Objective: To assess endothelin B receptor (ETbR) expression in human glaucomatous optic nerves and the spatial relationship between ETbR and astrocytes.

Methods: Twenty-six eyes from 16 glaucoma patients and 10 normal control subjects were immunohistochemically labeled with antibodies to ETbR. The immunoreactivity was quantified and compared between normal and glaucomatous eyes with an image analysis system. Tissues were also double-labeled for ETbR and astrocytes. In addition, the optic nerve of a monkey with regional degeneration induced by laser coagulation was examined with the same techniques.

Results: The frequency of positive ETbR immunoreactivity was higher in human glaucomatous optic nerves as compared with age-matched controls (9/16 vs 1/10, \( P = .02 \)). The ETbR immunoreactivity colocalized with astrocytic processes and was quantitatively higher in the glaucomatous eyes (\( P = .02 \)). In the monkey, the regions of degeneration showed increased ETbR associated with reactive astrocytes and was highest at the borders between normal areas and degeneration.

Conclusion: Increased ETbR immunoreactivity in diseased optic nerves and its association with astrocytes suggest that the glia-endothelin system may be involved in the pathologic mechanisms of neuronal degeneration.

Clinical Relevance: The study supports the clinical observation of endothelin involvement in glaucoma and provides direct evidence that the endothelin system is associated with glaucomatous pathologic abnormalities.

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In human diseases such as glaucoma. A jury that simulates the localized loss of RGC bundles seen based on focal laser axotomy and resultant optic nerve injury was also examined in a nonhuman primate experimental model. ETbR immunoreactivity and glial cell activation was compared with a group of age-matched normal controls. The tissues and 3 enucleated human glaucoma eyes. However, only 1 of the eyes with glaucoma in that study had primary glaucoma, and it was enucleated because of complications of endophthalmitis. The other 2 eyes had histories of secondary glaucoma following melanoma. These additional ocular pathologic abnormalities complicate the interpretation of that study’s findings.

In the present study, the expression of ETbR in optic nerve tissues was quantified with immunohistochemical double-labeling techniques. In addition, ETbR immunoreactivity and glial cell activation was also examined in a nonhuman primate experimental model based on focal laser axotomy and resultant optic nerve injury that simulates the localized loss of RGC bundles seen in human diseases such as glaucoma.

### METHODS

#### HUMAN SUBJECTS

Twenty-six postmortem donor eyes from 10 normal subjects (6 men, 4 women; average ± SD age, 82.2 ± 8.9 years) and 16 glaucoma patients (10 men, 6 women; average ± SD age, 82.7 ± 9.3 years) were obtained from the local branch of the Lions Eye bank (Lions Sight and Hearing Foundation, Devers Eye Institute, Portland, Ore). Consent to acquire and use the tissue was granted, and the study adhered to the tenets of the Declaration of Helsinki. The Table summarizes the demographic information from the most recent medical records of each subject. In brief, 13 glaucoma patients were diagnosed as having POAG, 1 had normal-tension glaucoma, and 2 were glaucoma suspects based on the latest diagnoses obtained from their medical records. The range of disease duration (ie, from initial recorded diagnosis to death) was 0.5 to 17 years. Visual field data was available for 9 of 16 eyes, although most intraocular pressure measurements were made in eyes receiving antiglaucoma medications. Because the duration and type of antiglaucoma medications varied considerably across the patient group, statistical analysis of their possible effects was inappropriate; thus, individual medications were not listed in the Table. We excluded from the study patients with a recorded history of diabetic retinopathy, retinal detachment, or other diseases that may affect the retina and optic nerve. All normal control eyes were verified first by the relatives of the donors and then.

### Table. Summary of Provided Patient Information and the Results of ETbR Immunoreactivity Grading

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<th>Diagnosis</th>
<th>Duration, y</th>
<th>Visual Field MD, dB</th>
<th>Visual Acuity</th>
<th>C/D</th>
<th>Max IOP</th>
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</tr>
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**Abbreviations:** C/D, cup-disc ratio; ellipsis, not applicable; ETbR, endothelin B receptor; ID, identification number; LTG, low tension glaucoma; Max IOP, maximum intraocular pressure; MD, mean deviation; POAG, primary open-angle glaucoma.

