Effect of Ischemia Duration on Autoantibody Response in Rats Undergoing Retinal Ischemia-Reperfusion

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Key Words

Ischemia-reperfusion • Retinal ganglion cells • Antibody microarray • Cyclophilin • $\alpha_5\beta_1$ -integrin • Glyceraldehyde-3-phosphate dehydrogenase

Abstract

Both the innate and the adaptive immune systems are involved in the pathogenic processes following ischemia-reperfusion injury. We analyzed the possible correlation between the duration of ischemia and autoantibody diversification in a model of ocular ischemia. Rats were subjected to 30, 45, or 90 min of ischemia, and retinal ganglion cell (RGC) density and antibody reactivity were analyzed via customized protein microarray slides. After ocular ischemia, significant alterations in antibody response were observed, while increasing exposure caused more severe RGC damage. Distinct antibody responses after ischemia were detected; these alterations comprised decreased reactivities against cyclophilin A and glyceraldehyde-3-phosphate dehydrogenase, possibly due to increased binding of circulating antibodies to debris material. Other antibodies, like those against $\alpha_5\beta_1$ -integrin or β_2 -adrenergic receptor, were upregulated after ischemia.

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Introduction

Transient retinal ischemia is a common model to induce retinal damage in rodents. In the majority of studies, it is achieved through a short-term intraocular pressure increase above the systolic level [1, 2]. In this model, degeneration of retinal ganglion cells (RGCs) and thinning of the inner retinal layers has been analyzed histologically [1, 2], and this damage has also been verified functionally, e.g. through electroretinography [3, 4].

Lafuente et al. [5] examined RGC survival rates after different durations of retinal ischemia. In this experiment, rats underwent 30, 45, 60, 90, or 120 min of retinal ischemia due to ligation of ophthalmic vessels, a method that leads to ischemia without increasing the intraocular pressure. Labeling of RGCs demonstrated significantly lower RGC densities in ischemic eyes compared to controls, at the earliest 5 days after the event. In a recent study, Jehle et al. [6] could confirm that 30, 45, 60, and 90 min of retinal ischemia lead to a significant decrease in retrogradely labeled RGCs. They used a technique to raise the intraocular pressure over the systolic blood pressure which results in retinal ischemia.

Table 1. All antigens used for the protein microarrays

Group	Antigen	Source
HSPs	α -A-crystallin	Sigma-Aldrich
	α -B-crystallin	Sigma-Aldrich
	β -S-crystallin	Sigma-Aldrich
	β -L-crystallin	Sigma-Aldrich
	γ -crystallin	Sigma-Aldrich
	HSP 27	Stressgen
	HSP 60	Sigma-Aldrich
	HSP 70	Sigma-Aldrich
CNS antigens	chaperonin 10 (GroES)	US Biological
	myelin basic protein (MBP)	Fluka and Sigma-Aldrich
	S 100 A4	Sigma-Aldrich
	neurotrophin-3 (NT-3)	R&D Systems
	neurotrophin-4 (NT-4)	R&D Systems
	amyloid precursor protein (APP)	R&D Systems
	neuron-specific enolase (NSE)	Sigma-Aldrich
	transthyretin (TTR)	Sigma-Aldrich
Structural antigens	contactin-1	R&D Systems
	spectrin	Sigma-Aldrich
	actin	Sigma-Aldrich
	vimentin	Progen
	glial fibrillary acidic protein (GFAP)	US Biological
Hormones	α -synuclein	Sigma-Aldrich
	γ -synuclein	Sigma-Aldrich
Cellular metabolism	vascular endothelial growth factor (VEGF)	Sigma-Aldrich
	insulin	Sigma-Aldrich
Plasma proteins	caspase 3	R&D Systems
	annexin V	Sigma-Aldrich
	ubiquitin	Sigma-Aldrich
	α_2 -macroglobulin (h α_2 M)	R&D Systems
Enzymes	albumin	Sigma-Aldrich
	fibrin	Sigma-Aldrich
	fibronectin	Sigma-Aldrich
Lipids	glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Sigma-Aldrich
	lysozyme	Sigma-Aldrich
	superoxide dismutase (SOD)	Sigma-Aldrich
Other proteins	cardiolipin	Sigma-Aldrich
	phosphatidylserine	Fluka
Enzymes	glutathione-S-transferase (GST)	Sigma-Aldrich
	clathrin	Sigma-Aldrich
	Jo-1	Sigma-Aldrich
	transferrin	Sigma-Aldrich
	cylophilin A	Sigma-Aldrich
	proteinase K	Sigma-Aldrich
	protein kinase C (PKC) inhibitor	Sigma-Aldrich
	$\alpha_5\beta_1$ -integrin	R&D Systems
	α -1-antitrypsin	Sigma-Aldrich
	β_2 -adrenergic receptor (ADRB2)	Sigma-Aldrich

