specific expression. The human sequence used to generate the oligonucleotide probe was obtained from GenBank (Accession number: Y00371) and the complementary sequence used was; 5′-ATC AAT ACC AAC TGC AGG TCC CTT GGA CAT-3′.

Western Blot Analysis

Protein was isolated from human and monkey retina extracts after homogenization of retinal tissues in reagent (Trizol; Gibco) and processing to remove RNA and DNA. The protein pellets isolated were washed at 4°C with 95% ethanol solution containing 0.3 M guanidine hydrochloride, and 100% ethanol, resuspended in 1% SDS and stored at −20°C until used. Total protein was determined by the micro-Bradford method using the Bradford reagent (Sigma, St. Louis, MO). For electrophoreses, protein extracts were thawed on ice, mixed with 3× sample buffer (NuPage; Novex, San Diego, CA) to obtain a protein concentration of 2.5 to 3.0 µg/µl and incubated for 5 minutes at 95°C. Denatured proteins were then fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) by a commercially available system (PhastSystem; Amersham-Pharmacia), using a 7.5% homogenous gel (PhastGel; Amersham–Pharmacia). Electrophoresed proteins were transferred to nitrocellulose membranes (Protran; Schleicher & Schuell, Keene, NH) after which membranes were washed briefly with 1× phosphate-buffered saline (PBS) and then incubated for 30 minutes at room temperature in blocking buffer made up of 0.2% I-Block (Tropix, Bedford, MA), 1× PBS, and 0.1% Tween-20. A monoclonal (mouse IgM) anti-human HSC70 antibody (Clone 13D3; Affinity BioReagents, Golden, CO), diluted 1:1000 with blocking buffer, was then added before incubation overnight at 4°C. Reacted membranes were rinsed twice with wash buffer (1× PBS; 0.2% Tween-20) and treated with a polyclonal biotinylated goat anti-mouse IgM antibody (Kirkegaard & Perry, Gaithersburg, MD) diluted 1:2000 with blocking buffer for 30 minutes at room temperature. The immunoreactivity signal was developed with a chemiluminescence detection kit (Western-Light Plus; Tropix) and captured on radiographic films (BIOMAX-MR, Kodak) during a 5- to 30-minute exposure and then digitized and quantified (as relative values after normalization), with the imaging workstation (NucleoVision; Nucleo-Tech).

RESULTS

Screening of retinal fovea-cDNA library clones with mixed cDNA probes prepared from retina of human donors indicated that approximately 1.6% (886/55,368) of mRNA sequences showed a consistent age-related decrease in gene expression. One of the clones (dd112), which is expressed at lower levels in retina of old human donors, also generated a reduced signal after Southern blot analysis of differentially expressed clones with retina probes of old rhesus monkeys, compared with youthful probes (data not shown). Sequencing of clone

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HSC70 oligonucleotide probe

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HTN, hypertension; SIDS, sudden infant death syndrome; MVA, motor vehicle accident; MI, myocardial infarction.

* Normalized values.
dd112 and FASTA analysis against GenBank submissions confirmed identity (99.6%) with human HSC70.

Reacting HSC70 cDNA insert against total retina RNA from different ages of either human (Fig. 1A) or rhesus (Fig. 1B), suggests that total HSC70 mRNA signal intensity declines during aging in the two species (Fig. 1A, compare 2-year-old and 94-year-old; Fig. 1B, old and young monkey). Comparison of normalized densitometric signals for retinal HSC70 RNA indicates that there was approximately a threefold decline from 2 to 94 years of age in the human sample (11.9 versus 3.7; Fig. 1A) and a twofold decline between young and old rhesus monkeys (5.0 versus 2.3; Fig. 1B). Thus, the decline in HSC70 mRNA levels seems to be a general feature of primate retinal aging. Two of the samples in Figure 1A show slightly higher bands (Fig. 1A, 55 and 61 years). The reason for this is not clear, but it could represent HSC70 cDNA probe homology with other HS70 isoforms. To minimize errors due to this possibility, we also performed Northern blot analysis using an HSC70-specific oligonucleotide probe. Northern analyses using the HSC70 mRNA-specific oligonucleotide also indicated that retinal HSC70 mRNA levels decline during aging (figure not shown). Regression analysis of all normalized Northern blot analysis signals obtained after reacting the HSC 70 cDNA-labeled and HSC70 oligo-labeled probes with total human retinal RNA indicates that there may be a twofold decline in

![Northern blot analysis of HSC70 mRNA levels in total retina RNA from (A) human donors ranging from 2 to 94 years of age and (B) rhesus monkeys aged 2 and 27 years. Five micrograms of total RNA was denatured and electrophoresed in denaturing 1.25% agarose-formaldehyde gels and transferred to nylon membranes. A 32P random-labeled HSC70 cDNA probe was reacted to immobilized RNA and membranes washed at high stringency (65°C; 0.2× SSC). After exposure to autoradiographic film, band signal intensities were normalized to individual 18s rRNA sample loading.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/)

