Retinal TUNEL-Positive Cells and High Glutamate Levels in Vitreous Humor of Mutant Quail with a Glaucoma-like Disorder

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PURPOSE. To investigate whether retinal cell death observed in an avian glaucoma-like disorder occurs by apoptosis and whether an increase in excitotoxic amino acid concentration in the vitreous humor is associated temporally with cell death in the retina.

METHODS. Presumptive retinal apoptotic nuclei were identified by histochomical detection of DNA fragmentation (by TdT-DUTP terminal nick-end labeling [TUNEL]), and vitreal concentrations of glutamate and several other amino acids were determined by high-pressure liquid chromatography with fluorometric detection in the al mutant quail (Coturnix coturnix japonica) in which a glaucoma-like disorder develops spontaneously.

RESULTS. TUNEL-labeled nuclei were located mostly in the ganglion cell layer (GCL) in the retina of mutant quails 3 months after hatching. However, labeled nuclei were also observed in the inner and outer nuclear layers. At 7 months, most TUNEL-positive nuclei were detected in the inner nuclear layer, whereas labeled cells in the GCL were reduced in number. No TUNEL-labeled nuclei were detected in the retina of control quails at any age. Vitreal concentrations of glutamate and aspartate were significantly increased in 1-month-old mutant quails compared with control animals. Concentrations decreased at 3 months, and no significant differences were observed between strains at 7 months.

CONCLUSIONS. Presumptive apoptotic cell death is detected from 3 months after hatching in mutant quails and is not restricted to retinal ganglion cells. Cell death appears just after a significant increase in excitotoxic amino acid concentrations in the vitreous humor, suggesting a correlation between both events. (Invest Ophthalmol Vis Sci. 1999; 40:990–995)

Glaucoma is a widespread human ocular disease, characterized by retinal ganglion cell degeneration, excavation of the optic nerve head, and in most cases, increase of intraocular pressure (IOP). Its cause remains unclear, and although increased IOP has long been thought to be the primary cause of ganglion cell degeneration and optic disc cupping, evidence from the studies of low-tension glaucoma suggests that it is neither necessary nor sufficient to induce the disease. Other mechanisms have been proposed that involve the neurosensory retina directly.1 Results in recent studies suggest that apoptotic mechanisms could be involved in the degeneration of ganglion cells1,2 and emphasize the potential neurotoxic role of glutamate.3,4 Abnormally high levels of glutamate have been detected in the vitreous of glaucomatous eyes of dogs, monkeys, and humans.5,6

The purpose of this work was to determine whether such particularities were also found in a hypopigmented mutant of the Japanese quail, Coturnix coturnix japonica, in which an ocular disease develops that has been described as resembling human closed-angle glaucoma, with increasing IOP (measured by cannulation), closure of the iridocorneal angle, and progressive degeneration of the ganglion cells.6–11 However, several morphologic characteristics such as enlargement of the retinal surface, abnormal corneal endothelium with degenerating cells, poorly differentiated cells with collapsed trabecular meshwork, and attachment of the anterior face of the iris to the posterior cornea, suggest that it also possesses some characteristics of human congenital glaucoma.12,13 Therefore, we searched for the presence and distribution of apoptotic nuclei and determined the vitreous concentration of glutamate during the development of glaucoma-like disease in mutant quail retina. Our results show that an increase of excitotoxic amino acid concentrations in vitreous could be temporally associated with the appearance of degenerating cells, suggested by the presence of nuclei positive for TdT-DUTP terminal nick-end labeling (TUNEL) in the mutant quail retina.

MATERIALS AND METHODS
Fifty-two mutant quails, with the mutant gene al, and 52 control quails Coturnix coturnix japonica were provided by the Institut National de la Recherche Agronomique (Jouy-en-Josas, France). Animals were maintained in a 14-hour-light–10-hour-dark photoperiod from hatching to death. Mutant quails were killed by decapitation at three stages of the disease determined previously:9 at 1 month, before the appearance of any morphologic signs of glaucoma; at 3 months, at the onset of the first pathologic signs of the disease, such as the appearance of buphthalmy; and at 7 months, when glaucoma, evidenced by marked buphthalmy, was well established. Control subjects were killed according to the same schedule. The experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
TUNEL Staining

