Upregulation of Antibody Response to Heat Shock Proteins and Tissue Antigens in an Ocular Ischemia Model

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PURPOSE. The aim of this study was to characterize the serum antibody reactivities occurring after ocular ischemia reperfusion. The time course of serum antibody responses was examined.

METHODS. Wistar rats were exposed to transient ocular ischemia by elevating intraocular pressure to 130 mm Hg for 60 minutes. Axonal damage was evaluated on optic-nerve sections 2 and 4 weeks later. Blood samples collected before and several times after ischemia were used for antibody detection via customized protein microarrays. Different tissue antigens, including heat shock proteins (HSPs) and crystallins, were selected based on previous identification of antibody reactivities in studies on ischemic events or ophthalmic diseases associated with ischemia. Antibody reactivity was compared using multivariate statistical techniques.

RESULTS. Significant axonal damage was observed 2 and 4 weeks after ocular ischemia (P < 0.05). Animals showed certain immunoreactivities against antigens even before ischemia, whereas many reactivities increased afterward. Significantly different responses were detected 2, 3, and 4 weeks after ischemia (P < 0.05). Antibody reactivity against actin, glial fibrillary acidic protein, HSP 27, vimentin, or spectrin continually increased.

CONCLUSIONS. Ischemia induced by acute intraocular pressure elevation led to complex changes in antibody reactivities in sera of treated animals. Upregulation of serum autoantibodies, especially against heat shock and structural proteins, progressively increased throughout the 4-week follow-up period, whereas others such as ubiquitin decreased. The upregulation of anti–HSP 27 antibodies might be an attempt to protect the tissue from ischemic damage. (Invest Ophthalmol Vis Sci. 2011;52:3468–3474) DOI:10.1167/iovs.10-5763

Retinal ischemia is involved in the pathogenesis of ophthalmic diseases, such as age-related macular degeneration,1 acute glaucoma,2 or diabetic retinopathy,3 that can lead to vision loss and are the major causes of blindness in industrialized countries such as Germany.4 An involvement of the immune system in the consequences of ischemic events is presently discussed. Increased levels of certain antibodies can be considered a risk factor (e.g., for cerebral5 or heart ischemia6). More precisely, antibodies against glial fibrillary acidic protein (GFAP) have been detected in the sera of patients with ischemic heart disease.7 Antibodies against heat shock proteins (HSPs) seem to be involved in myocardial ischemia or ischemic stroke.8,9 They are considered a risk factor for stroke, but they could also be metabolic consequences of ischemia and its mechanical effects.10 Anti-HSP antibodies could play an important role in postischemic events and possibly also facilitate further pathologic processes. Circulating anti-HSP antibodies may be induced by different mechanisms.11 These increased levels of specific antibodies against HSPs could react with surface-expressed HSP components12 and subsequently cause injury. Results of animal studies suggest a possible role of HSPs in the development of diseases related to retinal ischemia such as glaucoma. Wax et al.13 found that immunization with HSPs can elicit the loss of retinal ganglion cells (RGCs) and their axons in rats in vivo.

Since a dysregulation of the immune system might also be an influencing factor in some diseases involving retinal ischemia, such as glaucoma14,15 or age-related macular degeneration,16 it would be interesting and useful to study the postischemia antibody response in the animal model. What are the consequences of the immune system to ischemic events of the retina? They may be corrective processes or perhaps they can facilitate further pathology, as proposed for anti-HSP antibodies. Alterations in the immune response against HSPs, myelin basic protein (MBP), spectrin, actin, vimentin, GFAP, and crystallins were detectable in glaucoma patients.17–22 Circulating antibodies were also detectable in patients with age-related macular degeneration,23–25 including those against GFAP and crystallins,26 or patients with diabetic retinopathy.27,28 In monkeys with macular degeneration multiple autoantibodies against retinal proteins developed,29 hinting toward an autoimmune mechanism in this model.

