Purpose: To examine immunostaining of 60-kd and 27-kd heat shock proteins (HSP 60 and HSP 27), which are known to increase cell survival in response to stress, in glaucomatous retina and optic nerve head.

Methods: Six postmortem eyes from patients with primary open-angle glaucoma, 6 eyes from patients with normal-pressure glaucoma, and 6 eyes from age-matched normal subjects were studied by immunohistochemistry. The sections of the retina and optic nerve head were examined after immunostaining with antibodies to HSP 60 and HSP 27.

Results: The intensity of the immunostaining and the number of labeled cells for heat shock proteins (HSPs) were greater in retina sections from glaucomatous eyes than in sections from normal eyes from age-matched donors. Retinal immunostaining of HSP 60 was prominent in the retinal ganglion cells and photoreceptors, whereas immunostaining of HSP 27 was prominent in the nerve fiber layer and ganglion cells as well as in the retinal vessels. In addition, retinal immunostaining of these HSPs exhibited regional and cellular differences. Optic nerve heads of glaucomatous eyes exhibited increased immunostaining of HSP 27, but not HSP 60, which was mostly associated with astroglial cells in the lamina cribrosa.

Conclusion: The increased immunostaining of HSP 60 and HSP 27 in the glaucomatous eyes may reflect a role of these proteins as a cellular defense mechanism in response to stress or injury in glaucoma.

Clinical Relevance: These findings suggest that immunoregulation is an important component of glaucomatous optic neuropathy.

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Heat shock proteins (HSPs), also called stress proteins, are a group of highly conserved proteins that are constitutively expressed in most cells under normal physiological conditions. Heat shock proteins are classified into families on the basis of their molecular weight, including 90-kd (HSP 90), 70-kd (HSP 70), 60-kd (HSP 60), and small (25- to 30-kd) HSP families. They are thought to play a vital role in normal cellular function. One of the main roles that HSPs play is that of molecular chaperones. Specifically, HSPs have been shown to function in protein maturation events such as protein folding, unfolding, and translocation across membranes. In response to environmental stresses such as heat, anoxia, and exposure to cytokines, cells newly synthesize large quantities of HSPs. Because of their protective capacity, the increased expression of HSPs helps the cells to survive stressful conditions and also promotes recovery from stress.

Constitutive expression of HSPs occurs in the central nervous system in a variety of cell types, including oligodendrocytes, astrocytes, and neurons. The expression of HSPs in neuronal cells suggests that, since neurons are structurally and functionally very complex cells and exhibit nonmitotic characteristics, they may require constitutive levels of these proteins for protective purpose against various stresses, such as hypoxia, anoxia, and excessive excitatory stimulation.

In addition to their constitutive expression, the accumulation of HSPs in various cells of the nervous system during acute toxic-metabolic states and in a variety of degenerative, inflammatory, and neoplastic neurological diseases further suggests their role for neuronal survival. Up-regulation of HSP 27 has been shown after damage to peripheral nerves. For example, vagus nerve lesions...
MATERIALS AND METHODS

EYES

Six postmortem human eyes with a diagnosis of primary open-angle glaucoma (ages, 56-93 years), 6 eyes with a diagnosis of normal-pressure glaucoma (ages, 68-84 years), and 6 eyes from age-matched normal donors (ages, 61-91 years) were obtained from the Glaucoma Research Foundation (San Francisco, Calif), the National Disease Research Interchange (Philadelphia, Pa), the Mid-America Eye Bank (St Louis, Mo), and one of us (M.B.W.). Clinical findings of the patients were well documented and included intraocular pressure readings, optic disc assessments, and visual field tests (Table). Normal donors had no history of eye disease or diabetes. There was no infection or sepsis in any of the donors. The cause of death for all of the donors used in this study was myocardial infarction or cardiopulmonary failure.

The eyes were enucleated within 2 to 4 hours after death and processed and fixed within the following 6 to 12 hours in either 10% buffered formaldehyde or 4% paraformaldehyde. The posterior poles were dissected free of surrounding tissues, washed extensively in 0.2% glycine in phosphate-buffered saline at pH 7.4, embedded in paraffin, and oriented sagittally for 6-mm sections. Immunoperoxidase staining and double immunofluorescence labeling were used for localization of different epitopes of HSP 60 and HSP 27 to the retina and optic nerve head. Serial sections from the glaucomatous eyes and normal donor eyes were stained simultaneously to control variations in the immunostaining.

