Impaired Cytoprotective Mechanisms in Eyes with Pseudoexfoliation Syndrome/Glaucoma

Matthias Zenkel, Friedrich E. Kruse, Gottfried O. H. Naumann, and Ursula Schlötzer-Schrehardt

PURPOSE. Evidence suggests that chronically increased stress conditions in the anterior eye segment constitute major mechanisms involved in the pathobiology of pseudoexfoliation (PEX) syndrome. The expression of stress-related genes in eyes from patients with and without PEX syndrome/glaucoma was investigated to determine whether PEX syndrome is associated with an altered cellular stress response.

METHODS. CDNA array hybridization, quantitative real-time PCR, Western blot analysis, and immunohistochemistry were applied to analyze the mRNA and protein expression of stress-related genes in anterior segment tissues of PEX eyes, with and without glaucoma, and to compare them with normal and glaucomatous control eyes.

RESULTS. Hybridization of CDNA arrays identified a set of stress-related candidate genes for differential expression in PEX syndrome/glaucoma, of which 10 were confirmed by real-time PCR in ciliary processes and iris tissue. The expression of MAPKp38, heat shock proteins (HSP40, HSP60), and superoxide dismutase (SOD2) was increased up to threefold in PEX specimens. In contrast, a large set of cytoprotective gene products, including antioxidant defense enzymes (glutathione S-transferases mGST1 and GSTT1), ubiquitin-conjugating enzymes (UBE2A, UBE2B), the DNA repair protein MLH1, and the stress-inducible transcription factor GADD153, were found to be consistently downregulated up to threefold in PEX specimens on both the mRNA and protein levels.

CONCLUSIONS. The present findings provide evidence of alterations in cytoprotective mechanisms including antioxidant defense, proteasome function, endoplasmic reticulum–related stress response, and DNA repair in anterior segment tissues of PEX eyes. The resultant enhanced sensitivity and vulnerability to cellular stress conditions may therefore be one contributing factor in the pathobiology of PEX syndrome.

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PEX syndrome is a generalized disorder of the extracellular matrix and currently represents the most commonly identified specific cause of open-angle glaucoma. The pathologic process is characterized by the chronic, stable accumulation of abnormal fibrillar aggregates in the anterior segment and various extraocular tissues. Recent molecular biological evidence suggests that PEX syndrome is an elastic microfibrillopathy associated with an excessive production of elastic microfibril components, such as fibrillin-1 and latent transforming growth factor–binding proteins, enzymatic cross-linking processes, increased levels of transforming growth factor (TGF)-β1, and a proteolytic imbalance between matrix metalloproteinases and their inhibitors. A recent proteomic approach confirmed the presence of elastic fiber components within PEX deposits and provided evidence of further constituents, such as fibrillin-2, syndecan-3, versican, metalloproteases of the ADAM family, and complement factor C1q. However, the molecular mechanisms responsible for its excessive production and accumulation still remain elusive.

There is increasing evidence that cellular stress conditions, such as anterior chamber ischemia/hypoxia and oxidative stress, may constitute major mechanisms involved in the pathobiology of PEX syndrome, and several studies suggest impaired antioxidative protection mechanisms in patients with PEX. Significantly reduced levels of important antioxidative compounds, such as ascorbic acid and glutathione, and concomitantly increased levels of oxidative stress markers, such as 8-isoprostaglandine F2α and malondialdehyde, have been demonstrated in aqueous humor and lens epithelial cells of PEX eyes. Increased endothelin-1 and homocysteine levels in the aqueous humor of PEX eyes may further contribute to ischemic alterations and oxidative stress. In the serum of PEX subjects, vitamin C concentrations were much lower and concentrations of malondialdehyde were much higher than in control subjects. In addition, the activity of superoxide dismutase was significantly decreased, and products of protein oxidation were significantly increased in serum samples of patients with PEX compared with control patients.