*See text in the “Methods” section for details.
medical-record screening to exclude those with eye-related diseases. The length of the optic nerve attached to each donor eye varied between approximately 1 and 10 mm. All eyes were ice-chilled immediately after enucleation and fixed in 10% formaldehyde or 4% paraformaldehyde within approximately 24 hours. The fixation for the eyes lasted for approximately 24 to 48 hours, following which the tissue was processed for paraffin sections. Paraffin sections of 6-µm thickness were cut longitudinally through the anterior optic nerve, which also included approximately 4 mm of retina on either side of the optic nerve head. Additionally, in several cases, a sample of tissue was also processed for resin sections, which were then sectioned at a thickness of 1 µm transversely for axonal morphological observations.

LASER-INDUCED GANGLION CELL AXON DEGENERATION IN A NONHUMAN PRIMATE

All experimental methods and animal care procedures adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local institutional animal care and use committee.

The method has been described in detail previously.26 In brief, the left eye of 1 adult female rhesus monkey (Macaca mulatta) received retinal photocoagulation approximately 1 disc diameter from the optic nerve in a superotemporal location using an infrared diode laser (810 nm, OcuLight SL; Iridex, Mountain View, Calif). Multiple sessions of photocoagulation were performed at intervals of 2 to 4 weeks. After 6 treatments, a seventh laser treatment was administered using a green laser (532 nm, OcuLight GL; Iridex). Subsequent examination of the eye was performed at intervals of 2 to 4 weeks. After 6 treatments, a seventh laser treatment was administered using a green laser (532 nm, OcuLight GL; Iridex). Subsequent examination of the eye was performed at intervals of 2 to 4 weeks. After 6 treatments, a seventh laser treatment was administered using a green laser (532 nm, OcuLight GL; Iridex). Subsequent examination of the eye was performed at intervals of 2 to 4 weeks.

IMMUNOHISTOCHEMICAL LABELING

Antiserum of monoclonal mouse antihuman glial fibrillary acidic protein (GFAP) (1:100; Novocastra Laboratories Ltd, Newcastle upon Tyne, England) was used to identify the astrocytes. The ETbR was labeled by sheep antihuman antibody (1:750; United States Biological, Swampscott, Mass).

Paraffin sections from both the human and animal tissue were dewaxed and placed in 0.3% hydrogen peroxide in methanol for 30 minutes. The sections were then incubated with a mixture of 1% serum corresponding to the host species of secondary antibodies and 1% bovine serum albumin for 30 minutes. Primary antibody of GFAP was applied and slides were incubated at room temperature for 90 minutes. For ETbR, 24 hours at 4°C was used for the incubation. After three 10-minute rinses in 0.01 mol/L phosphate-buffered saline, biotinylated secondary antibody (1:100, Vectastain Elite ABC kit; Vector Laboratories, Inc, Burlingame, Calif) was applied for 30 minutes. This was followed by the avidin-biotin complex for another 30 minutes. The substrate chromagen staining process used 3,3′-diaminobenzidine (DAB Kit; Vector Laboratories, Inc) was used for up to 10 minutes (usually 2-5 minutes). The sections were counterstained with 0.1% Mayer hematoxylin (Sigma Diagnostics, St Louis, Mo) and mounted for microscopy.

The paraffin sections were processed in a manner similar to the avidin-biotin method described here until the secondary antibody was applied. At this time, the specific fluorophore-conjugated antiserum against the corresponding primary antibody (Vector Laboratories, Inc) was applied at a concentration of approximately 1:100 to 1:200 for 30 minutes at room temperature. The sections were then rinsed and mounted for viewing under a fluorescence microscope or a confocal microscope. We assessed co-stains of ETbR and GFAP labeled by corresponding secondary antibodies conjugated with different fluorophore dyes.

