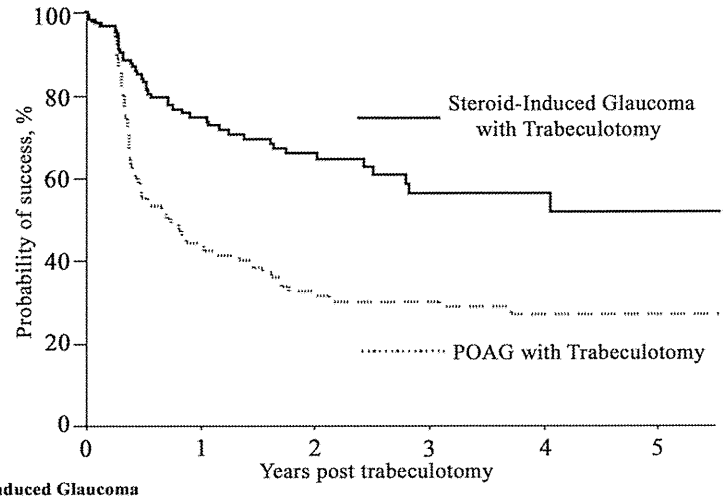


Steroid-Induced Glaucoma											
No. at risk	121	121	89	76	64	48	33	22	16	13	10
Failure	0	19	9	5	3	2	3	0	0	1	0
Censored	0	13	4	7	13	13	8	6	3	2	2
POAG											
No. at risk	108	108	57	45	37	28	24	22	17	16	12
Failure	0	47	11	6	5	2	0	1	1	0	0
Censored	0	4	1	2	4	2	2	4	0	4	3

FIGURE 2. Criterion B–based Kaplan-Meier survival curves of surgical outcomes in patients with steroid-induced glaucoma (solid line) vs primary open-angle glaucoma (POAG; dotted line) that underwent trabeculotomy. The steroid-induced glaucoma eyes had a significantly higher cumulative probability of success than the POAG eyes ($P < .0001$).

TABLE 2. Cox Proportional Hazards Model Determining Likelihood of Surgical Outcomes for Patients With Steroid-Induced Glaucoma and Primary Open-Angle Glaucoma who Underwent Trabeculotomy

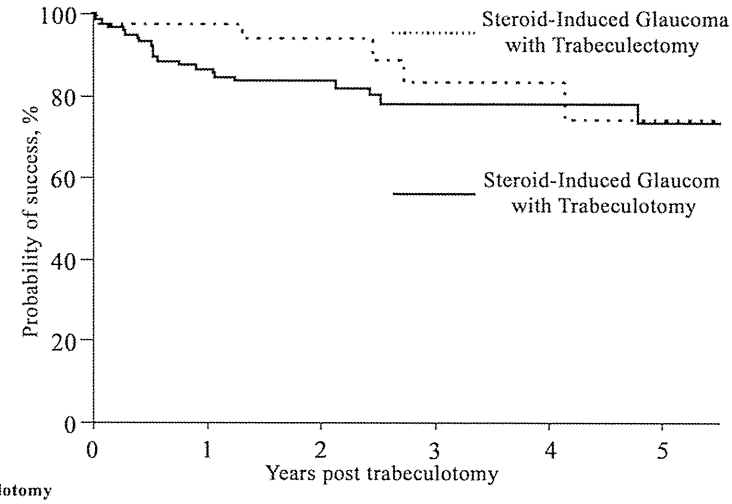
	Criterion A			Criterion B		
Variable	RR	95% CI	*P* Value	RR	95% CI	*P* Value
Steroid-induced glaucoma	0.409	0.223–0.735	.0027	0.451	0.286–0.706	.0005
Age (per year)	0.999	0.982–1.015	.8917	1.007	0.995–1.019	.2408
Preoperative IOP (per mm Hg)	1.004	0.977–1.029	.7557	0.993	0.972–1.013	.5115
Female	0.761	0.451–1.260	.2911	0.695	0.468–1.021	.0639
Previous cataract surgery	2.105	0.823–4.681	.1132	1.627	0.784–3.084	.1804
Combined sinusotomy	1.054	0.575–1.847	.8600	0.839	0.526–1.300	.4399

CI = confidence interval; IOP = intraocular pressure; RR = relative risk.

Institute, Cary, North Carolina, USA). Comparisons of the outcomes between the steroid-induced glaucoma with trabeculotomy group and the POAG with trabeculotomy group, as well as between the steroid-induced glaucoma with trabeculotomy group and the steroid-induced glaucoma with trabeculectomy group, were analyzed by the Kaplan-Meier survival curve and the log-rank test. To assess prognostic factors of steroid-induced glaucoma with trabeculotomy in univariate analysis, Kaplan-Meier survival-curve analysis and the log-rank test were used. To confirm the effects of prognostic factors and to identify the relative risk (RR) of surgical failure, multivariate prognostic factor analysis was performed with the Cox proportional hazards model. Multivariate factors were selected from variants with a probability (*P*) value of less than .15 shown by univariate analysis. A *P* value less than .05 was considered statistically significant.

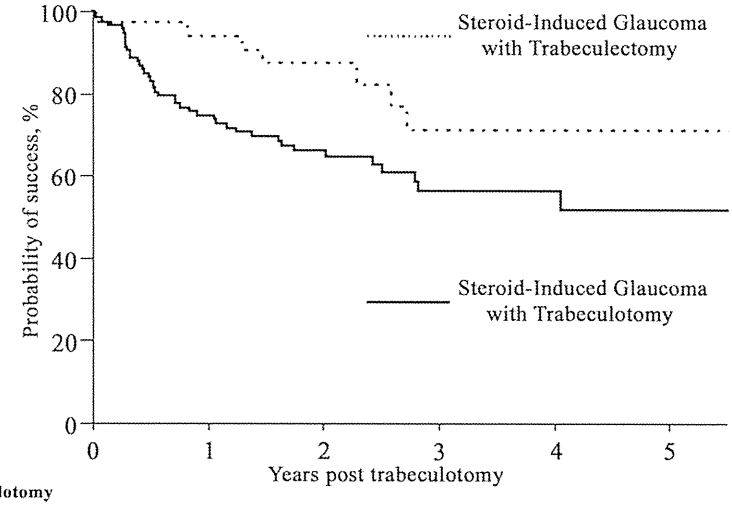
RESULTS

• PATIENT CHARACTERISTICS: In total, 163 patients (163 eyes) with steroid-induced glaucoma and 108 patients (108 eyes) with POAG satisfied the study criteria. All eligible patients were Japanese. Of the 163 eyes with steroid-induced glaucoma, 121 were included in the steroid-induced glaucoma with trabeculotomy group and 42 were included in the steroid-induced glaucoma with trabeculectomy group. Table 1 lists the characteristics of the enrolled patients.



Trabeculotomy											
No. at risk	121	121	100	89	77	63	42	32	26	22	20
Failure	0	8	7	3	0	2	1	0	0	0	1
Censored	0	13	4	9	14	19	9	6	4	2	5
Trabeculectomy											
No. at risk	42	42	32	31	28	23	17	14	11	10	8
Failure	0	1	0	1	0	1	1	0	0	1	0
Censored	0	9	1	2	5	5	3	3	1	1	2

FIGURE 3. Criterion A–based Kaplan-Meier survival curves of surgical outcomes in eyes with trabeculotomy (solid line) vs trabeculectomy (dotted line) for steroid-induced glaucoma. There was no significant difference in the cumulative probability of success between the eyes with trabeculotomy and trabeculectomy ($P = .3636$).



Trabeculotomy											
No. at risk	121	121	89	76	64	48	33	22	16	13	10
Failure	0	19	9	5	3	2	3	0	0	1	0
Censored	0	13	4	7	13	13	8	6	3	2	2
Trabeculectomy											
No. at risk	42	42	32	30	26	21	15	11	9	8	7
Failure	0	1	1	2	0	1	2	0	0	0	0
Censored	0	9	1	2	5	5	2	2	1	1	1

FIGURE 4. Criterion B–based Kaplan-Meier survival curves of surgical outcomes in eyes with trabeculotomy (solid line) vs trabeculectomy (dotted line) for steroid-induced glaucoma. The trabeculectomy group showed a significantly higher cumulative probability of success than the trabeculotomy group ($P = .0352$).