A short-term intraocular pressure elevation above ocular perfusion pressure is an established method to induce retinal ischemia in animals.30 Several studies describe RGC loss and optic-nerve damage in this model.30–34 Most studies used approximately 60 minutes of ischemia to achieve adequate RGC and axon damage to study successive pathogenetic mechanisms. Increased protein expressions of sortilin and p75 neurotrophin receptor, a member of the tumor necrosis factor receptor superfamily, were detected in rat retinas postischemia through elevated intraocular pressure35; they were coexpressed in Müller cells.36 Sortilin, a neurotrophin receptor, and the p75 neurotrophin receptor interact with proneurotrophins to form a complex capable of activating apoptotic signaling.37

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Yoneda et al.38 observed an increased expression of immunoreactive interleukin-1b approximately 12 hours after reperfusion in this animal model. The authors assume that there may be an invasion of immune cells into the retinal tissue, contributing to the ischemia-induced retinal damage in these animals.

We planned to examine the possible systemic autoantibody response against specific antigens in animals with retinal ischemia using customized microarrays. Western blot analyses or ELISAs are commonly used to study immune patterns in animal models,39–41 but have their limitations since only small sample volumes can be obtained from animals. Reproducibility of results or subsequent protein identifications are challenging tasks in Western blot analyses. Recent advantages in proteomics enable us to use antigen microarrays for antibody screening.42 Antigen arrays loaded with purified proteins have been used lately for antibody detection in different autoimmune or cancer animal models.43,44

The present study examined the IgG antibody reactivities in an animal model of ocular ischemia. Antibody reactivities against several purified proteins were analyzed up to 4 weeks after induced ischemia via customized protein microarrays. An upregulation of HSPs, especially HSP 27, an apoptosis inhibitor, was detectable. This upregulation might be an attempt to protect the tissue from ischemic damage.

**METHODS**

**Retinal Ischemia**

All animal experiments were conducted in conformity with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Further, the study was approved by the local animal care committee.

Male Wistar rats (n = 12; Charles River, Portage, MI) were intraperitoneally anesthetized with chloral hydrate (400–500 mg/kg) and the right pupil of each animal was dilated with topical atropine and phenylephrine. A stable body temperature was maintained through use of a heating pad. The anterior chamber of the right eye was cannulated with a 27-gauge needle attached to a 1 L bottle of balanced salt solution (sterile intraocular irrigating solution; Alcon Laboratories, Port Worth, TX). An intraocular pressure of 130 mm Hg was maintained for 60 minutes. Pressure-induced ischemia was confirmed by examining eyes for blanching and lack of arterial pulsation through an ophthalmoscope. After the needle was removed, the ocular fundus was observed to confirm reperfusion. Antibiotic eye ointment (Tobrex [tobramycin]; Alcon Labs) was applied to prevent potential infections. Ocular and body observations were conducted for 2 to 3 days to exclude behavioral and ocular changes. Six animals were killed after 2 weeks and six animals after 4 weeks.

**Blood Collection**

Blood was drawn before and 1, 2, 3, and 4 weeks after ocular ischemia, as described previously.11 At the first three points in time, samples were collected from all 12 animals, and after 3 and 4 weeks from the 6 remaining animals. All serum samples were subsequently used for antibody analysis.

**Histologic Examination**

Morphologic evaluation of the optic nerve can provide valuable information about possible axonal damage.45,46 Because of its exceptional correlation with the RGC density (Barnes G, et al. JOVS 2005;46:ARVO EAbstract 1315), a damage score was used to evaluate possible alterations of RGC axon density resulting from ischemia in this study.

Eyes of all animals were enucleated. Optic nerves were dissected 2 mm from the bulbus and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight. They were rinsed with 0.1 M sodium cacodylate buffer. After the remaining tissues were removed from the nerves, they were dehydrated in an ascending series of ethanol from 50% to 100%. Nerves were embedded in epoxy medium and stained with uranyl acetate. For staining of degenerated axons, 1-μm-thick cross-sections were cut from each nerve and stained in accordance with an adaptation of the protocol by Hollander et al.47 Optic-nerve cross-sections were stained with 1% p-phenylene diamine (Sigma-Aldrich, St. Louis, MO).