All mammalian cells possess an innate strategy for preventing stress-induced injury or cell death and for recovering from acute or chronic stress conditions by activating a host of cytoprotective mechanisms. Even in the presence of existing stress conditions in the anterior segment, previous studies showed a significant downregulation of protective genes, such as clusterin, a small heat shock protein–like chaperone, in anterior segment tissues of PEX eyes, indicating impaired cellular protection mechanisms.

Therefore, the purpose of this study was to investigate the expression of stress-related cytoprotective genes in anterior segment tissues of PEX eyes in more detail. CDNA array technology, quantitative real-time PCR, Western blot analysis, and immunohistochemistry were used to compare mRNA and protein expression of stress-related genes in PEX syndrome, without and with glaucoma, to normal and glaucomatous control eyes. The findings provide evidence of a dysregulation of antioxidative enzymes, ubiquitin-conjugating enzymes, a DNA repair enzyme, and an endoplasmic reticulum stress-related transcription factor in eyes with PEX syndrome/glaucoma and thus support the notion that impaired cytoprotective mechanisms may be involved in the pathophysiology of PEX syndrome.

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METHODS

Tissues

Tissues were obtained from three donor eyes with PEX syndrome without glaucoma (age, 79 ± 2.2 years; one female, two male) and three normal-appearing donor eyes (age, 82 ± 1.8 years; one female, two male). These eyes were obtained at autopsy and were processed within 8 hours after death. The donor eyes had no documented history of any ocular disease and the presence of PEX syndrome was diagnosed by macroscopic evidence of PEX deposits on lens, iris, ciliary processes, and zonules and confirmed by electron microscopic analysis of small tissue sectors. The absence of glaucoma in these eyes was confirmed by macroscopic evaluation of the optic disc and light microscopic analysis of optic nerve cross sections.

In addition, we used two eyes of patients with a documented history of PEX-associated open-angle glaucoma (77 and 81 years; one female, one male), two eyes of patients with primary open-angle glaucoma (POAG; 78 and 84 years; one female, one male), one eye with PEX-associated angle-closure glaucoma (85 years; female), and one eye with angle-closure glaucoma (ACG) without PEX syndrome (82 years; female). These eyes had to be surgically enucleated because of painful absolute glaucoma and were processed immediately after enucleation. The clinical characteristics of these eyes are presented in Table 1.

Anterior segment tissues were prepared under a dissecting microscope and shock frozen in liquid nitrogen. Total RNA and total protein were extracted using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Immunohistochemistry was performed on five eyes of donors with PEX syndrome (age, 72.1 ± 6.9 years; three female, two male), and five normal-appearing eyes of donors without PEX syndrome (age, 70.5 ± 6.5 years; three female, two male), which were also obtained at autopsy and cryopreserved within 8 hours after death.

Informed consent to tissue donation was obtained from the patients or, in the case of eyes obtained at autopsy, from their relatives, and the protocol of the study was approved by the local Ethics Committee and adhered to the tenets of the Declaration of Helsinki for experiments involving human tissues and samples.

cDNA Array Hybridization

A gene array (Atlas Human Stress Array; BD-Clontech, Mountain View, CA) containing 254 cDNAs of stress-related genes was used; a complete gene list is provided at http://www.clontech.com/support/tools.asp?product_tool_id=157578&tool_id=157579.

Representative cDNA libraries of PEX and control tissues were generated from 1 μg of total RNA as previously described and radioactively labeled (Atlas SMART Probe Amplification Kit; BD-Clontech) according to the manufacturer’s instructions. Equal counts per minute (cpm) of labeled cDNA from PEX and control tissues were hybridized to the array membranes with hybridization solution (Express Hyb; BD-Clontech) according to the manufacturer’s protocol. Membranes were exposed to imaging plates and analyzed using a phosphoimage imager and software (model BAS-2000; Fuji, Düsseldorf, Germany).