The steroid-induced glaucoma with trabeculotomy group was significantly younger ($P = .001$) and had a higher preoperative IOP ($P < .001$), a higher number of female patients ($P = .014$), a higher number of previous cataract surgeries ($P = .029$), and a lower number of combined sinusotomies ($P = .012$) than the POAG with

TABLE 3. Influence of Prognostic Factors on Survival Time of Steroid-Induced Glaucoma Patients who Underwent Trabeculotomy

Variable	Number of Patients	Criterion A		Criterion B	
		80% Survival Time (Days)	*P* Value[a]	80% Survival Time (Days)	*P* Value[a]
Gender			.1779		.0422
Female	62	>3850		305	
Male	59	385		146	
Age (years)			.9040		.2458
<30	51	852		206	
≥30	70	916		181	
Preoperative IOP (mm Hg)			.7443		.4387
<40	79	181		181	
≥40	42	840		200	
Diabetes mellitus			.1936		.0848
Yes	13	146		96	
No	108	816		291	
Hypertension			.9394		.9915
Yes	18	>2148		291	
No	103	852		195	
Combined sinusotomy			.2416		.9270
Yes	20	146		120	
No	101	916		260	
Previous cataract surgery			.0055		.1829
Yes	17	49		49	
No	104	1732		272	
Previous vitrectomy			<0.0001		.0050
Yes	6	10		10	
No	115	1742		260	
Cause of corticosteroid use					
Collagen disease			.3397		.9248
Yes	37	750		195	
No	84	1732		200	
Atopic dermatitis			.9929		.2449
Yes	21	278		162	
No	100	852		195	
Uveitis			.4674		.7942
Yes	25	>2519		177	
No	96	840		200	
Route of steroid administration					
Ocular instillation only			.6968		.1204
Yes	17	1732		1286	
No	104	852		181	
Posterior sub-Tenon's injection of TA			.9546		.3239
Yes	13	>1339		>1339	
No	108	916		200	
Intravitreal injection of TA			.1843		.4379
Yes	10	49		49	
No	111	916		206	
Oral administration			.7412		.6920
Yes	72	840		181	
No	49	1732		272	
Intravenous administration			.4050		.1883
Yes	3	>1580		>1580	
No	118	852		195	

Continued on next page

TABLE 3. Influence of Prognostic Factors on Survival Time of Steroid-Induced Glaucoma Patients who Underwent Trabeculotomy *(Continued)*

Variable	Number of Patients	Criterion A 80% Survival Time (Days)	Criterion A *P* Value[a]	Criterion B 80% Survival Time (Days)	Criterion B *P* Value[a]
Postoperative corticosteroid administration			.1987		.7335
>3 months	68	<3850		195	
≤3 months	53	372		200	

IOP = intraocular pressure; TA = triamcinolone acetonide.
[a]The *P* values are based on the log-rank test.

trabeculotomy group. However, there were no significant differences in patient characteristics between the steroid-induced glaucoma with trabeculotomy group and the steroid-induced glaucoma with trabeculectomy group.

• STEROID-INDUCED GLAUCOMA VS POAG: The mean follow-up periods were 38.4 ± 28.7 months in the steroid-induced glaucoma with trabeculotomy group and 49.8 ± 37.2 months in the POAG with trabeculotomy group (P = 0.010). Kaplan-Meier survival-curve analyses of the steroid-induced glaucoma with trabeculotomy group and the POAG with trabeculotomy group for criteria A and B are presented in Figures 1 and 2, respectively. The steroid-induced glaucoma with trabeculotomy group had a significantly higher cumulative probability of success for criteria A (P = .0008) and B (P < .0001). For criterion A, the probabilities of success 1, 2, 3, and 5 years after trabeculotomy in the steroid-induced glaucoma with trabeculotomy group and the POAG with trabeculotomy group were as follows: 86.5% vs 73.2%, 83.5% vs 63.0%, 78.1% vs 55.8%, and 73.5% vs 52.2%, respectively. For criterion B, the probabilities of success 1, 2, 3, and 5 years after trabeculotomy in the steroid-induced glaucoma with trabeculotomy group and the POAG with trabeculotomy group were as follows: 74.6% vs 44.7%, 66.1% vs 33.0%, 56.4% vs 30.6%, and 51.7% vs 27.5%, respectively. The number of eyes classified as surgical failures in the steroid-induced glaucoma with trabeculotomy group and the POAG with trabeculotomy group were 22/121 (18.2%) vs 45/108 (41.7%) for criterion A and 42/121 (34.7%) vs 73/108 (67.6%) for criterion B, respectively.

Since there were significant differences between the preoperative data of the steroid-induced glaucoma with trabeculotomy group and the POAG with trabeculotomy group, a Cox proportional hazards model including age, preoperative IOP, gender, previous cataract surgery, and combined sinusotomy was performed (Table 2). The multivariate model suggested that trabeculotomy in steroid-induced glaucoma eyes was independently associated with a better prognosis when compared with the same procedure in POAG eyes, even after adjusting for confounding factors (criterion A, RR = 0.409, P = .0027; criterion B, RR = 0.451, P = .0005).

• TRABECULOTOMY VS TRABECULECTOMY: The mean follow-up period in the steroid-induced glaucoma with trabeculectomy group was 37.1 ± 31.8 months (38.4 ± 28.7 months in the steroid-induced glaucoma with trabeculotomy group, P = .808). The Kaplan-Meier survival-curve analysis between the steroid-induced glaucoma with trabeculotomy group and the steroid-induced glaucoma with trabeculectomy group for criteria A and B are presented in Figures 3 and 4, respectively. No significant difference was found between the 2 groups for criterion A (P = .3636). The probabilities of success 1, 2, 3, and 5 years after surgery in the steroid-induced glaucoma with trabeculectomy group for criterion A were 97.6%, 94.3%, 83.8%, and 74.5%, respectively. The number of eyes classified as surgical failures in the steroid-induced glaucoma with trabeculectomy group was 5 (11.9%) for criterion A. However, the steroid-induced glaucoma with trabeculectomy group showed a significantly higher cumulative probability of success for criterion B (P = .0352). The probabilities of success 1, 2, 3, and 5 years after surgery in the steroid-induced glaucoma with trabeculectomy group for criterion B were 94.5%, 87.7%, 71.6%, and 71.6%, respectively. The number of eyes classified as surgical failures in the steroid-induced glaucoma with trabeculectomy group was 7 (16.7%) for criterion B.

• PROGNOSTIC FACTORS FOR FAILURE OF TRABECULOTOMY FOR STEROID-INDUCED GLAUCOMA EYES: The potential prognostic factors influencing survival time are listed in Table 3. Univariate analysis showed previous cataract surgery (P = .0055) and previous vitrectomy (P < .0001) to be significant prognostic factors for criterion A, and male gender (P = .0422) and previous vitrectomy (P = .0050) for criterion B. Diabetes mellitus (P = .0848) and ocular instillation of corticosteroid (P = .1204) were the factors with a P value of less than .15 for criterion B. The Cox proportional hazards model including these variables revealed that prognostic factors for surgical failure were

TABLE 4. Cox Proportional Hazards Model on Criteria A and B, Determining Likelihood of Surgical Outcomes for All 121 Patients With Steroid-Induced Glaucoma who Underwent Trabeculotomy

	Criterion A			Criterion B		
Variable	RR	95% CI	*P* Value	RR	95% CI	*P* Value
Previous cataract surgery	1.614	0.255–5.688	.5488		—	
Previous vitrectomy	5.340	1.037–38.655	.0452	3.898	1.108–10.688	.0360
Male		—		1.783	0.938–3.414	.0774
Diabetes mellitus		—		1.871	0.754–4.018	.1632
Corticosteroid administration other than ocular instillation		—		2.752	1.065–9.426	.0352

CI = confidence interval; IOP = intraocular pressure; RR = relative risk.

previous vitrectomy (RR = 5.340, P = .0452 for criterion A; RR = 3.898, P = .0360 for criterion B) and corticosteroid administration other than ocular instillation (RR = 2.752, P = .0352 for criterion B) (Table 4).

• POSTOPERATIVE COMPLICATIONS: In the steroid-induced glaucoma with trabeculectomy group, choroidal detachment occurred in 2 eyes (4.8%), flat anterior chamber requiring anterior chamber reformation occurred in 1 eye (2.4%), and hypotony maculopathy occurred in 7 eyes (16.7%). None of these complications occurred in either the steroid-induced glaucoma with trabeculotomy group or the POAG with trabeculotomy group. The progression of postoperative cataracts was observed in 9 eyes (7.4%) of the steroid-induced glaucoma with trabeculotomy group, 1 eye (0.9%) of the POAG with trabeculotomy group, and 4 eyes (9.5%) of the steroid-induced glaucoma with trabeculectomy group. No eyes encountered postoperative infectious blebitis or endophthalmitis.

DISCUSSION

THIS STUDY COMPARED THE SUCCESS RATES OF TRABECULOTOMY for steroid-induced glaucomatous eyes with those for trabeculectomy for steroid-induced glaucoma eyes, and for trabeculotomy for POAG eyes. Trabeculotomy showed a significantly higher cumulative probability of success in steroid-induced glaucoma patients than POAG patients for both criterion A (P = .0008) and criterion B (P < .0001). The probability of success in steroid-induced glaucoma eyes treated with trabeculotomy was comparable to that in steroid-induced glaucoma eyes treated with MMC trabeculectomy for criterion A (P = .3636), but was significantly lower for criterion B (P = .0352). Significant prognostic factors for surgical failure of trabeculotomy in steroid-induced glaucoma patients were previous vitrectomy for criteria A (RR = 5.340, P = .0452) and B (RR = 3.898, P = .0360), and corticosteroid treatment other than ocular instillation for criterion B (RR = 2.752, P = .0352).