Stained optic nerve cross-sections were photographed with a ×200 magnification using a light microscope (Leica, Allendale, NJ) attached with a digital camera. Three experienced investigators, masked to the protocol, graded the damage of optic nerves independently, by using an established optic-nerve damage score, ranging from 1 to 5.48 Nerves from ischemic eyes and corresponding control eyes were graded. All healthy appearing axons with no gliosis or axonal swelling were graded with 1. Optic-nerve sections graded with 2 had approximately 5% to 10% dark-stained axons and initial stages of gliosis were visible with some axonal swelling, whereas nerves at stage 3 had a greater number of dark-stained axons with the central part of the nerve involved and initial to mild gliosis. At grade 4, dark axon staining involved all parts of the nerve and 50% to 90% of the axons were already dead. Axonal damage was scored up to grade 5, where only fewer than 10% of axons were still alive and the gliosis, darkly stained axons, and axonal swelling made up most of the optic-nerve cross-section (apparent degeneration of all axons). Mean scores of optic nerves 2 and 4 weeks after ischemia were compared with corresponding control nerves using Student’s t-test.

**Protein Microarrays**

Approximately 20 commercially available purified proteins (Sigma-Aldrich or BioMol, Hamburg, Germany) were spotted onto nitrocellulose slides (Grace Bio-Laboratories, Bend, OR) using a noncontact spotter (Scienion, Berlin, Germany). All proteins were spotted in triplicates in a 1 μg/μL dilution. The selected antigens were chosen after researching the literature on proteins associated with ischemia. HSPs and GFAP were included based on findings in other ischemic diseases7–9 to analyze a possible involvement of autoantibodies against these antigens in our model of retinal ischemia. We included additional antigens based on previous identifications of specific antibody reactivities detected in glaucoma or age-related macular degeneration.18–20,26,27,49,50

Blocking of slides with 4% bovine serum albumin was followed by incubation with rat serum (dilution, 1:250). All slides were incubated with a fluorescent goat anti-rat IgG-labeled secondary antibody (Cy5; dilution, 1:500; Abcam, Cambridge, MA). Antibody–antigen reactivities on incubated slides were detected with an array scanner (Affymetrix, Santa Clara, CA). A software suite (TM4, Spotfinder, V3.1.1; Dana-Farber Cancer Institute, Boston, MA) was used for spot analysis, to convert scanned slide images into numerical data. Mean spot intensity per pixel was calculated followed by z-score calculation for each single spot by deducting the mean intensity of all spots from the spot intensity and then dividing through the overall SD of all spots from this study. The z-score is a standard score that allows the comparison of two scores from different normal distributions. The data are normalized globally, which means the score is based on a normalization to the total intensity.51 Z-score transformation statistics have been used in studies comparing gene expression differences via microarray.52

Z-scores were then used for statistical analysis through multivariate statistical techniques (Statistica software, V8.0; Statsoft, Tulsa, OK). For intergroup comparison one-way ANOVA followed by Tukey’s post hoc test were applied. The null hypothesis was rejected at P < 0.05. Mahalanobis distances were calculated between the antibody patterns at the time points after ischemia and the one before. The Mahalanobis distance is the distance of a case from the centroid in multidimensional space, defined by the correlated independent variables,53 providing a measure of how different the antibody patterns are from two time points. A greater distance stands for a larger pattern difference.
RESULTS

Optic-Nerve Cross-Section Scoring

Eyes that were exposed to 60 minutes of ocular ischemia presented axonal loss after 2 and 4 weeks. Damaged, dark axons were observed on optic-nerve cross-sections of ischemic eyes that were exposed to 60 minutes of ocular ischemia (Fig. 1B). Exemplary optic nerves from the control (C) and the ischemic eye (D, E) of an animal at 4 weeks. Dark-stained axons and some gliosis can be observed on cross-sections of the ischemic nerves (B, D, and E). (F) Mean scores of optic-nerve damage depicting nerves without ischemia (black columns) compared with nerves after an ischemic event (gray columns) with SD (n = 6 per group). Nerve damage scores at both points in time were significantly different when compared by t-test (P < 0.05).

Antibody Reactivities

Antibody reactivities against some of the spotted antigens detectable in rat sera, collected before ischemia as well as 1, 2, 3, and 4 weeks after immunization, are displayed in Figure 2. One week after the ischemic event no significant difference in overall antibody reactivity compared with the baseline patterns was detectable (P = 0.06; Mahalanobis distance: 1.9). Significant differences in overall antibody reactivity, against all antigens, was detectable 2 (P = 0.00006, distance: 5.2), 3 (P = 0.005, distance: 5.8), and 4 weeks (P = 0.009, distance: 4.4) after the induction of ocular ischemia.

