The High-Mobility Group Box-1 Nuclear Factor Mediates Retinal Injury after Ischemia Reperfusion

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PURPOSE. High-mobility group protein B1 (Hmgb1) is released from necrotic cells and induces an inflammatory response. Although Hmgb1 has been implicated in ischemia/reperfusion (IR) injury of the brain, its role in IR injury of the retina remains unclear. Here, the authors provide evidence that Hmgb1 contributes to retinal damage after IR.

METHODS. Retinal IR injury was induced by unilateral elevation of intraocular pressure and the level of Hmgb1 in vitreous humor was analyzed 24 hours after reperfusion. To test the functional significance of Hmgb1 release, ischemic or normal retinas were treated with the neutralizing anti-Hmgb1 antibody or recombinant Hmgb1 protein respectively. To elucidate in which cell type Hmgb1 exerts its effect, primary retinal ganglion cell (RGC) cultures and glia RGC cocultures were treated with Hmgb1. To clarify the downstream signaling pathways involved in Hmgb1-induced effects in the ischemic retina, receptor for advanced glycation end products (Rage)-deficient mice (RageKO) were used.

RESULTS. Hmgb1 is accumulated in the vitreous humor 24 hours after IR. Inhibition of Hmgb1 activity with neutralizing antibody significantly decreased retinal damage after IR, whereas treatment of retinas or retinal cells with Hmgb1 induced a loss of RGCs. The analysis of RageKO versus wild-type mice showed significantly reduced expression of proinflammatory genes 24 hours after reperfusion and significantly increased survival of ganglion cell layer neurons 7 days after IR injury.

CONCLUSIONS. These results suggest that an increased level of Hmgb1 and signaling via the Rage contribute to neurotoxicity after retinal IR injury. (*Invest Ophthalmol Vis Sci.* 2011;52:7187-7194) DOI:10.1167/iovs.11-7793

Retinal ischemia-reperfusion (IR) injury is a clinical entity that remains a common cause of visual impairment and blindness in the industrialized world due to relatively ineffective treatment.¹ Inflammation is a pathologic hallmark of IR injury and is spatiotemporally related to the occurrence of

delayed cell death.¹⁻¹⁰ Recently published studies and our own data using transmission electron microscopic analysis indicate that retinal ischemia results in a prolonged period of neuronal cell death with a high level of necrotic cells at an early stage of pathology.^{11,12} The danger signals (so-called damage-associated molecular patterns [DAMPs]) liberated from necrotic cells act through pattern recognition receptors, initiating an additional innate immune response.¹³⁻¹⁵ Thus, DAMPs emanating from necrotic cells may pose an additional risk to surrounding cells postreperfusion.

High-mobility group box-1 (Hmgb1) protein is the prototypic DAMP molecule and has been implicated in several inflammatory disorders.^{16,17} It is an abundant chromatin protein that acts as a cytokine when released in the extracellular milieu by necrotic cells.¹⁶⁻²⁰ Levels of Hmgb1 protein are often increased in areas of ischemic insult that mediate ischemia-associated inflammatory response and damage, whereas neutralization of the extracellular Hmgb1 released by ischemic damaged cells protects against IR injury.²¹⁻²⁸ Hmgb1 induces an inflammatory response directly through the pattern recognition receptors such as Toll-like receptor 4 (Tlr4) and receptor for advanced glycation end products (Rage).¹⁶⁻²⁰ It was shown that suppression of Hmgb1 signaling through Rage and Tlr4 significantly improves outcome in the brain, heart, liver, and the kidney after IR injury.²¹⁻³¹ Previously, we demonstrated that Tlr4 signaling plays a significant role in retinal ischemia.³² Here, we investigated the effects of Hmgb1 and Rage signaling on the level of inflammation and neuronal death in the ischemic retina.

METHODS

Animals

All experiments and postsurgical care were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Association for Research in Vision and Ophthalmology statement for use of animals in ophthalmic and vision research, and according to the University of Miami Institutional Animal Care and Use Committee approved protocols. RageKO mice were a kind gift from Dr. Joseph Bidwell (Indiana University School of Medicine, Indianapolis, IN).³³ RageKO mice were backcrossed for 14 generations on a C57BL/6 background.³⁴ Therefore, we used age-matched C57BL/6 mice as controls. All animals used in our experiments were 3-month-old male mice or 10- to 14-day-old pups.

Cell Culture

Retinal ganglion cells (RGCs) were isolated according to the two-step immunopanning method.¹¹ Briefly, the whole retinas were incubated in papain solution (16.5 U/mL) for 30 minutes. In the next step, macrophage and endothelial cells were removed from the cell suspension by panning with the antimacrophage antiserum (Accurate Chemical, Westbury, NY). RGCs were specifically bound to the panning plates containing anti-Thy1.2 antibody and released by trypsin incubation.

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Supported in part by National Eye Institute (NEI) Grant R21EY020613, American Heart Association Scientist Development Grant 0735014B (DI), and NEI Grant P30 EY014801 to the University of Miami Department of Ophthalmology.

Submitted for publication April 26, 2011; revised June 30, 2011; accepted July 28, 2011.