![HSC70 mRNA levels in human retina. Total RNA (5 μg) isolated from donors of different ages were denatured, electrophoresed on 1.2% agarose-formaldehyde gel, transferred to nylon membranes and probed with (A) HSC70 cDNA probe generated by RT-PCR (n = 15 individuals) and (B) HSC70-specific oligonucleotide probe (n = 11 individuals). The values plotted are the normalized Northern signals. The R² values are derived from trendlines and represent unadjusted least square fits. Significance was set at P < 0.05.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/)

**FIGURE 1.** Northern blot analysis of HSC70 mRNA levels in total retina RNA from (A) human donors ranging from 2 to 94 years of age and (B) rhesus monkeys aged 2 and 27 years. Five micrograms of total RNA was denatured and electrophoresed in denaturing 1.25% agarose-formaldehyde gels and transferred to nylon membranes. A 32P random-labeled HSC70 cDNA probe was reacted to immobilized RNA and membranes washed at high stringency (65°C; 0.2× SSC). After exposure to autoradiographic film, band signal intensities were normalized to individual 18s rRNA sample loading.

**FIGURE 2.** HSC70 mRNA levels in human retina. Total RNA (5 μg) isolated from donors of different ages were denatured, electrophoresed on 1.2% agarose-formaldehyde gel, transferred to nylon membranes and probed with (A) HSC70 cDNA probe generated by RT-PCR (n = 15 individuals) and (B) HSC70-specific oligonucleotide probe (n = 11 individuals). The values plotted are the normalized Northern signals. The R² values are derived from trendlines and represent unadjusted least square fits. Significance was set at P < 0.05.

**DISCUSSION**

It is relevant to define age-related retinal gene expression alterations, because such changes may be associated with late-onset neurodegenerative and retinal disease susceptibility.5,51,52 Because primate-specific retinal region differences may also predispose to primate-specific age-related diseases such as ARMD, identification of relevant retina-expressed genes altering their activity during aging, may be important in understanding the basis for susceptibility to age-related retinal disorders.

The similarity between human and nonhuman primate retinal gene expression has been previously evaluated, and the expression pattern of genes associated with human retinal diseases, are similar in both human and nonhuman primate retinal regions.33 Aged rhesus monkeys exhibit many of the age-associated diseases seen in humans, including adult-onset diabetes, diabetic retinopathy, cataracts, and drusen,34,35 suggesting that the retinal aging process in both species are similar. In addition, monkey tissue can be obtained at time of death, eliminating postmortem time-related mRNA degradation as a potential source of error. Thus, analysis of nonhuman primate retina, which has a retinal fovea-periphery regional...
HSC70 gene expression in the human and rhesus monkey retina apparently decreases by approximately twofold, from youth to old age (Fig. 1). Alterations in HS70 gene family member expression in nonretinal systems have been previously documented. For example, decreases in the amount of inducible HSP70 mRNA after stress are seen in the liver and cardiovascular systems of aged rodent,14 and fibroblasts39 and in vitro in fibroblasts derived from old humans.13 These results suggest that the age-related decline in HS70 mRNA transcription is not limited to the retina.

Non-specific alterations in mRNA levels can occur from a number of variables, including heat shock or fever, ischemia, systemic disease, and mRNA degradation due to delays from time of death until tissue preservation. These variables can result in considerable variation in Northern expression, a factor that may in part explain the scatter surrounding the regression line (Fig. 2) and the P obtained in Figure 2B. This phenomenon has also been observed for age-related tissue inhibitor of matrix metalloproteinase (TIMP)-3 deposition in Bruch’s membrane.32 Thus, caution is required in interpreting individual sample results. Our finding that HSC70 mRNA levels apparently decline during human aging is supported by the observation that this pattern of expression also occurs in the rhesus monkey retina. The results are consistent across several rhesus monkeys and two independent related species is therefore helpful in confirming observations involving age-related changes in human gene expression. A caveat, however, is that related species such as rhesus monkeys are also subject to stresses similar to those experienced by humans, and it is therefore advisable to sample multiple individuals of both species for a clearer picture of the expression pattern of any particular stress or age-related gene.

The level of HSC70 protein is also lower in the retina of older primates (Fig. 3). A lower level of this protein has also been observed in the photoreceptors of older rats,15 suggesting that age-related HS70 gene expression changes may be general to mammalian systems. HSC70 protein intracellular levels are primarily regulated at the transcriptional level.44,45 Thus, decreases in HSC70 mRNA during the aging process are likely to translate to reductions in intracellular HSC70 protein.

HSC70 protein plays a role in the translocation and folding of proteins after synthesis in the endoplasmic reticulum46 and in regulating protein degradation through the ubiquitination pathway.47 Our observation as well as that reported earlier in rat photoreceptors,15 is therefore noteworthy, because it is possible that the altered HSC70 expression during aging modifies a number of critical processes that ultimately influence the stress response mechanism of the retina, including increased susceptibility to stress-induced apoptosis.48 It is interesting that there is a relatively strong reported association of atherosclerosis with ARMD.5,49 The noted decline in vascular HSP70 expression50 raises the possibility that a common mechanism could yield both cardiovascular and retinal disease. A decrease in retinal HSC70 expression, added to a system functionally compromised by other conditions predisposing to age-related diseases, could contribute to the late clinical appearance of these conditions. This link should be investigated.

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References