Studies on the retina ischemia model have rarely focused on the systemic immune response, although recent findings suggest a complex role of the immune system in accelerating repair and tissue remodeling after ischemia-reperfusion in certain organs [7–9]. Several studies link autoantibodies to ischemic diseases [10–12], suggesting that they play a role in events following retinal ischemia and possibly also facilitate further pathologic processes. Yuan et al. [13] observed a correlation of enhanced levels between anti-heat shock protein (HSP) 70 and anti-HSP 60 antibodies and chronic myocardial ischemia. However, the presence of autoantibodies in ocular ischemia needs to be studied in detail.

In a previous study, we analyzed autoantibody alterations up to 4 weeks after 60 min of retinal ischemia [14]. The aim of the present study was to compare the serum antibody response after various periods of retinal ischemia (particularly the severity of the damage). We selected antigens that led to differences in autoantibody response in our preceding analysis as well as additional antigens associated with ischemia [14–16]. Customized protein microarrays were used for antibody detection. This is a quite novel approach that allows the analysis of rather global antibody patterns against purified antigens [17, 18].

In order to study the effect of ischemia duration on autoantibody response, we compared the serum antibody patterns of rats that underwent 30, 45, or 90 min of ocular ischemia followed by reperfusion.

Experimental Procedures

All procedures were approved by the Animal Care Committee of the University of Freiburg. The animal studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Communities Council Directive of November 24, 1986 (86/609/EEC). Adult male Brown Norway rats (200–250 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). Rats had free access to food and water and were held in cages in temperature-controlled rooms with a controlled 12-hour light-dark cycle.

Operations were performed under general anesthesia with isoflurane/O₂. During recovery from anesthesia, animals were placed in separate cages and antibiotic ointment (Refobacin®; Merck, Darmstadt, Germany) was applied on the ocular surface. The body temperature was maintained at 37 ± 0.5°C via a heating pad and monitored with a rectal thermometer probe throughout the surgery.

Ischemia-Reperfusion Model

Rats were anesthetized as described above, and the anterior chamber was cannulated with a 30-gauge needle connected to a

reservoir containing 0.9% NaCl. The intraocular pressure in the left eye was increased to 120 mm Hg for 30 (n = 10), 45 (n = 9), or 90 min (n = 5). Retinal ischemia was confirmed by retinal edema and stasis in retinal arteries. The needle was removed at the end of the intraocular pressure elevation. Rats that were not reperfused within 5 min, those with incomplete ischemia, or those with suspicion of trauma of the crystalline lens were excluded from the experiments.