Disclosure: G. Dvorianchikova, None; E. Hernandez, None; J. Grant, None; A.R.C. Santos, None; H. Yang, None; D. Ivanov, None

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tion. RGCs were grown in serum-free basal media (Neurobasal/B27 media; Invitrogen, Carlsbad, CA). Mixed glial cells were prepared from the brains of neonatal (postnatal day 2) mice as previously described.²³

Oxygen and Glucose Deprivation Model

RGCs were deprived of oxygen using an anaerobic chamber (0% O₂, 5% CO₂, and 95% N₂) and glucose- and sodium pyruvate free basal media (Neurobasal media; Invitrogen) for 4 hours at 37°C. After oxygen and glucose deprivation (OGD), the culture medium was exchanged for fresh basal media (Neurobasal/B27), and the neurons were further incubated for 24 hours in a 5% CO₂ atmosphere. Parallel cultures were exposed to oxygenated media in a normoxic incubator (37°C; atmosphere 5% CO₂) to serve as sham controls.

Transient Retinal Ischemia

Retinal ischemia was induced for 60 minutes by introducing into the anterior chamber of the eye a 33-gauge needle attached to a normal (0.9% NaCl) saline-filled reservoir raised above the animal to increase intraocular pressure (IOP, which increased to 120 mm Hg). The contralateral eye was cannulated and maintained at normal IOP to serve as a normotensive control. Body temperature was maintained at 37 ± 0.5°C. Complete retinal ischemia, evidenced by a whitening of the anterior segment of the eye and blanching of the retinal arteries, was verified by microscopic examination.

Treatment with Neutralizing Antibody against Hmgb1 or with Recombinant Hmgb1

The neutralizing anti-Hmgb1 antibody (30 µg per mouse in 200 µL of PBS) was administered by intraperitoneal (IP) injection 15 minutes before ischemia.²³ Controls received the same amount of a nonspecific mouse IgG. Recombinant Hmgb1 (total 1 µg in 2 µL of PBS, catalog number: 1690HM; R&D Systems, Inc., Minneapolis, MN) was administered by intravitreal injection as described in Yoneda et al.³⁵ Controls received the same amount of PBS.

Western Blot Analysis

Samples containing 20 µg of total retinal proteins were loaded, and the proteins were size-separated in sodium dodecyl sulfate polyacrylamide gel (SDS PAGE). Proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) and incubated with HMGB1 primary antibody (1:1000, ab18256; Abcam, Cambridge, MA). Proteins recognized by the antibody were revealed by an ultrasensitive chemiluminescent substrate system (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Protein Research Products) according to instructions (Thermo Fisher Scientific, Rockford, IL). Briefly, luminol/enhancer and stable peroxide solutions were mixed at a 1:1 ratio to prepare the substrate working solution. PVDF membrane was incubated 5 minutes in a working solution. Quantification of the protein bands was performed using image acquisition and analysis software (Quantity One; Bio-Rad Laboratories, Hercules, CA). The amounts of expressed protein were calculated by comparison with a standard curve. A standard curve was constructed using purified recombinant Hmgb1 protein of known concentration diluted in PBS.

Neuronal Death Assay

After OGD, necrotic and apoptotic cells were determined using an apoptosis assay kit (Vybrant Apoptosis Assay Kit #2; Invitrogen). Cells were imaged using a confocal microscope (Leica TSL AOBSP5; Leica Microsystems, Exton, PA) and counted using image analysis software (MetaMorph; Molecular Devices, Sunnyvale, CA). The percentage of necrotic cells (annexin V and propidium iodide) and apoptotic cells (only annexin V) relative to the total number of cells was determined for ten independent images.

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde (PFA) solution in PBS (pH 7.4) and blocked with 5% normal goat serum with 0.1% Tween-20 in PBS (pH 7.4). Cells were then incubated with FITC-conjugated beta III tubulin or antiactive caspase-3 (1:250; Cell Signaling Technology). The cells were incubated with goat anti-rabbit fluorescent dye (Alexa Fluor 546, 1:1000; Invitrogen) for 1 hour. Negative controls were incubated with secondary antibody only.

Real-Time PCR

Real-time PCR analysis was performed as previously described^{9,10} using gene-specific primers (Table 1). Specifically, total RNA was extracted from retinas using a resin spin-column system (Nanoprep; Stratagene, Carlsbad, CA) and reverse transcribed with reverse transcriptase (SuperScript III; Invitrogen) polymerase to synthesize cDNA. Real-time PCR was performed using a commercial PCR detection system (iCycler iQ; Bio-Rad, Hercules, CA) and quantitative real-time PCR (QuantiTect SYBR Green PCR MasterMix; Qiagen, Valencia, CA). Relative expression was calculated by comparison with a standard curve after normalization to the housekeeping gene β -actin expression chosen as control.