Several studies have demonstrated surgical results for steroid-induced glaucoma patients.[10,11,13–15,17,23–27] For example, Sihota and associates[10] reported that 9 eyes with steroid-induced glaucoma that required trabeculectomy with MMC showed normal IOP levels after surgery. Several reports[11,23–27] demonstrated that filtering surgery was successful for IOP management in steroid-induced glaucomatous eyes after intravitreal injection of triamcinolone acetonide. Krishnan and associates[13] found that all of 3 eyes with triamcinolone-induced IOP elevation were successfully treated with viscocanalostomy. For laser trabeculoplasty, Ricci and associates[14] and Viola and associates[15] reported that argon laser trabeculoplasty was effective in all cases in their studies, and selective laser trabeculoplasty was shown to lower IOP in 5 of 7 eyes.[17] However, these reports on surgical treatment for steroid-induced glaucoma included only a small number of cases and lacked control groups and details of long-term prognosis. Our previous study lacked control groups but showed that trabeculotomy reduced IOPs to 21 mm Hg or less in 14 eyes with steroid-induced glaucoma.[12] To our knowledge, our present multicenter study reports on the largest number of steroid-induced glaucoma patients.

Trabeculotomy showed a better prognosis in steroid-induced glaucoma eyes than POAG eyes in the present study. The accumulation of extracellular matrices in trabecular meshwork has been believed to cause increased outflow resistance of the aqueous humor in steroid-induced glaucoma patients. This is because histochemical data demonstrate abnormally accumulated extracellular matrices such as type IV collagen, heparin sulfate, proteoglycan, and fibronectin in the trabecular meshwork of steroid-induced glaucoma patients.[9] Because the main target of trabeculotomy for IOP reduction is the relief of outflow resistance in the trabecular meshwork, the consistency between the surgical target and the pathologic lesion might explain the effectiveness of surgery for steroid-induced glaucoma eyes.

The surgical success of trabeculotomy for steroid-induced glaucoma was comparable to the success of MMC trabeculectomy for steroid-induced glaucoma for criterion

A. Trabeculectomy has a potential risk of late-onset infection of the filtering bleb.[28–31] A previous multicenter case-control study suggested that the use of systemic corticosteroid and juvenile-onset glaucoma should be included among the risk factors for late-onset infection after filtering surgery.[32] Moreover, younger patients are more susceptible to steroid-induced IOP elevation.[4,33,34] Trabeculotomy might be more beneficial for younger patients with steroid-induced glaucoma, from the viewpoint of late-onset infection, than trabeculectomy because of the nonfiltering surgery.

Trabeculectomy had a significantly higher probability of success than trabeculotomy using criterion B in the present study. Thus, many steroid-induced glaucoma eyes treated with trabeculotomy had postoperative IOPs of 18 to 20 mm Hg, which might be too high to prevent progressive visual field changes for glaucomatous eyes with advanced progressive visual field defects. The Advanced Glaucoma Intervention Study[35] found that visual field loss in eyes with advanced open-angle glaucoma progresses further if postoperative IOP of 18 mm Hg or higher is more frequent. These findings imply that trabeculectomy rather than trabeculotomy is more favorable for controlling IOP in steroid-induced glaucoma eyes with advanced visual field loss.

Even when other types of glaucoma are included, the prognostic factors for the surgical failure of trabeculotomy have not been sufficiently identified. Our previous report indicated that higher preoperative IOP results in poorer prognosis in eyes with POAG or exfoliative glaucoma.[19] Higher preoperative IOP might reflect the severity of glaucoma, resulting in a poorer response to reduce IOP. The present study showed that higher preoperative IOP was not a prognostic factor for surgical failure of trabeculotomy for steroid-induced glaucoma, while corticosteroid administration other than ocular instillation was shown to be a prognostic factor in criterion B. These data might reflect the fact that the severity of steroid-induced glaucoma depends on the route of corticosteroid administration rather than the preoperative IOP levels. In addition, previous vitrectomy was a prognostic factor for surgical failure for both criteria. Although we have no conclusive explanation, it is conceivable that vitrectomy causes the elevation of inflammatory factors or growth factors in the aqueous humor. Vitrectomized eyes might lead to recurrent fibrosis in the outflow pathway of the trabecular meshwork that was created by the trabeculotomy.

This study had some limitations caused by the retrospective design. First, the selection bias of the type of surgery performed for steroid-induced glaucoma eyes might have affected the surgical result. In fact, all 6 vitrectomized eyes were treated with trabeculotomy. Because of conjunctival scarring after vitrectomy, trabeculotomy rather than trabeculectomy might have been the surgery of choice for vitrectomized eyes. Second, as higher IOP is a prognostic factor for failure of trabeculotomy for POAG eyes,[19] POAG patients with higher IOP might have been treated with trabeculectomy rather than trabeculotomy; this might have increased the cumulative probability of success in POAG with trabeculotomy.

In conclusion, this study demonstrates that trabeculotomy might be more effective for steroid-induced glaucoma eyes than POAG eyes. Moreover, the surgical success in steroid-induced glaucoma eyes is comparable to the outcome of trabeculectomy unless more substantial IOP reduction is necessary, in which case trabeculectomy would be a better option. IOP reduction of steroid-induced glaucoma patients with previous vitrectomy or with corticosteroid administration other than ocular instillation might be more resistant to trabeculotomy. Trabeculotomy should be considered as an option for the surgical management of steroid-induced glaucoma, although future prospective studies are necessary to validate our findings.

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Biosketch

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ORIGINAL ARTICLE

Deleterious Role of Anti-high Mobility Group Box 1 Monoclonal Antibody in Retinal Ischemia-reperfusion Injury

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ABSTRACT

Purpose: To investigate the effect of anti-high mobility group box 1 (HMGB1) monoclonal antibody (mAb) against ischemia-reperfusion injury in the rat retina.

Materials and Methods: Retinal ischemia was induced by increasing and then maintaining intraocular pressure at 130 mmHg for 45 min. An intraperitoneal injection of anti-HMGB1 mAb was administered 30 min before ischemia. Retinal damage was evaluated at 7 days after the ischemia. Immunohistochemistry and image analysis were used to measure changes in the levels of reactive oxygen species (ROS) and the localization of anti-HMGB1 mAb. Dark-adapted full-field electroretinography (ERG) was also performed.

Results: Pretreatment with anti-HMGB1 mAb significantly enhanced the ischemic injury of the retina. HMGB1 expression increased at 6–12 h after ischemia in the retina. After the ischemia, production of ROS was detected in retinal cells. However, pretreatment with anti-HMGB1 mAb increased the production of ROS. On the seventh postoperative day, the amplitudes of both the ERG a- and b-waves were significantly higher in the vehicle group than in the groups pretreated with anti-HMGB1 mAb.

Conclusions: The current *in vivo* model of retinal injury demonstrated that anti-HMGB1 mAb plays a large deleterious role in ischemia-reperfusion injury. In order to develop neuroprotective therapeutic strategies for acute retinal ischemic disorders, further studies on anti-HMGB1 mAb function are needed.

Keywords: Anti-HMGB1 mAb, Retinal ischemia, Reactive oxygen species, Electroretinogram, Retinal thickness

INTRODUCTION

Ischemic injury to the retina is a major cause of visual loss and morbidity. As these morbidities are difficult to treat, research into various potential treatments is currently ongoing.[1–5] Ischemia-reperfusion injury involves many signaling mechanisms that ultimately result in necrotic and apoptotic cell death.[6] Delayed neuronal cell death in the brain and retina secondary to transient ischemic injury occurs, in part, by apoptosis.[7,8] During or after ischemia, reactive oxygen species (ROS) can be produced in large quantities and act as cytotoxic metabolites.[9] ROS can provoke cell death by reacting with cell components that lead to necrosis, or by activating specific targets that trigger apoptosis.

High-mobility group box-1 (HMGB1) protein was originally described 30 years ago as a nonhistone DNA-binding protein with high-electrophoretic mobility.[10] HMGB1 is a nuclear protein involved in nucleosome stabilization and gene transcription.[11] It is known that these functions are essential for survival, as HMGB1-deficient mice have been shown to die of hypoglycemia within 24 h of birth.[12] HMGB1 is

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found in almost all eukaryotic cells, and its presence has been confirmed in the rodent retina.[13] HMGB1 has also been implicated in the mechanism of ischemic brain damage.[14–20] In a stroke model, short hairpin (sh) RNA-mediated HMGB1 down-regulation in the brain reduces the severity of lesions.[15] Intravenous injection of the anti-HMGB1 monoclonal antibody (mAb) causes a dramatic reduction in the infarct size in the stroke model.[17]

The purpose of the present study was to investigate the role of anti-HMGB1 and its specific expression in retinal ischemia-reperfusion injury.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats weighing 200–250 g were obtained from Charles River Japan (Yokohama, Japan). Rats were permitted free access to standard rat food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animal care and all experiments were conducted in accordance with the approved standard guidelines for animal experimentation of the Kagawa University Faculty of Medicine, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anti-HMGB mAb or IgG2a was injected by three different methods. Intraperitoneal injection of anti-HMGB1 monoclonal antibody (mAb) (200 μg)[17] or class-matched control mAb (IgG2a) (200 μg) against *Keyhole Limpet* hemocyanin was administered 30 min before ischemia. Anti-HMGB mAb (200 μg) or IgG2a (200 μg) was administered intravenously immediately and 6 h after reperfusion. The pupil was dilated with topical phenylephrine hydrochloride and tropicamide; anti-HMGB mAb (20 μg) or IgG2a (20 μg) was injected into the vitreous space 30 min before ischemia.