Histological Examination

RGCs were retrogradely labeled 4 days after retinal ischemia by stereotactic injection of the fluorescent tracer FluoroGold® (Fluorochrome, Denver, Colo., USA), dissolved in dimethylformamide, into the superior colliculi of anaesthetized rats using a stereotactic device (Stoelting, Germany). Rats were sacrificed 6 days after labeling by CO₂ (i.e. 10 days after ischemia induction). The eyes were removed and fixed in paraformaldehyde. The retinas were dissected, mounted on gelatin-coated glass slides, and embedded. The number of FluoroGold-positive RGCs was quantified under a fluorescence microscope (AxioImager; Carl Zeiss, Jena, Germany). They were counted in 12 distinct areas of 40,000 μm^2 each in a blinded fashion. The RGC density of each ischemia group was compared to the values of the control group using a two-tailed Student's t test (Statistica V8; Statsoft, Tulsa, Okla., USA). Null hypotheses were rejected at $p < 0.05$.

Protein Microarrays

Blood was collected from all animals through heart puncture 10 days after ischemia. Samples were also collected from an age-matched control group of healthy animals (n = 10).

Fifty different highly purified antigens (1 $\mu\text{g}/\mu\text{l}$ diluted in PBS buffer with 1.5% trehalose) were obtained from Sigma-Aldrich (Munich, Germany), BioMol (Hamburg, Germany), or R&D Systems (Wiesbaden, Germany). Antigen spotting was performed with a noncontact microarray spotter (sciFLEXARRAYER S3; Scienion, Berlin, Germany). Antigens that were related to ischemic events in the literature, like S-100, myelin basic protein (MBP), glial fibrillary acidic protein, GST, neuron-specific enolase, ubiquitin, cardiolipin, or HSPs, were selected [15, 16, 19, 20]. Some additional antigens were chosen because animals that underwent 60 min of ischemia in a previous study showed an immune response against these antigens [14].

Microarray preparation and incubation were performed in accordance with previous protocols (table 1) [14]. Briefly, all antigens were spotted in triplicate and spotted slides were blocked with 4% BSA before incubation with rat serum (dilution 1:250). They were later incubated with a goat anti-rat IgG secondary antibody (Cy-5 labeled; dilution 1:500; Abcam, Cambridge, Mass., USA). Antibody reactivity was detected with an array scanner (Affymetrix, Santa Clara, Calif., USA) before Spotfinder software TM4 (V3.1.1; Dana-Faber Cancer Institute, Boston, Mass., USA) was employed for spot analysis. The mean spot intensity was calculated to compute z-scores for each single spot.

Detection of potential antibody markers and changes in antibody reactivity was performed by multivariate statistical analysis using Statistica software. Differences among the four independent animal groups were calculated by ANOVA, and Tukey's post hoc test was assessed for p value calculation. Null hypotheses were rejected at $p < 0.05$. Mahalanobis distances were calculated to examine the difference in antibody patterns between the ischemia

Fig. 1. a RGCs labeled in a retrograde fashion from a control eye as well as from eyes exposed to 30, 45, and 90 min of retinal ischemia, showing apparent RGC loss. **b** Mean RGC density ($\pm$ SD and $\pm$ SE) in all four groups. There was a significant difference between the RGC counts of the control group and those of all ischemia groups (30 min: $p = 0.04$; 45 min: $p = 0.0002$; 90 min: $p = 0.0002$).

group and the control group. This is a measure of the divergence between several groups based on multiple characteristics (in this case the antibody responses against several antigens). For each period of ischemia, the distance to the control group was calculated.

Due to the lower number of animals in the 90-min group, this group was excluded from further biomarker calculations. Canonical roots were calculated between the control and the 30-min and 45-min groups to display the overall difference in antibody pattern between those groups. The closer the points are to each other, the more similar the antibody patterns of these animals are.

Results

RGC density was analyzed in retinal flatmounts 10 days after the ischemic event. A mean RGC density of $2,322 \pm 77$ cells/ mm^2 (mean $\pm$ SD) was counted in control eyes. Cell density decreased with ischemia duration (fig. 1). Thirty minutes of ocular ischemia led to a significantly lower mean RGC density ($2,092 \pm 216$ cells/

Fig. 2. Exemplary protein microarray sections incubated with a control serum sample (a) or samples of animals after 30 min (b), 45 min (c), and 90 min (d) of ischemia. Antigens were all spotted in triplicates. The antibody reactivities against cyclophilin (I 1), α -A-crystallin (I 4), HSP 60 (II 3), GST (II 5), GAPDH (III 1), and SOD (IV 3) are displayed.

