Expression of Nitrotyrosine and Oxidative Consequences in the Trabecular Meshwork of Patients with Primary Open-Angle Glaucoma

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PURPOSE. To evaluate the possible correlation between the visual field defects in patients with primary open-angle glaucoma (POAG) and the expression and enzymatic activity of nitric oxide synthase (NOS) isoenzymes and nitrotyrosine in trabecular meshwork (TM) samples.

METHODS. TM specimens were collected from 146 patients with POAG by using standard filtration surgery. Visual field defects were evaluated by perimeter. Expression of endothelial (e)NOS and inducible (i)NOS were evaluated by quantitative RT-PCR. Constitutive (Ca2+-dependent) and iNOS (Ca2+-independent) activities were measured by the conversion of L-[14C]arginine to L-[14C]citrulline. In four TM specimens from POAG-affected eyes and in three human donor control eyes, 3-nitrotyrosine was localized by immunohistochemistry. The marker of lipid peroxidation malondialdehyde (MDA) was measured by the thioharbituric acid test in samples of aqueous humor (AH) from 48 patients with either POAG or cataracts.

RESULTS. The results showed an upregulation of iNOS and a downregulation of calcium-dependent NOS correlated with visual field defects. Expression and activity of iNOS increased in parallel with visual field defects. However, constitutive activity decreased as the visual field defect increased. Nitrotyrosine was observed only in the cells of the TM specimens from eyes with severe POAG.

CONCLUSIONS. The increased expression and activity of iNOS in the TM of patients with POAG are proportional to the visual field defect and could lead to the increased of nitrotyrosine levels which may serve as marker of oxidative stress in the progression of cell death of the TM in POAG. (Invest Ophtalmol Vis Sci. 2008;49:2506–2511) DOI:10.1167/iovs.07-1363
TABLE 1. Demographic Characteristics of Patients Enrolled in the Study

<table>
<thead>
<tr>
<th>Control</th>
<th>POAG</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
<td>AH</td>
</tr>
<tr>
<td></td>
<td>TM</td>
</tr>
<tr>
<td>Mean age (±SD)</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>IOP (mm Hg; mean ± SD)</td>
<td>14.2 ± 2.1*</td>
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</table>

AH, aqueous humor; TM, trabecular meshwork.

* P < 0.05.

expression.14 Similar effects were observed in bovine TM subjected to pressure.15

It has been shown that cytotoxicity associated with high levels of NO are due to the formation of the powerful oxidant peroxynitrite, (ONOO−) by its interaction with superoxide anion (O2•−). ONOO− can cause cell damage or death16 by lipid peroxidation of cell membranes,17 oxidation of sulfhydryl groups,18 and oxidation or nitration of various amino acid functional groups, such as tyrosine.19 A major reaction with proteins is the formation of nitrotyrosine (NT).20 Studies in patients with POAG have provided evidence of an increase in oxidative DNA damage,21 in relationship with the lack of genes regulating the function of antioxidant, free-radical neutralizing defenses.22

We hypothesized that in vivo iNOS overexpression in the chronic progress of POAG could contribute to TM cell damage, through protein nitration by reactive peroxynitrite. This process can be an important link in the chain of events leading to the oxidative damage observed in severe POAG.21,22 Therefore, the purpose of this study was to evaluate the possible correlation between the visual field defects in patients with POAG and the expression and enzymatic activity of NOS isoenzymes and NT in TM samples.

