Angiographic findings are useful for VKH diagnosis and evaluation of therapeutic effects [1, 5, 7–9, 12]. ICG angiography in acute phase VKH revealed a dark background, which was indistinct, and had fewer choroidal vessels and filling delays during the early phase.

We also examined the quantitative assessment of the choroidal circulation in VKH. Compared to controls, acute phase VKH patient choroidal τ values were significantly longer in the absence of ocular perfusion pressure changes, suggesting a vascular resistance increase and a disturbance of choroidal circulation during this phase. Histopathological studies of VKH in the acute phase have demonstrated that the choroid is markedly thickened and infiltrated by lymphocytes, epithelioid and giant cells [4]. Therefore, choroidal blood flow might decrease significantly, corresponding to edema or compression of the vessels by cell infiltration in the VKH acute phase. In the VKH recovery phase, choroidal τ was significantly shortened and returned to normal without changes in ocular perfusion pressure, indicating a choroidal vascular resistance decrease and improved circulation that is most likely due to the decreased inflammation.

In two patients, the choroidal τ values shortened without disappearance of serous retinal detachment. This suggests that choroidal filling velocity is not related to the presence of serous retinal detachment. In one patient, although choroidal background in the ICG seemed improved during the 2 weeks after corticosteroid treatment, the choroidal τ value was still high. With corticosteroid tapering, recurrence of serous retinal detachment was observed. Thus choroidal background improvement in ICG angiography may solely be due to disappearance of serous retinal detachment alone. However as seen in this case, the choroidal inflammation still remained and recurrence followed. This discrepancy between improvement of choroidal background and a long choroidal τ indicates that choroidal τ, which may reflect quantitative assessment of choroidal circulation, is important as an indicator of VKH treatment efficacy and as a prognostic factor for recurrence of serous retinal detachment.

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Another feature of CGD involves poor wound healing.[10] Therefore, subretinal granulation tissue mass in our case may represent an abnormal reparative response to previous chorioretinal injury. The absence of infection or granuloma, intravitreal pro-inflammatory cytokines, and improvement with immunosuppression suggests that his ocular disease is probably the result of aberrant inflammatory responses. Routine ocular biopsy is not recommended as part of standard ophthalmic evaluation.

R R Buggage
Laboratory of Immunology, National Eye Institute, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

R M Bauer II
Winn Army Hospital, Fort Stewart, GA, USA

S M Holland
Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

C I Santos
Department of Ophthalmology, University of Puerto Rico, San Juan, Puerto Rico

C-C Chan
Laboratory of Immunology, National Eye Institute, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Correspondence to: Chi-Chao Chan, MD, National Eye Institute, National Institutes of Health, 10 Center Drive, Bldg 10, Room 10N103, Bethesda, MD 20892-1857, USA; chanc@nei.nih.gov

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Ocular decompression retinopathy following trabeculectomy with mitomycin C associated with familial amyloidotic polyneuropathy

Familial amyloidotic polyneuropathy (FAP), a disorder inherited in autosomal dominant fashion, is characterised by systemic accumulation of polymerised mutated amyloidogenic TTR (ATTR) in peripheral nerves and in organs.[1] We report two patients with FAP ATTR Y114C (a point mutation, from tyrosine to cysteine, at codon 114) who developed diffuse retinal haemorrhages immediately after uncomplicated trabeculectomy with mitomycin C (MMC).

Case reports

A woman underwent vitrectomy for vitreous opacity associated with FAP ATTR Y114C in the left eye when she was 34 years old and in the right eye when she was 35 years old. Neurological examination revealed that she had polyneuropathy and autonomic dysfunction, and she underwent liver transplantation at 34 years old. Thereafter, intraocular pressure (IOP) in the left eye gradually increased and visual field loss progressed. Trabeculectomy with MMC in the left eye was performed when she was 39 years old. Preoperative IOP was 43 mm Hg despite maximal medical therapy. On the next postoperative day, IOP was 5 mm Hg, and fundus examination revealed scatter retinal haemorrhages in the periphery and posterior pole (fig 1). The haemorrhages had completely resolved and visual acuity was 20/20 in the left eye.

A woman underwent vitrectomy for vitreous opacity associated with FAP ATTR Y114C when she was 45 and trabeculectomy in the right eye at another hospital when she was 46, respectively. At 48, vitrectomy in the left eye was performed. She underwent liver transplantation at 50. Thereafter, IOP in the left eye gradually increased and non-penetrating trabeculectomy with MMC was performed when she was 51. However, IOP increased postoperatively, and trabeculectomy with MMC was added. IOP before the additional trabeculectomy was 37 mm Hg despite maximal medical therapy. On the next postoperative day, IOP was 5 mm Hg, and fundus examination revealed scatter retinal haemorrhages in the periphery and posterior pole involving the fovea. Although haemorrhages had almost resolved during the follow up period, visual acuity decreased from 20/20 to 20/50.

Comment

Retinal haemorrhages are a rare complication after filtration surgery.[2–6] Fechtner *et al* reported this condition for the first time under the descriptive term of ocular decompression retinopathy.[2] Affected patients are typically young, but not exclusively, with relatively high preoperative IOP, a very low postoperative IOP, and advanced cupping. Elevated IOP levels are generally significantly high in cases of secondary glaucoma related to FAP,[7] and diffuse retinal haemorrhages immediately after uncomplicated trabeculectomy with MMC are noted in both cases. One possible mechanism is hypothesised to be caused by a loss of autoregulation of retinal vessels, which overwhelms their capacity to respond to changes in IOP, resulting in retinal haemorrhages.[2] Our cases had an autonomic dysfunction as a systemic symptom, further increasing the susceptibility to such a phenomenon. Also, although both

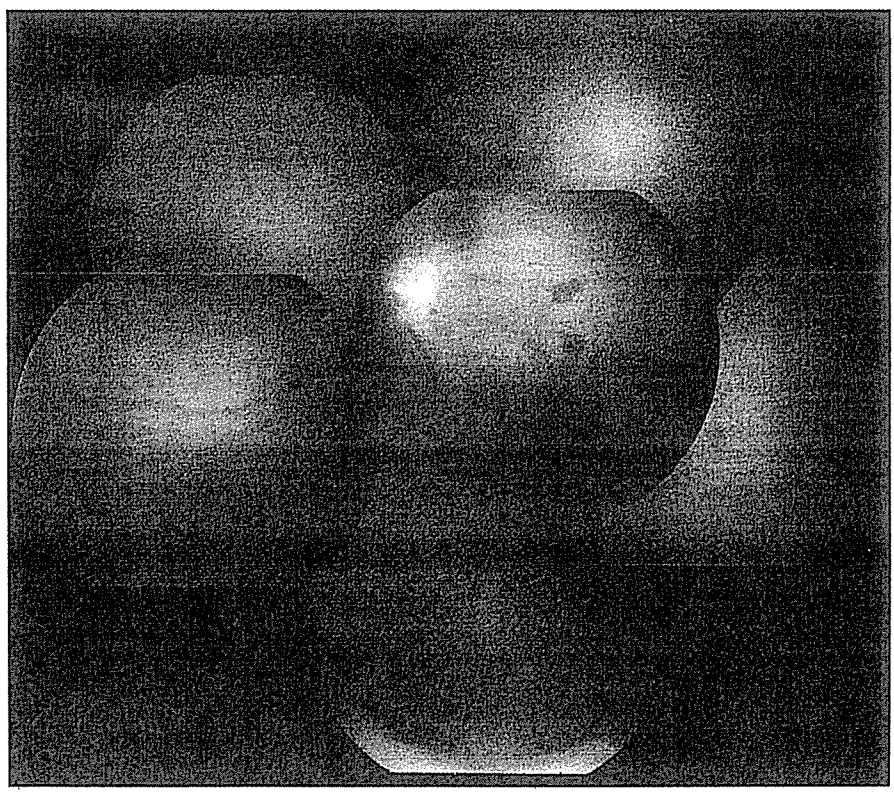

Figure 1 Scatter retinal haemorrhages in the periphery and posterior pole on the first postoperative day.

cases underwent trabeculectomy after vitrectomy, there were no complications, such as the collapse of eye, caused by a vitreous condition. Visual prognosis is usually benign after the resolution of haemorrhages; however, cases with poor visual acuity as shown in case 2 have also been described. Taken together, because clinical features in glaucoma secondary to FAP are related to the pathogenesis of this rare complication after trabeculectomy, early postoperative hypotony should be avoided to prevent it.

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M Wakita, T Kawaji, E Ando, T Koga, M Inatani, H Tanihara
Department of Ophthalmology and Visual Science, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

Y Ando
Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

Correspondence to: Takahiro Kawaji, MD, Ophthalmology and Visual Science, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan; kawag@white.plala.or.jp

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Dapsone induced haemolytic anaemia in patients treated for ocular cicatricial pemphigoid

Ocular cicatricial pemphigoid (OCP) is a systemic autoimmune disease of unknown aetiology. It causes a chronic, scarring conjunctivitis and frequently affects other mucous membranes. Definitive diagnosis is made by immunofluorescent staining of conjunctival tissue demonstrating IgG, IgM, and or IgA in the basement membrane.[1] Dapsone is an immunomodulating sulphonamide and has widely been used in the treatment of mild to moderate OCP.[2–4] All patients treated with dapsone show varying degrees of haemolysis.[5] Haemolytic anaemia, requiring withdrawal of therapy, has been shown to occur in approximately 10% of patients.[2 5]

The medical records of 12 patients treated with dapsone for ocular cicatricial disease were reviewed. Eleven of these patients were treated with dapsone as first line therapy and one as second line therapy; 11 patients had ocular cicatricial pemphigoid (OCP) and one had idiopathic cicatrising disease. There were an equal number of male and females in this group with a mean age of 72 years (range 55–89 years). The daily dose of dapsone was consistent at 50 mg twice daily taken orally. Mean follow up time while on dapsone therapy was 19 months with a range of 1–60 months.

Six (50%) patients had reticulocytosis including four (33%) with clinically significant haemolytic anaemia with a raised mean cell volume and a steady fall in haemoglobin from baseline. The development of the haemolytic anaemia was not dose dependent and all the patients had a diagnosis of OCP (table 1).

Our cohort of patients had a much higher rate of haemolytic anaemia than previously published reports.[2 5] Although our study sample is small, the proportion of patients with haemolytic anameia is striking. Patients who are glucose-6-phosphate hydrogenase (G6PD) deficient are known to be more at risk of developing haemolytic anaemia.[4] We do not routinely check for this rare deficiency in our department. Clinically significant dapsone induced haemolytic anaemia may occur more frequently than previously expected and clinicians should be acutely aware of any downward trend in haemoglobin from baseline. We no longer use dapsone as a first line agent in the management of OCP.

M S Wertheim, J J Males, S D Cook, D M Tole
Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX, UK

Correspondence to: Michael S Wertheim, Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX, UK; drwertie@hotmail.com

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Bilateral juxtapapillary choroidal neovascularisation associated with interferon alfa treatment of a metastatic cutaneous melanoma

Interferon alfa (IFNα) is commonly used in the treatment of many neoplastic diseases owing to its antiproliferative and immunomodulatory effects. IFNα is used in adjuvant therapy of melanoma stage IIa/b or higher.[1] A wide variety of ocular adverse events related to IFN therapy have been reported during the past decades.[2 3] A case of bilateral juxtapapillary choroidal neovascularisation is described here.