Real-Time RT-PCR

First-strand cDNA synthesis from 1 μg of total RNA and quantitative real-time PCR were performed with a thermal cycler and software (MyIQ; Bio-Rad, Munich, Germany), as previously described. PCR reactions (25 μL) were run in triplicate and contained 2 μL of the 1:5 diluted first-strand cDNA, 0.4 μM each of upstream- and downstream primer, 3.0 to 4.0 mM MgCl₂, and master mix (SYBR Green Supermix; Bio-Rad). Exon-spanning primer (MWG Biotech, Anzing, Germany), designed by means of Primer 3 software, and PCR conditions are summarized in Table 2. For quantification, serially diluted standard curves of plasmid-cloned cDNA were run in parallel, and amplification specificity was checked by using melting curve and sequence analyses (Prism 3100 DNA sequencer; Applied Biosystems, Inc. [ABI], Foster City, CA). For normalization of gene expression levels, mRNA ratios relative to the housekeeping gene GAPDH were calculated.

Western Blot Analysis

Protein concentrations were determined (Micro BCA Protein Assay kit; Perbio Science, Bonn, Germany) with bovine serum albumin as a standard. Two micrograms of total protein was separated by SDS-PAGE under reducing conditions, and immunoblot analysis was performed as previously described, using antibodies against human UBE2A/2B, mGST1, GSTT1, or MLH1 (listed later) diluted 1:200, 1:250, 1:250, or 1:100, respectively, and two different antibodies against GADD153 (mouse, clone 9C8: Abcam, Cambridge, UK; or rabbit polyclonal: Biovision, Mountain View, CA; each diluted 1:50). Equal loading was verified with anti-human β-actin antibody (mouse, clone AC-15: Sigma-Aldrich, Munich, Germany) diluted 1:5000. In negative control experiments, the primary antibody was replaced by PBS. Immunodetection was performed with horseradish peroxidase-conjugated secondary antibodies (Chemicon, Egham, UK) diluted 1:5000 and chemiluminescence detection (Chemiluminescent; Chemicon). Signals were analyzed and quantified (ChemiSmart 5000 chemiluminescence detection system and software; Vilber Lourmat, Eberhardzell, Germany). For standardization, each blot included one lane with an equal mixture of three individual protein samples from control patients, which was set to 100%. For normalization of protein expression levels, signal intensity ratios relative to the housekeeping gene β-actin were calculated.

Immunohistochemistry

Light microscopic indirect immunofluorescence labeling was performed on ocular tissue sections, as previously described, using antibodies against UBE2A/2B (rabbit polyclonal; Bethyl Laboratories, Montgomery, TX), mGST1 (mouse polyclonal; Abnova, Taipei, Taiwan), GSTT1 (mouse polyclonal; Abnova), MLH1 (mouse, clone G168-15; BD Biosciences, Heidelberg, Germany), or GADD153 (mouse, clone 9C8; Abcam). Antibody binding was detected by Alexa 488-conjugated secondary antibodies (Invitrogen-Molecular Probes, Eugene, OR), and nuclear counterstaining was performed with propidium iodide or DAPI (4′,6-diamino-2-phenylindole; Sigma-Aldrich, St. Louis, MO). In negative control experiments, the primary antibody was replaced by PBS or equimolar concentrations of an irrelevant primary antibody.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Glaucoma</th>
<th>IOP&lt;sub&gt;max&lt;/sub&gt;</th>
<th>C/D Ratio</th>
<th>Medication</th>
<th>Ocular Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. FH (L)</td>
<td>77/F</td>
<td>Sec. OAG, PEX</td>
<td>36</td>
<td>1.0</td>
<td>Azopt, Travatan</td>
<td>None</td>
</tr>
<tr>
<td>2. FS (R)</td>
<td>81/M</td>
<td>Sec. OAG, PEX</td>
<td>45</td>
<td>1.0</td>
<td>Timolol, Alphagan</td>
<td>Filtration surgery (7 years ago)</td>
</tr>
<tr>
<td>3. FS (L)</td>
<td>85/F</td>
<td>Sec. ACG, PEX</td>
<td>42</td>
<td>0.95</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4. LF (R)</td>
<td>78/F</td>
<td>POAG</td>
<td>35</td>
<td>1.0</td>
<td>Timolol, Pilocarpin</td>
<td>Filtration surgery (6 years ago)</td>
</tr>
<tr>
<td>5. HG (R)</td>
<td>84/M</td>
<td>POAG</td>
<td>48</td>
<td>1.0</td>
<td>Pilocarpin, Isopto-carbachol</td>
<td>Laser trabecuoplasty (10 years ago)</td>
</tr>
<tr>
<td>6. BE (R)</td>
<td>82/F</td>
<td>Sec. ACG, CRVO</td>
<td>52</td>
<td>1.0</td>
<td>None</td>
<td>Cataract surgery (4 years ago)</td>
</tr>
</tbody>
</table>