Ischemia

Rats were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Abbott, Abbott Park, IL) followed by a topical administration of 0.4% oxybuprocaine hydrochloride. The anterior chamber of the right eye was cannulated with a 27-gauge infusion needle connected to a reservoir containing normal saline. The intraocular pressure (IOP) was raised to 130 mmHg for 45 min by elevating the saline reservoir. Only the right eye of each rat was subjected to ischemia. Retinal ischemia was indicated by whitening of the iris and fundus. The left eyes served as the sham-treated controls, with these eyes undergoing a similar procedure that did not include elevation of the saline bag, thus maintaining normal ocular tension. Rectal and tympanic temperatures were maintained during the operation at approximately 37°C via the use of a feedback-controlled heating pad (BRC, Nagoya, Japan). After restoration of blood flow, temperature was maintained continuously at 37°C.

HISTOLOGICAL EXAMINATION

For histological examination, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) 7 days after ischemia. Eyes were enucleated and stored in a 4% paraformaldehyde solution for 24 h at room temperature. The retinas were removed and embedded in paraffin, and thin sections (5-μm thickness) were cut using a microtome. Each retina was mounted on a silane-coated glass slide and then stained with hematoxylin and eosin (HE).

Morphometric analysis was performed to quantify ischemic injury. These sections were selected randomly in each eye. Light microscopic examination was performed by a person with no prior knowledge of the treatments. A microscopic image of sections obtained within 0.5–1 mm of the optic disc was scanned. For each computer image, the number of cells in the ganglion cell layer (GCL) was counted. The thicknesses of the inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and outer plexiform layer (OPL) for the entire frame were measured. The number of cells in the GCL was normalized as linear cell density (cells per millimeter). Thicknesses of the IPL, INL, ONL, and OPL were obtained by calculating the mean value of seven measurements in each eye. Similarly, the linear cell density in the GCL was also determined by calculating the mean value of seven measurements. For each animal, the right eye parameter was normalized to that of the intact left eye and shown as a percentage.

ELECTRORETINOGRAMS (ERGS)

ERG responses were measured after overnight dark adaptation (at least 6 h) using a recording device (Mayo Corporation, Aichi, Japan) 7 days after ischemia. Rats were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceuticals, Osaka, Japan). All procedures were performed in dim red light, with all rats kept warm during the procedure. The LED corneal electrode was set vertical to the cornea center. A reference electrode was set subcutaneously on the forehead and the ground connection was set on the base of the tail. An LED stimulator LS-W controlled the stimulus duration and intensity during the 11-step intensity series, which ranged from 0.0003–30 cds/m^2. The ERG response was amplified using an AC amplifier ML135 (Bio Amplifier, AD Instruments, NSW, Australia) with a bandwidth of 0.3–500 Hz and amplification of 2,000 times. The ischemic damage to the retina was

evaluated as the percentage of the a- and b-wave amplitudes of the ischemic right eyes as compared to the non-ischemic left eyes.

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Eyes were enucleated at 6, 12 or 24 h after 45 min of ischemia. Eyes were then fixed in 4% paraformaldehyde in the PBS and embedded in paraffin. Retinal sections (5 μm) were rinsed in 100% ethanol twice for 5 min each, followed by a separate 95% ethanol and 90% ethanol rinse for 3 min each. The sections were then washed using PBS, pH 7.4, three times for 10 min each and treated with 0.3% Triton X-100 in PBS, pH 7.4, for 1 h. After further washing three times for 10 min each with PBS, pH 7.4, sections were then blocked in 3% normal horse serum and 1% bovine serum albumin (BSA) in PBS for 1 h in order to reduce nonspecific labeling. Sections were incubated overnight at 4°C in PBS with either 2.0 mg/mL of monoclonal antibody against HMGB1[17] which served as the primary antibody. After washing in PBS for 50 min, sections were then immersed in the second antibody conjugated to horseradish peroxidase for 1 h at room temperature. Images were acquired using 40× objective lenses (DXM 1200; Nikon, Tokyo, Japan). Adobe PhotoShop v. 5.0 was used to adjust the brightness and contrast of the images.

FLUORESCENT LABELING OF ROS

To investigate the production of ROS, we intraperitoneally injected 5 mg/kg dihydroethidium (DHE; Sigma-Aldrich, St. Louis, MO) in 5% dimethyl sulfoxide (DMSO) in PBS 15 min before ischemia. A 0.3-mL aliquot of distilled water, 200 μg anti-HMGB1 mAb, or 200 μg IgG2a was administered intraperitoneally 30 min before ischemia. Eyes were enucleated 15 min after ischemia and then embedded in OCT compound (Sakura Finetek, Torrance, CA), after which cryosections (20 μm) were prepared. Sections were examined with a microscope (Radiance 2100/Rainbow, Carl Zeiss, München, Germany) using a laser set (excitation laser 514 nm; emission laser >580 nm).

Statistical Analysis

Fluorescence was quantified by automated image analysis with Image-Pro Plus software (version 4.0, Media Cybernetics, The Imaging Expert, Bethesda, MD). For each section, mean fluorescence was calculated from five separate high-power fields per eye. A threshold was set to define positive labeling.

All data are presented as the mean ± SD. Data were analyzed using an independent Student's *t*-test and ANOVA followed by Tukey-Kramer post-hoc testing corrected for multiple comparisons. Statistical analysis was performed using SPSS for Windows (SPSS Inc, Chicago, IL). A p value of < 0.05 was considered statistically significant.

RESULTS

Histologic Changes in the Retina after Ischemia with Anti-HMGB1

Figure 1A shows a normal retina. Light microscopic photographs were taken 7 days after ischemia and treatment with IgG2a (Figure 1B) or anti-HMGB1 (Figure 1C). In animals pretreated with IgG2a, the GCL cell number was reduced to 73.9 ± 16.2% of the control; the IPL thickness was reduced to 67.7 ± 14.6% of the control; the INL thickness was reduced to 82.8 ± 13.5% of the control; the OPL thickness was reduced to 88.6 ± 30.8% of the control; and the ONL thickness was reduced to 88.1 ± 13.0% of the control ($n=7$; Figure 1D). In animals pretreated with anti-HMGB1 mAb, the GCL cell numbers were 67.7 ± 16.8% of the control ($p=0.50$); the IPL thickness was 51.6 ± 12.3% of the control ($p=0.02$); the INL thickness was 69.0 ± 6.8% of the control ($p=0.03$); the OPL thickness was 51.9 ± 10.8% of the control ($p=0.01$); and the ONL thickness was 72.4 ± 13.7% of the control ($p=0.049$) ($n=7$; Figure 1D).

Treatment with intravenous injection of IgG2a or anti-HMGB1 mAb twice (immediately and 6 h after reperfusion) reduced the retinal thickness dramatically (n = 4, each group) (Figure 2).

Treatment with local administration of IgG2a or anti-HMGB1 mAb 30 min before ischemia was similar to the results with intraperitoneal injection of anti-HMGB1 mAb ($n=4$, each group) (Figure 3).

EFFECT OF ANTI-HMGB1 ON NORMAL RETINA

Animals were killed at 7 days after the intraperitoneal injection of anti-HMGB1 mAb or IgG2a. Treatment with anti-HMGB1 did not affect the retinal thickness in normal rat (Figure 4) ($n=4$, each group).

EFFECT OF ANTI-HMGB1 ON RETINAL FUNCTION

Scotopic ERG was recorded to evaluate anti-HMGB1 mAb effects on retinal function. A representative example of function is seen in Figure 5A. Mean amplitudes of the a- and b-wave are shown in Figure 5B. We observed a statistically significant difference between the three groups ($n=5$, each group).