Case report

A 48 year old woman reported acute vision loss in her left eye (LE) 1 week after starting treatment with IFNα for a cutaneous metastasic melanoma. She had been receiving IFNα, 5 million international units (MIU), subcutaneously three times a week. On examination, visual acuity (VA) was right eye (RE) 20/50 and LE 20/60. Funduscopy showed bilateral optic disc oedema and subretinal haemorrhages in inferior temporal and nasal arcades. To rule out any cause of papilloedema a brain computed tomography was performed, which was normal. One month later, IFNα doses were increased to 8 MIU; VA decreased to RE 20/100 and LE counting fingers at 9 feet. Funduscopy showed bilateral optic disc oedema, bilateral juxtapapillary serous retinal detachment, and subretinal haemorrhages (fig 1A, B).

Table 1 Patients with haematological complications from dapsone

Patient	Age (years)	Sex	Diagnosis	Dapsone dose	Complication
1	77	M	OCP	50 mg twice daily	Reticulocytosis (with normal Hb)
2	78	M	OCP	50 mg twice daily	Haemolytic anaemia*
3	67	M	OCP	50 mg twice daily	Reticulocytosis (with normal Hb)
4	60	F	OCP	50 mg twice daily	Haemolytic anaemia*
5	89	F	OCP	50 mg twice daily	Haemolytic anaemia*
6	85	F	OCP	50 mg twice daily	Haemolytic anaemia*

OCP, ocular cicatricial pemphigoid, Hb, haemoglobin, *dapsone withdrawn.

Posterior Vitreous Detachment Induced by Nattokinase (Subtilisin NAT): A Novel Enzyme for Pharmacologic Vitreolysis

Akiomi Takano,[1] *Akira Hirata,*[1] *Kazuya Ogasawara,*[2] *Nina Sagara,*[1] *Yasuya Inomata,*[1] *Takahiro Kawaji,*[1] *and Hidenobu Tanihara*[1]

Purpose. To investigate the effects of intravitreal injection of nattokinase (subtilisin NAT), a serine protease that is produced by *Bacillus subtilis* (natto), for induction of posterior vitreous detachment (PVD).

Methods. Different doses of nattokinase (1, 0.1, or 0.01 fibrin-degradation units [FU]) or physiologic saline as a control were injected into the vitreous cavity of rabbit eyes. Scanning electron microscopy was used to observe the retinal surfaces of four rabbit eyes per concentration. Histologic alterations were assessed by light microscopy, using four eyes from each group. Electroretinography (ERG) was performed to observe retinal function, ranging from 1 hour to 1 week after the nattokinase (1 or 0.1 FU) or saline solution administration, using four eyes from each group at each time point. Also, findings in all rabbits were monitored by slit lamp examination and by indirect ophthalmoscopy with a 20-D lens.

Results. Scanning electron microscopy showed smooth retinal surfaces, indicating the occurrence of PVD at 30 minutes after intervention in all the experimental eyes injected with 0.1 or 1.0 FU nattokinase, but none of the control eyes. Light microscopy and ERG analysis showed no critical change even after the use of 0.1 FU nattokinase, an amount sufficient to induce PVD. However, toxicity in the forms of preretinal hemorrhage and ERG changes was noted with the higher dose (1 FU) of nattokinase.

Conclusions. The results suggested that nattokinase is a useful enzyme for pharmacologic vitreolysis because of its efficacy in inducing PVD. (*Invest Ophthalmol Vis Sci.* 2006;47: 2075–2079) DOI:10.1167/iovs.05-0130

From the [1]Department of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan; and the [2]Japan Bio Science Laboratory Co., Ltd., Oita, Japan.

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Corresponding author: Hidenobu Tanihara, Department of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjo, Kumamoto 860-8556, Japan; tanihara@pearl.ocn.ne.jp.

The vitreous is composed of a network of two major constituents, collagen, and hyaluronan.[1] In patients with proliferative vitreoretinal disorders, the formation and recurrence of proliferative tissue at the vitreoretinal interface often cause a high incidence of vitreous hemorrhage, retinal detachment, and resultant visual loss after vitreous surgeries. The current treatment for vision-threatening proliferative vitreoretinal diseases consists of vitrectomy to remove the proliferative tissue and its scaffold of vitreous from the retinal surface surgically.

However, the surgical procedure for the creation of posterior vitreous detachment (PVD) is associated with a potential risk of the onset of retinal breaks and hemorrhage. In addition, even after vitreous surgeries, the occurrence and progression of cell proliferation on the residual vitreous gel sometimes cause serious postoperative complications, such as proliferative vitreoretinopathy (PVR). Therefore, the development of an enzymatic treatment for the complete removal of the vitreous (and associated proliferative tissue) is desirable, to reduce the incidence of complications during and after vitreous surgeries.[2–5]

The vitreous is composed mainly of collagen and hyaluronan; therefore, hyaluronidase has been recommended for intravitreal injection to facilitate vitreous liquefaction and vitreous hemorrhage absorption.[6,7] Plasmin has been also reported to be an effective adjunct for the induction of PVD.[8–14] Previous reports on the clinical use of plasmin purified from autologous blood showed the usefulness of enzyme-assisted vitrectomy for vitreoretinal diseases.[15–18]

Nattokinase (subtilisin NAT) is a serine protease composed of 275 amino acids that is produced by *Bacillus subtilis* (natto).[19] It has potent fibrinolytic activity, enhances plasminogen activators, and inactivates a plasminogen activator inhibitor.[20–22] It also has fibrinolytic activity when administered orally and is widely available in processed and health foods containing natto (fermented soybean) extracts.[21] Therefore, we hypothesized that nattokinase might be an effective pharmacologic adjunct to surgery to induce PVD in patients with vitreoretinal disorders.

In the present study, we determined the efficacy and the safety of nattokinase for pharmacologic vitreolysis.

Materials and Methods

Experimental Animals

Japanese adult albino rabbits (Kyudo, Kumamoto, Japan), 12 weeks old and weighing 2.0 kg, were used in the study. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Committee on Animal Research of Kumamoto University.

Nattokinase Preparation

Nattokinase was supplied by Japan Bio Science Laboratory Co., Ltd. (JBSL; Oita, Japan). It was purified from fermented soybean extract

(product name NSK-SD; JBSL) including rich nattokinase produced by *B. subtilis* (natto), as follows. The fermented soybean extract (NSK-SD) was dissolved in 2 mM calcium acetate, dialyzed overnight in 10 mM phosphate buffer (pH 7.0), adsorbed on a pre-equilibrated Sepharose column (CM-Sepharose Fast Flow; GE Healthcare, Piscataway, NJ), and finally washed and eluted with a linear gradient of 10 mM phosphate buffer (pH 7.0) containing 500 mM sodium chloride. To obtain the active enzyme fraction, the sample was gel-filtrated in a Sephacryl column (S-100 HR; GE Healthcare) equilibrated with 10 mM phosphate buffer (pH 7.5) containing 150 mM of sodium chloride. The active enzyme fraction was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and confirmed as a single band.

Nattokinase Activity

Nattokinase activity was measured with a fibrin degradation assay developed by JBSL. First, 0.4 mL of 0.72% fibrinogen (fibrinogen fraction I, type I-S, product number F8630; Sigma-Aldrich, St. Louis, MO) was placed in a test tube (15-mm inner diameter × 150-mm length) with 1.4 mL of 50 mM borate buffer (pH 8.5) containing 0.9% sodium chloride and incubated in a constant-temperature water bath at 37 ± 0.3°C for 5 minutes. Then, 0.1 mL of a 20-U/mL thrombin solution (product number T6634; Sigma-Aldrich) was added. The solution was incubated at 37 ± 0.3°C for exactly 10 minutes, 0.1 mL of sample solution was added, and incubation continued at 37 ± 0.3°C. This solution was again mixed after 20 and 40 minutes. At exactly 60 minutes, 2 mL of 200 mM trichloroacetic acid was added, mixed, and the solution was incubated at 37 ± 0.3°C for 20 minutes. This solution was placed in a microcentrifuge tube and centrifuged at 15,000*g* for 5 minutes. Then, 1 mL of supernatant was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01-per-minute increase in absorbance at 275 nm of the reaction solution (excluding acid-insoluble material).

The substrate (fibrinogen) used in this assay is naturally derived, and so there can be considerable differences in quality among product lots. This makes it difficult to determine absolute values for enzyme activity. Therefore, JBSL also supplies a standard enzyme for use in the assay. The measured value divided by the labeled standard enzyme activity was used as a correction factor. All assay values were then multiplied by this correction factor. Measurement of human plasmin activity by this assay showed that 1 unit of human plasmin (Calbiochem, La Jolla, CA) had an activity equivalent to 1 FU. These activities were measured by JBSL, and the prepared nattokinase was used immediately after measurement of the activity.

Anesthesia

The rabbits were anesthetized for each procedure with pentobarbital (20 mg/kg injected intravenously) and ketamine hydrochloride (20 mg/kg injected intramuscularly).

Administration of Nattokinase

Rabbits were randomized into four groups. Group 1 received 1 FU nattokinase, group 2 received 0.1 FU, group 3 received 0.01 FU, and group 4 received physiologic saline (BSS Plus; Alcon Surgical, Tokyo, Japan) in one eye. The pupils were dilated with a mixture of 0.5% tropicamide and 0.5% phenylephrine hydrochloride. Nattokinase activity was adjusted by dilution with physiologic saline. After filter sterilization using a 0.22-μm membrane filter, 1-mL syringes of nattokinase dilutions were prepared (1 FU/0.1 mL, 0.1 FU/0.1 mL, and 0.01 FU/0.1 mL). A vitrectomy contact lens was placed on the cornea, then under an ophthalmic microscope, a 30-gauge needle attached to a syringe was inserted at a point 2 mm from the corneal limbus. Then, 0.1 mL of nattokinase solution was carefully and slowly injected into the center of the vitreous cavity. Control eyes were injected in a similar manner with 0.1 mL physiologic saline. Because of increased intraocular pressure after injection, anterior chamber paracentesis was performed immediately with a 20-gauge ophthalmic knife to normalize the pressure and prevent the solution from coming out of the eye.

Scanning Electron Microscopy

Sixteen eyes were studied with scanning electron microscopy (SEM). Nattokinase (1, 0.1, or 0.01 FU) or saline was injected into the vitreous cavity of four rabbit eyes per concentration. At 30 minutes after injection, the eyes were enucleated and fixed with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer at 4°C. One hour later, the eyes were cut circumferentially at the limbus to make posterior cups and then placed in fixative solution (the same one as above) overnight. The posterior cup was cut across and vertically with a razor to prepare samples for SEM. The samples were placed in 2% tannic acid (Wako Pure Chemicals, Osaka, Japan) and allowed to stand overnight at 4°C. Then, the samples were washed six times with phosphate-buffered saline at 20-minute intervals, placed in 2% osmium on ice, and allowed to stand for 70 minutes. After recovery of the 2% osmium, the samples were washed three times with distilled water, dehydrated in an ethanol series (50% for 15 minutes, 70% for 15 minutes, 90% for 15 minutes, 95% for 15 minutes, and twice in 99.5% for 30 minutes), and immersed in *t*-butyl alcohol (20 minutes, three times). After freezing, the samples were freeze dried, mounted on an aluminum stage with double-sided carbon tape, coated with gold (JFC-1200; JEOL, Tokyo, Japan), and examined with a scanning electron microscope (JSM-5800 LV; JEOL).

Light Microscopy and Electroretinography

To evaluate the short-term effects of intraocular injection of nattokinase, 30 minutes after intravitreal injection of nattokinase (1, 0.1, or 0.01 FU) or saline solution as a control, four eyes from each group were used for a light microscopic study of the retina. The eyes were fixed with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer for 24 hours and embedded in paraffin, and horizontal sections were made and stained with hematoxylin and eosin.