For immunohistochemical staining of ETbR, we used the Tyramide Signal Amplification kit (TSA Biotin System; NEN Life Sciences Productions, Inc, Boston, Mass) to enhance the signal. The procedures were generally the same as those described earlier until biotinylated secondary antibody was applied. After that, biotinyl tyramide signal amplification reagent was used before and after streptavidin–horseradish peroxidase solution (30 minutes for each) and followed by the chromogenic visualization with either fluorescence or 3,3′-diaminobenzidine.

Negative controls for immunohistochemistry for both the avidin-biotin method and the fluorescence method were performed by omitting the corresponding primary antibody from the solution. For the tyramide signal amplification, the biotinyl tyramide signal amplification reagent was omitted during the procedure. Additional antigen absorption control was performed to test the specificity of the ETbR antibody. In brief, all the staining procedures were the same except that ETbR antibody was preincubated together with 10 times the titer concentration of ETbR antigen (Alomone Laboratories Ltd, Jerusalem, Israel) at 4°C overnight before being applied on the slides.

It was noted that the immunohistochemical stain for the ETbR is either present or absent on individual sections when observed under a fluorescence microscope and the distribution of the stain was nonuniform throughout the sections. These features make automated threshold-based quantification very difficult. This is in counterdistinction to the GFAP stains used for identification of glial cells. We have previously used automated quantification of staining intensities for GFAP where relative levels of stain intensity denoted progressive activation of retinal astrocytes27 but elected to use masked observers to assess the presence or absence of ETbR in these sections. Therefore, quantitative comparisons of ETbR IR in normal and glaucomatous human tissues were performed by grading the extent and the density of the stain in the sections under a light microscope by 2 masked observers (L.W., G.G.). Five grades (1-5) were predetermined: 1, no stain was visible at ×10 magnification; 2, stain was scarcely distributed within one ×10 field and high magnification lens (×40) was needed to identify the stain; 3, moderately distributed ETbR stain was observed within 1 or 2 fields at ×10; 4, strong ETbR IR was found in more than 2 fields; and 5, ETbR IR was distributed throughout the optic nerve and peripapillary retina. Each observer graded all of the sections in a masked fashion. The presence or absence of ETbR was easily apparent, and there were no instances in which the observers disagreed; however, the relative grades had slight variations, and in cases of disagreement, the lower score was used.

At a temperature of approximately 80°C, the sections were stained with 1% toluidine blue in the phosphate buffer (pH 7.0-7.4) for 3 minutes, followed by adding a few drops of Sorensen buffer for another 2 minutes. The slides were rinsed with distilled water and air-dried.

STATISTICS

Statistical comparison of the immunoreactivity between glaucoma eyes and the age-matched control eyes was made by applying a nonparametric Mann-Whitney U test, for which a critical level of .05 was considered statistically significant. The frequencies of ETbR positive individuals between the 2 groups were also evaluated with χ² test.
RESULTS

METHODOLOGICAL CONTROLS

Negative controls for both the avidin-biotin and the fluorescence methods (performed by omitting the primary antibody of ETbR) showed no immunoreactivity within the optic nerve. Antigen absorption to the ETbR eliminated all the positive stains within the fascicles shown in the normal stain but remained positive for septal connective tissues, particularly within the monkey optic nerve. Thus, only the immunohistochemi-

Figure 1. Representative photomicrographs of optic nerves of human eyes with glaucoma and normal eyes labeled immunohistochemically with endothelin B receptor (ETbR) antibody. Arrowheads indicate the positive stain in the fascicles; asterisks, the septal connective tissue (hematoxylin). A, Negative control. B, One normal optic nerve (H46N) shows no ETbR immunoreactivity (IR) in the fascicle. A glaucoma suspect, H18G (C), and a patient with low-tension glaucoma, H12G (D), show abundant ETbR IR within the fascicles. E, A patient (H109G) with 10-year glaucoma history and severe visual field loss shows no ETbR IR in the fascicles that have greatly shrunk. F, Another patient with a long-term glaucoma history shows localized distribution of ETbR. Note that most of the ETbR IR is not associated with the hematoxylin-stained nuclei in all the photographs.
cal stain of ETbR within the fascicles of both human and monkey tissue was evaluated.