Fig. 3. a Serum antibody reactivity matrices against the tested antigens in control sera and sera of ischemic animals (30, 45, and 90 min). Columns depict rat sera, rows show antigens, and colors correspond to antibody reactivity. A separation of ischemic and control samples was observed. **b** Differences in antibody reactivity between the control group and each ischemia group for the eight antigens against which the strongest effects were observed are displayed (mean $\pm$ SE).

mm², $p = 0.04$). A further significant RGC reduction followed from 45 min of ischemia. The mean cell density of these eyes was $1,567 \pm 252$ cells/mm² ($p = 0.0002$). The most severe ganglion cell loss was observed in retinal flat-mounts after eyes underwent 90 min of ischemia. Only 33 ± 7 cells/mm² were detectable in this group ($p = 0.0002$).

A distinct difference in staining intensity was observed for samples from animals that underwent 30, 45,

or 90 min of retinal ischemia compared to control samples ($p < 0.005$; fig. 2).

Calculation of the Mahalanobis distances revealed the greatest conformity in antibody reactivities between the control group and animals with 30 min of ischemia, with a distance of 6.7. The 45-min ischemia group displayed a greater distance (Mahalanobis distance: 7.5) and the most different patterns were detected between controls and the 90-min ischemia group (Mahalanobis distance: 7.9),

Fig. 4. **a** The six most prominent biomarkers distinguishing between the control and the 30- and 45-min ischemia groups. The x-axis shows the six biomarkers and the y-axis the intensity of the antibody reactivity. **b** p values between the 30-min or 45-min group and the control group for the six calculated biomarkers between those groups. Box plots displaying the mean antibody reactivities (mean $\pm$ SE) against cyclophilin A (**c**), $\alpha_5\beta_1$ -integrin (**d**), and β_2 -adrenergic receptor (**e**) in the control and 30- and 45-min ischemia groups.

where about 85% of RGCs died off within 2 weeks. From these date we can conclude that not only the number of RGCs but also the antibody response is affected by ischemia duration. The eight antigens with the most prominent alterations between the four groups were cyclophilin A, $\alpha_5\beta_1$ -integrin, superoxide dismutase, glutathione-S-transferase, β_2 -adrenergic receptor, glyceraldehyde-3-phosphate dehydrogenase, transthyretin, and contactin-1 (fig. 3b).

When we compared the 30-min, 45-min, and control groups, downregulations of antibodies against cyclophilin A, glyceraldehyde-3-phosphate dehydrogenase,

and α -A-crystallin were observed in the ischemia groups. However, antibodies against $\alpha_5\beta_1$ -integrin, superoxide dismutase, and β_2 -adrenergic receptor were upregulated in the 30- and 45-min ischemia groups in comparison to controls (fig. 4a, b).

Downregulation of antibodies against cyclophilin was observed in animals exposed to 30 min ($p = 0.003$) and 45 min ($p = 0.004$) of ischemia compared to healthy animals (fig. 4c). The box plot in figure 4d displays an increase in anti- $\alpha_5\beta_1$ -integrin antibodies in ischemic animals, which was significant in the 45-min group ($p = 0.0004$). Autoantibodies against β_2 -adrenergic receptor

Fig. 5. Canonical roots of the control (black) and 30-min (green) and 45-min (red) ischemia groups. The canonical roots were plotted for each animal and show the similarity of the antibody patterns of these animals. A clear separation between the three groups was observed.

were significantly upregulated in animals exposed to 45 min of ischemia compared to controls ($p = 0.009$; fig. 4e). The canonical roots show a separation between the 30-min, 45-min, and control groups (fig. 5).