(L), left eye; (R), right eye.
Statistics

Data are presented as the mean ± SD. Statistical evaluation of significant differences between groups of patients was performed with the Student’s t-test for pair-wise comparison. P < 0.05 was considered statistically significant.

RESULTS

Differential Gene Expression

As a first step in exploring the role of stress-related gene products in the pathogenesis of PEX syndrome, we used a gene microarray (Atlas Human Stress Array; BD-Clontech) to monitor simultaneously the expression of 234 key molecules of the cellular stress response, including antioxidative enzymes, chaperone molecules, antiapoptotic proteins, DNA repair proteins, and stress-regulated signal transducers, in eyes with PEX-associated glaucoma (n = 3) compared with glaucomatous control eyes (n = 3). Because of the low availability of RNA from ciliary process and iris tissues, we first generated 12 representative cDNA libraries (three PEX and three control libraries for each tissue type) by exponential cDNA amplification, which were then hybridized to the arrays (Fig. 1). Signal intensities were normalized by the method of centralization, which seems best

![FIGURE 1. Human stress array hybridized with α-32P-labeled cDNA probes synthesized from ciliary process total RNA of an eye with (A) angle-closure glaucoma without PEX syndrome (patient 6, Table 1) compared to (B) PEX-associated ACG (patient 3, Table 1). The far left duplicate lane contains nine housekeeping genes, three negative controls, and four calibration markers (from the top), indicating equal expression of housekeeping genes and a highly specific hybridization. Data are representative of results in three independent experiments (n = 3). Spot numbers are in parentheses. MAPKp38 (1), SOD2 (2), HSP27 (3), HSP40 (4), HSP60 (5), UBE2A (6), UBE2B (7), nGST1 (8), GSTT1 (9), ERCC1 (10), MLH1 (11), and GADD153 (12), were differentially expressed in all ciliary process and iris specimens.](image-url)
sulted for the use with low-density arrays and the comparison of normal with pathologic conditions in ex vivo tissues. No signals were observed for the negative control spots indicating highly specific hybridization, whereas the nine housekeeping genes yielded signals of equal intensity in PEX and control tissues. Only those genes with expression levels that were highly specific hybridization, whereas the nine housekeeping genes yielded signals of equal intensity in PEX and control tissues. Only those genes with expression levels that were significantly upregulated and 10 were downregulated in iris and ciliary process specimens of PEX eyes compared with control eyes (Table 3). Consistently upregulated genes included a mitogen activated protein kinase (MAPKp38), manganese superoxide dismutase (SOD2), and several heat shock proteins (HSP27, HSP40, HSP60). A large set of genes consistently downregulated in PEX tissues included several glutathione S-transferase (GST) isozymes including GSTT-1, GSTM1, GSTP1, microsomal GST-1 (mgGST1), two ubiquitin-conjugating enzymes (UBE2A and UBE2B), DNA repair enzymes (MLH1, ERCC1), and the stress-inducible transcription factor GADD153. The cyclin-dependent kinase inhibitor 1A (CDKN1A) and topoisomerase I displayed a tissue-specific differential expression pattern, limited to ciliary body or iris specimens, respectively. Dysregulation of genes was observed in both PEX-associated open-angle and angle-closure glaucoma and appeared to be independent of prior medical or surgical treatment.