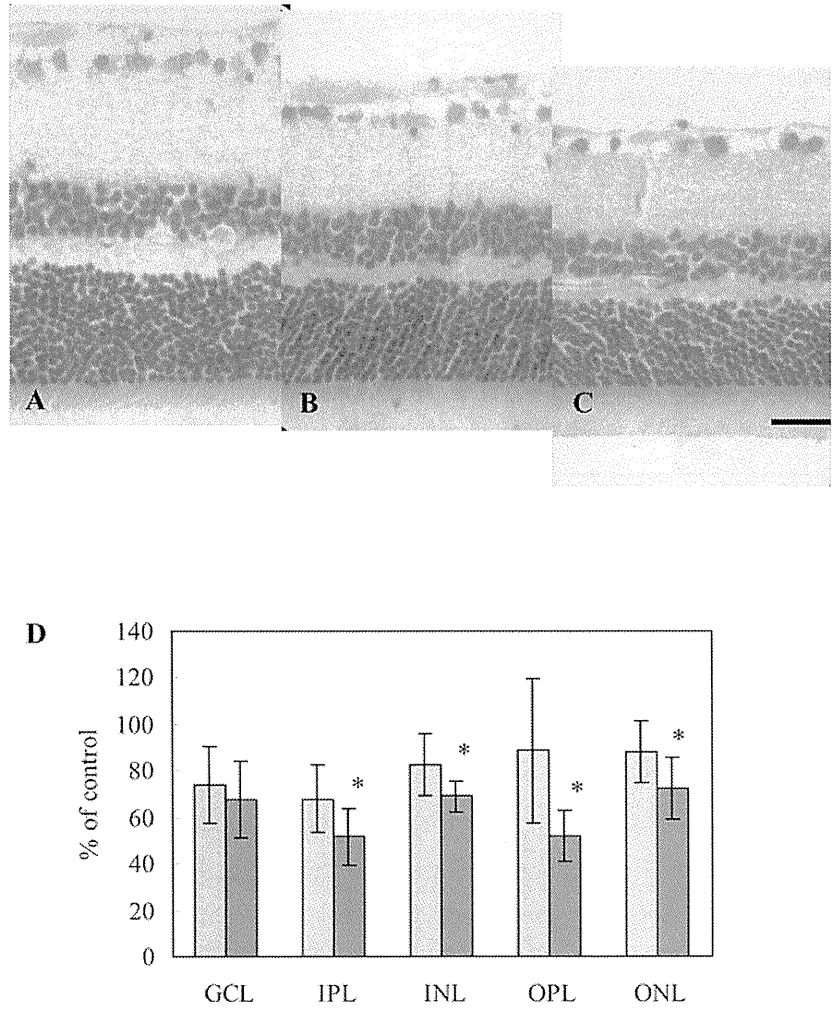

FIGURE 1 Light micrographs of a cross-section through normal rat retina (A) and at 7 days after ischemia when treated with control class-matched mAb (anti-*Keyhole Limpet* hemocyanin mAb, IgG2a) (B) or anti-HMGB1 mAb (C). Percentages indicate change relative to control values for the number of GCL cells and for the IPL, INL, ONL, and OPL thicknesses 7 days after ischemia when treated with IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD (*$p < 0.05$). Scale bar = 20 μm.

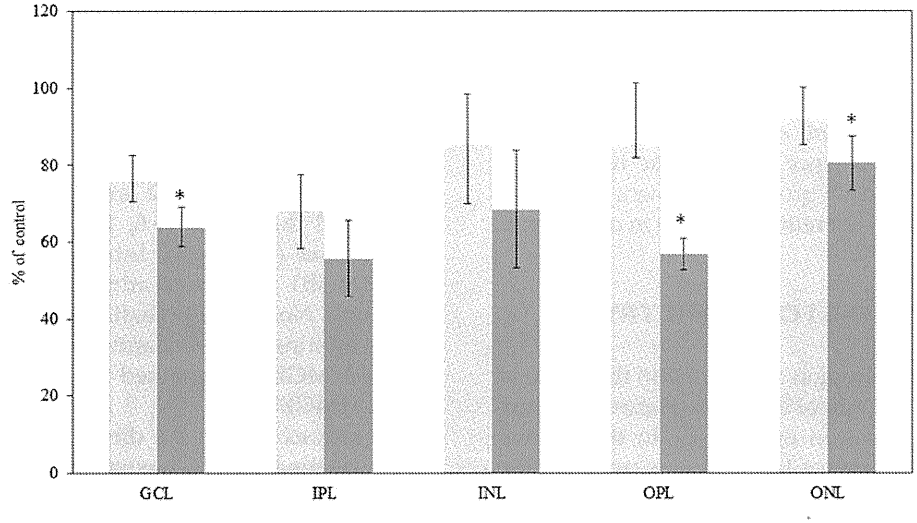

FIGURE 2 Percentages indicate change relative to control values for the number of RGC cells and for IPL, INL, ONL, and OPL thickness 7 days after ischemia when treated with intravenous injections of IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD (*$p < 0.05$).

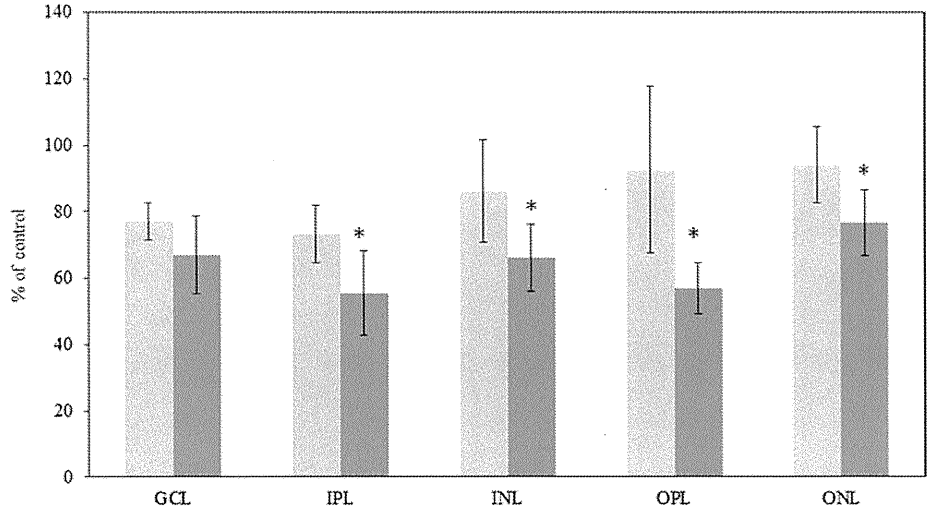

FIGURE 3 Percentages indicate change relative to control values for the number of RGC cells and for IPL, INL, ONL, and OPL thickness 7 days after ischemia when treated local administration of IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD (*$p < 0.05$).

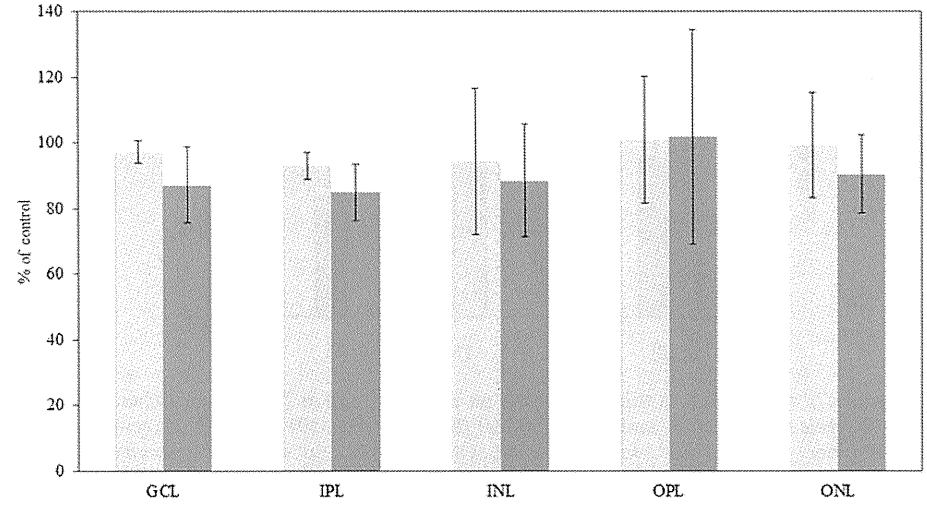

FIGURE 4 Percentages indicate change relative to control values for the number of RGC cells and for IPL, INL, ONL, and OPL thickness 7 days after treatment with IgG2a ( ) or anti-HMGB1 ( ). Results are expressed as the mean ± SD.

Scotopic ERG was measured 7 days after the intraperitoneal injection of anti-HMGB1 mAb. There was no significant difference between the two groups (Figure 6A, B) ($n=5$, each group). Treatment with anti-HMGB1 did not affect the retinal function in normal rat.

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We examined the expression of HMGB1 in the retina at 6, 12, and 24 h after 45 min of ischemia (Figure 7). Figure 3A shows the localization of HMGB1 in the normal retina. Immunostaining for HMGB1 was noted in the ONL in the normal retina. However, immunostaining for HMGB1 in the post-ischemic retina (Figure 7B–D) was detected in not only the ONL but also in the INL and GCL. A high degree of edema was noted in the post-ischemic retina.

We examined the effect of anti-HMGB1 mAb on endogenous HMGB1 expression in the normal retina and the retina at 6, 12, and 24 h after 45 min of ischemia (Figure 8). Intraperitoneal injection of anti-HMGB1 mAb was administered 30 min before ischemia. Normal eye balls were enucleated after 30 min administration of anti-HMGB1. Administration of anti-HMGB1 suppressed the expression of endogenous HMGB1.