Antibody reactivities against vimentin and spectrin continually increased after the ischemic event, whereas reactivities against actin, GFAP, and HSP 27 decreased at 4 weeks (but never below baseline levels) after an initial increase. Antibody reactivity against ubiquitin, on the other hand, decreased immediately after retinal ischemia, followed by a certain decrease in reactivity later on (but never below baseline levels). Antibody reactivities against spectrin and vimentin, on the other hand, continually increased throughout the 4-week follow-up period.

Clustering of serum samples, seen in Figure 4, was based on the eight most different antigens. We did not see a complete separation of points in time, but an overall trend of separating baseline and 1-week samples from samples collected 2 to 4 weeks after the ischemic event.

DISCUSSION

In this study we examined the antibody response after retinal ischemia. To study the possible autoantibody production can be interesting and pertinent to disease associations for several reasons. It can signify epiphenomenon, biochemical associations and clues to etiology, and possibly suggest a direct causative basis for the pathophysiology for certain diseases. Some autoantibodies cause cell damage through binding to the cell surface receptor or enzymatic complex followed by the induction or blockage of cell surface molecules. We propose that certain antigens with upregulated antibody response are linked to normally hidden self-antigens that induce antibody responses after ischemic damage to the retina.

Tissue injury through ischemia reperfusion can occur in different organs including the eye. Ischemia describes a pathologic situation involving an inadequate tissue blood flow. During an ischemic event the tissue cannot be adequately supplied with nutrients and oxygen. Some studies propose that serum antibodies are involved in ischemia/reperfusion injury. In a study by Fleming et al., autoimmunity-prone mice have enhanced and accelerated intestinal tissue damage after mesenteric ischemia/reperfusion damage. The authors hypothesize that autoantibodies present in the sera of mice with systemic...
autoimmunity can initiate organ injury induced through ischemia reperfusion. In these experiments autoantibodies, such as anti-dsDNA and antihistone antibodies, can instigate ischemia/reperfusion injury in animals. Anti-HSP 60 antibodies are known to accelerate arteriosclerosis in mice by recognizing surface-expressed HSPs, thereby causing endothelial damage.57

Retinal ischemia is a widely used model to achieve RGC loss or axonal damage and to consequently test potential therapeutic approaches.58–60 Immunologic effects of retinal ischemia have not yet been studied in detail. Our results indicate that retinal ischemia leads to a complex systemic antibody response. For several antibodies we saw a continuous increase during the first weeks after the ischemic event (Table 1), whereas the animals showed detectable optic-nerve damages (Fig. 1). In our study the most different antibody reactivities were upregulations, compared with baseline (Fig. 3), including reactivities against GFAP, actin, HSP 27, and vimentin. Based on these antigens we were able to distinguish samples collected before and 1 week after ischemia from samples taken 2 weeks after the ischemic event or later (Fig. 5). The most

| Table 1. Spotted Antigens Are Listed and Categorized in Protein Classes |
|---------------|---------------|---------------|---------------|---------------|
| Class         | Antigen       | 1 Week        | 2 Weeks       | 3 Weeks       | 4 Weeks       |
| Heat shock proteins | HSP 27        | ▲             | ▲             | ▲             | ▼             |
|                | HSP 60        | ▲             | ▲             | ▲             | ▼             |
|                | HSP 70        | ▲             | ▲             | ▲             | ▼             |
| Tissue antigens | MBP           | ▲             | ▲             | ▲             | ▼             |
|                | MOG           | ▲             | ▲             | ▲             | ▼             |
| Structural antigens | Spectrin     | ▲             | ▲             | ▲             | ▼             |
|                | Actin         | ▲             | ▲             | ▲             | ▼             |
|                | Vimentin      | ▲             | ▲             | ▲             | ▼             |
|                | GFAP          | ▲             | ▲             | ▲             | ▼             |
| Crystallins    | α-Crystallin  | ▲             | ▲             | ▲             | ▼             |
|                | α-A-Crystallin| ▲             | ▲             | ▲             | ▼             |
|                | α-B-Crystallin| ▲             | ▲             | ▲             | ▼             |
|                | β-Crystallin  | ▲             | ▲             | ▲             | ▼             |
|                | γ-Crystallin  | ▲             | ▲             | ▲             | ▼             |
| Cellular metabolism | GST         | ▲             | ▲             | ▲             | ▼             |
|                | Ubiquitin     | ▲             | ▲             | ▲             | ▼             |
|                | Annexin V     | ▲             | ▲             | ▲             | ▼             |
|                | Transferrin   | ▲             | ▲             | ▲             | ▼             |
| Plasma         | Albumin       | ▲             | ▲             | ▲             | ▼             |

Upregulations and subsequent decreases of antibody reactivities throughout the course of the study are displayed. Only antiubiquitin antibody levels decreased below baseline. ▲, upregulation; ▼, decrease (compared with previous antibody levels). GST, glutathione S-transferase; MOG, myelin oligodendrocyte glycoprotein.