Four retinas from four quails of each strain killed at each of the three stages (i.e., 12 mutant and 12 control quails) were prepared for TUNEL analysis by use of an in situ apoptosis detection kit (Apoptag; Oncor, Gaithersburg, MD). The eyes were fixed in Bouin fixative for 48 hours at 4°C, embedded in paraffin, and 10-μm thick vertical sections were cut. Endogenous peroxidase was inactivated by incubating sections with 2% H2O2 for 5 minutes. Sections were preincubated in the equilibration buffer (provided in the kit) for 30 seconds at room temperature and were then treated with terminal deoxynucleotidyl transferase (TdT) and digoxigenin deoxyuridine triphosphate (dUTP) for 1 hour at 37°C. They were rinsed in buffer provided in the kit (Stop/WA; Oncor) for 30 minutes at 37°C. Retinas were incubated with a peroxidase-coupled antidigoxigenin antibody for 30 minutes at room temperature. The 3'-OH DNA tail was detected by incubating retinas with a diaminobenzidine-H2O2 solution and counterstained with methyl green for 10 minutes. Controls were made by omitting TdT during the first step of the labeling procedure. No labeling was observed in control sections. We estimated the proportion of TUNEL-positive ganglion cells during the time course of the disease by expressing the results as a percentage of the number of labeled cells in all sections from one stage, compared with the total number of cells located within the ganglion cell layer (GCL) observed in the same sections at the same stage.

Semithin Sections

Degeneration of retinal ganglion cells was estimated by comparing the thickness of the nerve fiber layer in mutant and control semithin sections. Eight eyes of 7-month-old animals, four from different mutant quails and four from different control quails, were removed and immersed for 2 hours in 0.1 M cacodylate buffer (pH 7.3) containing 2.5% glutaraldehyde. After a rinse in 0.2 M cacodylate buffer for 24 hours at 4°C, retinas were dissected and postfixed in 1% osmium tetroxide for 1 hour. After dehydration and embedding in epoxy resin, 1-μm semithin sections were collected in both strains from the same region of the retina near the optic nerve head. To measure the optic nerve layer thickness, 20 semithin sections at 5-μm intervals were examined in each eye in both strains. The mean thickness of each strain was compared using the Student’s t-test.

Glutamate and Other Amino Acid Assays

Twelve vitreous bodies, one vitreous body per quail, were collected at each stage from each strain (i.e., 36 from mutant and 36 from control quails). Glutamate concentrations were determined by high-pressure liquid chromatography (HPLC) with a fluorescence monitor (RF-551; Shimadzu, Tozart and Matignon, Courtaboeuf, France) using a slightly modified method described previously. Individual vitreous bodies were sonicated in 0.2 M perchloric acid containing 0.1% Na2S2O5 and 0.1% EDTA. Homogenates were centrifuged at 15,000g for 5 minutes at 4°C and the supernatant used for glutamate determination. The stock reagent contained 27 ng of phthalaldehyde, 1 ml methanol, 5 μl β-mercaptoethanol, and 9 ml 0.1 M sodium tetraborate. The working solution was prepared by diluting the stock solution with 0.1 M sodium tetraborate (1:5 v/v). Precolumn derivation was performed by mixing 10 μl sample (1:4 dilution in perchloric mixture as described above) with 90 μl working o-phthalaldehyde reagent. This final mixture (10 μl) was injected into the HPLC system. Separation of glutamate, glutamine, γ-aminobutyric acid (GABA), asparagine, aspartate, threonine, taurine, and tyrosine was achieved by reversed-phase liquid chromatography (Hypersil C18, 3-μm column, 150 × 4.6 mm; Touzard and Matignon, Courtaboeuf, France). The mobile phase (pH 7.8), flow rate (1 ml/min), consisted of water-acetonitrile-methanol (70:5:25 vol/vol/vol) containing 0.1 M dibasic sodium phosphate buffer. Excitation and emission wavelengths were set at 335 nm and 425 nm.

For precise evaluation of the within- and between-assay coefficients of variation (CV) for amino acid quantification, samples of pooled vitreous bodies were prepared and analyzed three times the same day (within CV) and on 4 different days (between CV). The CVs were never greater than 13%. Results were expressed in nanograms per milligram vitreous humor.
Individual data were analyzed by global Kruskal-Wallis analysis, followed when significant ($P < 0.05$) by individual interstage comparisons with the nonparametric Mann-Whitney test.