Immunohistochemistry

For immunohistochemistry of a neuron-specific nuclear protein (Neuronal Nuclei [NeuN]) in flat-mounted retinas, eyes were enucleated on euthanatization by CO₂ inhalation under anesthesia, incised at the ora serrata, immersion fixed in a 4% PFA solution (in PBS, pH 7.4) for 1 hour, after which the retinas were removed. The retinas were cryoprotected overnight in 30% sucrose followed by three freeze thaw cycles, rinsed 3 × 10 minutes in 0.1 M PBS, and blocked by 5% donkey serum, 0.1% Triton X-100 in 0.1 M Tris buffer (TB) for 1 hour, and incubated overnight with monoclonal NeuN antibody (dilution 1:300, MAB377X; Millipore, Billerica, MA), followed by a fluorescent dye (Alexa Fluor 647; Invitrogen) goat anti-mouse IgG (heavy and light chains) secondary fluorescent antibodies (Invitrogen). After three 10-minute rinses in 0.1 M TB, retinas were flat-mounted, coverslipped, and imaged using a confocal microscope (Leica TSL AOBSP5).

TABLE 1. List of PCR Primers

Gene		Oligonucleotides
<i>Il1b</i>	Forward	GACCTTCAGGATGAGGACA
	Reverse	AGGCCACAGGTATTTGTGC
<i>Il6</i>	Forward	ATGGATGCTACCAACTGGAT
	Reverse	TGAAGGACTCTGGCTTTGTCT
<i>Tnf</i>	Forward	CAAAATTGAGTGACAAAGCCTG
	Reverse	GAGATCCATGCCGTTGGC
<i>Vcam1</i>	Forward	GTGGTGTGTGACAATGACC
	Reverse	ACGTCAGAACACCGAATCC
<i>Ccl2</i>	Forward	AGGTCCTGTCTAGCTTCTG
	Reverse	ATTTGGTTCCGATCCAGGTT
<i>Ccl5</i>	Forward	AGCAGCAAGTGCTCCAATCT
	Reverse	ATTTCTTGGGTTTGCTGTGC
<i>Cxcl10</i>	Forward	GCTGCAACTGCATCCATATC
	Reverse	CACTGGGTAAAGGGAGTGA
<i>Icam1</i>	Forward	TGGTGATGCTCAGGTATCCA
	Reverse	CACACTCTCCGGAACGAAT
<i>Cybb</i>	Forward	GACTGCGGAGAGTTTGAAG
	Reverse	ACTGTCCACCTCCATCTTG
<i>Ncf1</i>	Forward	CGAAGAAGTTTCGGGAACAG
	Reverse	AGCCATCCAGGAGCTTATGA
<i>Ncf2</i>	Forward	CTACCTGAGCCAGTTGAGC
	Reverse	AGCGCCAGCTTCTTAGACAC
<i>Nos2</i>	Forward	CAGAGGACCCAGAGACAAGC
	Reverse	TGCTGAAACATTTCCTGTGC
<i>Actb</i>	Forward	CACCCTGTGCTGCTCACC
	Reverse	GCACGATTTCCTCTCAG

FIGURE 1. Hmgb1 is accumulated in the vitreous humor and retina of animals suffering from retinal ischemia. (A) Representative Western blot. (B) Level of Hmgb1 in vitreous humor of sham-operated and ischemic eyes 24 hours after reperfusion. Results are expressed as a percentage of corresponding value in the sham-operated eye $\pm$ SEM ($^*P < 0.05$, $n = 10$).

To evaluate the penetration of the anti-HMGB1 antibody into retina, the rabbit anti-HMGB1 antibody (ab18256; Abcam; 30 μ g per mouse in 200 μ L) or PBS was administered by IP injection 15 minutes before IR injury. To complete the immunostaining, 24 hours after surgery, animals were humanely euthanized and retinas were dissected, after which anti-Hmgb1 antibodies were evaluated in the flat-mounted retina. For this purpose, whole retinas were fixed in methanol/20% dimethyl sulfoxide, treated with 1.5% hydrogen peroxide in methanol, and incubated for 30 minutes with a commercial universal reagent (ImmPRESS Anti-Rabbit Ig [peroxidase] Polymer Detection Kit; Vector Laboratories, Burlingame, CA). A commercial peroxidase was used as substrate (NovaRED Peroxidase Substrate Kit; Vector Laboratories).

Counting of NeuN-Positive Ganglion Cell Layer Neurons

NeuN-positive neurons in the ganglion cell layer (GCL), including RGCs and displaced amacrine cells, were imaged by confocal microscopy in flat-mounted retinas. Individual retinas were sampled randomly to collect a total of 20 images located at the same eccentricity in the four retinal quadrants using a $\times 20$ objective lens. NeuN-positive neurons were counted semiautomatically using a software application module (MetaMorph; Molecular Devices). Cell loss in the ischemic retinas was calculated as a percentile of the mean cell density in fellow control eyes.