Also, to investigate the adverse effects of nattokinase, we obtained scotopic full-field electroretinograms (ERGs) at times ranging from 1 hour to 1 week after the nattokinase (1 or 0.1 FU) or saline administration, using four eyes from each group at each time point. The pupils were dilated and dark adapted for 30 minutes before recordings. White-light–emitting diode built-in contact lens electrodes (LW-102; Tomey, Waltham, MA) were put on the cornea, and an electrode was attached to the forehead after the hair was shaved from the area. A ground electrode was attached to the ear. Stimuli (6000 cd/m^2, 0.5 ms) were delivered, and ERGs were recorded (LE-2000; Tomey). Statistical analyses were performed on a computer (StatView, ver. 5.0; SAS Institute, Inc., Cary, NC). In addition, a light microscopic study of the retina was performed 1 week after the administration of 1 or 0.1 FU nattokinase or saline solution in four eyes from each group.

Slit Lamp Examination and Indirect Ophthalmoscopy

Findings in all rabbits were monitored by slit lamp examination and by indirect ophthalmoscopy with a 20-D lens.

Results

Scanning Electron Microscopy

Figure 1 shows an example of the retinal surface observed by SEM. In all the saline-injected eyes, SEM showed very dense vitreous fibers covering the entire area of the retinal surface (Fig. 1, top left). All eyes treated with 0.01 FU nattokinase still showed numerous vitreous fibers covering the retinal surface (Fig. 1, top right). These fibers were attached to the retina, particularly in the area of the medullary rays. In contrast, all eyes in the groups treated with 0.1 or 1 FU nattokinase exhib-

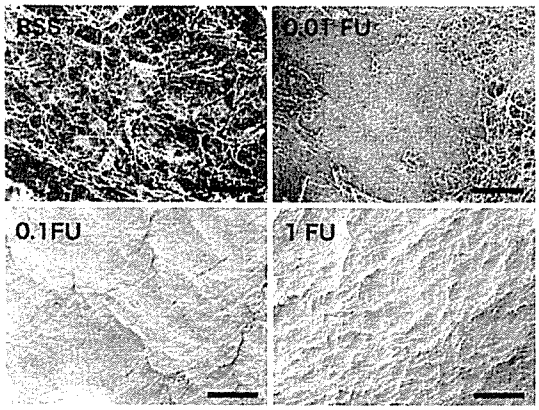

FIGURE 1. Scanning electron microscopy (SEM) image of the retinal surface 30 minutes after an intravitreal injection of physiologic saline (*top left*) or 0.01 (*top right*), 0.1 (*bottom left*), or 1 (*bottom right*) FU nattokinase. In the saline-injected eyes, SEM showed very dense vitreous fibers covering the retinal surface. In contrast, the eyes treated with 0.1 or 1 FU nattokinase exhibited smooth retinal surfaces, indicating the occurrence of posterior vitreous detachment. Bar, 50 μm.

ited smooth retinal surfaces without any remnant of vitreous fibers, except in the regions of the medullary rays (Fig. 1, bottom).

Light Microscopy and ERG

Figure 2 shows an example of the retinal section 30 minutes after the administration of nattokinase. All eyes showed intact retinal surfaces and no abnormalities compared with control eyes.

Figure 3 shows the ERG findings for tested eyes. The ERG a- and b-waves in control eyes showed no significant reductions in the mean amplitudes from the preoperative examination ($P > 0.1$). In eyes treated with 0.1 FU nattokinase, the a-wave showed no significant reductions. Temporary reduction of the b-wave was observed 1 day after the administration of 0.1 FU nattokinase ($P = 0.021$); however, amplitude recovered 1 week after the intervention ($P = 0.170$). In eyes injected with 1 FU nattokinase, the b-wave amplitude 1 day and 1 week after injection showed significant reductions ($P = 0.012$ and 0.045, respectively). The a-wave 1 week after injection also showed significant reduction ($P = 0.006$). The histology of the retina 1 week after administration of saline solution or 0.1 FU nattokinase showed no adverse change. One week after 1 FU nattokinase, however, slight thinning of the inner plexiform layer was observed (Fig. 4).

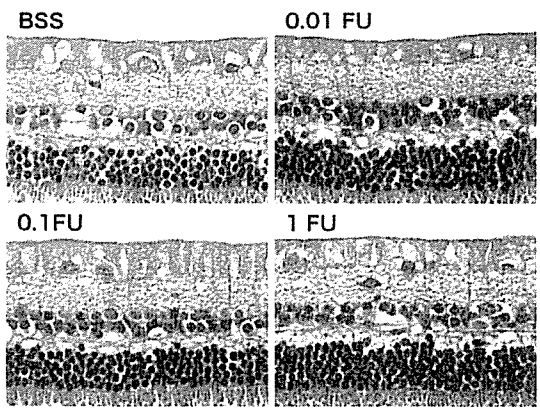

FIGURE 2. Light micrograph of rabbit retinas 30 minutes after an intravitreal injection of saline solution (*top left*) or 0.01 (*top right*), 0.1 (*bottom left*), or 1 (*bottom right*) FU nattokinase. There were no abnormalities compared with the control eyes. Magnification, ×400.

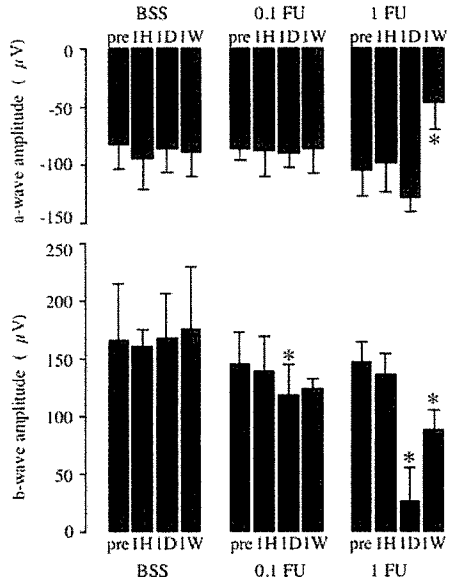

FIGURE 3. The mean (±SD) of ERG a- and b-waves before and after nattokinase injection (*$P < 0.05$). Data are shown for injection of physiologic saline or 0.1 or 1 FU nattokinase. pre, before injection; 1 H, 1 hour; 1 D, 1 day; and 1 W, 1 week after injection.

Slit Lamp Examination and Indirect Ophthalmoscopy

Slit lamp examination and indirect ophthalmoscopy during follow-up showed no evidence of hemorrhage, retinal detach-

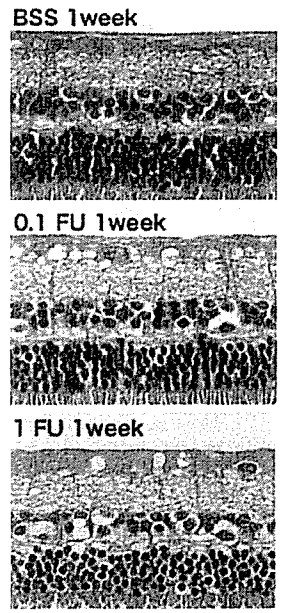

FIGURE 4. Light micrograph of rabbit retinas 1 week after an intravitreal injection of saline solution (*top*) or 0.1 (*middle*) or 1 FU nattokinase (*bottom*). Histology 1 week after the administration of saline or 0.1 FU nattokinase showed no adverse change. One week after injection of 1 FU nattokinase, however, slight thinning of the inner plexiform layer was observed. Magnification, ×400.

ment, or any other complications after the use of 0.01 FU nattokinase in all the tested eyes. Also, all eyes injected with 0.1 FU nattokinase, which was sufficient to induce PVD, showed no abnormalities compared with control eyes. However, 1 day after injection of 1 FU nattokinase, we observed mild preretinal hemorrhages near the optic disc in all the eyes. One week after injection of 1 FU nattokinase, the mild hemorrhages were still observed, but the area gradually reduced.

Discussion

Safer surgical procedures for removing the vitreous in the treatment of vitreoretinal diseases such as proliferative diabetic retinopathy (PVR) and numerous macular diseases are constantly being sought. Among them, enzyme-assisted vitrectomy has been advocated as a useful surgical modality for the treatment of vitreoretinal diseases. Hyaluronidase, which breaks down hyaluronan, facilitates vitreous liquefaction and vitreous hemorrhage absorption; however, it is known that hyaluronidase does not induce PVD.[23,24] In contrast, it has been reported that autologous plasmin is useful in inducing PVD and in facilitating vitreoretinal surgery in clinical cases.[15–18] However, the purification of autologous plasmin requires sterile facilities and trained personnel to perform. Thus, the development of another form has been regarded as important in the treatment of many proliferative vitreoretinal diseases and macular diseases.

In this study, ultrastructural observation with SEM revealed a loss of vitreous fibers on the retinal surface, suggesting the occurrence of PVD induced by nattokinase. Also, concerning the safety of the nattokinase injection, the histology of the retina 30 minutes after the administration of several doses of nattokinase showed no significant damage. In addition, ERGs after the injection of amounts of nattokinase sufficient to induce PVD revealed no critical reductions in the a- and b-wave amplitudes. These results suggest that using an adequate dose of nattokinase as a pharmacologic adjunct to pharmacologic vitreolysis and/or enzyme-assisted vitrectomy in patients with vitreoretinal disorders is very promising.

The mass purification of this *B. subtilis* (natto)-derived enzyme is achieved with an easy and convenient protocol in comparison with plasmin. The clinical application of this enzyme will enable us to perform pharmacologic vitreolysis and/or enzyme-assisted vitrectomy even in medical centers without the facilities or trained personnel to purify autologous plasmin, and in patients requiring urgent surgery. In addition, unlike with autologous plasmin, we can obtain uniform enzymatic activity by the use of purified nattokinase among individual cases. These advantages make nattokinase a reasonable alternative in all medical centers for performing safer and less invasive treatment for vitreoretinal disorders.

Nattokinase hydrolyzes collagen fiber, which is a matrix component of the vitreous, indicating effectiveness in liquefying the vitreous gel when injected into the vitreous cavity. In the present study, SEM showed smooth retinal surfaces without vitreous fibers, suggesting strong and broad cleavage of some materials in the interface between the retina and vitreous by nattokinase. Regarding the mechanisms of PVD induction, our previous study suggested that the activation of endogenous matrix metalloproteinase-2 by exogenous plasmin is associated with the induction of PVD.[25] Despite having considerably different amino acid sequences, nattokinase and plasmin share some common features: both are serine proteases and both have a high affinity for fibrin. Nattokinase not only has potent fibrinolytic activity but also enhances plasminogen activators and inactivates a plasminogen activator inhibitor.[20–22] Therefore, regarding the PVD-inducing mechanism, we think that nattokinase may have two major effects: one is the direct effect of liquefying the vitreous gel by its proteolytic activity and the other is the indirect effect of increasing the plasmin activity that induces the vitreoretinal dehiscence. In further studies, the details of the mechanisms related to nattokinase's PVD-inducing effects should be revealed. Concerning the therapeutic dose of nattokinase from this study, we think that 0.1 FU is adequate for pharmacologic vitreolysis. After administration of 1 FU nattokinase, the experimental eyes showed preretinal hemorrhage and histologic alteration. Although we did not determine the relationship between these findings, it may not be a direct toxic effect on ocular tissues, but rather the result of sudden separation of the posterior vitreous cortex from the retina that was induced by the higher dose. Further study of the optimal concentration of nattokinase and the mechanism of its adverse effects should be conducted. Further study is also necessary to determine an adequate exposure time to this enzyme and any adverse effects after an injection of nattokinase combined with a mechanical vitrectomy.