IMMUNOHISTOCHEMICAL ANALYSIS

For immunostaining of HSP 60, mouse monoclonal antibodies to human HSP 60, which were either a gift from Radhey S. Gupta, PhD (McMaster University, Hamilton, Ontario) or purchased (StressGen, Victoria, British Columbia) were used at 1:200 dilution and 1:1000 dilution, respectively. A rabbit polyclonal anti-human HSP 27, a gift from Kanefusa Kato, PhD (Institute for Developmental Research, Aichi, Japan) was used at 1:200 dilution, and a mouse monoclonal anti-human HSP 27 (Sigma-Aldrich Corp, St Louis, Mo) was used at 1:1000 dilution for immunostaining for HSP 27. Additional monoclonal and polyclonal antibodies against human HSP 27 (StressGen) were used at 1:1000 and 1:400, respectively. Non-immune rabbit and mouse serum samples (Sigma-Aldrich Corp) were used to replace the primary antibodies to serve as negative controls.

For immunoperoxidase staining, sections from normal and glaucomatous eyes were deparaffinized, rehydrated, and pretreated with 0.3% hydrogen peroxide in phosphate-buffered saline to decrease endogenous peroxidase activity. Primary antibodies were localized by immunoperoxidase using a commercial kit (Vector Laboratories, Burlingame, Calif). The biotinylated secondary antibody was incubated on the sections for 30 minutes, washed with phosphate-buffered saline containing 0.1% bovine serum albumin, and reacted with streptavidin-horseradish peroxidase conjugated for 30 minutes. After several washes, color was developed by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Corp) as cosubstrate for 3 to 7 minutes. Sections were counterstained with hematoxylin and coverslipped with a mounting medium (Permoun; Fischer, Pittsburgh, Pa).

To study colocalization of HSPs, we performed a double immunofluorescence procedure. Sections were incubated with a mixture of mouse antibody against HSP 60 and rabbit antibody against HSP 27 at 1:100 dilution for 30 minutes. The sections were then incubated with a mixture of rhodamine red- and Oregon green–labeled secondary antibodies (Molecular Probes, Eugene, Ore) for another 30 minutes. Negative controls were performed by replacing the primary antibody with nonimmune serum or by incubating sections with each primary antibody followed by the inappropriate secondary antibody to determine that each secondary antibody was specific to the species against which it was made.

Slides were examined and documented with a fluorescence microscope (Olympus, Tokyo, Japan) equipped with bright-field illumination and epifluorescence light. Images were recorded on 400 ASA color print film or 400 ASA black-and-white print film (Eastman Kodak Company, Rochester, NY). Images were also recorded by means of a digital camera (Diagnostic Instruments, Sterling Heights, Mich) attached to the microscope.

The stained sections were examined by 2 observers (G.T. and M.R.H.) in a masked fashion regarding the identity or diagnosis of the donors. Qualitative evaluation of the immunostaining (negative, faint, or increased immunostaining) in the optic nerve head and different layers of the retina was independently recorded by these observers. Judgment of increased immunostaining was made only when both observers independently agreed.

result in a time-dependent up-regulation of HSP 27 in vagal motor and ganglion sensory neurons.16 Transsection of the sciatic nerve similarly results in a 9-fold up-regulation of HSP 27 messenger RNA and protein in ganglion cells.17

In the retina, the expression of HSPs is developmentally regulated through ocular organogenesis.18,19 The most commonly studied retinal HSPs are the members of HSP 70 family, which are rapidly induced by hyperthermic, light, or ischemic injury in rat and rabbit retinas.20-25 However, studies of retinal expression of other HSPs are limited. For example, heat-induced retinal expression of small HSPs, including HSP 23 and HSP 27, has been shown in Drosophila melanogaster.26 In addition, retinal synthesis of a small HSP, HSP 30, increases after optic nerve crush in goldfish.27

Since elevated titers of serum autoantibodies to HSP 60 and HSP 27 were found in many patients with glaucoma,28,29 we studied HSP 60 and HSP 27 immunoreactivity in postmortem glaucomatous eyes in comparison with normal donor eyes. Our observations disclosed increased immunostaining of these HSPs in the retina and optic nerve head of the glaucomatous eyes, which suggests that HSP 60 and HSP 27 may be components of a natural defense mechanism that are activated in glaucomatous optic neuropathy.
The examination of retina sections by immunohistochemical analysis demonstrated low intensity of immunostaining of HSP 60 and HSP 27 in retina from normal donor eyes. The immunostaining was noticeably increased for both HSPs in glaucomatous eyes with either primary open-angle glaucoma or normal-pressure glaucoma. Immunostaining with different antibodies to either HSP 60 or HSP 27 from 2 different sources yielded identical results. Control sections stained with nonimmune serum to replace the primary antibodies did not exhibit immunostaining.