Statistical Analysis

Statistical analyses of real-time PCR and cell density data were performed with one-way ANOVA, followed by the Tukey test for multiple comparisons. In the case of single comparisons, the Student's *t*-test was applied. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Hmgb1 Is Released in the Vitreous Humor after Retinal Ischemia and Contributes to IR Injury

To investigate the role of Hmgb1 in retinal ischemia, we induced unilateral retinal ischemia in wild-type (WT) mice by raising IOP above normal systolic levels and evaluating neuronal survival. High IOP-induced transient retinal IR is one of the most frequently used models to investigate molecular mechanisms contributing to neuronal ischemic injury.³⁶ To study the changes of Hmgb1 levels after retinal ischemia, samples of vitreous humor were collected 24 hours after reperfusion, resolved by SDS PAGE, transferred to nitrocellulose, and probed with antibodies against the Hmgb1. We observed an increase in Hmgb1 protein content in the vitreous humor of

ischemic eyes (28 ± 2 ng per vitreous) compared with sham-operated controls (5 ± 1 ng per vitreous) (Fig. 1).

To test the functional significance of Hmgb1 release in retinal ischemia, the neutralizing anti-Hmgb1 antibody or control nonspecific mouse IgG was injected IP 15 minutes before ischemia. Since IR-induced degeneration of neurons in the GCL is biphasic, with a primary degeneration occurring within 24 hours after reperfusion and a secondary degeneration progressing over several days,³⁷ we detected cumulative damage from both waves of degeneration by direct counting of NeuN-labeled GCL neurons of flat-mounted retinas 1 week after reperfusion. Comparison of neuronal loss among anti-Hmgb1 antibody, IgG-treated mice, and untreated mice revealed a significant difference in neuronal resistance to IR injury in the anti-Hmgb1 treated group. The percentage of surviving GCL neurons in the IR retinas was significantly higher in mice injected with the neutralizing anti-Hmgb1 antibody ($97 \pm 1\%$) compared with those injected with a nonspecific mouse IgG ($70 \pm 6\%$, $P < 0.01$) and untreated mice ($65 \pm 6\%$, $P < 0.001$) (Figs. 2A, 2B). To evaluate the penetration of the antibody against Hmgb1 into the retina, we stained the parallel flat-mounted ischemic and sham-operated retinas with a horseradish peroxidase labeled secondary antibody. Twenty-four hours after administration of anti-Hmgb1 and retinal IR injury, we detected anti-Hmgb1 antibody in the ischemic retina. In contrast, no signal was found in mice treated with PBS (Fig. 2C).

To study whether Hmgb1 alone mediates similar damage as IR injury, we injected recombinant Hmgb1 (rHmgb1) intravitreally and evaluated the survival of GCL neurons 7 days after treatment. The analysis of rHMGB1-treated animals showed a decrease ($83 \pm 3\%$) in cell numbers in the GCL compared with PBS-treated controls ($102 \pm 5\%$, $P < 0.01$) (Fig. 3A). Since Hmgb1 as a danger signal alone is expected to trigger inflammation, we investigated the activation of the proinflammatory markers *Ccl2*, *Cxcl10*, *Il1 β* , *Nos2*, *Tnf*, and *Icam1* 24 hours after injection of rHMGB1 at the level of RNA expression by quantitative PCR. We observed transcriptional upregulation of the *Tnf* and *Il1 β* cytokines as well as *Ccl2* and *Cxcl10* chemo-

FIGURE 2. Blockade of Hmgb1 by a neutralizing anti-Hmgb1 antibody protects retina against IR injury. (A) The percentage survival of GCL neurons in the IR retinas treated with neutralizing anti-Hmgb1 antibody, control IgG, and in retinas of untreated animals 7 days after IR ($^*P < 0.05$, six animals per group). (B) Representative confocal images of NeuN-labeled GCLs (green) in flat-mounted sham-operated and ischemic retinas 7 days after reperfusion. (C) Immunostaining of rabbit Ig demonstrating presence of the anti-Hmgb1 antibody into the ischemic retina 24 hours after reperfusion. For comparison, stainings of flat-mounted retinas from mice treated with PBS is shown (left).

FIGURE 3. Recombinant Hmgb1 (rHmgb1) mediates death of GCL neurons. **(A)** The percentage survival of GCL neurons in the retinas treated with rHmgb1 or PBS (** $P < 0.01$, $n = 5$). **(B)** Retinas of WT mice that were exposed in vivo to rHmgb1 showed enhanced expression of proinflammatory genes (* $P < 0.05$, ** $P < 0.01$, $n = 6$).

kines (Fig. 3B). Thus, our results suggest that intravitreal injection of rHmgb1 activates a proinflammatory response in retina. To elucidate in which cell type Hmgb1 exerts its detrimental effect, we turned to cell cultures. We assayed RGC survival in glia RGC cocultures in which RGCs, isolated according to the two-step immunopanning method, were plated directly on a glial monolayer. These cultures were challenged with rHmgb1 or PBS and the number of activated caspase-3/beta III Tubulin-positive RGCs was counted after 24 hours. Treatment with rHmgb1 decreased the number of beta III Tubulin-positive neurons, indicating that RGCs are dying if cultures are treated with rHmgb1 (Figs. 4A, 4B). We next performed experiments with RGC primary cultures to establish the direct role of Hmgb1 in neuronal vulnerability. RGCs were treated

with rHmgb1 or PBS and then assessed for levels of necrotic and apoptotic cells and survival after 24 hours. Quantification of RGC death showed significantly higher cell death in cultures treated with rHmgb1 versus PBS (Figs. 4C, 4D). To determine whether the observed effects were due to contamination by astrocytes and/or microglia/macrophages, isolated cells were tested by morphology using microscopy (Fig. 4D), as well as by real-time PCR, to validate the abundance of neuronal marker and to determine the level of contamination by nonneuronal cells such as glia and macrophages. We assessed the relative abundance of the marker genes for RGCs (*Thy1*), astrocytes (*S100b*), and microglia/macrophage (*Cd11b*) cells in RGCs purified by immunopanning. Real-time PCR did not detect contamination with microglia/macrophages (*Cd11b*), and as-