**RESULTS**

**TUNEL Method**

No TUNEL-positive cells were observed in the retina of control quails at any of the three stages studied (Figs. 1A, 2A and 3A) and in the retina of 1-month-old mutant quail (Fig. 1B). TUNEL-positive nuclei were detected mainly in the GCL in 3-month-old mutant quails (Figs. 2B, 2C). Labeled cell bodies were estimated at almost 50% (49%) of the total number of cells located in the GCL in retinal sections examined (Table 1). TUNEL-labeled cells were also found in the inner and outer nuclear layers (Fig. 2B). DNA fragments were observed as dark aggregates in many nuclei at the highest magnification (Fig. 2D). At 7 months, the number of ganglion cells appeared reduced and more spaced in the mutant than in the control quails. At this stage, TUNEL-positive nuclei were still observed in the GCL (estimated at almost 20% (18.7%) of total cells remaining in the GCL in retinal sections examined), but many more labeled nuclei were located in the inner nuclear layer than were seen in previous stages, and the outer nuclear layer was unlabeled (Fig. 3B).
The loss of ganglion cells during the disease was confirmed by a statistically significant (P < 0.001) 39% reduction of the thickness of the nerve fiber layer, which contains axons of retinal ganglion cells, in 7-month-old mutant quails (9.2 ± 2.4 μm) compared with the thickness in control quails (15 ± 0.7 μm; Figs. 4A, 4B).

**Amino Acid Assays**

Results of assays are reported in Table 2. Although no significant changes were found in control quails at any age, an elevation in glutamate and aspartate concentrations was detected in 1-month-old mutant quails that was approximately 1.5 times that in 1-month-old control quails. A progressive decrease in vitreous glutamate and aspartate was observed at 3 months in the mutant quail, attaining control levels at 7 months. The increase in amino acid vitreous concentrations observed in 1-month-old mutant quails was found only for glutamate and aspartate. The time course of other amino acid concentrations varied according to the amino acid in question. There was no significant difference in asparagine and tyrosine levels between the two strains at any age, and levels of GABA and glutamine decreased from 3 months in mutant quails to particularly low levels at 7 months. Taurine and threonine increased significantly at 3 months, followed by a decrease at 7 months in mutant quails.

### TABLE 1. TUNEL-Positive Cells in Mutant Quails

<table>
<thead>
<tr>
<th>Age of Mutant Quails</th>
<th>Number of TUNEL-Positive</th>
<th>Total Number of Cells</th>
<th>% of TUNEL-Positive Cells</th>
<th>Number of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>944</td>
<td>1926</td>
<td>49</td>
<td>20</td>
</tr>
<tr>
<td>7 months</td>
<td>214</td>
<td>1141</td>
<td>18.7</td>
<td>20</td>
</tr>
</tbody>
</table>

No data is reported for 1 month because no TUNEL-positive cells were observed.

**Observation of the Nerve Fiber Layer**

Glaucoma is a frequently occurring ocular disease that causes degeneration of ganglion cells, and subsequently, impairment of the visual field. The causes of this disease remain unclear, but some ocular dysfunctions (principally an increase in IOP) are often associated with it. In the present work, we have observed presumptive apoptotic nuclei (detected by TUNEL) in the retina of the mutant quails which, from 3 months after hatching, manifest a glaucoma-like disease, with an increase in IOP and degeneration of ganglion cells. Apoptosis, which is characterized morphologically by condensation and aggregation of chromatin and nuclear shrinkage, is described as a process of programmed cell death that occurs naturally during development. In adulthood, it is found in a variety of diseases including glaucoma, in which labeled nuclei are generally found exclusively in the GCL.

In mutant quail, however, the TUNEL-positive nuclei were observed not only in the GCL but also in the outer and inner nuclear layers. These results are not seen commonly in this disease, although a photoreceptor loss has sometimes been associated with spontaneous glaucoma in humans, and a reduction of cells located in the inner nuclear layer has been observed in the retina of mutant quails and in the retina of monkeys with induced glaucoma. Moreover, because the percentage of degenerating cells seemed especially elevated in our animal model, we cannot exclude the hypothesis that retinas of hypopigmented mutant quails are particularly susceptible to cellular death. The reasons for such dramatic cellular susceptibility remain unknown, but it would be interesting, in that connection, to study the proteins involved in apoptosis. Among these, Bcl-2 is a particularly interesting candidate, because the inactivation of the bcl-2 gene leads to excessive apoptotic cellular death and depigmentation subsequent to a dysfunction in melanin synthesis.

A variety of stimuli are thought to initiate or activate apoptosis in glaucoma. Thus, increased IOP and/or organelle accumulation at the optic nerve head could block axoplasmic flow, hindering the circulation of trophic factors. Further studies must be undertaken to determine the degree of involvement of increased IOP in cellular degeneration in mutant quail. However, some data suggest that glutamate neurotoxicity could be also involved in cellular degeneration in glaucoma. It has been shown that an intravitreal injection of glutamate agonists such as kainic acid or N-methyl-D-aspartate can induce apoptosis-like cell death, and glutamate seems to be in excess in the vitreous humor of dogs, monkeys, and humans with glaucoma. We observed also an elevation of vitreal concentrations of glutamate and aspartate (an excitatory amino acid resembling closely glutamate and exhibiting excitotoxic...
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**References**