To verify the cDNA array data and to quantify the mRNA expression levels of those genes that were differentially expressed in all iris and ciliary body specimens of the PEX eyes examined, quantitative real-time PCR was performed on iris and ciliary process cDNA from eyes with PEX syndrome (n = 3) and PEX-associated glaucoma (n = 3) compared with normal (n = 3) and glaucomatous control eyes (n = 3). This approach confirmed a significant upregulation of MAPKp38 (2.0-fold), SOD2 (2.5-fold), HSP40 (2.2-fold), and HSP60 (2.6-fold) in iris and ciliary process tissues of PEX eyes (P < 0.005), whereas the difference in expression of HSP27 was not statistically significant (Fig. 2). The expression of UBE2A (2.3-fold), UBE2B (2.1-fold), mgGST1 (3.4-fold), and GSTT1 (2.4-fold) displayed a significant downregulation in iris and ciliary process tissue of PEX eyes compared with control eyes (P < 0.005). Differential expression of these genes appeared to be independent of the presence or absence of glaucoma, because it was consistently found in ciliary process and iris tissue of all eyes with PEX syndrome, without or with glaucoma. In contrast, a statistically significant downregulation of the DNA repair protein MLH1 (2.3-fold, P < 0.005) and the transcription factor GADD153 (2.6-fold, P < 0.008) could be confirmed in iris and ciliary process specimens of eyes with PEX glaucoma but not in eyes with PEX syndrome. Although ERCC1 displayed a tendency toward reduced expression in PEX glaucoma specimens, the difference was not statistically significant.

### Differential Protein Expression

To further analyze the protein expression and localization of those gene products that were significantly downregulated on the mRNA level in PEX tissues, indicating impaired protective mechanisms, we performed Western blot analyses of ciliary protein extracts and immunohistochemistry on sections of PEX and control eyes.

By immunoblot analysis, UBE2A/B, mgGST1, GSTT1, and MLH1 were detected in all ciliary body samples with an apparent molecular mass of 18, 24, 31, and 82 kDa, respectively (Fig. 3A). Specific immunoreactivity was abolished, when PBS was used instead of the primary antibody (data not shown), and immunodetection of the housekeeping gene β-actin showed equal loading of the samples (Fig. 3B). Comparison of the protein content by densitometry of the immunoreactive bands (Fig. 3C) revealed that the levels of UBE2A/B, mgGST1, and GSTT1 were significantly downregulated 2.5-, 2-, and 1.7-fold, respectively, in ciliary body samples from eyes with PEX syndrome and PEX-associated glaucoma compared with normal and glaucomatous control eyes (n = 3 for each group, P < 0.005). In consistency with mRNA data, the reduced expression of MLH1 protein was statistically significant in PEX-associated glaucoma only (2.2-fold, P < 0.005). However, GADD153 could have been upregulated in PEX glaucoma.