FIGURE 4. Hmgb1 mediates RGC death in vitro. **(A)** Glia mediates the effect of HMGB1. Primary RGCs isolated by two-step immunopanning procedure from retinas and cocultured with glia were treated with rHmgb1 (1 $\mu\text{g/mL}$, 24 hours). RGC survival was assessed by counting the number of activated caspase-3/beta III Tubulin-positive cells ($n = 50$; ** $P < 0.01$). **(B)** Representative confocal images. In merged images (yellow), beta III Tubulin-positive RGCs (green) colocalized with activated caspase-3 (red). **(C)** Treatment with rHmgb1 increases cell death in the pure primary RGC cultures. The percentage of necrotic cells and apoptotic cells relative to the total number of cells was determined for each of 10 images (** $P < 0.01$). **(D)** RGC death was determined using annexin V as a marker of apoptotic cells and annexin V/propidium iodide (PI) to identify necrotic cells. **(E)** Relative mRNA abundance for contaminating cell markers versus *Thy1*.

trocytes were detected only with relative abundance of *S100b/Thy1* marker genes mRNA ranging between 1 and 3% (Fig. 4E). Thus, the calculated efficiency of RGC purification was 97% to 99%. Importantly, RGC death was significantly higher in glia RGC coculture treated with rHmgb1 ($28 \pm 2\%$ RGC apoptosis) compared with the primary RGC cultures treated with rHmgb1 ($15 \pm 2\%$ RGC apoptosis, $P < 0.05$) (Fig. 4). Thus, glia are required for the harmful effect of Hmgb1.

Inactivation of Rage Promotes Survival of GCL Neurons after Ischemic Injury

Rage is a member of the Ig superfamily of cell surface receptors that is activated directly by Hmgb1 and is a mediator of inflammation and damage.^{16,17} In the brain and retina, Rage is present on neurons, glia, and endothelial cells.^{38,39} To study the changes in Rage expression after ischemia, we used the OGD model of ischemia in vitro and the in vivo model of transient retinal ischemia. Primary RGCs were deprived of oxygen and glucose for 4 hours in an anaerobic chamber. For in vivo experiments, we induced retinal ischemia by unilateral elevation of IOP for 1 hour by direct anterior chamber cannulation. The changes in expression of Rage were assessed 24 hours postreperfusion by quantitative RT-PCR. We did not observe statistically significant changes in Rage expression in RGCs and retina 24 hours postreperfusion (Fig. 5).

To evaluate the effect of Rage on the severity of retinal ischemia, we took advantage of a previously characterized Rage knockout (RageKO) mouse model.³⁴ A testing has demonstrated that RageKO mice are indistinguishable from the wild-type (WT) littermates in every aspect (retinal morphology, total number of GCL neurons, etc.), showing no phenotypic abnormalities (data not shown). We induced retinal ischemia in the WT and RageKO mice and whole retina flatmounts were stained for the neuronal marker NeuN to quantify the number of surviving neurons in the GCL 7 days after reperfusion. We observed IR-induced loss of retinal neurons in both WT and RageKO retinas. However, when neuronal loss in the GCL was compared between WT and RageKO mice, significant differences in neuronal viability became apparent. Retinas from experimental eyes of WT mice had significantly lower numbers of surviving NeuN-positive neurons ($69 \pm 2\%$) in the GCL compared with RageKO mice ($95 \pm 3\%$, $P < 0.01$). In fact, RAGE-deficient mice appeared to be completely protected from IR injury in that no significant difference was found between ischemic and sham-operated retinas in the number of viable GCL neurons (Figs. 6A, 6B).

To elucidate the downstream signaling pathway involved in elevated resistance to ischemia in RageKO retinas, we com-

FIGURE 5. Rage expression in the ischemic and normal retina. Expression of Rage was not changed in retina and RGCs after ischemic stress.

FIGURE 6. Rage deficiency results in neuroprotective effects in the GCL of retinas after IR. (A) The percentage survival of GCL neurons in the IR retinas of WT and RageKO animals 7 days after IR. The percentage of NeuN-labeled neurons was compared between sham-operated and ischemic eyes of WT and RageKO animals 7 days after IR (** $P < 0.01$, $n = 6$). (B) Representative confocal images of NeuN-labeled GCLs (green) in flat-mounted controls and ischemic retinas 7 days after reperfusion.

pared the expression of several proinflammatory genes, known to be involved in IR-induced cytotoxicity, in ischemic versus sham-operated eyes for both WT and RageKO mice. Transcriptional upregulation of the cytokines *Il1 β* , *Il6*, *Tnf*; the chemokines *Ccl2*, *Ccl5*, and *Cxcl10*; the cell adhesion molecules *Icam1*, *Vcam1*, and the *Nos2* (*iNos*) gene; as well as *Cyb*, *Ncf1*, and *Ncf2* genes encoding subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase protein complex was evident in all experimental eyes 24 hours after reperfusion (Fig. 7). In RageKO mice, however, the expression of *Tnf*, *Il6*, *Ccl2*, *Cxcl10*, *Vcam1*, *Nos2*, and *Ncf2* was significantly reduced relative to WT. These data suggest that, as a result of the absence of RAGE signaling, the inflammatory response observed in the retina 24 hours after reperfusion was significantly suppressed in the retinas of RAGE-deficient mice.