In conclusion, our findings suggest that nattokinase may be a useful pharmacologic adjunct in vitreous surgery.

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Thioredoxin inhibits NMDA-induced neurotoxicity in the rat retina

Yasuya Inomata,*† Hajime Nakamura,‡ Masaki Tanito,§ Akie Teratani,‡ Takahiro Kawaji,† Norihiko Kondo,* Junji Yodoi* and Hidenobu Tanihara†

**Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto, Japan*

†Departments of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan

‡Thioredoxin Project, Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan

§Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma, USA

Abstract

Thioredoxin (TRX) plays a variety of redox-related roles in organisms. To investigate its function as an endogenous redox regulator in NMDA-induced retinal neurotoxicity, we injected NMDA with TRX, mutant TRX or saline into the vitreous cavity of rat eyes. Retinal ganglion cells were rescued by TRX, compared with saline, when evaluated by retrograde labeling analysis at 7 days after NMDA injection. TRX, but not its mutant form, prevented NMDA-induced apoptosis in the retina, as measured by terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling. The induction of caspase 3 and 9, but not caspase 8, by NMDA was significantly lower in TRX-treated eyes than in saline-treated eyes. NMDA-induced activation of the MAPKs, p38 kinase and c-Jun N-terminal kinase after 6 h and of the MAPK kinases (MKKs) MKK3/6 and MKK4 after 3 h was markedly suppressed in retinal ganglion cells by TRX but not by the mutant form. NMDA-induced increases in protein carbonylation, nitrosylation and lipid peroxidation were also suppressed in TRX-treated eyes. We concluded that the intravitreous injection of TRX effectively attenuated NMDA-induced retinal cell damage and that suppression of oxidative stress and inhibition of apoptotic signaling pathways were involved in this neuroprotection.

Keywords: apoptosis, mitogen-activated protein kinases, NMDA, oxidative stress, retinal ganglion cells, thioredoxin.

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Apoptotic cell death is a feature of various eye diseases, including retinal ischemia (Levin and Louhab 1996; Rosenbaum *et al.* 1998) and glaucoma (Levin and Louhab 1996). Neurotoxicity induced by excitatory amino acids, such as glutamate, is a major mechanism underlying retinal damage in such diseases (Schwarcz and Coyle 1977; Sucher *et al.* 1997). This excitotoxicity has been shown to be mediated by over-stimulation of both NMDA-type and non-NMDA-type glutamate receptors in the retina (Lam *et al.* 1999), with NMDA-type receptors being most strongly implicated in neuronal cell death (Bonfoco *et al.* 1995). A number of cells in the eye, including retinal ganglion cells (RGCs) and amacrine cells, express this receptor (Brandstatter *et al.* 1994). Indeed, administration of NMDA to rodent eyes hyperstimulates glutamate receptors and induces retinal damage, with free radicals, including reactive oxygen species, playing an important role in this process (Siliprandi *et al.* 1992; El-Remessy *et al.* 2003). This animal model has been widely used to investigate the mechanism of retinal neuronal cell death and the role of apoptosis, and to evaluate neuroprotective factors and biological drugs (Inomata *et al.* 2003a,b; Hartwick *et al.* 2004; Wehrwein *et al.* 2004). The MAPK superfamily, which consists of c-Jun N-terminal kinase (JNK), p38 kinase (p38) and extracellular signal-regulated kinase, is stimulated by various cellular stresses. Recently, roles for the p38 and JNK signal pathways were

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Address correspondence and reprint requests to Hajime Nakamura, Thioredoxin Project, Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, 54 Shogoin-Kawaharacho, Sakyo, Kyoto, 606-8507, Japan.
E-mail: hnakamur@kuhp.kyoto-u.ac.jp

Abbreviations used: DM, double-mutant; GCL, ganglion cell layer; INL, inner nuclear layer; JNK, c-Jun N-terminal kinase; MDA, malondialdehyde; MKK, MAPK kinase; p, phospho; p38, p38 kinase; RGC, retinal ganglion cell; rh, recombinant human; TRX, thioredoxin; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling; WT, wild-type.

reported in NMDA-induced RGC apoptosis (Manabe and Lipton 2003; Munemasa *et al.* 2005).

Thioredoxin (TRX), which is a ubiquitous 12-kDa multifunctional protein, plays a variety of redox-related roles in organisms ranging from *Escherichia coli* to humans (Holmgren 1985). Human TRX was originally cloned as a soluble factor released from human T-cell leukemia virus-I-transformed T cells (Tagaya *et al.* 1989; Yodoi and Uchiyama 1992). It has two redox-active cysteine residues in its active center, Cys^{32}-Gly-Pro-Cys^{35}, which undergo reversible oxidation–reduction reactions catalysed by an NADPH-dependent enzyme, TRX reductase (Holmgren 1985). TRX is induced by various oxidant stresses, including viral infection and ischemia/reperfusion, and secreted from cells (Nakamura *et al.* 1997; Kondo *et al.* 2004). TRX plays a crucial role in the redox regulation of transcriptional factors such as nuclear factor-kappa B and shows antioxidative, anti-inflammatory and antiapoptotic effects (Nakamura *et al.* 1997, 2001). Overexpression or administration of TRX protects against neuronal damage associated with oxidative stress (Takagi *et al.* 1999; Bai *et al.* 2003; Hattori *et al.* 2004). Moreover, TRX effectively inhibits retinal damage including light-induced photic injury and ischemia/reperfusion injury (Shibuki *et al.* 1998; Tanito *et al.* 2002b,c). Current information suggests that TRX regulates apoptosis through a wide variety of mechanisms, including scavenging reactive oxygen species, regulating cytochrome *c* release from mitochondria, and regulating MAPKs and their upstream regulators (Masutani *et al.* 2005).

The purpose of the current study was to investigate the potential neuroprotective effects of TRX in NMDA-induced retinal damage *in vivo*. To further elucidate the mechanisms involved, we assessed terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL), the activity of caspase 3, 8 and 9, the phosphorylation of the MAPKs p38 and JNK and the MAPK kinases (MKKs) MKK3/6 and MKK4, and the oxidation status of proteins in terms of their carbonylation, nitrosylation and lipid peroxidation in NMDA-treated retinas.

Materials and methods

Antibodies

Rabbit polyclonal antibodies against JNK, phospho (p)-JNK, MKK3/MKK6, p-MKK3/MKK6, MKK4 and p-MKK4 were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies against p38 and p-p38 were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals

Male 9-week-old Wistar rats weighing 180–200 g were housed at 25°C under a 12-h light–dark cycle, and given water and food *ad libitum*. All studies were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

NMDA-induced retinal damage

Intravitreal injection of NMDA (Sigma Chemical Co.) was performed as previously reported (Inomata *et al.* 2003a,b). Briefly, rats were anesthetized by injecting 50 mg/kg pentobarbital intraperitoneally, the right pupil was dilated with phenylephrine hydrochloride and tropicamide drops, and 20 nmol of NMDA was injected into the vitreous cavity. Injections were performed under a microscope using a 33-gauge needle connected to a microsyringe and the needle was inserted approximately 1 mm behind the corneal limbus.

Thioredoxin treatment

Recombinant human (rh) TRX was provided by Ajinomoto (Kawasaki, Japan; Mitsui *et al.* 1992). Preparation of 6× histidine-tagged wild-type (WT) TRX and double-mutant (DM) TRX, in which the two cysteines at positions 32 and 35 were replaced with serine, was as described previously (Liu *et al.* 2004). A 100-μg sample of rhTRX, WT-TRX, DM-TRX or saline as a vehicle control, mixed with NMDA in a total volume of 3 μL, was injected into the vitreous cavity.

Recombinant human TRX was labeled using an Alexa Fluor 594 Labelling Kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's protocol, and was used to assess the distribution in the retina. Aliquots (10 μg) were injected in a total volume of 2 μL into the vitreous cavity. The eyes were enucleated 1, 6, 12 or 24 h later, fixed in 4% paraformaldehyde and embedded in optical cutting temperature compound. Sections (10 μm thick) were cut with a cryostat and examined by fluorescence microscopy.

Retrograde labeling of retinal ganglion cells

At 4 days after the NMDA injection, retrograde labeling of RGCs was performed as described previously (Inomata *et al.* 2003a,b). Briefly, rats were anesthetized, their heads immobilized and Fluoro-Gold (Fluorochrome, Englewood, CO, USA) was microinjected bilaterally into the superior colliculi. After 3 days (7 days after the NMDA injection), the eyes were enucleated and fixed in 4% paraformaldehyde for 1 h. The retinas were divided by six radial cuts, removed from the sclera and mounted on slides. The numbers of Fluoro-Gold-labeled RGCs were counted in the locations described previously (Inomata *et al.* 2003a,b). Briefly, labeled RGCs were counted in two fields in the central area (1 mm from the optic disc) and two fields in the peripheral area (4 mm from the optic disc).

Terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling

Rats were killed with an overdose of sodium pentobarbital 24 h after the NMDA injection, and their eyes were immediately enucleated and fixed in 4% paraformaldehyde in phosphate-buffered saline. TUNEL was performed using a fluorescent apoptosis-detection system (Promega, Madison, WI, USA) on 5-μm-thick paraffin-embedded sections. TUNEL-positive cells were counted in the locations described previously (Inomata *et al.* 2003a,b), i.e. the ganglion cell layer (GCL) and the inner nuclear layer (INL) 1.0–1.5 mm from the optic disc.

Caspase assays

Caspase protease activities were measured as reported previously, with slight modifications (Ueda *et al.* 1998). Caspase 3, 8 and 9 cleaved the fluorogenic peptides *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin, *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin and *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin, respectively. Briefly, the retinas were removed after NMDA injection, immediately homogenized in lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA and 10 mM EGTA, and centrifuged at 20 000 *g* for 15 min. The supernatants were incubated with 50 μM fluorogenic peptide at 37°C. Release of 7-amino-4-trifluoromethyl coumarin was monitored using a fluorescence microplate reader (Gemini EM; Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 380 and 460 nm, respectively. For caspase 3 and 8, 1 U was defined as the release of 0.13 pmol 7-amino-4-trifluoromethyl coumarin/min/mg retinal protein. For caspase 9, 1 U was defined as the release of 0.12 pmol 7-amino-4-trifluoromethyl coumarin/min/mg retinal protein (Ueda *et al.* 1998).

Western blot analysis

Eyes were enucleated at various times after NMDA injection. The retinas were then removed and homogenized in cell lysis buffer (Kinase Assay Kit: Cell Signaling Technology). The lysates were centrifuged at 20 000 *g* for 15 min at 4°C. Protein concentrations in the supernatants were determined using a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (20 μg/lane) were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes. After blocking in Tris-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin, the membranes were incubated overnight at 4°C with 1 : 1000 dilutions of primary antibodies against p38, p-p38, JNK, p-JNK, MKK3, p-MKK3/MKK6, MKK4 or p-MKK4, followed by a peroxidase-linked second antibody (Amersham Pharmacia, Buckinghamshire, UK; 1 : 5000). Chemiluminescence was detected with an enhanced chemiluminescence western blot detection kit (Amersham Pharmacia).