### Table 3. Differentially Expressed Genes in Ocular Tissues of Eyes with PEX-Associated Glaucoma

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession</th>
<th>Iris Tissue</th>
<th>Ciliary Processes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>UBE2A</td>
<td>X76981</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>UBE2B</td>
<td>M74816</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>mgGST1</td>
<td>J03746</td>
<td>2.4</td>
<td>5.0</td>
</tr>
<tr>
<td>GSTT1</td>
<td>X79389</td>
<td>2.2</td>
<td>2.6</td>
</tr>
<tr>
<td>GSTM1</td>
<td>X68676</td>
<td>2.0</td>
<td>1.1</td>
</tr>
<tr>
<td>GSTP1</td>
<td>M24485</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>ERCC1</td>
<td>M13194</td>
<td>2.1</td>
<td>4.2</td>
</tr>
<tr>
<td>MLH1</td>
<td>U07418</td>
<td>2.0</td>
<td>3.2</td>
</tr>
<tr>
<td>GADD153</td>
<td>S40706</td>
<td>2.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Topoiso</td>
<td>J03250</td>
<td>2.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Data were collected after hybridization of cDNA from three individual pairs (A, 1 and 4; B, 2 and 5; C, 3 and 6; Table 1) of PEX and control tissues to the gene array (Atlas Human Stress Array; BD-Clontech, Palo Alto, CA) and are expressed as the approximate level of differential expression in PEX tissues (±fold change). The pairs of eyes were matched by age, gender, and type of glaucoma.
Immunofluorescence signals for GSTs (Figs. 4A–D) displayed a widespread expression in all anterior segment tissues of normal control eyes. Signals for both GSTT1 and mGST1 were found in almost all cell types, particularly in the vascular endothelia of the iris, conjunctiva, episclera, and ciliary body; signals in the ciliary epithelium were particularly pronounced along the tips of the ciliary processes. The levels of GSTT1 and mGST1 protein expression were generally weaker or hardly detectable in anterior segment tissues, particularly the iris, ciliary body, and trabecular meshwork, of all PEX eyes examined. Consistent with their known subcellular localization, signals for UBE2A/B indicated a ubiquitous but variable nuclear, perinuclear, or cytoplasmic localization in anterior segment tissues of normal eyes. Staining was most pronounced in epithelial, endothelial, stromal, and muscle cells of the iris and appeared markedly reduced in all PEX eyes examined. Staining for MLH1 was exclusively nuclear and was seen in epithelial, endothelial, stromal, and muscle cells of the cornea, conjunctiva, trabecular meshwork, iris, and ciliary body. Nuclear signals for MLH1 were clearly weaker or hardly detectable in iris and ciliary body tissue of three of five PEX eyes examined. Although GADD153 was not detectable by Western blot analysis and usually is expressed at low levels under normal conditions, a weak but distinct immunoreactivity was observed in many cell nuclei of the cornea, conjunctiva, trabecular meshwork, iris, and ciliary body of normal eyes, which showed marked interindividual differences in expression levels. The overall number of immunopositive cells was clearly diminished, however, in the iris and ciliary body of all PEX eyes examined (Figs. 4I, 4J).

Antibody binding was abolished when an irrelevant monoclonal antibody or PBS was used instead of the primary antibodies (data not shown).

**DISCUSSION**

Although the exact pathogenesis and etiology of PEX syndrome have still not been elucidated, it is believed that cellular stress conditions, such as oxidative stress and ischemia/hypoxia, constitute major mechanisms involved in its pathobiology. Despite the pronounced oxidative milieu and hypoxic conditions in the anterior chamber of PEX eyes, the expression of the extracellular chaperone clusterin was significantly downregulated in all anterior segment tissues of PEX eyes examined (Figs. 4A–D). In contrast to the upregulation of MAPKp38, SOD2, HSP40, and SOD60, the expression of UBE2A, UBE2B, mGST1, and GSTT1 displayed a statistically significant downregulation in PEX eyes only (n = 3 for each group; *P < 0.005, **P < 0.001, ***P < 0.008 compared with control group).

![Figure 2](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAAEAAAABCAYAAAAfFcSJAAAADUlEQVR42mP8/A8wG7AAAAABJRU5ErkJggg==)

**FIGURE 2.** Quantitative determination of mRNA expression levels of stress-related genes in (A) ciliary processes and (B) iris tissue using real-time PCR. Data were normalized to GAPDH and are expressed as copies of the gene of interest per copies of GAPDH. The values represent the mean ± SD of results in three independent experiments comparing PEX syndrome (PEXS) and PEX-associated glaucoma (PEXG) with normal (control) and glaucomatous (GL) eyes. In contrast to the upregulation of MAPKp38, SOD2, HSP40, and HSP60, the expression of UBE2A, UBE2B, mGST1, and GSTT1 displayed a statistically significant downregulation in PEXS and PEXG eyes. MLH1 and GADD153 were significantly downregulated in PEXG eyes only (n = 3 for each group; *P < 0.005, **P < 0.001, ***P < 0.008 compared with control group).
stress-related protective gene products in PEX eyes. These genes, including glutathione S-transferases, ubiquitin-conjugating enzymes, DNA repair enzymes, and a stress-induced transcription factor, are normally upregulated and activated by various stress conditions, but were found to be consistently downregulated in ocular tissues of patients with PEX syndrome/glaucoma. Downregulation of these genes, on both the mRNA and protein levels, supports the notion that reduced cytoprotective mechanisms are involved in PEX pathogenesis, as has been reported for other fibrotic and neurodegenerative diseases.20,21