DISCUSSION

Retinal IR injury results in a prolonged period of cell death with a high level of necrosis at an early stage of pathology.^{11,12,36} Previously, we demonstrated that necrotic cell death contributes to IR injury.¹¹ It was shown that danger signals (DAMPs) liberated from necrotic cells act through pattern recognition receptors, initiating an additional innate immune response.^{13–15} Thus, DAMPs emanating from necrotic cells in the retina may pose an additional risk to surrounding cells postreperfusion. Hmgb1 is the prototypic DAMP molecule and has been implicated in IR injury of the brain, heart, liver, kidney, and lung as well as in several inflammatory disorders.^{16,17,21–31} Here, we demonstrated that Hmgb1 and its receptor Rage contribute to retinal damage after IR.

Hmgb1, a DNA-binding nuclear protein, is released not only actively after cytokine stimulation but also passively during necrotic cell death.^{16–20} Hmgb1 can promote inflammatory responses and damage by numerous mechanisms.^{16,17,20} Importantly, Hmgb1 is elevated in the serum of patients with mechanical trauma, stroke, acute myocardial infarction, acute respiratory distress, and liver transplantation, as well as were released from ischemic tissue in animal models of the brain, heart, liver, kidney, and lung IR injury.^{21–31} At the same time, interference with Hmgb1 by a neutralizing antibody, Hmgb1 box A, or a short-hairpin RNA ameliorated damage in these tissues.^{21–26,28,31} Our data indicate that Hmgb1 is massively released into the extracellular space and accumulates in the vitreous humor after retinal IR injury, whereas inhibition of Hmgb1 activity with neutralizing antibody decreased retinal damage after IR injury. To study whether Hmgb1 alone mediates similar damage as IR injury, we injected intravitreally

FIGURE 7. Rage deficiency suppresses induction of proinflammatory markers after IR. Gene expression was assessed using real-time PCR in sham-operated controls and experimental retinas 24 hours after IR. For each gene, results are expressed as a percentage of the corresponding value in the sham-operated eye $\pm$ SEM after normalization to β -actin (* P < 0.05, n = 6).

recombinant Hmgb1 and evaluated the GCL neuron survival. The analysis of rHmgb1-treated mice showed increased damage of GCL neurons 7 days after injection. Increased levels of chemokines and cytokines in rHmgb1-treated retinas 24 hours after injection could explain this result.^{4,35,40} Astrocytes and microglia are generally believed to be the primary or sole source of chemokines and neurotoxic cytokines in the retina.^{3,9,41–45} Thus, increased RGC death in rHmgb1-treated glia RGC coculture verifies the previous statement. This result is consistent with findings obtained in other tissues. It was previously shown that Hmgb1 injection promotes inflammation and damage in kidney, liver, heart, and brain.^{21,22,24,25,46} Importantly, the observed RGC death after rHmgb1 treatment suggests that Hmgb1 can directly promote neuronal death.

Thus, Hmgb1 can mediate damage in the ischemic retina both directly and indirectly.

To elucidate the downstream signaling pathways involved in Hmgb1-induced effects in the ischemic retina, we used mice that lacked Hmgb1 receptor. Hmgb1 is known to interact directly and activate Tlr4 and Rage.^{16–20} We have previously demonstrated that Tlr4 mediates inflammation and damage after retinal IR injury.³² In addition, our results suggest that the deleterious effects of endogenous Hmgb1 on IR injury in retina could be explained at least in part by engagement of Rage because retinas from RageKO mice were significantly protected from ischemia as comparable to that of WT mice. Lowered activity of genes encoding the cytokines *Tnf* and *Il6*, the chemokines *Ccl2* and *Cxcl10*, the cell adhesion molecule

Vcam1, the inducible NO-synthase Nos2 and Ncf2, a subunit of the reactive oxygen species producing enzymes NAD(P)H, in RageKO animals suggests that the reduced level of neurotoxic cytokines, inflammatory mediators, and the diminished ability for inflammatory cells to infiltrate the retina could elicit a neuroprotective effect after ischemia.^{4,9,40,47–50} The similarity between proinflammatory responses of ischemic retinas and rHmgb1 treated retinas and results that follow from our studies of Tlr4 and Rage knockouts in retinal ischemia would suggest that Hmgb1 signals through Rage and Tlr4 receptors in retinal IR injury. However, we cannot exclude Hmgb1 signaling through other membrane receptors beside Rage and Tlr4 in retinal ischemia because treatment with neutralizing anti-Hmgb1 antibody demonstrated a better survival of GCL neurons compared with RageKO and Tlr4KO.³² For example, it was shown that the complex of Hmgb1 and Il1 β has greater proinflammatory activity than Il1 β alone and interacts with the Il1r.⁵¹ An important role of Il1r in mediating ischemic damage in the retina was shown in previous studies.^{3,35}