Discussion

In this animal model, retinal ischemia was induced for 30, 45, or 90 min via intraocular pressure elevation. In accordance with previous studies where retinal ischemia was induced in animal models, we detected a severe increase in RGC damage with increasing duration of ischemia [5, 6]. Although models of retinal ischemia have been studied extensively, little focus has been placed on immunological changes. So far, it has been shown that several cytokines, including interleukin, are upregulated in the retina shortly after ischemia [21, 22]. In a previous study, our group detected autoantibody alterations up to 4 weeks after 60 min of retinal ischemia [14]. The results presented here confirm that retinal ischemia-reperfusion damage leads to detectable alterations in autoantibody reactivity. The objective of this study was to examine the correlation between the length of the ischemic event and autoantibody diversification. We observed significant alterations in antibody reactivity including decreased reac-

tivities against cyclophilin A and glyceraldehyde-3-phosphate dehydrogenase (fig. 4). Decreased reactivities in animals that underwent ischemia compared to controls could reflect loss of circulating IgG antibodies in these animals because they have bound to altered tissue, as already shown by Zephir et al. [23].

In addition, downregulation of antibodies against cyclophilin A was observed (fig. 4c). The cytoplasmic cyclophilin A protein is distributed in neurons throughout the mammalian brain including the visual cortex and the retina [24–26]. It has a fundamental role in cell metabolism [27]. Loss of circulating anti-cyclophilin A antibodies in animals that underwent longer ischemia periods may be a consequence of more severe ischemia-reperfusion damage which leads to increased binding of these antibodies to damaged retinal tissue.

Increased reactivities, as observed for antibodies against $\alpha_5\beta_1$ -integrin or β_2 -adrenergic receptor in our study, could be initiated by an ischemic compromise of the blood-retina barrier that certain antigens leak into the peripheral circulation with the consequence of antibody formation, as demonstrated for the central nervous system [28]. Ischemic animals had higher levels of anti- $\alpha_5\beta_1$ -integrin antibodies (fig. 4d). Integrins are heterodimers consisting of an α and a β subunit. The β_1 and the α_5 subunits were detectable in retina from human donor eyes, including vessels, various layers of the neural retina [29], and trabecular meshwork [30]. Proliferative diabetic retinopathy vessels expressed integrins [31], and increased β_1 -integrin expression in retina from diabetic patients was demonstrated [32]. This alters the interaction of vascular endothelial cells with their basement membranes in the direction of firmer cell-matrix adhesion, compromises the migration and replication critical to the re-endothelialization process, and contributes to microvascular damage and closure. The observed increased levels of antibodies against $\alpha_5\beta_1$ -integrin in our study could possibly be explained by increased systemic $\alpha_5\beta_1$ -integrin levels due to leakage through the retina-blood barrier. As a consequence, animals in our study might have developed increased antibody levels against this protein.

Autoantibodies against β_2 -adrenergic receptor were significantly upregulated in animals exposed to 45 min of ischemia (fig. 4e). β -Adrenergic receptors are present on endothelial cells of the retina [33], ciliary epithelial cells, trabecular meshwork, and the optic nerve head [34–36]. Ninety percent of the β -adrenergic receptors in the iris-ciliary body are of the β_2 subtype [35]. Autoantibodies against β_2 -adrenergic receptor have also been shown

to be present in patients with myasthenia gravis [37, 38], ocular hypertension, and primary open-angle glaucoma [39], indicating an involvement in ocular pathology, for the latter in presumed ischemic neuronal apoptosis.

Conclusions

Ischemia duration not only influences RGC density but also leads to differences in autoantibody response. Complex antibody response alterations following retinal ischemia include upregulations of the antibody response

against $\alpha_5\beta_1$ -integrin and β_2 -adrenergic receptor, as well as downregulations of antibodies against cyclophilin A and glyceraldehyde-3-phosphate dehydrogenase with increasing ischemia duration.

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