In normal eyes, faint immunostaining of HSP 60 was observed in a few retinal ganglion cells and photoreceptors. However, in glaucomatous eyes, the intensity of the immunostaining and the number of stained cells for HSP 60 were greater than in normal eyes. In the eyes with primary open-angle glaucoma, some retinal ganglion cells, but not all, strongly stained for HSP 60. In the eyes with normal-pressure glaucoma, in addition to increased immunostaining in the retinal ganglion cells and photoreceptors, immunostaining was observed in some cells located in the inner nuclear layer of the retina (Figure 1). No prominent staining for HSP 60 was associated with retinal vasculature or nerve fiber layer.

Although clearly positive immunostaining of HSP 27 was observed in the vascular wall in normal retinas, there was faint immunostaining of HSP 27 in retinal tissue (Figure 2). As shown in Figure 2, B, few retinal ganglion cells stained for HSP 27 in normal retinas, and no staining was observed in the nerve fiber layer. However, the intensity of the immunostaining and the number of stained cells for HSP 27 were greater in glaucomatous eyes than in normal eyes. The increased immunostaining of HSP 27 in glaucomatous eyes was prominent in the nerve fiber layer as well as in retinal ganglion cells (Figure 2).

Immunostaining of HSP 60 and HSP 27 with the use of adjacent retina sections from an eye with normal-pressure glaucoma is shown in Figure 3. Immunostaining of HSP 60 was prominent in the retinal photoreceptors but not around the retinal vasculature, whereas immunostaining of HSP 27 was prominent around the blood vessels but not in the retinal photoreceptors. Double immunofluorescence labeling of HSP 60 and HSP 27 in a glaucomatous eye as well as negative controls are shown in Figure 4. Similar to the immunoperoxidase staining, no labeling for HSP 60 was associated with retinal vasculature, but there was prominent immunostaining for HSP 27 around the retinal blood vessels. Most of the retinal ganglion cells were heavily stained for both HSP 60 and HSP 27. However, some of the retinal ganglion cells exhibited marked staining for only one of the HSPs.

The immunostaining of HSPs in retinal ganglion cells exhibited regional differences that seemed to be related to their localization with respect to vascular structures. For example, immunostaining of HSP 27 in retinal ganglion cells close to blood vessels (Figure 2, D) exhibited a lower intensity than that in the cells far from blood vessels (Figure 2, B, E, and F). In addition to the regional differences, there was an apparent difference between the immunostaining of individual retinal ganglion cells (Figure 1, D, and Figure 4, A, B, D, and E).

Immunostaining of HSP 60 in the optic nerve head was similarly faint in normal and glaucomatous eyes (Figure 5). However, immunostaining of HSP 27 increased markedly in the optic nerve head of glaucomatous eyes with either primary open-angle glaucoma or normal-pressure glaucoma compared with age-matched controls. The increased HSP 27 immunostaining was mostly located in the laminar region and associated with astroglial cells as assessed by morphological examination (Figure 6).

Immunohistochemical analysis in postmortem eyes demonstrated that retinal immunostaining of HSP 60 and HSP 27 was greater in glaucomatous eyes than in eyes from age-matched normal donors. In addition, optic nerve heads of glaucomatous eyes exhibited increased immunostaining of HSP 27, but not HSP 60, which was associated mostly with astroglial cells.