In conclusion, our data strongly support the hypothesis that Hmgb1 plays an important role in retinal IR injury. The effects of Hmgb1 in retinal ischemia could be explained, at least in part, by engagement of Rage and Tlr4, resulting in the activation of proinflammatory pathways and enhanced retinal injury. The current findings clearly indicate that a neutralizing anti-Hmgb1 antibody may be a specific tool to interfere with both inflammation and cell death in retinal ischemia and might represent a novel therapeutic strategy in IR injury.

Acknowledgments

The authors thank Bernd Arnold (German Cancer Research Center, Heidelberg, Germany) and Joseph Bidwell (Indiana University School of Medicine, Indianapolis, IN) for the kind gift of RageKO mice, and Gabriel Gaidosh for his expert technical assistance.

References

- Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M, Melena J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res*. 2004;23:91–147.
- Lipton P. Ischemic cell death in brain neurons. *Physiol Rev*. 1999;79:1431–1568.
- Hangai M, Yoshimura N, Yoshida M, Yabuuchi K, Honda Y. Interleukin-1 gene expression in transient retinal ischemia in the rat. *Invest Ophthalmol Vis Sci*. 1995;36:571–578.
- Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, Eisel U. Neurodegenerative and neuroprotective effects of tumor necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci*. 2002;22:RC216.
- Hangai M, Yoshimura N, Hiroi K, Mandai M, Honda Y. Inducible nitric oxide synthase in retinal ischemia-reperfusion injury. *Exp Eye Res*. 1996;63:501–509.
- Chen YG, Zhang C, Chiang SK, Wu T, Tso MO. Increased nuclear factor-kappa B p65 immunoreactivity following retinal ischemia and reperfusion injury in mice. *J Neurosci Res*. 2003;72:125–131.
- Nishiwaki A, Ueda T, Ugawa S, Shimada S, Ogura Y. Upregulation of P-selectin and intercellular adhesion molecule-1 after retinal ischemia-reperfusion injury. *Invest Ophthalmol Vis Sci*. 2003;44:4931–4935.
- McCluskey P, Powell RJ. The eye in systemic inflammatory diseases. *Lancet*. 2004;364:2125–2133.
- Dvorianchikova G, Barakat D, Brambilla R, et al. Inactivation of astroglial NF-kappaB promotes survival of retinal neurons following ischemic injury. *Eur J Neurosci*. 2009;30:175–185.
- Dvorianchikova G, Agudelo C, Hernandez E, Shestopalov VI, Ivanov D. Phosphatidylserine-containing liposomes promote maximal survival of retinal neurons after ischemic injury. *J Cereb Blood Flow Metab*. 2009;29:1755–1759.
- Dvorianchikova G, Barakat DJ, Hernandez E, Shestopalov VI, Ivanov D. Liposome-delivered ATP effectively protects the retina against ischemia-reperfusion injury. *Mol Vis*. 2010;16:2882–2890.
- Fujita R, Ueda M, Fujiwara K, Ueda H. Prothymosin-alpha plays a defensive role in retinal ischemia through necrosis and apoptosis inhibition. *Cell Death Differ*. 2009;16:349–358.
- Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*. 2010;10:826–837.
- Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm*. 2010;2010:672395. Epub 2010 July 13.
- Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol*. 2007;81:1–5.
- Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol*. 2008;26:367–388.
- Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005;5:331–342.
- Biscetti F, Straface G, De Cristofaro R, et al. High-mobility group box-1 protein promotes angiogenesis after peripheral ischemia in diabetic mice through a VEGF-dependent mechanism. *Diabetes*. 2010;59:1496–1505.
- Tang D, Kang R, Zeh HJ 3rd, Lotze MT. High-mobility group box 1 and cancer. *Biochim Biophys Acta*. 2010;1799:131–140.
- Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*. 2002;418:191–195.
- Andrassy M, Volz HC, Igwe JC, et al. High-mobility group box-1 in ischemia-reperfusion injury of the heart. *Circulation*. 2008;117:3216–3226.
- Faraco G, Fossati S, Bianchi ME, et al. High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. *J Neurochem*. 2007;103:590–603.
- Muhammad S, Barakat W, Stoyanov S, et al. The HMGB1 receptor RAGE mediates ischemic brain damage. *J Neurosci*. 2008;28:12023–12031.
- Tsung A, Sahai R, Tanaka H, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med*. 2005;201:1135–1143.
- Wu H, Ma J, Wang P, et al. HMGB1 contributes to kidney ischemia reperfusion injury. *J Am Soc Nephrol*. 2010;21:1878–1890.
- Liu A, Jin H, Dirsch O, et al. Release of danger signals during ischemic storage of the liver: a potential marker of organ damage? *Mediators Inflamm*. 2010;2010:436145. Epub 2010 Dec 21.
- Evankovich J, Cho SW, Zhang R, et al. High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. *J Biol Chem*. 2010;285:39888–39897.
- Li J, Gong Q, Zhong S, et al. Neutralization of the extracellular HMGB1 released by ischaemic damaged renal cells protects against renal ischaemia-reperfusion injury. *Nephrol Dial Transplant*. 2011;26:469–478.
- Kruger B, Krick S, Dhillon N, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. *Proc Natl Acad Sci USA*. 2009;106:3390–3395.
- Kaczorowski DJ, Nakao A, Vallabhaneni R, et al. Mechanisms of Toll-like receptor 4 (TLR4)-mediated inflammation after cold ischemia/reperfusion in the heart. *Transplantation*. 2009;87:1455–1463.
- Yang QW, Xiang J, Zhou Y, Zhong Q, Li JC. Targeting HMGB1/TLR4 signaling as a novel approach to treatment of cerebral ischemia. *Front Biosci (Schol Ed)*. 2010;2:1081–1091.
- Dvorianchikova G, Barakat DJ, Hernandez E, Shestopalov VI, Ivanov D. Toll-like receptor 4 contributes to retinal ischemia/reperfusion injury. *Mol Vis*. 2010;16:1907–1912.
- Philip BK, Childress PJ, Robling AG, et al. RAGE supports parathyroid hormone-induced gains in femoral trabecular bone. *Am J Physiol Endocrinol Metab*. 2010;298:E714–E725.