Immunohistochemistry

At 6 h after NMDA injection, the eyes were enucleated, fixed in 4% paraformaldehyde in phosphate-buffered saline and soaked in a cryoprotective 20% sucrose solution overnight at 4°C before being frozen in optical cutting temperature compound (Tissue-Tek; Sakura Finetechnical Co. Ltd, Tokyo, Japan). Cryostat sections (10 μm thick) were mounted on glass slides coated with poly-L-lysine, incubated with Protein Block Solution (DAKO, Glostrup, Denmark) and then incubated overnight at 4°C with either anti-p-p38 antibody (1 : 100) or anti-p-JNK antibody (1 : 100). The sections were washed three times and incubated with Alexa Fluor 488-labeled anti-rabbit IgG (Molecular Probes; 1 : 200) at 25°C for 1 h. They were then washed three times, exposed to one drop of Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) to stain the nuclei and examined by fluorescence microscopy.

Protein carbonylation assay

The level of protein oxidation induced by NMDA was measured using an OxyBlot kit (Chemicon, Temecula, CA, USA), which is a sensitive immunodetection method for carbonyl groups, as described previously (Tanito *et al.* 2002a). Retinas were removed 18 h after NMDA injection and homogenized with ice-cold phosphate-buffered saline (20 mM, pH 7.4). The homogenates were centrifuged at 20 000 *g* for 15 min and the supernatants were used to prepare 2,4-dinitrophenyl-hydrazone derivatives, according to the manufacturer's protocol. The samples were separated on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking, the membrane was incubated initially with a primary antibody specific for 2,4-dinitrophenyl and then with a peroxidase-linked secondary antibody. Chemiluminescence was detected with an enhanced chemiluminescence western blot detection kit (Amersham Pharmacia). An image scanner was used to scan each lane and the intensities of protein bands were analysed by using NIH IMAGE 1.63 software according to the software tutorial.

Nitrotyrosine enzyme immunoassay

The formation of peroxynitrite ($ONOO^-$), which is short-lived at physiological pH but easily nitrosylates tyrosine residues, was assessed in retinas by measuring the concentration of nitrotyrosine using an enzyme immunoassay kit (Cell Sciences, Norwood, MA, USA), according to the manufacturer's protocol. Retinal samples were prepared as for the protein carbonylation assay and assays were performed in duplicate.

Lipid peroxidation assay

We determined lipid peroxidation induced by NMDA by measuring malondialdehyde (MDA) in retinal samples with a Bioxytech LPO-586 assay kit (Oxis International, Portland, OR, USA), according to the manufacturer's protocol. Samples (100 μL), adjusted to contain 100 μg retinal protein, were prepared from retinas as for the protein carbonylation assay. *N*-methyl-2-phenylindole was used as the chromogen and measurements were made at an absorbance of 586 nm. We used 1,1,3,3-tetramethoxypropane as an external standard and the level of lipid peroxide was expressed as pmol MDA/mg protein.

Statistical analysis

The data presented as bar graphs show the means ± SD of at least four independent experiments. The differences between groups were evaluated by one-way ANOVA followed by Scheffe's post-hoc test for multiple comparisons. p values < 0.05 were considered statistically significant.

Results

Distribution of thioredoxin in the retina after intravitreous injection

To examine the distribution of TRX in the retina after injection, 10 μg Alexa-labeled rhTRX was injected into the vitreous cavity. After 1 h, Alexa-labeled rhTRX was detected in all retinal layers, including the retinal pigment epithelium layer (Fig. 1). The distribution had not changed significantly 12 h after injection. At 24 h, labeling increased in the rod inner segment and the rod outer segment, and decreased in the inner plexiform layer and the outer plexiform layer.

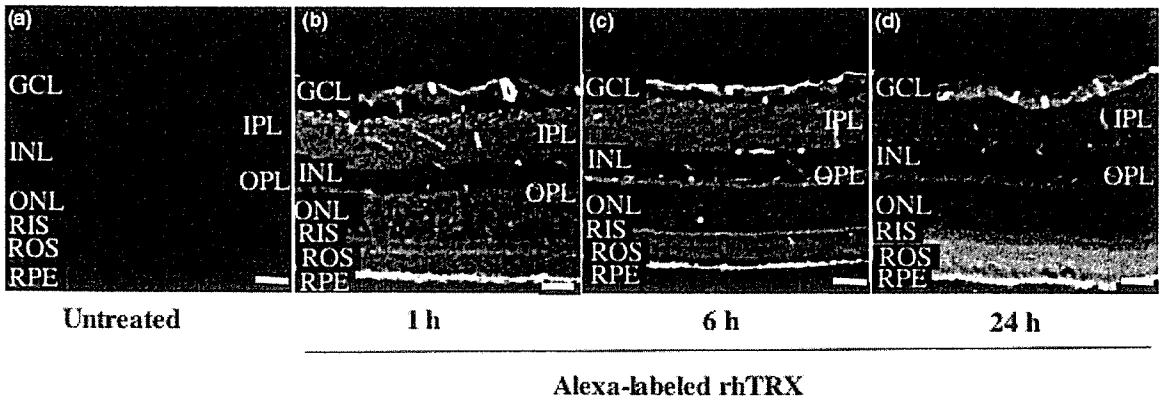

Fig. 1 Distribution of thioredoxin (TRX) in retinal tissue. Photographs of representative retinal sections were examined by fluorescence microscopy for labeling due to Alexa 594-TRX. Sections were from untreated eyes (a) or eyes treated with 10 μg recombinant human (rh)TRX injected into the vitreous cavity 1 h (b), 6 h (c) or 24 h (d) previously. Alexa labeling was detected in all retinal layers, including the retinal pigment epithelium (RPE) layer, at 1 h and up to 24 h after injection. Scale bars, 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RIS, rod inner segment; ROS, rod outer segment.

Neuroprotective effects of thioredoxin on retinal ganglion cells using retrograde labeling

To investigate whether exogenous TRX could protect RGCs from NMDA-induced cell death, we carried out retrograde labeling experiments with Fluoro-Gold (Fig. 2). Compared with a density of 1006 ± 55 RGCs/mm^2 in control eyes treated with saline alone, the density of RGCs was significantly lower in eyes examined 7 days after NMDA injection (135 ± 15 cells/mm^2; $p < 0.001$). However, in eyes treated with TRX, the effect of NMDA on the density of RGCs after 7 days was significantly suppressed, with 210 ± 19 cells/mm^2 ($p < 0.05$) and 570 ± 38 cells/mm^2 ($p < 0.05$) in eyes treated with 100 or 400 μg rhTRX, respectively (Fig. 2d).

Effects of thioredoxin on NMDA-induced apoptosis

We investigated whether TRX inhibited NMDA-induced apoptosis by assessing the numbers of TUNEL-positive cells 24 h after NMDA injection in retinal sections. As shown previously, TUNEL-positive cells were present in the INL and GCL after 24 h (Fig. 3a; Inomata *et al.* 2003a,b). However, in eyes treated with rhTRX or WT-TRX, the total numbers of TUNEL-positive cells in the retinal sections were significantly lower compared with vehicle-treated ($p < 0.001$) and DM-TRX-treated eyes ($p = 0.044$ for rhTRX and 0.002 for WT-TRX; Fig. 3e). Moreover, when the numbers of TUNEL-positive cells in specific parts of the retina were counted, significant differences were found in both the GCL and INL between eyes treated with rhTRX and WT-TRX compared with vehicle ($p < 0.001$). The differences in the numbers of apoptotic cells were also significant when eyes treated with DM-TRX were compared with eyes treated with rhTRX in the GCL ($p = 0.003$) or INL ($p = 0.049$), or compared with eyes treated with WT-TRX in the GCL ($p = 0.006$) or INL ($p = 0.002$; Figs 3f and g). These results indicated that the redox-active site sequence Cys-Gly-Pro-Cys in TRX might play an important role in its antiapoptotic effect.

Inhibition of the mitochondrial apoptotic pathway by thioredoxin

As caspase activity is generally required for apoptosis, we assessed the effect of TRX on caspases induced by NMDA. We measured the induction of these caspases at 6, 12, 18 and 24 h after NMDA injection, and the induction of caspase 3 peaked at 18 h and caspase 9 and 8 peaked at 12 h, respectively (data not shown). In eyes treated with NMDA and vehicle, caspase 3 was induced in the retina after 18 h (73.3 ± 23.8 U) but this activity was significantly inhibited in TRX-treated retinas (38.8 ± 21.8 U; $p = 0.035$; Fig. 4c). NMDA also induced caspase 9 in the retina after 12 h (18.5 ± 5.3 U) but this activity was significantly inhibited in TRX-treated eyes (11.4 ± 4.5 U; $p = 0.045$; Fig. 4a). By contrast, NMDA failed to induce caspase 8 (Fig. 4b) and we observed no significant differences in caspase 8 activity between vehicle and rhTRX treatment after NMDA administration. These results indicated that NMDA-induced apoptosis was mediated by the mitochondrial pathway (Cao *et al.* 2001) and that TRX exerted an antiapoptotic effect on this pathway.

Thioredoxin inhibits NMDA-induced phosphorylation of p38 and c-Jun N-terminal kinase

We next asked whether the MAPKs p38 and JNK, which might form part of the upstream signaling cascades in the mitochondrial apoptosis pathway, were involved in the cellular response to NMDA, and whether they were affected

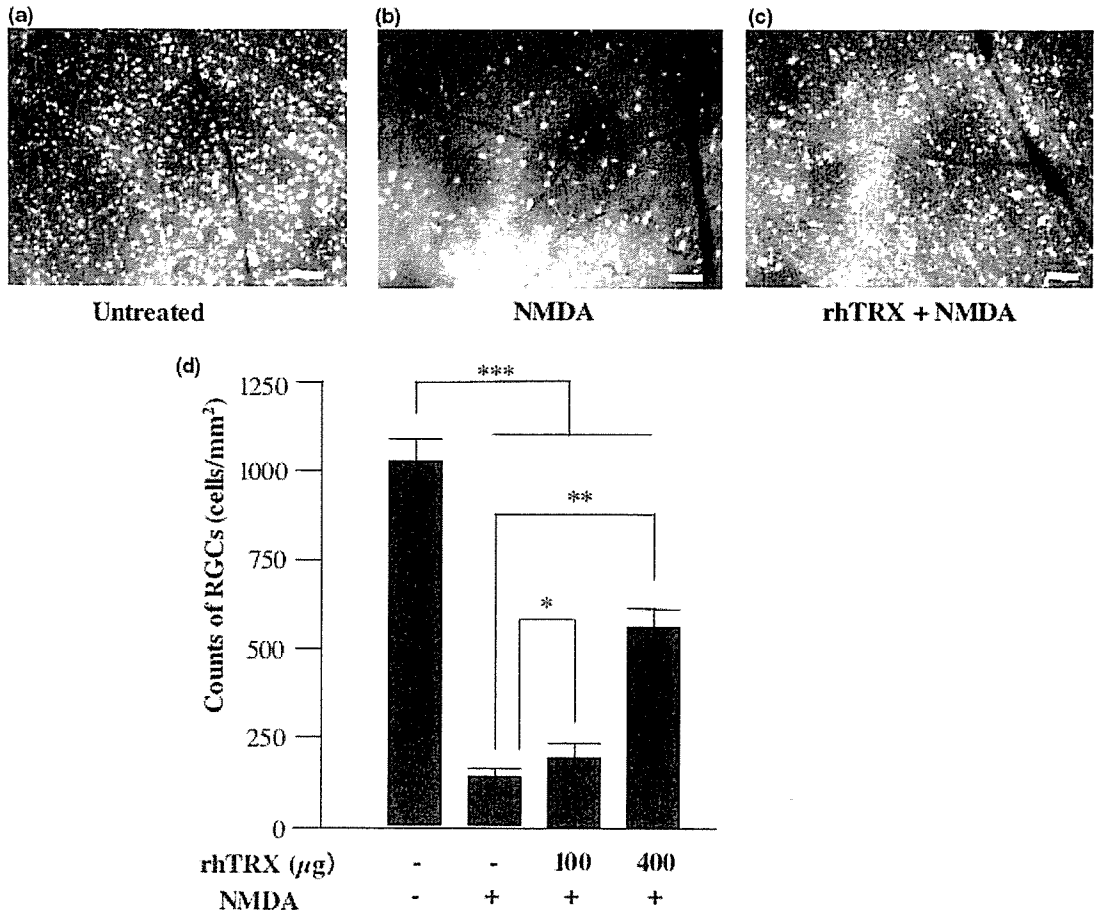

Fig. 2 Density of retinal ganglion cells (RGCs) 7 days after NMDA injection. Fluorescence microscopic photographs of representative retinal sections in untreated (a) and NMDA-treated (b and c) eyes simultaneously injected with vehicle (b) or 100 μg recombinant human thioredoxin (rhTRX) (c). Scale bars, 50 μm. (d) Mean ± SD cell densities are shown as bar graphs ($n = 4$ in each group). The statistical significance of differences between groups was calculated by one-way ANOVA followed by Scheffe's post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