METHODS

Study Population

Human tissue was handled according to the Declaration of Helsinki and the Local Committee on Ethics in experimentation with human samples. Institutional review and approval were obtained, and all enrolled patients provided informed written voluntary consent. One hundred forty-six consecutive patients with POAG, aged 55 to 85 years (mean ± SD, 71 ± 12) were included. Table 1 summarizes the demographic distribution. Only one eye per patient was included in the study. The duration of the disease was 4 to 12 years. We included all patients who fulfilled the following inclusion criteria: a diagnosis of POAG, patients older than 50 years, access to at least three reliable baseline preoperative visual fields, and no retinal or neurologic disease that may have affected the visual field. Exclusion criteria included ocular disease other than POAG, normal-tension glaucoma, pseudoexfoliation or pigmentary syndrome, previous eye surgery and the inventory gene-specific primers: β-actin: HS99999903; eNOS: HS99999904; iNOS: HS99999905; NOS2: HS99999906; CYP1B1: HS99999907; and INOS: HS00167257; and iNOS: HS00167166. The cycling conditions were

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n = 38) and MD worse than −20 dB (MD < −20 dB; glaucoma; n = 38). IOP was determined using the Goldmann applanation tonometer (GAT; Haag-Streit, Konz, Switzerland; Table 2).

TM Specimen Collection

The experimental protocol required removal of the TM specimens during trabeculectomy. The surgical technique has been described elsewhere.23 A large flap was used (scleral flap size: 8:10 × 5-6 mm, flap thickness: two thirds scleral thickness; excised inner block size: 6:5 × 4 mm) allowing us to obtain a large specimen. The TM specimens were obtained according to standard surgical procedures: to remove all specimens, a 45° knife was used to cut a 6:5 × 4-mm button of conjunctiva and TM. The TM was then dissected under microscopic control. TM specimens were either snap frozen in liquid nitrogen and stored at −80°C until assay or fixed in 10% neutral-buffered formalin.

NO Synthase Activity

Calcium dependent and independent NOS activities were measured by the conversion of l-[14C]-arginine to l-[14C]-citrulline, as previously described.24,25 Each TM specimen was assayed independently. Frozen specimens were homogenized by sonication (Vibracell; Sonics & Materials, Inc., Newtown, CT) in an ice-cold buffer (pH 7.4) containing Tris-HCl (50 mM), sucrose (320 mM), dithiothreitol (1 mM), leupeptin (10 μg/mL), soybean trypsin inhibitor (10 g/mL), and aprotinin (2 μg/mL), followed by centrifugation at 10,000g for 20 minutes 4°C. NOS activity was determined in cell extracts under conditions (substrate and calcium concentration) of maximal activity, to assess indirectly the amount of enzyme. The samples (40 μL of supernatant) were incubated at 37°C for 10 minutes in a buffer (KH2PO4, 50 mM; MgCl2, 1 mM; CaCl2, 0.2 mM; L-valine, 50 mM; L-citrulline, 1 mM; L-arginine, 20 mM; and dithiothreitol 1.5 mM) containing l-[14C]-arginine (0.1 μCi/mL; GE Healthcare, Iberica, Spain). The reaction was terminated by removing the substrate by the addition of 1 mL of 1:1 H2O resin (Dowex AF 500W-8; Bio-Rad Laboratories SA, Madrid Spain). The activity of the calcium-dependent NOS was calculated from the difference between l-[14C]-citrulline produced from control samples and samples containing ethylene glycol-bis(aminohydroxy ether) N,N'-tetra acetic acid (EGTA, 1 mM). The activity of the calcium-independent isoenzyme was determined from the difference between samples with EGTA and samples containing 1 mM N′-monomethyl-l-arginine (L-NMMA). The l-[14C]-bound radioactivity was counted using a liquid scintillation counter (LS-6500; Beckman Instruments, Fullerton, CA).

Quantitative Real-Time RT-PCR
cNOS and iNOS expression was detected by real-time RT-PCR. Three TM specimens from each group of patients were pooled. Total mRNA was extracted and purified (RNasy Mini Kit; Qiagen, Crownley, UK), according to the manufacturer’s protocol with residual DNA removed by DNase digestion. cDNA was synthesized using a high capacity kit (Applied Biosystem. Inc. [ABI], Foster City, CA). PCR amplification (Assays-on-Demand Gene Expression Products; ABI) was performed with master mix (TaqlMan Universal PCR Master Mix; ABI) and the inventory gene-specific primers: β-actin: HS99999903; eNOS: HS00167257; and iNOS: HS00167166. The cycling conditions were