We also examined the direct effect of anti-HMGB1 mAb on retinal HMBG1 expression (Figure 9). Normal eye balls were enucleated at 12 h after intravitreous injection of anti-HMGB1. Immunostaining for HMGB1 was not detected.

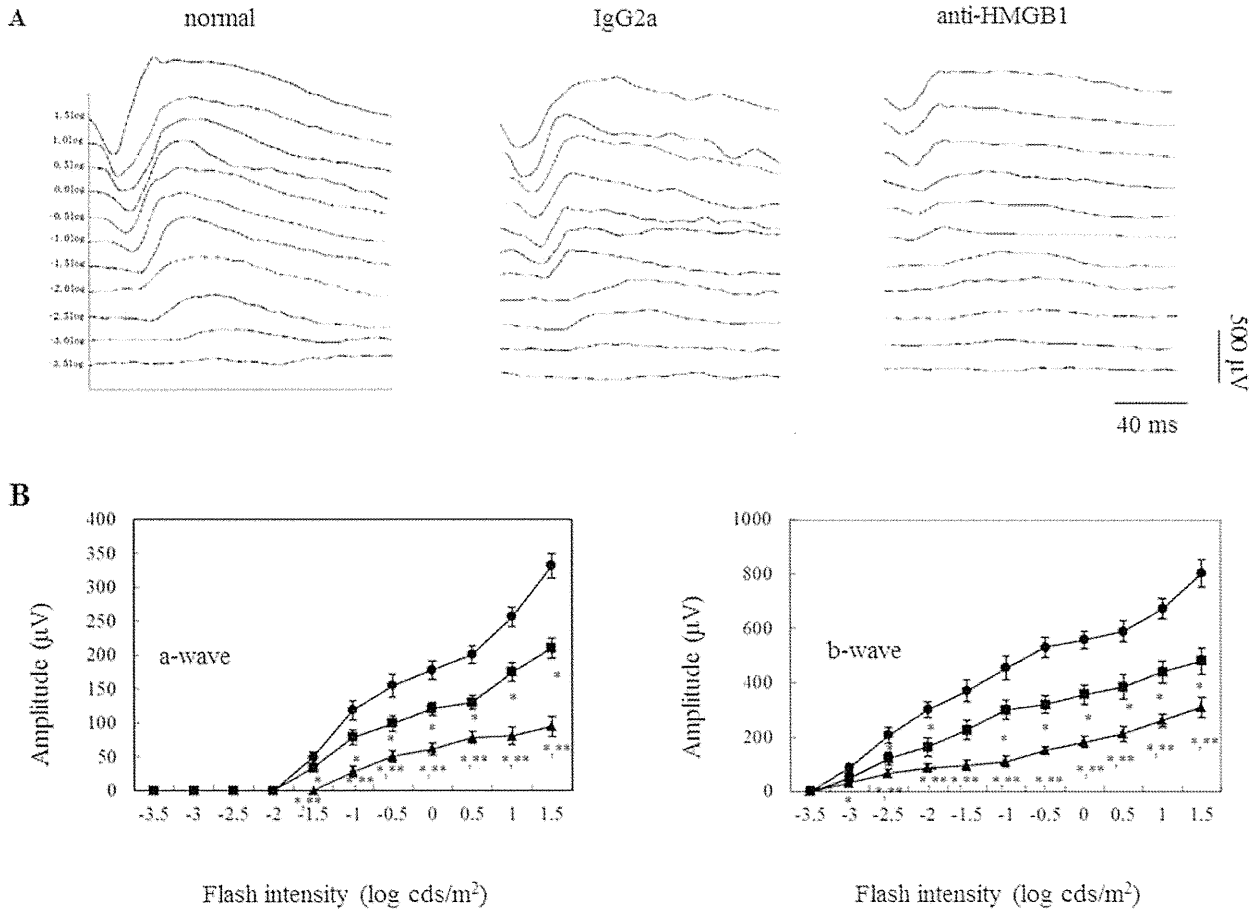

FIGURE 5 (A) Representative scotopic ERGs at baseline and at 7 days after ischemia when treated with control mAb or anti-HMGB1 mAb. (B) Amplitudes for a- and b-waves plotted as a function of flash intensity. Pretreatment with anti-HMGB1 mAb markedly reduced the amplitudes. Results are expressed as the mean ± SD. •: normal, ▪: IgG2a, ▲: anti-HMGB1. *$p<0.05$ versus normal retina. **$p<0.05$ versus ischemic retina with IgG2a.

ROS ACTIVATION BY ISCHEMIA

We used DHE staining to test whether ROS were enhanced by treatment with 200 μg anti-HMGB1 mAb. DHE specifically reacts with intracellular O_2^-, a ROS, and is converted to the red fluorescent compound ethidium in nuclei. In the post-ischemic retina, DHE fluorescence was clearly up-regulated in the retinal neuronal cells, and this up-regulation was efficiently enhanced by anti-HMGB1 mAb (Figure 10A–C). Figure 9D shows the quantified specific retinal DHE fluorescence. The mean ROS activation was significantly increased by treatment with anti-HMGB1 mAb ($n=4$, each group).

DISCUSSION

This study shows that, compared to the IgG2a treatment, pretreatment with anti-HMGB1 mAb significantly enhanced the ischemic injury of the retina. The results also showed that there was expression of HMGB1 mAb in the retina after ischemia-reperfusion injury.

A recent study showed that HMGB1 inhibited glial glutamate transport by GLAST in mouse gliosomes and suggested that HMGB1 can increase extracellular glutamate levels in ischemic brain.[21] We previously reported that anti-HMGB1 mAb suppressed ischemia-reperfusion-induced brain injury in a transient middle cerebral artery occlusion model in rats.[17] Based on these findings, we predicted that neutralizing mAb could be used to inhibit HMGB1 activity, and thus to significantly decrease the progression of the retinal ischemia-reperfusion injury. However, use of the neutralizing anti-HMGB1 mAb treatment in the present study remarkably increased the retinal damage following ischemia-reperfusion. This was due to an increased production of ROS caused by the anti-HMGB1 mAb treatment. Therefore, it might be possible that elevated levels of HMGB1 had neuroprotective effects against retinal ischemia- reperfusion injury. It has also been reported that treatment with anti-HMGB1 mAb increased ischemia-reperfusion

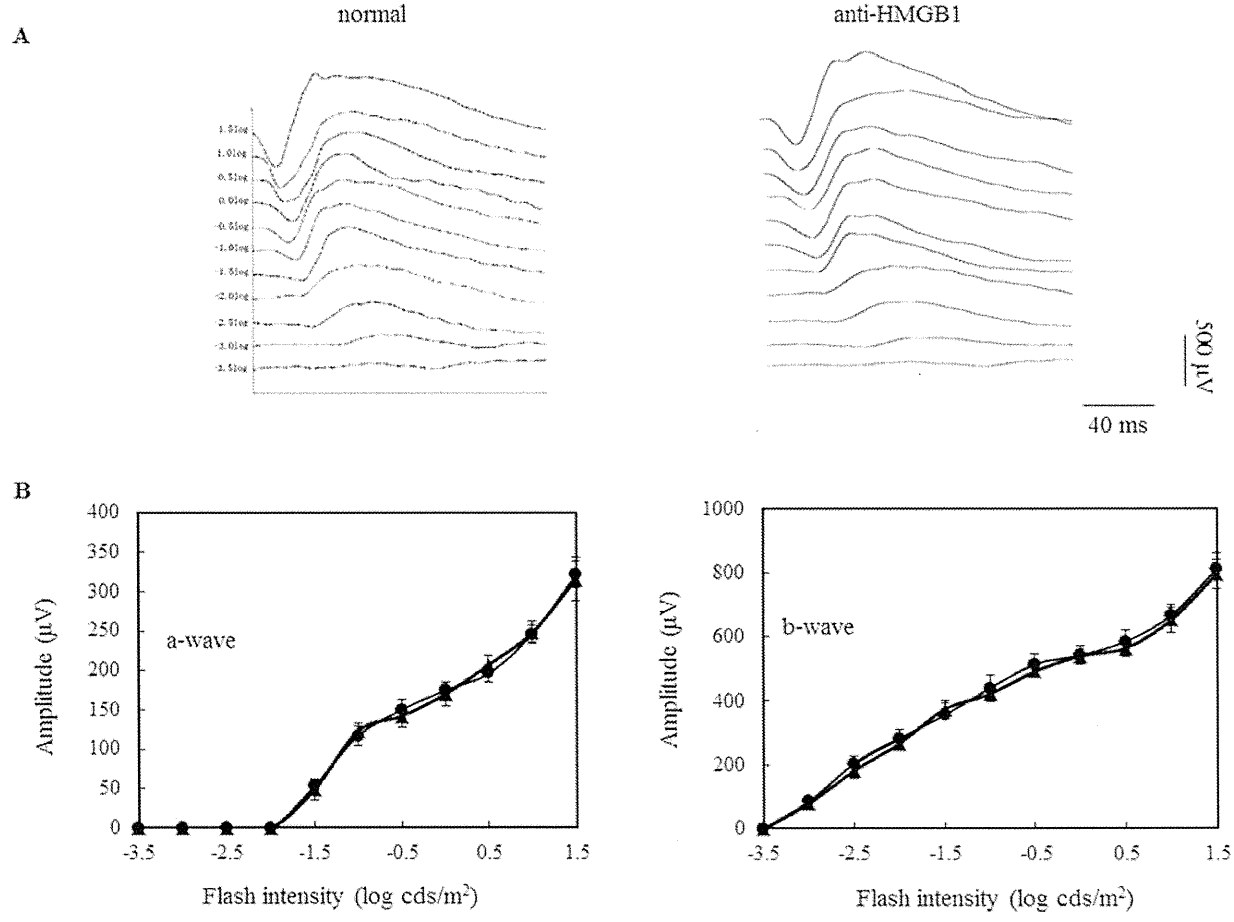

FIGURE 6 (A) Representative scotopic ERGs 7 days after treatment with anti-HMGB1. (B) Amplitudes for a- and b-waves plotted as a function of flash intensity. Results are expressed as the mean ± SD. •: normal, ▲: anti-HMGB1.