by the administration of TRX. In western blot analysis, the level of p-p38 was shown to gradually increase after NMDA injection, with maximal expression seen after 6 h (Fig. 5a). The level of p-p38 was clearly lower in vehicle-treated eyes than in eyes that were also treated with rhTRX and WT-TRX (Fig. 5b). The expression of p38 did not change after NMDA injection in either sample. No change in p-p38 or p38 was observed in eyes injected with vehicle alone compared with untreated eyes (data not shown). The results of immunohistochemical labeling for p-p38 showed that, at 6 h after NMDA injection, positive cells were present in the GCL and INL in eyes treated with vehicle but the labeling was clearly reduced in rhTRX-treated eyes (Figs 5c–h).

Western blot analysis showed that the levels of both p-JNK-1 and p-JNK-2 gradually increased after NMDA injection, and reached a maximum after 6 h (Fig. 6a). In eyes treated with rhTRX or WT-TRX as well as NMDA, the levels of phosphorylation of both JNK-1 and JNK-2 were clearly lower than those in eyes treated with vehicle or DM-TRX (Fig. 6b). The expression of JNK did not change after NMDA injection. No changes in p-JNK or JNK were observed in eyes injected with vehicle alone compared with untreated eyes (data not shown). The results of immunohistochemical labeling for p-JNK (Figs 6c–h) showed that, at 6 h after NMDA injection, positive cells were observed in the GCL in vehicle-treated eyes and the labeling was clearly inhibited in eyes that were also treated with rhTRX.

Inhibition of NMDA-induced phosphorylation of MAPK kinases by thioredoxin

We further investigated the phosphorylation of the kinases upstream of MAPKs (i.e. MKK3/MKK6, which activates p38, and MKK4, which activates JNK) by western blot analysis. The levels of p-MKK3/MKK6 (Fig. 7a) and

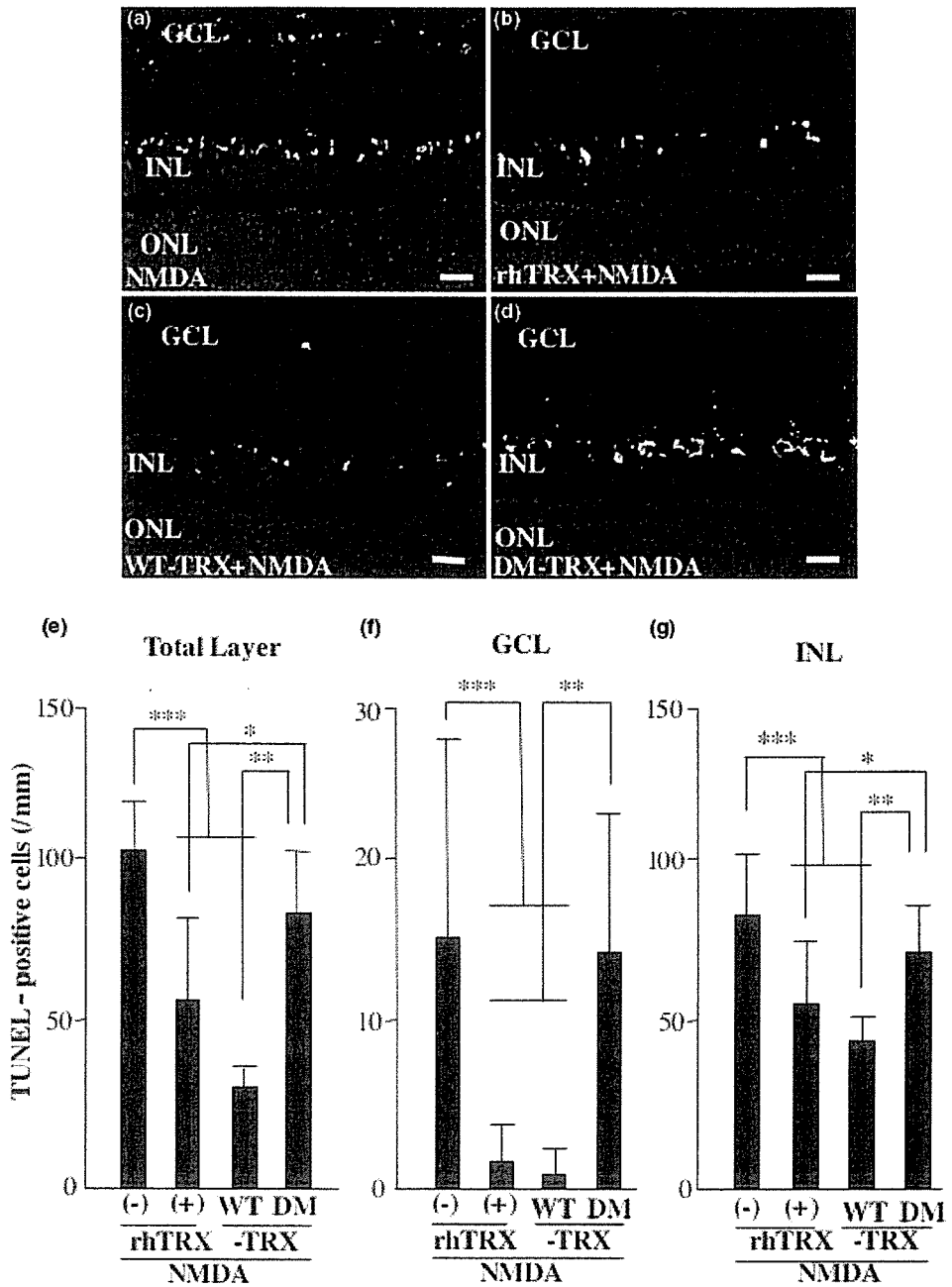

Fig. 3 Effect of thioredoxin (TRX) treatment on terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) in retinal sections 24 h after NMDA injection. Representative retinal specimens from eyes treated with NMDA and (a) vehicle, (b) 100 μg recombinant human (rh)TRX, (c) 100 μg wild-type (WT)-TRX or (d) 100 μg double-mutant (DM)-TRX. Green and red signals indicate TUNEL-positive cells and cell nuclei stained by propidium iodide, respectively (a–d). Scale bars, 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (e–g) Bar graphs show the mean ± SD densities of TUNEL-positive cells in sections from eyes treated with vehicle ($n = 9$), rhTRX ($n = 8$), WT-TRX ($n = 5$) and DM-TRX ($n = 5$) assessed in all retinal layers (e), in the GCL (f) and in the INL (g). The statistical significance of differences between groups was calculated by one-way ANOVA followed by Scheffe's post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

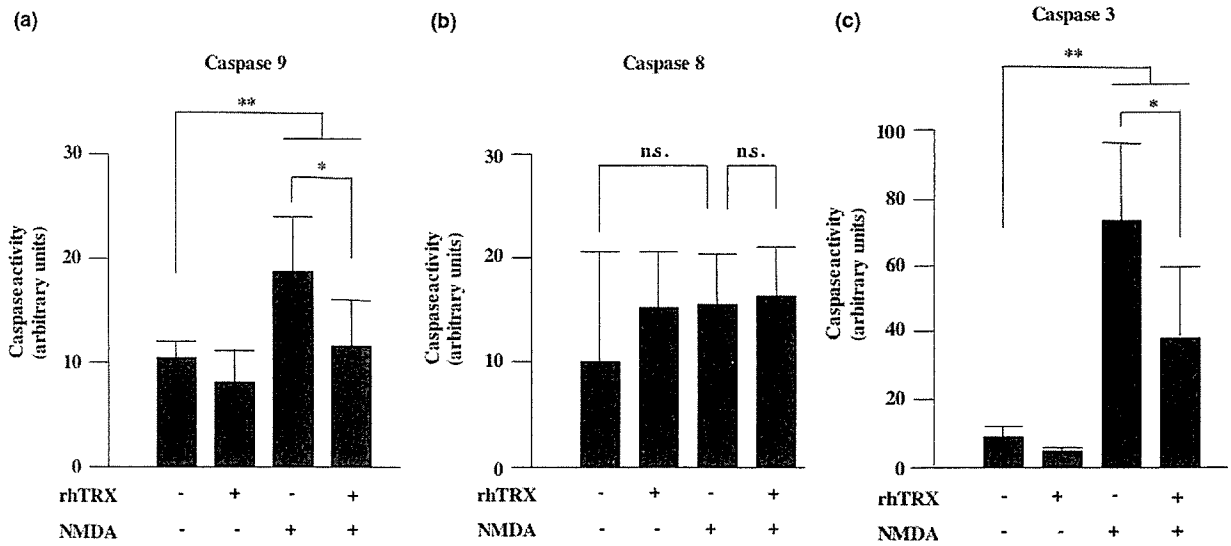

Fig. 4 Caspase activity in the retina after NMDA treatment. The activity of (a) caspase 9, (b) caspase 8 and (c) caspase 3 at 12, 12 and 18 h, respectively, after injection with NMDA and either vehicle or 100 μg recombinant human thioredoxin (rhTRX), as indicated. Bar graphs show the mean activity in arbitrary units (± SD; $n = 5$ in each group). The statistical significance of differences between groups was calculated by one-way ANOVA followed by Scheffe's post-hoc test. $^*p < 0.05$ and $^{**}p < 0.001$. n.s., not significant.

p-MKK4 (Fig. 7b) increased immediately after NMDA injection, and these kinases were maximally phosphorylated after 3 h. In eyes treated with rhTRX, WT-TRX, DM-TRX or vehicle simultaneously with NMDA injection (Figs 7a and b), the levels of p-MKK3/MKK6 and p-MKK4 in rhTRX-treated or WT-TRX-treated eyes were lower than those in vehicle-treated or DM-TRX-treated eyes after 3 h. These data indicate that TRX modulates signaling pathways upstream of p38 and JNK, and that the active site of TRX might be crucial in this activity.