TABLE 2. IOPs in the Four Groups of Patients with POAG

<table>
<thead>
<tr>
<th>MD (dB)</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>&gt;−6</td>
<td>35</td>
<td>20.6 ± 1.8</td>
<td>18–23</td>
</tr>
<tr>
<td>6 to −12</td>
<td>35</td>
<td>21.6 ± 1.9</td>
<td>19–24</td>
</tr>
<tr>
<td>13 to −20</td>
<td>38</td>
<td>25.1 ± 2.9</td>
<td>20–30*</td>
</tr>
<tr>
<td>&lt; −20</td>
<td>38</td>
<td>28.3 ± 4.0</td>
<td>22–40*</td>
</tr>
</tbody>
</table>

n, number of patients.

* P < 0.05 vs. >−6 dB group.

Perimetry was performed during the 4 weeks before surgery (Octopus tG1; Interzeag AG, Schlieren, Switzerland). We divided the patients into four groups based on mean defect (MD): MD better than −6 dB (MD > −6 dB; mild visual field loss; n = 35), MD between −6 and −12 dB (MD −6 to −12 dB; moderate visual field loss; n = 35), MD between −13 and −20 dB (MD −13 to −20 dB; severe visual field loss;
Immunohistochemical staining was performed as previously described. Mouse monoclonal antibody against 3-NT (sc-32,757; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:75 dilution. Briefly, deparaffinized and hydrated sections were incubated in blocking solution (TBT [Tris-buffered saline] [TBS] 0.5 M [pH 7.4], containing 3% [wt/vol] BSA and 0.05% [vol/vol] Triton X-100) for 30 minutes at room temperature, to reduce nonspecific binding. The sections were incubated overnight at 4°C in a humidified chamber. The slides were washed for 5 minutes in TBS. Immunodetection was performed with biotinylated anti-mouse immunoglobulins followed by streptavidin conjugated with alkaline phosphatase and a fuchsin chromogen for red staining (LSAB2 kit; Dako Corp., Carpinteria, CA). The sections were lightly counterstained with Mayer's hematoxylin. Final mounting was in water-soluble medium (Glycergel; Dako). Negative controls were treated in the same manner but with the omission of the primary antibodies. With the observer blinded to the experimental conditions, a series of three images of equal surface of 3NT-stained TMs were delineated with an operator-controlled cursor. Sections were analyzed with an image analyser (Image processing and analysis in Java Image; National Institutes of Health, Bethesda, MD).

**Statistical Analysis**

Results were expressed as the mean ± SE. Statistical analysis was performed with Student’s t test. Correlations between IOP and iNOS expression were evaluated by using the Student’s t test. The results were considered statistically significant at *P < 0.05.*

**RESULTS**

Expression of iNOS mRNA and Calcium-Independent NOS Activity in Samples from Patients with POAG

In POAG specimens from patients with MD between −13 to −20 dB and MD < −20 dB groups compared with those of the MD > −6 dB group (Fig. 1A).

In vitro analysis of calcium-independent NOS activity in the soluble fraction of these samples paralleled these findings (Fig. 1B). POAG caused the appearance of iNOS expression and activity in TM, which increased with the degree of severity. As shown in Figure 1, iNOS appeared when MD reaches severe visual field loss −13 to −20 dB (300 ± 30 pg/min per mg protein) and < −20 dB (400 ± 15 pg/min per mg protein). Spearman ρ showed significant correlation among iNOS expression and IOPs (Fig. 2) in patients with terminal (MD < −20 dB).
FIGURE 2. Scatterplots of the relationship between the relative expression of iNOS in TM of patients with severe POAG and their IOPs. Spearman ρ showed significant correlation (r = 0.41, P = 0.15) in patients with blinding (MD < −20 dB) POAG (□) but not in patients with MD −13 to −20 dB (□).

dB) POAG (r = 0.61, P = 0.002), but not in patients with MD −13 to −20 dB (r = 0.41, P = 0.15).