We did not observe marked differences in the HSP 60 and HSP 27 immunostaining between eyes with normal-pressure glaucoma and those with primary open-angle glaucoma. This may suggest that increased expression of these HSPs may be, at least in part, independent of the level of intraocular pressure, and therefore related to tissue stress or damage in all eyes with glaucoma. Induction of HSPs in the central nervous system in response to several environmental stresses, including ischemia, has been suggested to be an early response against stress and restoration of damaged areas in the brain after injury. Increased expression of HSPs in astrocytes within the affected area has been similarly implicated so as to increase neuronal survival. The increased immunostaining of HSP 60 and HSP 27 in the retina and optic nerve head of the glaucomatous eyes therefore may suggest that these proteins play a role as a defense mechanism of stressed or injured neurons in glaucoma. In addition, there were regional differences in the immunostaining of retinal HSPs as well as differ-

### Clinical Data of Postmortem Glaucomatous Eyes*

<table>
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<th>Patient No./ Sex/Age, y</th>
<th>Diagnosis</th>
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*C/D indicates vertical cup-to-disc ratio; VF, visual field; POAG, primary open-angle glaucoma, and NPG, normal-pressure glaucoma.
ences between HSP immunostaining of individual retinal ganglion cells. This may correspond to local and individual differences in the susceptibility of neuronal cells to damage in various regions of the retina. Such a preferential response to stress appears consistent with our limited understanding of the patterns of glaucomatous defects. However, the precise assessment of the relationship between HSP immunostaining and corresponding functional or anatomical damage in glaucomatous eyes needs further study.

Figure 1. Immunoperoxidase staining of heat shock protein (HSP) 60 in human retina. A and B, Retina sections from normal donor eyes. C and D, Retina sections from eyes with primary open-angle glaucoma. E and F, Retina sections from eyes with normal-pressure glaucoma. C and E are from eyes with moderate glaucomatous damage; D and F, from eyes with advanced glaucomatous damage. There was faint immunostaining of HSP 60 in some retinal cells including retinal ganglion cells (arrows) in normal retina. However, glaucomatous eyes with either primary open-angle glaucoma or normal-pressure glaucoma exhibited increased immunostaining. In addition to the immunostaining of retinal ganglion cells and photoreceptors in the eyes from patients with normal-pressure glaucoma, some cells located in the inner nuclear layer (black arrowheads) exhibited immunostaining. Note in D that one retinal ganglion cell was immunostained (black arrow) but another adjacent cell was not (white arrowhead) (v indicates vessel; nlf, nerve fiber layer; and p, photoreceptors) (chromagen, 3,3'-diaminobenzidine tetrahydrochloride; nuclear counterstain with hematoxylin; magnification bars represent 150 µm).
The ability of neuronal and glial cells to increase HSP expression in response to stress is key to their survival. After transection, up-regulated HSP 27 in dorsal root ganglion cells of the sciatic nerve is transported to injured axons and may promote survival and contribute to alterations in the cytoskeleton associated with axonal growth.17 Similar to peripheral nerves,16,17 up-regulation of HSPs occurs after optic nerve crush.27 Additionally, it has been shown

Figure 2. Immunoperoxidase staining of heat shock protein (HSP) 27 in human retina. A and B, Retina sections from normal donor eyes. C and D, Retina sections from eyes with primary open-angle glaucoma. E and F, Retina sections from eyes with normal-pressure glaucoma. C and E are from eyes with moderate glaucomatous damage; D and F, from eyes with advanced glaucomatous damage. Note in A that, despite clearly positive immunostaining of HSP 27 in retinal vessels, there was faint immunostaining in retinal tissue including a few retinal ganglion cells (arrows) in normal retina. The arrow in B points to a stained retinal ganglion cell in normal retina. In comparison with normal eyes, glaucomatous eyes with either primary open-angle glaucoma (C and D) or normal-pressure glaucoma (E and F) exhibited increased immunostaining, which was most prominent in the retinal nerve fiber layer. Immunostaining of retinal ganglion cells for HSP 27 exhibited differences between different cells. Notice in D that a retinal ganglion cell close to a vessel was not as heavily immunostained as other retinal ganglion cells shown in B, E, and F (v indicates vessel; nfl, nerve fiber layer; and p, photoreceptors) (chromagen, 3,3'-diaminobenzidine tetrahydrochloride; nuclear counterstain with hematoxylin; magnification bars represent 150 µm).

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Figure 3. Immunoperoxidase staining of heat shock protein (HSP) 60 and HSP 27 in adjacent retina sections through the same blood vessel from an eye with advanced normal-pressure glaucoma. A, Retina section exhibiting immunostaining of HSP 60. B, Retina section exhibiting immunostaining of HSP 27. Immunostaining of HSP 60 was positive in the retinal photoreceptors but negative around the retinal blood vessel. Immunostaining of HSP 27 was positive around the same blood vessel as shown in A but negative in the retinal photoreceptors. There was significant immunostaining for HSP 60 and HSP 27 in the retinal ganglion cells (arrows) (v indicates vessel; vit, vitreous; and p, photoreceptors) (chromagen, 3,3′-diaminobenzidine tetrahydrochloride; nuclear counterstain with hematoxylin; magnification bar represents 150 µm).