34. Constien R, Forde A, Liliensiek B, et al. Characterization of a novel EGFP reporter mouse to monitor Cre recombination as demonstrated by a Tie2 Cre mouse line. *Genesis*. 2001;30:36–44.
35. Yoneda S, Tanihara H, Kido N, et al. Interleukin-1 β mediates ischemic injury in the rat retina. *Exp Eye Res*. 2001;73:661–667.
36. Lafuente MP, Villegas-Perez MP, Selles-Navarro I, Mayor-Torroglosa S, Miralles de Imperial J, Vidal-Sanz M. Retinal ganglion cell death after acute retinal ischemia is an ongoing process whose severity and duration depends on the duration of the insult. *Neuroscience*. 2002;109:157–168.
37. Selles-Navarro I, Villegas-Perez MP, Salvador-Silva M, Ruiz-Gomez JM, Vidal-Sanz M. Retinal ganglion cell death after different transient periods of pressure-induced ischemia and survival intervals. A quantitative in vivo study. *Invest Ophthalmol Vis Sci*. 1996;37:2002–2014.
38. Pachydaki SI, Tari SR, Lee SE, et al. Upregulation of RAGE and its ligands in proliferative retinal disease. *Exp Eye Res*. 2006;82:807–815.
39. Barile GR, Schmidt AM. RAGE and its ligands in retinal disease. *Curr Mol Med*. 2007;7:758–765.
40. Ubogu EE, Cossoy MB, Ransohoff RM. The expression and function of chemokines involved in CNS inflammation. *Trends Pharmacol Sci*. 2006;27:48–55.
41. Davies MH, Eubanks JP, Powers MR. Microglia and macrophages are increased in response to ischemia-induced retinopathy in the mouse retina. *Mol Vis*. 2006;12:467–477.
42. Langmann T. Microglia activation in retinal degeneration. *J Leukoc Biol*. 2007;81:1345–1351.
43. Tezel G. TNF- α signaling in glaucomatous neurodegeneration. *Prog Brain Res*. 2008;173:409–421.
44. Anderson PJ, Watts HR, Jen S, et al. Differential effects of interleukin-1 β and S100B on amyloid precursor protein in rat retinal neurons. *Clin Ophthalmol*. 2009;3:235–242.
45. Kilic U, Kilic E, Matter CM, Bassetti CL, Hermann DM. TLR-4 deficiency protects against focal cerebral ischemia and axotomy-induced neurodegeneration. *Neurobiol Dis*. 2008;31:33–40.
46. Oyama Y, Hashiguchi T, Taniguchi N, et al. High-mobility group box-1 protein promotes granulomatous nephritis in adenine-induced nephropathy. *Lab Invest*. 2010;90:853–866.
47. Sun GY, Horrocks LA, Farooqui AA. The roles of NADPH oxidase and phospholipases A2 in oxidative and inflammatory responses in neurodegenerative diseases. *J Neurochem*. 2007;103:1–16.
48. Neufeld AH, Kawai S, Das S, et al. Loss of retinal ganglion cells following retinal ischemia: the role of inducible nitric oxide synthase. *Exp Eye Res*. 2002;75:521–528.
49. Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res Brain Res Rev*. 1999;30:77–105.
50. Broisat A, Riou LM, Ardisson V, et al. Molecular imaging of vascular cell adhesion molecule-1 expression in experimental atherosclerotic plaques with radiolabelled B2702-p. *Eur J Nucl Med Mol Imaging*. 2007;34:830–840.
51. Sha Y, Zmijewski J, Xu Z, Abraham E. HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *J Immunol*. 2008;180:2531–2537.