**HUMAN NORMAL AND GLAUCOMA GROUPS**

In the normal group, there was no ETbR IR visible within the axonal fascicles except in 1 case (H11N), where a trace amount of ETbR IR was seen by both observers. However, 9 of 16 glaucomatous optic nerves showed ETbR IR within the axonal bundles ($\chi^2=5.99, P=.02$). The ETbR IR appeared mostly as small isolated spots or tiny dots, but some were star-shaped. The staining pattern of ETbR IR–positive cells viewed under high magnification often revealed a hollow center with a lack of central staining. The spatial localization of ETbR IR within cell soma was generally not closely associated with the counterstained nuclei. Figure 1 shows the ETbR IR in the axon bundles of 4 of the glaucoma patients (Figure 1C-F) and a normal subject (Figure 1B). An example of a negative control is shown in Figure 1A. Further quantitative analysis confirmed that there was a significant increase in ETbR IR in the glaucomatous optic nerves compared with controls (Mann-Whitney U test, $P<.02$; Table).

Double-labeling the ETbR IR–positive glaucomatous optic nerve with GFAP showed that almost all of the ETbR within the fascicles was colocalized with the GFAP (Figure 2). However, many of the GFAP-positive sites, in particular, those adjacent to the astrocytic nuclei, were not colocalized with ETbR. It suggests that the ETbR may exist only on the astrocytic processes. Because the resolution of fluorescence stain was insufficient to allow further detailed observation, we compared the morphological characteristics of ETbR IR with that of GFAP IR stained with the avidin-biotin method; we also compared them with the axons stained by toluidine blue. These nonfluorescence stained sections can be viewed at a higher magnification ($\times100$) with better resolution. As shown in Figure 3, the ETbR staining pattern morphologically matches well with the astrocytic processes demonstrated by GFAP stain and the axons stained with toluidine blue. The central hollowed pattern of ETbR IR represents the astrocytic processes enclosing an axon; the star-like pattern represents astrocytic processes filling extracellular space formed by several neighboring axons.

**LASER-INDUCED AXONAL DEGENERATION**

In the monkey eye that underwent focal laser treatment, regional axon bundle degeneration was produced in the retina and further developed into the optic nerve as has been described previously. Optic nerve cross-sections from approximately 2 mm retrobulbar showed a wedge-shaped area with axonal degeneration (Figure 4A). Within the wedge-shaped area of the defect, astrocytes proliferated and axons were almost abolished, however, only the astrocytes adjacent to and overlapping the border of the degeneration expressed ETbR (Figure 4B), as demonstrated with a co-stain of GFAP. Figure 5 shows that both GFAP IR and ETbR IR were markedly increased in the border area of degeneration (Figure 5C and D) compared with the relatively normal regions (Figure 5A and B). The morphological pattern and dis-
As a predominant cellular component in the central nervous system, astrocytes directly contact neurons. In brain, the ramified processes of astrocytes may surround the nodes of Ranvier of myelinated fibers where the highest density of potassium and sodium ion channels are located. In the optic nerve, astrocyte processes in the endoneurium may contact axons directly. This glial-neuronal structural connection underscores the importance of astrocytes both in neuronal physiologic features and in the pathophysiologic features of diseases. In glaucoma, the astrocytes have been demonstrated to have a variety of altered molecular products, such as extracellular matrix components, and induction of nitric oxide. The current finding of increased ETbR IR in the processes of reactive astrocytes in the diseased optic nerve provides further evidence that astrocytes are involved in the pathological mechanisms of neural injury. And for the first time, these data provide histopathological evidence linking the glia-ET system with human POAG.