**Glutathione S-Transferases**

The GSTs represent a family of detoxification enzymes that catalyze the conjugation of glutathione to a wide variety of reactive compounds and products of oxidative stress, converting them to less harmful compounds.22 Eight families of cytosolic GSTs and five membrane-bound isoenzymes (mGSTs) have been identified. Certain GSTs display glutathione peroxidase activity against free lipid hydroperoxides (GSTA, GSTT), DNA hydroperoxides (GSTM, GSTT), and phospholipid hydroperoxides (mGSTs), playing a pivotal role in protecting cells from oxidative damage. GSTs have been shown to be widely expressed in ocular tissues23–25 and their protective role against oxidative injury has been implicated, for instance, in cataractogenesis, retinal degeneration, and age-related macular degeneration.26

According to their decisive role in cytoprotection, GSTs are upregulated in response to oxidative stress in vivo and in vitro.22 Unexpectedly, several GST isoforms were found to be consistently downregulated in ocular tissues of patients with PEX, indicating an impaired protective function against oxidative insult in PEX eyes, possibly leading to increased levels of reactive compounds and subsequent damage to ocular tissues. In fact, increased levels of lipid peroxidation products, such as 8-isoprostaglandin F2α (8-IPGF), malondialdehyde (MDA), and thiobutyric acid reactive species (TBARS), the major breakdown product of lipid peroxides, were significantly increased in aqueous humor and serum samples of patients with PEX.5,7,11,12 Since mGST1 activity scavenges phospholipid hydroperoxides, like arachidonic acid hydroperoxides, the precursor stage of 8-IPGF,27 its decreased expression in anterior segment tissues may account for increased aqueous concentrations of 8-IPGF in PEX eyes. Oxidation of membrane lipids may also result in degeneration and disruption of cell membranes, which has been observed by electron microscopy in cells involved in the PEX process (e.g., iris...
pigment epithelial cells, leading to pigment liberation and dispersion).¹

**Ubiquitin-Conjugating Enzymes**

Proteasome-mediated protein degradation is responsible for the clearance of short-lived regulatory and abnormal proteins (e.g., misfolded or oxidized proteins).²⁻⁶ The modification of proteins with ubiquitin constitutes an important cellular mechanism for targeting proteins for degradation by the 26S proteasome.²⁹,³⁰ Conjugation of ubiquitin to target proteins is usually accomplished by the sequential action of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. The role of the ubiquitin-conjugating enzymes E2 is to transfer ubiquitin from the E1 active site to the E3 active site, which finally is transferred from E3 to the substrate.

The ubiquitin-conjugating enzymes UBE2A and UBE2B, mammalian homologues of the yeast RAD6/UBC2 protein, are highly conserved, homologous proteins with 96% identity and redundant, but not identical functions.³¹ Their fundamental biological importance, as well as their ability to compensate for the absence of each other, is supported by the fact that, in contrast to embryonically lethal double-knockout mice, single-knockout mice are viable and display a mild phenotype.³² UBE2A/B interact with their E3 enzymes (human homologues of yeast RAD18, UBR1, and BRE) and are involved in cellular processes as diverse as DNA repair, gene silencing, selective

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**FIGURE 4.** Immunofluorescence labeling of stress-related proteins in anterior segment tissues of eyes with PEX syndrome (right) and normal control eyes (left). Green: positive signals; red and blue: nuclear counterstains propidium iodide and DAPI, respectively. (A, B) Staining for GSTT1 in the epithelium and stroma of ciliary processes of normal and PEX eyes. (C, D) Staining for mGST1 in iridal vessels (arrows) of normal and PEX eyes. (E, F) UBE2A/B immunoreactivity in iridal tissue of normal and PEX eyes. (G, H) Nuclear staining for MLH1 in the ciliary epithelium of normal and PEX eyes. (I, J) Nuclear staining for GADD153 in iris tissue of normal and PEX eyes. BV, blood vessel; CE, ciliary epithelium; CS, ciliary stroma; DIL, dilator muscle; IPE, iridal pigment epithelium; IS, iris stroma. Original magnification, ×200.
proteolysis, and degradation of proteins via the N-end rule pathway. 
UBE2A/B have also been shown to function in several pathologic processes (e.g., in basal and atrophy-induced muscle degeneration), which are usually associated with cellular increases in UBE2A/B gene expression, whereas in patients with lung cancer, decreased UBE2B mRNA expression has been associated with a reduced DNA repair capacity.