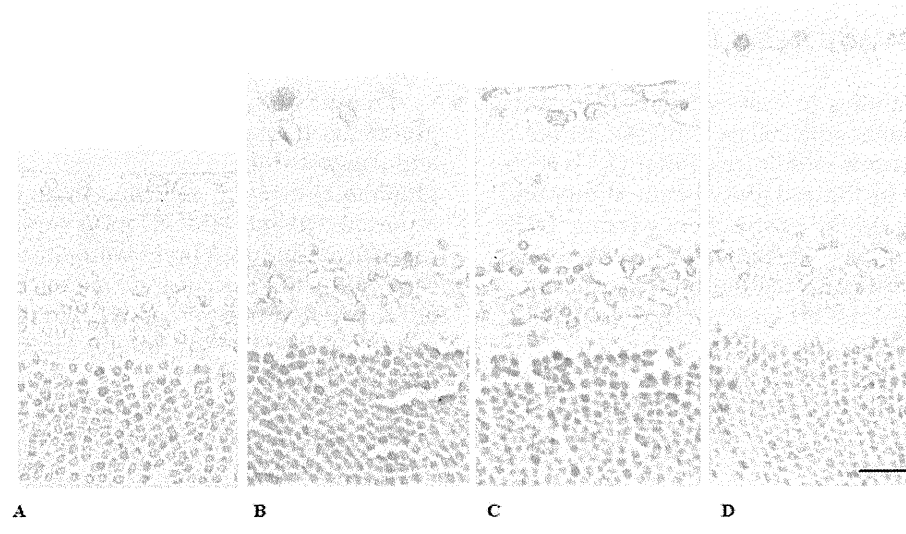

FIGURE 7 Immunohistochemical staining of HMGB1 expression in the retina. Retinal sections from normal animals (A) or 6 h (B), 12 h (C), or 24 h (D) after ischemia. Scale bar = 20 μm.

injury in the rat heart.[22] Therefore, it appears that the effect of anti-HMGB-1 mAb depends on the organ involved.

When the IOP is increased, glutamate is released from the retina during and after the ischemia.[3,23,24] The major causes of the cell death that occur after activation

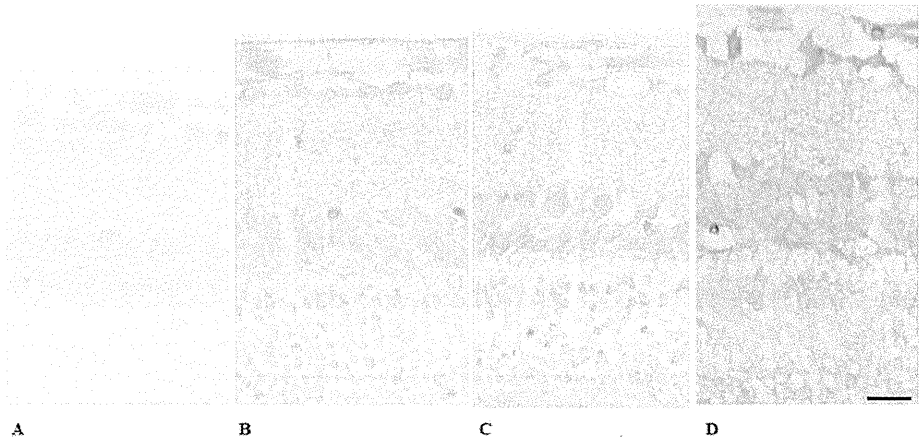

FIGURE 8 The effect of anti-HMGB1 mAb on endogenous HMGB1 expression. Intraperitoneal injection of anti-HMGB1 mAb was administered 30 min before ischemia. Retinal sections from normal animals (A) or 6 h (B), 12 h (C), or 24 h (D) after ischemia. Scale bar = 20 μm.

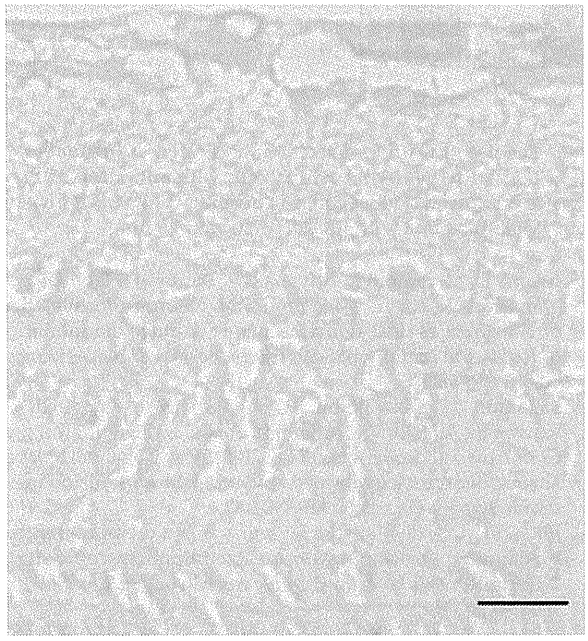

FIGURE 9 The direct effect of anti-HMGB1 mAb on endogenous HMGB1 expression in the normal retina. Scale bar = 20 μm.

of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors are related to the influx of calcium into the cells and the generation of free radicals.[25] Excessive accumulation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) can have a wide range of detrimental effects, including inhibition of mitochondrial function, reduction of cellular ATP levels, enhancement of ROS production, and activation of cellular proteases and nitric oxide (NO) synthase.[26] Since production of ROS was increased by anti-HMGB1 mAb treatment in the current study, anti-HMGB1 mAb played a large deleterious role in the resultant ischemia-reperfusion injury. In the present study, there was an increase in the HMGB1 level in the retina after the ischemia-reperfusion injury. These results suggest that endogenous HMGB1 released from retinal cells may modulate ischemia-reperfusion injury in the retina. Therefore, the anti-HMGB1 mAb treatment increased the delayed neuronal death.

We evaluated the functional retinal damage after ischemia-reperfusion injury by measuring the ERG a- and b-wave amplitudes. The b-wave of the ERG has been identified as a particularly sensitive index of retinal ischemia both in humans[25] and in experimental models of retinal ischemia in vitro.[27] After the anti-HMGB1 mAb treatment, there was a decrease in the thickness of ONL following ischemia-reperfusion, with the a-wave of the ERG also lower than that noted in the eyes treated with IgG2a. There was a good correlation between the ERG for both a- and b-waves and the histological results. It has been reported that administration of pentobarbital enhance the a- and b-wave of the ERG.[28,29] Under the anesthesia, many factors indirectly affecting the retinal activity could not be completely excluded.

It has been reported in previous studies that HMGB1 is expressed in GCL, INL, ONL, the inner and outer segments of photoreceptors, and in the retinal pigment epithelial cells in normal retina.[30,31] However, the current immunohistochemical study showed that HMGB1 was present in the ONL in normal retinas, which may be due to the use of different antibodies in the various studies (monoclonal antibody vs. polyclonal antibody).

HMGB1 passively released from necrotic cells.[32] Cell death was frequently observed in both the GCL and the INL after 3 h of ischemia-reperfusion.[33] In the present study, we demonstrated that immunostaining for HMGB1 in the post-ischemic retina was detected in not only the ONL but also in the INL and GCL. HMGB1 may play a key role in the protection of retinal injury after ischemia-reperfusion.