Effects of thioredoxin on NMDA-induced oxidative stress

It has been proposed that oxidative stress is the trigger for various types of cellular response, including the activation of MAPKs and apoptotic signaling cascades. Therefore, we finally analysed the levels of oxidative stress induced in the retina by NMDA injection. Western blot and densitometric analysis showed that total levels of protein carbonylation in retinal samples gradually increased 6, 12 and 24 h after NMDA injection compared with the NMDA-untreated eyes (1.35-, 2.18- and 2.98-fold, respectively; Fig. 8a). At 18 h, retinal levels of protein carbonylation were markedly lower in rhTRX-treated compared with vehicle-treated eyes (0.55-fold; Fig. 8b). Interestingly, change of band intensities by rhTRX treatment was not uniform among proteins. As examples, the protein bands indicated by the upper and lower arrows in Fig. 8(b) decreased more than other bands in rhTRX-treated eyes (0.41- and 0.43-fold, respectively). Enzyme immunoassay revealed that the concentration of nitrotyrosine 18 h after NMDA injection was 113.1 ± 20.1 pmol/mg, which was significantly higher than the value in untreated eyes (57.7 ± 14.9 pmol/mg; $p < 0.001$; Fig. 8c). The NMDA-induced increase in nitrotyrosine concentration was significantly suppressed in the rhTRX-treated eyes (89.7 ± 4.3 pmol/mg; $p = 0.014$). There was no significant difference between the concentration of nitrotyrosine in vehicle-treated and rhTRX-treated eyes that had not been injected with NMDA. MDA was also significantly increased by NMDA treatment, reaching levels of 613.0 ± 204.2 pmol/mg compared with 168.0 ± 4.9 pmol/mg ($p < 0.001$) after 18 h (Fig. 8d). Considering protein carbonylation and nitrotyrosine, the NMDA-induced increase in MDA in the retina was significantly lower in rhTRX-treated eyes (360.7 ± 68.0 pmol/mg; $p = 0.019$). There was no significant difference between the MDA level in vehicle-treated and rhTRX-treated eyes that had not been injected with NMDA.

Discussion

We have shown that the intravitreous injection of TRX provided neuroprotection for rat retinal cells against NMDA-induced death. To date, effective cytoprotection by exogenously administered TRX protein has been shown in a variety of diseases (Nakamura *et al.* 2001; Hattori *et al.* 2004; Liu *et al.* 2004; Ichiki *et al.* 2005). However, the tissue distribution of TRX administered *in vivo* has not been studied extensively. In the current study, Alexa-labeled rhTRX injected into the vitreous cavity was detected in all retinal layers, including the retinal pigment epithelium, 1 h after

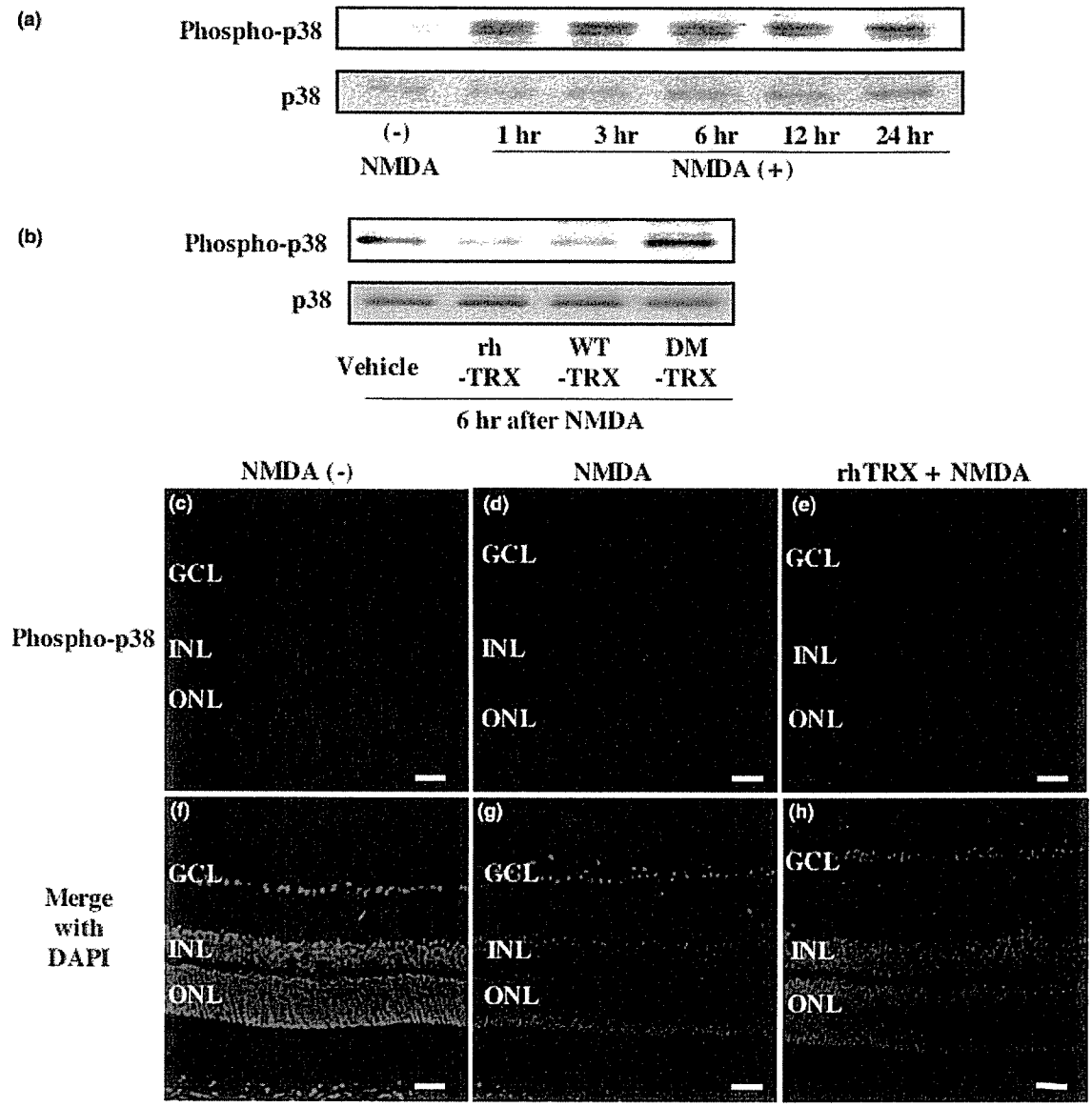

Fig. 5 Effect of thioredoxin (TRX) on NMDA-induced p38 kinase (p38) phosphorylation. Western blots showing (a) the time course of p38 phosphorylation after injection with 20 nmol NMDA and (b) the effect of TRX on p38 phosphorylation at 6 h after NMDA injection. Vehicle or 100 μg recombinant human (rh)TRX, wild-type (WT)-TRX or double-mutant (DM)-TRX was injected with NMDA. (c–h) Representative retinal sections labeled immunohistochemically for phospho (p)-p38 (c–e) or p-p38 merged with 4′,6-diamidino-2-phenylindole (DAPI) staining (f–h) from control eyes (c and f) or eyes treated with NMDA 6 h earlier, either with vehicle (d and g) or with rhTRX (e and h). Strong labeling for p-p38 is present in the ganglion cell layer (GCL) and inner nuclear layer (INL) in vehicle-treated eyes (d and g) and is reduced in rhTRX-treated eyes (e and h). Scale bars, 50 μm. ONL, outer nuclear layer.

injection and up to 24 h later (Fig. 1). Surprisingly, TRX labeling in the outer retina (i.e. the rod inner segment, rod outer segment and retinal pigment epithelium) increased over time (Fig. 1; 24 h), suggesting that some active-transport system might be involved in its accumulation. This is in line with a previous report showing the cytoprotective effects of TRX injected intravitreally in a model of light-induced photoreceptor cell damage (Tanito *et al.* 2002a). Although the underlying mechanism is to be further investigated, this suggests a high bioavailability of TRX in the retinal tissue.

Retrograde labeling showed that the density of RGCs was significantly higher in eyes treated intravitreally with rhTRX simultaneously with NMDA injection than in those treated with saline (Fig. 2), suggesting that TRX inhibited the

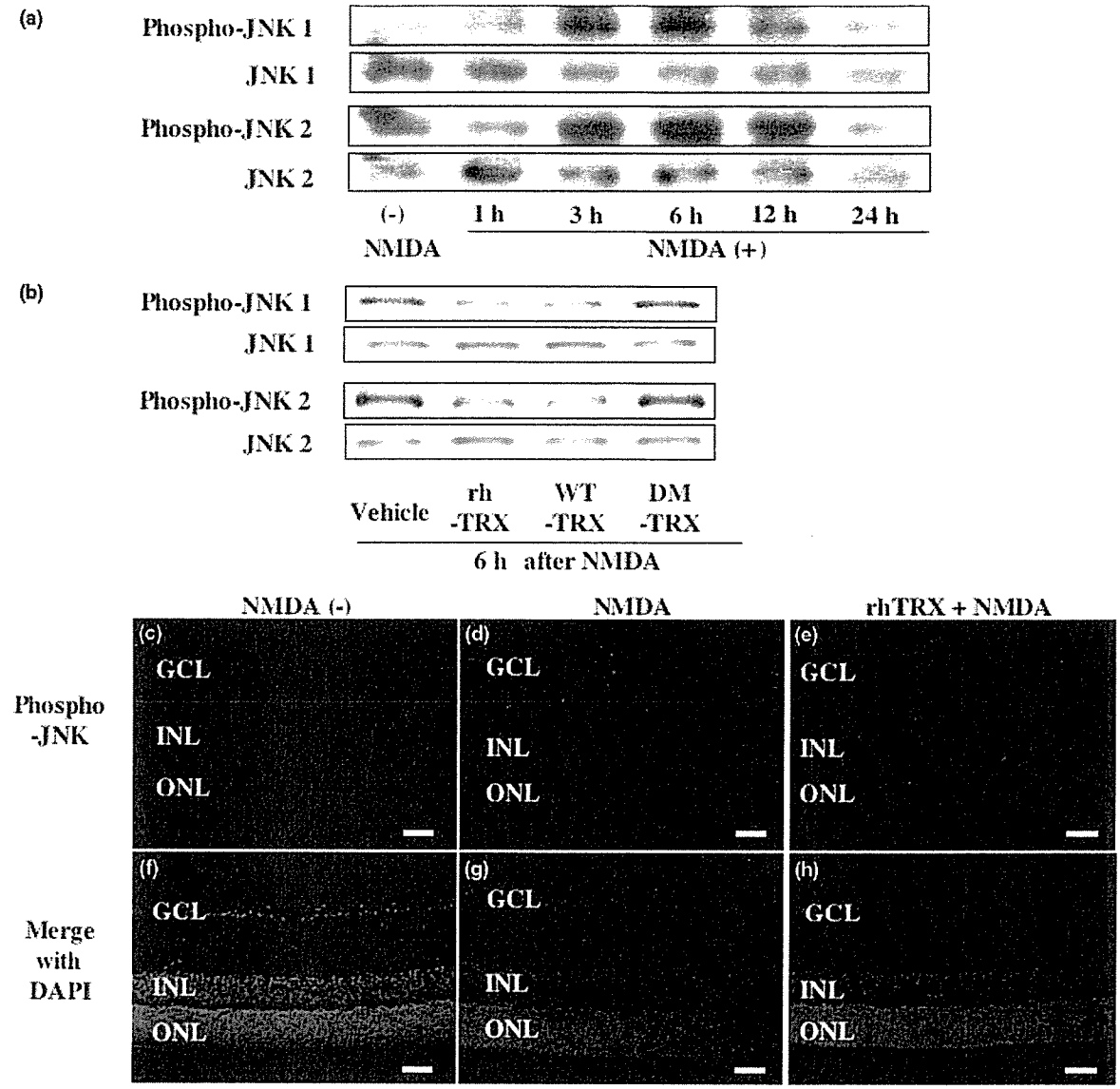

Fig. 6 Effect of thioredoxin (TRX) on NMDA-induced c-Jun N-terminal kinase (JNK) phosphorylation. Western blots showing (a) the time course of JNK phosphorylation after injection with 20 nmol NMDA and (b) the effect of TRX on JNK phosphorylation at 6 h after NMDA injection. Vehicle or 100 μg recombinant human (rh)TRX, wild-type (WT)-TRX or double-mutant (DM)-TRX was injected with NMDA. Representative retinal sections labeled immunohistochemically for phospho (p)-JNK (c–e) or p-JNK merged with 4′,6-diamidino-2-phenylindole (DAPI) staining (f–h) from control eyes (c and f) or eyes treated with NMDA 6 h earlier, either with vehicle (d and g) or with rhTRX (e and h). Strong labeling for p-JNK is present in the ganglion cell layer (GCL) in vehicle-treated eyes (d and g) and is lower in rhTRX-treated eyes (e and h). Scale bars, 50 μm. INL, inner nuclear layer; ONL, outer nuclear layer.