Located on blood vessels, both endothelin A and B receptors (ETbR) mediate local vasoactive effects of the endothelin peptides. Endothelin B receptor has also been localized in nonvascular tissues, for example, on glial cells in neural tissue. Under pathological conditions, increased expression of ETbR in the astrocytes has been found in both brain and optic nerve following mechanical, pharmacological, and ischemic insults. This association of ETbR with neural injury is likely due to several factors, including that endogenous endothelin production increases during neural injury and that ETbR mediates many of the detrimental effects of endothelin on neural tissues. The latter may occur via mechanisms that increase intracellular calcium, inhibit gap junction permeability, and indirectly act through excitatory neurotransmitter pathways by inhibiting glutamate transport. In a study on the mechanisms of optic nerve damage induced by administration of ET-1 into the vitreous cavity, disruption of anterograde axonal transport was shown to be mediated by ETbR. Optic nerve injury in rabbits, induced by acutely applying ET-1 into retrobulbar space via an osmotic pump, was accompanied by hypertrophy of astrocytes with ETbR-positive re-

Figure 3. Astrocytic processes within the extra-axonal space (endoneurium) of an optic nerve labeled immunohistochemically with endothelin B receptor (A) and glial fibrillary acidic protein (B) in a human glaucoma eye. C, Axons in the optic nerve with toluidine blue stain. The processes within a region surrounded by multiple axons appear to be star-shaped or solid dots (arrowheads). The process enclosing an axon appear to be ring-shaped (arrows). Asterisks denote the soma of the astrocytes (original magnification, ×100).

Figure 4. A, A wedge-shaped area of degeneration (light gray area indicated by arrowheads) was developed within the retrobulbar optic nerve after focal laser photocoagulation in the monkey. Within the area, most of the axons disappeared (p-phenylene-diamine, Sigma Diagnostics, St Louis, Mo, ×2.5). B, A rectangle-shaped area from relatively normal areas to the degeneration shows different immunoreactivity levels of endothelin B receptor (ETbR). In the normal region (left one third), there is little ETbR. Within the region of degeneration adjacent to the normal area shows the highest expression of ETbR (brown dots within the mid one-third area marked with 2 lines of arrowheads). The ETbR decreases at the center of the degeneration (right one third).
Similar changes were found in the optic nerve with significant retinal ganglion axon loss after chronic release of ET-1 into the retrobulbar in nonhuman primates. In another recent study on experimental glaucoma, ETbR expression was enhanced in the astrocytes of glaucomatous optic nerves. As such, increased ETbR in the astrocytes of the glaucomatous optic nerve is likely to be associated with pathophysiologic events that result in glaucomatous optic neuropathy. This in turn may be the result of increased production of endogenous ET-1 in the plasma and ocular fluid.

It should be noted that there was no clear association between the level of ETbR expression and the severity of clinical or histological signs of optic nerve damage. A likely explanation for this dissociation is that ETbR may only be expressed during a certain stage of the disease. In addition, the effects of various antiglaucoma medications on ETbR expression are unknown, so the diversity of antiglaucoma medication among the patients in this study may have further complicated this relationship. Another important consideration is that ETbR in the vasculature can be down-regulated under stress, that is, opposite to the change in astrocytes. We have similarly found that ETbR expression can increase within neural bundles of the primate optic nerve but decrease in the vascularized septal tissue in the nerves with experimental ischemia (unpublished observation, L.W.). Therefore, quantification of the total tissue level of ETbR, by methods such as Western blot, might actually fail to detect changes and should thus be interpreted with caution.

The expression of ETbR IR within the astrocytes located at the border of the degenerative region in the monkey experiment is interesting. It has been proposed that some loss of RGCs in glaucoma occurs by secondary de-
generation, a pathological process whereby a primary neural injury leads to additional damage of adjacent healthy neurons. However, detailed pathogenic mechanisms are not yet well described. In 2 experimental studies of secondary degeneration in the optic nerve, glial cells have been proposed to be involved. In addition, the “activated” glial cells are often found at the border of the lesion. It may be speculated that the reactive astrocytes at the border of degeneration in the monkey optic nerve seen in this study are likely linked to a potential secondary injury process via the endothelin system. Such a hypothesis can only be clarified by detailed sequential follow-up of ETBr and glial cells in association with neural damage in experimental models. After all, the results from both the human and the monkey experiment may prompt a potential treatment for the glaucomatous optic nerve injury.

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