In the eye, UBE2A/B and other ubiquitin-conjugating enzymes have been particularly localized to the lens, and ubiquitin-conjugating activity including UBE2A/B activity is upregulated in lens epithelial cells in response to oxidative stress.

Therefore, the reduced expression of both UBE2A and UBE2B in PEX eyes may be associated with a reduced capacity for DNA repair and ubiquitin-mediated protein degradation.

Transcription Factor GADD153 and DNA Repair Protein MLH1

Growth arrest and DNA damage-inducible gene 153 (GADD153) is a leucine zipper transcription factor, which is specifically induced by stress conditions disturbing the function of the endoplasmic reticulum (ER), such as oxidative stress and hypoxia.

Conditions interfering with ER function lead to the accumulation of unfolded proteins in the ER, as has been shown for myocilin aggregates in patients with myocilin-caused glaucoma, but may be associated with formation of extracellular aggregates and amyloid fibers.

In addition, ER-associated stress and protein misfolding have been implicated in various common diseases such as cataract, glaucoma, diabetes, and neurodegenerative disorders.

Whereas the expression of ER-related stress proteins including GADD153 tended to be upregulated in most conditions, a specific oxidative stress-induced downregulation of GADD153 has been demonstrated in neuronal cells in vitro and in vivo in Alzheimer patients.

These findings suggested that a disturbance of the ER-related stress response has a major impact on neuronal cell injury. Similarly, the downregulation of GADD153 in PEX eyes, as demonstrated in this study, may reduce the cells' capacity to respond to ER-associated stress and dysfunction and may be even involved in the accumulation of protein aggregates in the extracellular space.

The mammalian mismatch repair (MMR) is a complex system for recognizing and repairing replication errors and various types of DNA damage.

Heterodimeric MLH (Escherichia coli MutL homologue) proteins are recruited to mismatch-containing DNA and mediate various downstream events leading to repair of the lesion. As MLH1 appears to serve as an essential subunit for all functional MLH heterodimers, MLH1 deficiency may have a major impact on the MMR system and has been associated with increased mutagenesis and various types of sporadic cancer. Because alterations in the capacity of cells to repair DNA damage play an important role in several age-related diseases, including neurodegenerative disorders, the downregulation of MLH1 in PEX tissues, as demonstrated in this study, suggests that a defective DNA repair capacity may be also involved in the pathogenesis of PEX syndrome.

Although evidence of any causative relationship between gene dysregulation and PEX pathophysiology is still lacking, the reduced capacity of cells to protect against oxidative challenge may well lead to impaired cellular function and finally to the abnormal extracellular matrix metabolism characteristic of PEX syndrome. At present, it is not clear which pathogenetic factors initiate the pathologic cascade. TGF-β1, which is significantly increased in the anterior chamber of PEX eyes, has been shown to inhibit proteosomal activity and to suppress activities of antioxidative enzymes, including GSTs, leading to increased cellular levels of lipid peroxidation products.

On the other hand, oxidative stress has been shown to interfere with proteasome function and to increase extracellular matrix accumulation via TGF-β and MAPKp38 signaling in various in vitro settings and fibrotic disorders.

Whatever the pathologic sequence may be, the present findings provide evidence for alterations in cellular cytoprotective mechanisms including antioxidative defense, proteasome function, ER-related stress response, and DNA repair in anterior segment tissues of PEX eyes. The resultant enhanced sensitivity and vulnerability to cellular stress, such as oxidative stress and hypoxia/ischemia, may therefore be one contributing factor in the pathobiology of PEX syndrome, but this possibility must be corroborated by functional studies, to provide conclusive evidence of causality.

References