Figure 4. Double immunofluorescence labeling for heat shock protein (HSP) 60 (red) and HSP 27 (green) in retina sections from an eye with primary open-angle glaucoma and moderate glaucomatous damage. A and D, Immunostaining of HSP 60. B and E, Immunostaining of HSP 27. C and F, Colocalization of HSP 60 and HSP 27. G and H, Negative controls of the HSP 60 and HSP 27 immunostaining, respectively. Immunostaining of HSP 60 was negative, but immunostaining of HSP 27 was positive around the blood vessels. Glial cells lining the internal limiting membrane were also positive for HSP 27. Most of the retinal ganglion cells were stained for both HSP 60 and HSP 27 (arrows). However, notice a retinal ganglion cell in B and C (arrowheads) that was positive for HSP 60 but negative for HSP 27. Also notice a retinal ganglion cell in D and F (arrowheads) that was positive for HSP 27 but negative for HSP 60 (v indicates vessel; vit, vitreous; and nfl, nerve fiber layer) (magnification bar represents 150 µm).
Figure 5. Immunoperoxidase staining of heat shock protein (HSP) 60 in human optic nerve head. A, Optic nerve head section from a normal donor eye. B, Optic nerve head section from an eye with primary open-angle glaucoma and advanced glaucomatous damage. C, Optic nerve head section from an eye with normal-pressure glaucoma and advanced glaucomatous damage. There was no prominent immunostaining of HSP 60 in the optic nerve head of normal donor eyes. Glaucomatous eyes did not exhibit a prominent difference in the immunostaining of HSP 60 compared with normal eyes (v indicates vessel; vit, vitreous; and lc, lamina cribrosa) (chromagen, 3,3′-diaminobenzidine tetrahydrochloride; nuclear counterstain with hematoxylin; magnification bar represents 1 mm).

Figure 6. Immunoperoxidase staining of heat shock protein (HSP) 27 in human optic nerve head. A, Optic nerve head section from a normal donor eye. B, Optic nerve head section from an eye with primary open-angle glaucoma and advanced glaucomatous damage. C, Optic nerve head section from an eye with normal-pressure glaucoma and advanced glaucomatous damage. There was immunostaining of HSP 27 around the vessels in the optic nerve head of normal donor eyes. Glaucomatous eyes exhibited a prominent difference in the immunostaining of HSP 27 compared with normal eyes. The increased immunostaining was mostly located in the laminar area (v indicates vessel; vit, vitreous; lc, lamina cribrosa; Bm, Bruch membrane; and cs, cavernous spaces of Schnabel degeneration) (chromagen, 3,3′-diaminobenzidine tetrahydrochloride; nuclear counterstain with hematoxylin; magnification bar represents 1 mm).
in rabbits that HSPs can be transported in axons between retinal ganglion cells and optic nerve. In addition to increased immunostaining of HSP 27 in the optic nerve head and cell body of retinal ganglion cells in glaucomatous eyes, increased immunostaining in the retinal nerve fiber layer may indicate the feasibility of HSP transport between optic nerve head and retinal ganglion cells to increase survival of the injured axons in glaucomatous eyes.

Heat shock proteins are highly antigenic, and immune responses to HSPs are implicated in the development of a number of human autoimmune diseases. Although increased expression of HSPs in glaucomatous eyes may serve initially to protect cells from further destruction and facilitate repair, they subsequently may recruit immune responses that contribute to the progression of disease.33,34 Glial cells of the retina and optic nerve head are antigen-presenting cells.35-36 Since these glial cells become activated in glaucomatous eyes, enhanced expression of HSPs in these eyes may be an immunostimulatory signal, which leads to a break in immune tolerance that occurs under normal conditions.37 Increased titers of autoantibodies to HSP 60 and/or HSP 27 in many patients with glaucoma38,39 therefore may represent a generalized response to tissue stress and/or damage, which subsequently may contribute to disease progression by diminishing the protective abilities of native HSPs.

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REFERENCES