Expression of eNOS mRNA and Calcium-Dependent NOS Activity in Samples from Patients with POAG

eNOS mRNA expression was significantly lower in the TM specimens from MD > 20 dB compared with those of other groups (Fig. 3A). Ex vivo analysis of calcium-dependent NOS activity in the soluble fraction of these samples clearly indicated a decrease in activity (5 ± 2 pg/min per mg protein; Fig. 3B). POAG caused a decrease in physiological NOS activity in the TM when MD reached severe visual field loss at −15 to −20 dB (6.3 ± 1.5 pg/min per mg protein) in comparison with that in the MD < −6 dB (140 ± 20 pg/min per mg protein) and MD −6 to −12 (120 ± 15 pg/min per mg protein) eyes.

Oxidative Consequences in AH of Patients with POAG

POAG caused the accumulation of the mediator of lipid peroxidation malondialdehyde (MDA) in AH of patients with POAG with terminal disease (MD < 20 dB; Fig. 4). The levels of MDA in AH of cataract patients were similar to those in the other MD groups.

Localization of Peroxynitrite by 3 NT Staining

In TM specimens from eyes with MD between −13 and −20 dB (24.2 ± 1.2 mm Hg; Fig. 5A) and with MD < −20 dB (IOP: 27 ± 1.9 mm Hg; Fig. 5B), moderate to intense labeling intensity was found in many but not all cells of the uveal, corneoscleral meshwork, and juxtacanalicular tissue. Only a few cells of the inner wall endothelium of SC were stained. In the control section (IOP: 15.3 ± 0.9 mm Hg), no signal was detected (Fig. 5C).

The 3-NT immunostaining quantification obtained from image processing and analysis were: control (n = 3): 54.4 ± 2 AU, arbitrary units; Fig. 5C); MD between −13 and −20 dB (n = 3): 80.7 ± 4 AU (P < 0.05 vs. control, Fig. 5A); and MD ≤ −20 dB (n = 3): 151.8 ± 17 AU (P < 0.05 vs. MD between −13 and −20 dB and control, Fig. 5B). A correlation between the results of the immunostaining quantification and the IOP in each group was observed (Table 3).

DISCUSSION

The reported results demonstrate that iNOS expression and activity increase in TM of patients with POAG, in parallel with visual field defects. Of interest, the expression of eNOS and activity of Ca2+-dependent NOS decreases as the visual defect increases. The induction of the cytotoxic, high-output enzymatic source of NO, and the functional decrease in the physiological NOS leads to the accumulation of MDA in AH, a marker of lipid damage by free radicals. Finally, this is the first study, to our knowledge, that shows the expression of the powerful

FIGURE 3. (A) Expression of eNOS mRNA in TM of patients with POAG. The total mRNA was extracted from specimens from each group of patients, subjected to real-time quantitative PCR, and normalized to β-actin levels. Relative expression of mRNA was calculated comparing the values to those obtained in TM samples from patients with MD between 0 and 6 (MD > −6 dB). (B) Calcium-dependent NOS activity in TM from patients with POAG. MD: mean deviation visual field index. The data represent the mean ± SE of five specimens. *P < 0.05 vs. control (MD > −6 dB), by Student’s t-test.

FIGURE 4. MDA levels in aqueous humor of patients with POAG. The data represent the mean ± SEM of 12 samples. *P < 0.05 vs. cataract. (Student’s t-test).
oxidant peroxynitrite (by 3-NT staining) in the TM of patients with severe to blinding POAG.

The induction of iNOS in the TM from severe POAG along with MDA accumulation in the AH is concomitant with extensive and prolonged release of NO metabolites, which finally lead to a production of other oxidant species such as peroxynitrite.