The current study showed, for the first time, that treatment with anti-HMGB1 mAb increased ischemia-reperfusion injury in the rat retina. Further investigations are needed to clarify the mechanism of anti-HMGB1 mAb in retinal ischemia-reperfusion

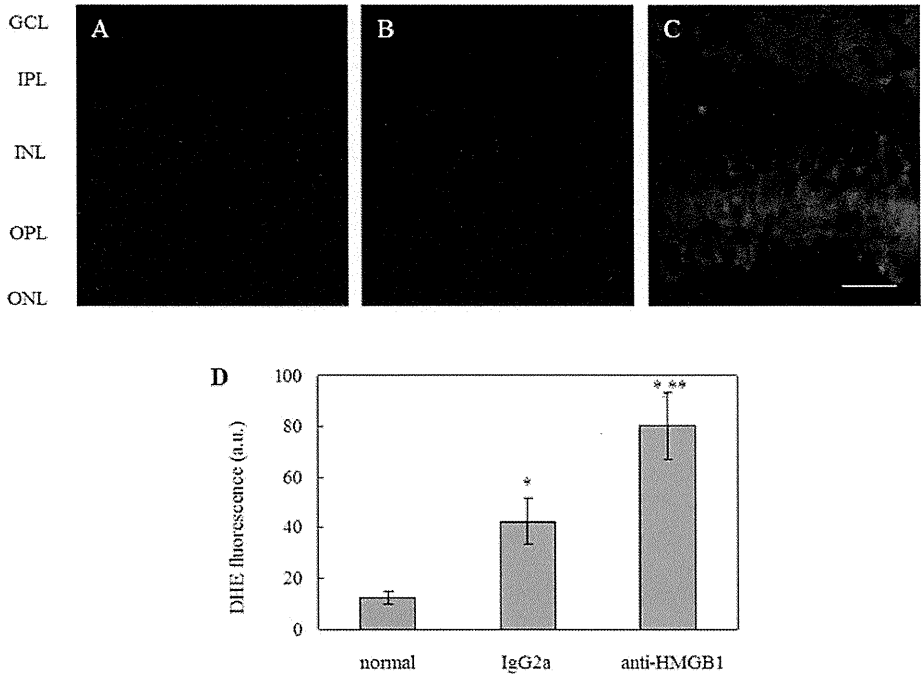

FIGURE 10 Effect of anti-HMGB1 mAb pretreatment on the release of ROS. The use of DHE to detect ROS indicated up-regulation of retinal neuronal cells in the retina after ischemia (IgG2a (B) as compared to normal retina (A)). Pretreatment with anti-HMGB1 mAb enhanced the level of ROS (C). (D) Quantified specific retinal DHE fluorescence is expressed for sections in arbitrary units (AU) for each group. Data express the mean ± SD; $*p < 0.05$ normal retina. $**p < 0.05$ versus ischemic retina with IgG2a. Scale bar = 20 μm.

injury. Additionally, anti-HMGB1 mAb function needs be further explored, as this could potentially lead to the development of neuroprotective therapeutic strategies for acute retinal ischemic disorders.

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LABORATORY INVESTIGATION

D-Allose as ischemic retina injury inhibitor during rabbit vitrectomy

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Abstract
Purpose To investigate the protective effects of D-allose, a rare sugar, on pressure-induced ischemia during vitrectomy in the rabbit eye.
Methods The rabbits underwent pars plana vitrectomy, and continuous intraocular irrigation at a perfusion pressure of 140 mmHg was performed for 45 min. Intraocular pressure was regulated by adjusting the height of a bottle of balanced saline solution containing D-allose. Morphometric studies were performed to study the effects of D-allose on the histological changes induced by ischemia in the rabbit retina. Electroretinograms (ERGs) were taken before and 1 and 7 days after vitrectomy. Nitroblue tetrazolium was used as an index of superoxide anion ($O_2^{\cdot-}$) generation. Data were analyzed by use of the unpaired Student's *t* test.
Results Seven days after ischemia, significant reductions in both number of ganglion cells and the thickness of the inner plexiform layer were observed. D-Allose significantly inhibited ischemic injury of the inner retina ($P < 0.05$). On postoperative day 7, amplitudes of ERG b-waves were significantly lower in the control group than in the D-allose group ($P < 0.05$). D-Allose suppressed the production of $O_2^{\cdot-}$.
Conclusions Intraocular irrigation with D-allose during vitrectomy may protect the retina against ischemia-induced damage.

Keywords D-Allose · Neuroprotection · Retinal ischemia · Superoxide anion · Vitrectomy

Introduction

Rare sugars are monosaccharides that exist in nature in limited quantities only. Whereas naturally abundant monosaccharides such as D-glucose and D-fructose are few in number, more than 50 kinds of rare sugars have been identified. D-Allose is a rare aldo-hexose sugar produced from D-psicose by enzymes from microorganisms [1]. Murata et al. [2] examined the scavenging activity of D-allose using electron spin resonance. They showed that D-allose inhibits the production of reactive oxygen species (ROS) in a dose-dependent manner. Furthermore, we recently reported that D-allose might protect neurons against retinal ischemia–reperfusion injury by reducing extracellular glutamate and attenuating oxidative stress [3].

Improvements in the techniques of pars plana vitrectomy (PPV) have resulted in better prognosis for vision-threatening eye diseases such as macular hole, epiretinal membrane, retinal detachment, and proliferative diabetic retinopathy (PDR). However, optic nerve atrophy after successful PPV for diabetic retinopathy is one of the most serious complications and can lead to blindness [4]. Because the extent of autoregulation in the retina and optic nerve head is limited, high infusion pressures during

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vitreous surgery may cause retinal ischemia and lead to damage of the retina and optic nerve.

During or after ischemia, ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-) can be produced in large quantities and act as cytotoxic metabolites. ROS have been implicated in the process of apoptosis, because treatment of cells with ROS can result in this form of cell death, whereas application of antioxidants can prevent it under some conditions [5, 6]. ROS scavengers have been shown to be neuroprotective against ischemia in acute experimental models [7].

The purpose of this study was to investigate the protective effects of D-allose on pressure-induced ischemia during vitrectomy in the rabbit eye.

Materials and methods

Animals

Female New Zealand White rabbits, weighing 2.0–2.4 kg, were obtained from Kitayama Labs KK (Nagano, Japan). The rabbits were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Animal care and experiments followed the standard guidelines for animal experimentation of Kagawa University Faculty of Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were divided into two groups according to the intraocular irrigating solutions they received: the vehicle group, using a balanced saline solution (BSS) alone, and the D-allose group, using BSS + D-allose (2%).

Vitrectomy and subsequent vitreous perfusion

A 250-ml bottle of intraocular irrigating solution (BSS; Alcon Laboratories, Fort Worth, TX, USA) was suspended approximately 100 cm above the eye level of each rabbit, and connected to a 20-gauge infusion cannula through a 200-cm-long tube. The infusion cannula was inserted through the sclerotomy in the inferonasal quadrant 3 mm posterior to the limbus, and sutured in place. A vitreous cutter (MVS XX; Alcon) was placed through the sclerotomy in the supratemporal quadrant, and vitrectomy was then performed for 10 min. The vitreous cavity was irrigated during vitrectomy either with BSS alone or with BSS + D-allose. Adequate illumination was provided by a paraxial light operation microscope without intraocular fiberoptic illumination. If retinal tear or retinal detachment occurred during vitrectomy, those animals were excluded from the experiment. After completion of the vitrectomy, the cutter was removed and the sclerotomy wound was tightly sutured using a 7-0 vicryl. Intraocular pressure (IOP) was raised to 140 mmHg for 45 m by elevating the solution bottle. Retinal ischemia was confirmed by whitening of the fundus. Rectal temperature was monitored throughout the surgery. Because body temperature during vitrectomy in acute ischemic eyes may affect ischemia-induced retinal damage [8, 9], rectal and tympanic temperature was maintained at approximately 37°C by use of a feedback-controlled heating pad (BRC, Nagoya, Japan) during the operation. After restoration of blood flow, the temperature was continuously maintained at 37°C. Eyes were treated topically with levofloxacin (Santen Pharmaceutical, Osaka, Japan) after surgery to prevent postoperative infection.

Electroretinograms (ERGs)

Before surgery, ERG responses were measured after 20 min of dark adaptation using a recording device (Mayo, Aichi, Japan). The procedure was repeated on postoperative days 1 and 7. ERGs were recorded by positioning the rabbit in a box and placing a xenon lamp 15 cm in front of the eye. A flash of maximum intensity 20 J provided photostimulation. A contact lens electrode was placed on the cornea, and a reference electrode subcutaneously at the vertex. ERGs were taken of both eyes for each animal. The a- and b-wave amplitudes of each operated eye were shown as a percentage of those in the pre-operated eye.

Histological examination

For histological examination, the rabbits were anesthetized by intraperitoneal injection of ketamine and xylazine 1 week after ischemia and perfused intracardially with phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in PBS. Eyes were removed and embedded in paraffin, and thin sections (5-μm thickness) were cut using a microtome. Each eye was mounted on a silane-coated glass slide and then stained with hematoxylin and eosin (HE).

Morphometric analysis was performed to quantify ischemic injury. The sections for analysis were selected randomly for each eye. A microscopic image of each section within 0.5–1 mm of the optic disc was scanned. In each computer image, the thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL) at the tire frame were measured. Finally, in each eye, the thicknesses of the IPL and INL were obtained as the mean values of five measurements. For each animal, these values for the right eye were normalized to those for the intact left eye and shown as a percentage.