FIGURE 3. Differences in IgG antibody reactivity (with SD) of serum samples collected one (1), two (2), three (3), and four weeks (4) after retinal ischemia compared with baseline. The eight antigens displayed in this graph revealed the most alterations.

FIGURE 4. Reactivity matrices using eight antigens, previously identified as the most different ones, separating ischemic and healthy animals. The rows depict the antigens (GFAP, HSP 27, γ-crystallin, actin, vimentin, albumin, spectrin, and β-L-crystallin) and the columns depict the rat sera. The box colors represent the groups in different points of time. Colors correspond to antibody reactivity, ranging from blue (= low antibody reactivity), to green (= medium reactivity), to yellow (= strong antibody reactivity).
different IgG antibody reactivities (compared with baseline) were observed 3 weeks after ischemia induction.

GFAP, an intermediate filament protein, is associated with retinal glial cells. Müller cells are the principal glial cells of the neural retina and support regular neuronal functions.54 GFAP expression in Müller cells increases through retinal detachment.62 Immunohistochemical studies demonstrated that the expression of GFAP is induced in Müller cells after a retinal ischemic event.63,65 Zhang et al.60 could detect an upregulation of GFAP protein and mRNA in retinas postischemia.62 Müller cells in postischemic retinas of mice expressed GFAP.64 A systemic anti-GFAP antibody increase, which was evident in our study, was also reported in other ischemic diseases. A study of patients with ischemic heart disease detected increased antibody levels against GFAP in patients' sera.7

HSP 27 has been reported to interact with cytoskeletal proteins. It is hypothesized that the accumulation of these proteins after ischemia may have an important role in the stabilization of cytoskeletal structures and/or their recovery from disruption. Ischemia reperfusion leads to a disruption of actin microfilaments65,66 which are found in the cytoplasm of cells. Ischemia can lead to redistribution of actin throughout the cytoplasm,67 potentially causing increased antiactin antibodies. An increased level of antiactin antibodies was detected in this study (Fig. 5). An increase of systemic antibody reactivity against HSPs, especially against HSP 27, was also apparent in our study (Fig. 5). Ischemic retinas also showed an increase in HSP 27–positive RGCs68 and expression of HSP 27 is known to be increased after retinal ischemia,69 occurring in both neuronal and nonneuronal retinal cells and even after cerebral ischemia.70 These studies indicate that a similar ischemic induction of stress-related proteins might occur in retinas. Yokoyama et al.71 demonstrated that resistance of RGCs to ischemia/reperfusion injury is enhanced by electrical application of HSP 27 protein solution into the vitreous immediately after the ischemic event. The authors explicate that, through this technique, HSP 27 proteins are able to enter and protect RGCs, given that they are considered to have a neuroprotective function.72 Both HSP 27 and HSP 70 are inhibitors of apoptotic cell death.73 HSP 27 can maintain both the redox homeostasis and mitochondrial stability of cells. It is capable of binding to both proteins, cytochrome c, after its release from mitochondria, and procaspase-3, preventing the formation of apoptosisome complexes.74,75 HSP 27 is also capable of blocking Daxx (death-associated protein 6)-mediated apoptosis. Daxx directly binds to the death domain of Fas, a widely expressed cell death receptor.76 Possibly the increase of antibodies against HSPs we detected in our animals could be an indicator for the loss of necessary tissue protection through ischemia/reperfusion damage. Better understanding of the role of the immune system in postretinal ischemia events may in the future create novel and innovative therapeutic approaches.

In conclusion, we revealed alterations in immune reactivities, especially upregulations of antibodies against heat shock and structural proteins, as a result of ocular ischemia reperfusion. Elevations of anti-HSP antibodies might be a reaction of the tissue to ischemic stress.

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References