**Table 2. Amino Acid Concentrations in the Vitreous Bodies of Mutant and Control Quails**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Control Quails</th>
<th>Mutant Quails</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Month</td>
<td>3 Months</td>
</tr>
<tr>
<td>Glutamate</td>
<td>26.3 ± 3.8</td>
<td>26.8 ± 3.6</td>
</tr>
<tr>
<td>Aspartate</td>
<td>14.6 ± 2</td>
<td>9.2 ± 1†</td>
</tr>
<tr>
<td>Asparagine</td>
<td>4.9 ± 0.5</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>GABA</td>
<td>29.5 ± 6.6</td>
<td>10.3 ± 1.7†</td>
</tr>
<tr>
<td>Glutamine</td>
<td>231.7 ± 25.7</td>
<td>204.4 ± 15.6</td>
</tr>
<tr>
<td>Taurine</td>
<td>205.8 ± 28</td>
<td>253.2 ± 15.7</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.4 ± 0.3</td>
<td>2.8 ± 0.3†</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>7.3 ± 0.7</td>
<td>5.4 ± 0.5</td>
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* Statistical comparison between control quails and mutant quails at the same age (*P < 0.05). Comparisons by Kruskall-Wallis test; n = 12 for each age.
† Statistical comparison between the age considered and the previous age (*P < 0.05). Comparisons by Kruskall-Wallis test; n = 12 for each age.
Bothnia Dystrophy Caused by Mutations in the Cellular Retinaldehyde-Binding Protein Gene (RLBP1) on Chromosome 15q26

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PURPOSE. To determine the chromosomal location and to identify the gene causing a type of retinitis punctata albescens, called Bothnia dystrophy, found in a restricted geographic area in northern Sweden.

METHODS. Twenty patients from seven families originating from a restricted geographic area in northern Sweden were clinically examined. Microsatellite markers were analyzed in all affected and unaffected family members. Direct genomic sequencing of the gene encoding cellular retinaldehyde-binding protein was performed after the linkage analysis had been completed.

RESULTS. Affected individuals showed night blindness from early childhood with features consistent with retinitis punctata albescens and macular degeneration. The responsible gene was mapped to 15q26, the same region to which the cellular retinaldehyde-binding protein gene has been assigned. Subsequent analysis showed all affected patients were homozygous for a C to T substitution in exon 7 of the same gene, leading to the missense mutation Arg234Trp. Analysis of marker haplotypes suggested that all cases had a common ancestor who carried the mutation.

CONCLUSIONS. A missense mutation in the cellular retinaldehyde-binding protein gene is the cause of Bothnia dystrophy. The disease is a local variant of retinitis punctata albescens that is common in northern Sweden due to a founder mutation.

Retinitis pigmentosa (RP) is a clinically and genetically heterogeneous group of retinal dystrophies characterized by early night blindness and later loss of peripheral and central vision. Pigment deposition in the retina and attenuation of the retinal blood vessels are observed. The diagnosis is confirmed by an abnormal or extinguished electroretinogram (ERG). Retinitis pigmentosa loci have been mapped to numerous chromosomal locations, and mutations in many different genes have been found in patients. An updated list of disease loci is available on the RetNet web site: http://utsph.sph.uth.tmc.edu/retnet.

A unique atypical variant of RP, known among clinicians in northern Sweden for decades as Västerbotten dystrophy or Bothnia dystrophy (refers to the area adjacent to the Gulf of Bothnia), is described in this report. Affected individuals have had night blindness since early childhood, retinitis punctata albescens (RPA), and macular degeneration. Retinitis punctata albescens is associated with RP and characterized by numerous punctate whitish-yellow spots in the fundus. We demonstrate by linkage analysis that the disease gene is localized to 15q26. Subsequently we show that a missense mutation in the cellular retinaldehyde-binding protein gene (RLBP1) is present in a homozygous state in 20 patients from seven Västerbotten families with Bothnia dystrophy.

METHODS

Ophthalmologic Examinations

Inclusion criteria for the families in this study were ophthalmologic records of retinal disease and more than one family member showing early onset night blindness, fundus appearance similar to RPA with small white dots in central fundus, macular degeneration, and lack of for RP typical bone spicules in peripheral retina. Standard ophthalmologic examination and fundus photography were carried out in all affected individuals and selected siblings. Dark adaptation tests and full-field ERGs were performed in selected cases. The study followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all subjects.

Genotyping

Extraction of genomic DNA and analysis of microsatellite markers were performed as described by Balciuniene et al.