The decrease in eNOS function is in agreement with previously published data in POAG, where the population of TM endothelial cells is markedly decreased compared with that of age-matched healthy subjects.6,7 Taking into account the cytoxic effects of iNOS and particularly of ONOO−, we hypothesized that the increased production of NO by iNOS present in the TM of patients with POAG could contribute to the death of TM cells. The double-faced effect of NO also appears to increase in this disease. The fact that the TM of patients with POAG contains less eNOS than that of normal control subjects suggests that the NO pathway is affected. Of interest, some studies suggest that eNOS gene polymorphisms act as an additional risk factor in the development of endothelial dysfunction in glaucoma.8,9

We have demonstrated the presence of ONOO− by showing positive NT immunoreactivity in cells from uveal, corneoscleral meshwork, and juxtacanalicular tissues. Such a distribution within the TM from patients with severe POAG indicates a long-term exposure to peroxynitrite.10

Mechanisms by which POAG induces iNOS on the human TM remain to be elucidated. In vitro studies of perfused anterior segments of human donor eyes have shown that NO production increases after elevation of the pressure gradient over the TM accompanied by an upregulation of iNOS gene expression.11 In agreement, our study demonstrated a statistically significant correlation between iNOS expression and IOP values in patients with terminal POAG. Indeed, we observed a significantly increased NT in severe POAG, which may serve as a marker of loss of constitutive eNOS activity. These changes lead to the increased NT in severe POAG, which may serve as a marker of oxidative stress in the progression of cell death in POAG.

The TM from POAG donors showed upregulation of several genes involved in inflammatory and acute-phase responses, including the expression of selectin E, the first molecular marker for glaucomatous TM.6,7 Furthermore, a genetic association between the iNOS gene and POAG, based on genotyping of the CCTTT-microsatellite in the inducible NOS regulatory sequence, has been found.36

Indeed, in the TM of patients with glaucoma oxidative DNA damage that correlates significantly with IOP and with visual field defects and increased expression of oxidative stress markers have been described.37

The formation of large amounts of oxygen and nitrogen reactive species during stress may also initiate lipid peroxidation, as it has been demonstrated to occur in heart and liver,38 and we have demonstrated to occur in the anterior chamber in the present study. In addition, an increase in lipid peroxidation may be due to an insufficiency of the protective antioxidant systems (mainly GSH), which are also depleted by ONOO−, decreased antioxidant potential,41 and peroxidized lipids.12 In addition, the TM from POAG donors demonstrated downregulated expression of the antioxidants paraoxonase 3 and ceruloplasmin.42

The membrane lipid peroxidation due to an oxyradical attack on membrane fatty acids results in the formation of several cytotoxic aldehyde products, such as MDA44 and has been described to play an important role in the pathogenesis of several degenerative diseases.45

This process is of particular interest, since the high-output isoform of NO synthase (iNOS) has been implicated in cellular toxicity in many cell systems. Once iNOS is expressed, the formation of large amounts of oxygen and nitrogen reactive species may account for the oxidation of cellular components—membrane lipid peroxidation due to the oxyradical attack on membrane fatty acids resulting in the formation of several cytotoxic aldehyde products, such as MDA.44,45

In conclusion, the expression of iNOS and the activity of the inducible Ca2+-independent iNOS in the TM of patients with POAG are proportional to the visual field defect as well as the loss of constitutive eNOS activity. These changes lead to the increased NT in severe POAG, which may serve as a marker of oxidative stress in the progression of cell death in POAG. Further studies should determine whether specific inhibition of iNOS would be of therapeutic benefit in this condition of vast overproduction of NO.

Table 3. 3-NT Immunostaining Quantification in Patients with POAG

<table>
<thead>
<tr>
<th>Group of Patients</th>
<th>IOP (mm Hg)</th>
<th>3-NT (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.3 ± 0.9</td>
<td>54.4 ± 2</td>
</tr>
<tr>
<td>MD −13 and −20 dB</td>
<td>24.2 ± 1.2*</td>
<td>80.7 ± 4*</td>
</tr>
<tr>
<td>MD &lt; −20 dB</td>
<td>27.0 ± 1.9*</td>
<td>151.8 ± 17†</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. control.
† P < 0.05 vs. control and MD −13 and −20 dB.