NMDA-induced loss of RGCs. After NMDA injection, there were significantly fewer TUNEL-positive cells in both the GCL and INL in rhTRX-treated and WT-TRX-treated eyes than in saline-treated and DM-TRX-treated eyes (Fig. 3). This provided additional evidence of the antiapoptotic effect of TRX on NMDA-induced neurotoxicity and suggested that the conserved cysteine residues in the redox-active site in TRX might have an important role in this process. These results support the evidence of previous studies that demonstrated a protective effect of TRX in neuronal cell apoptosis, both *in vivo* (Tanito *et al.* 2002a; Chiueh *et al.* 2005) and *in vitro* (Bai *et al.* 2002). Actually, NMDA receptor itself contains several cysteines in the structure and has some sensitivity on the thiol-mediated redox regulation function (Lipton *et al.* 2002; Herin and Aizenman 2004). The possibility that TRX may modulate the ligand binding and

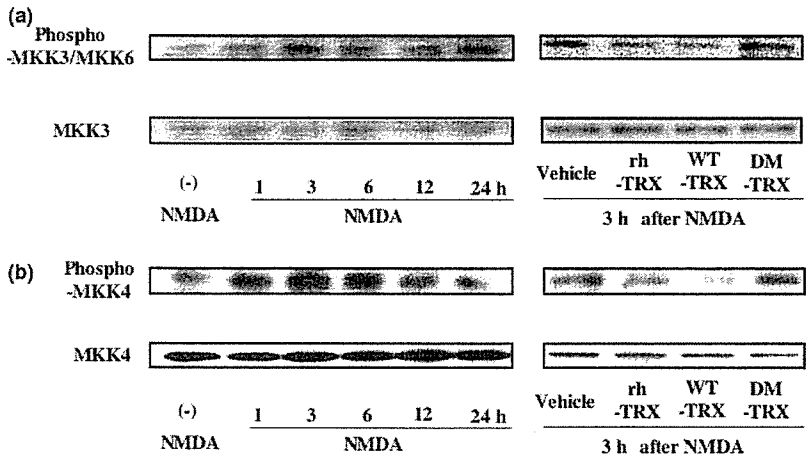

Fig. 7 Effect of thioredoxin (TRX) on NMDA-induced MAPK kinase (MKK) phosphorylation. Western blots showing the phosphorylation of MKK3/MKK6 (a) and MKK4 (b) in retinal samples. The left panels show time courses for phosphorylation after injection with 20 nmol NMDA. The right panels show the effects of injecting vehicle or 100 μg recombinant human (rh)TRX, wild-type (WT)-TRX or double-mutant (DM)-TRX with NMDA. Samples were collected 3 h after the injection.

signal transduction by redox regulation is to be clarified in the future.

The marked activation of caspase 9 and 3, but not caspase 8, in the retina by NMDA (Fig. 4) suggested that it induces apoptosis via the mitochondrial pathway, as has been previously suggested (Budd *et al.* 2000). This NMDA-induced activation of caspase 9 and 3 was significantly inhibited by rhTRX. A previous study showed that this apoptotic pathway could be inhibited by the endogenous mitochondrial form of TRX, TRX-2 (Tanaka *et al.* 2002). Our results showed that exogenous TRX-1 could also inhibit the mitochondrial apoptotic pathway; thus, to elucidate the mechanism involved, we examined the effect of TRX on molecules implicated in upstream signaling in this pathway.

Several lines of evidence have suggested that the mitochondrial apoptotic pathway in neurons is specifically regulated by the MAPKs p38 and JNKs (Mielke and Herdegen 2000; Bossy-Wetzel *et al.* 2004). In models of NMDA-induced retinal damage, possible links between the activation of p38 and JNKs, and apoptosis in RGCs, have been reported recently (Manabe and Lipton 2003; Munemasa *et al.* 2005). We used western blot analysis and immunohistochemistry to demonstrate that the phosphorylation of both p38 and JNKs increased after NMDA injection in whole retinal samples, and that these phosphorylation events were specifically localized in the GCL (Figs 5 and 6), in agreement with previous reports. These increases in phosphorylation were clearly suppressed by rhTRX and WT-TRX but not by DM-TRX (Figs 5 and 6). The kinases upstream of p-38 and JNKs have not been extensively studied in NMDA-induced apoptosis in RGCs. Using western blot analysis, we showed that the phosphorylation of both MKK3/MKK6 and MKK4 increased after NMDA injection, and at earlier time points than the phosphorylation of p38 and JNKs (Fig. 7), indicating that the activation of specific MKKs and MAPKs is involved in the signal-transduction pathway leading to apoptosis in RGCs. This increase in MKK phosphorylation was also clearly suppressed by rhTRX and WT-TRX but not DM-TRX (Fig. 7). Thus, our findings strongly suggest that the suppression of these signaling cascades, specific to NMDA-induced apoptosis, is involved in the cytoprotective mechanism of TRX and that its redox-active region is crucial for its effect on these kinase cascades.

It is well established that various types of oxidative stress trigger the activation of MAPK cascades (Choi *et al.* 2004) and, subsequently, the mitochondrial apoptosis pathway (Ueda *et al.* 2002). In the current study, all the oxidative-stress markers analysed, including protein carbonylation, tyrosine nitrosylation and lipid peroxidation, were enhanced by injecting NMDA into retinal tissue (Fig. 8). These increases in oxidative-stress markers were clearly suppressed by TRX (Fig. 8), suggesting that it effectively attenuated the oxidative stress induced by NMDA. Interestingly, some proteins were better protected by TRX from the carbonylation than others (Fig. 8), suggesting a presence of specific target proteins for neuroprotection by TRX, but this needs to be tested. TRX has been reported to scavenge hydroxyl radicals or singlet oxygen by itself (Das and Das 2000) and to eliminate hydrogen peroxide in cooperation with peroxiredoxins (Chae *et al.* 1994); the latter are also abundantly expressed in retinal tissue, including the inner retina (Tanito *et al.* 2005b). Peroxynitrite ($ONOO^-$) is produced by the accumulation of superoxides and nitric oxides, and causes lipid peroxidation, mitochondrial dysfunction and, eventually, cell death (Coyle and Puttfarcken 1993). Accordingly, the elimination of reactive oxygen species in the extracellular space or at the cellular membrane, and inhibition of subsequent reactive nitrogen species and lipid peroxide formation, might be involved in the antioxidative effects of TRX in this study. An imbalance in the extracellular redox status changes the thiol content at the external surface of the cell membrane, and affects the intracellular redox status of both the TRX and glutathione systems; these changes are sensed by p38 (Filomeni *et al.* 2003). In the intracellular

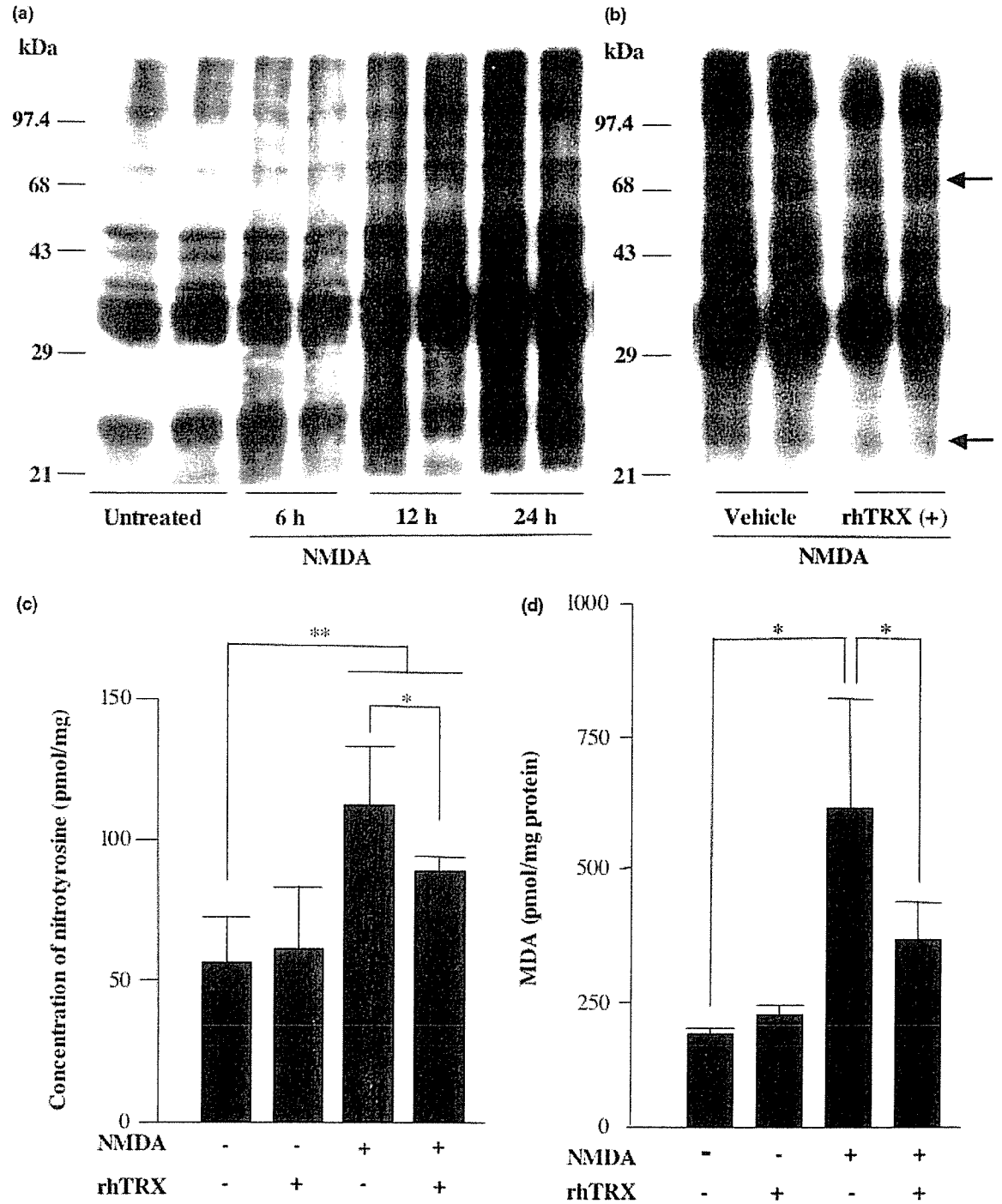

Fig. 8 Effects of thioredoxin (TRX) on NMDA-induced markers of oxidative stress. Western blots showing (a) the time course of protein carbonylation after injection with 20 nmol NMDA and (b) the effect of injecting vehicle or 100 μg recombinant human (rh)TRX with NMDA. Samples were collected 18 h after the injection. (c) Enzyme immunoassay for nitrotyrosine and (d) the measurement of malondialdehyde in retinal samples collected 18 h after injection with NMDA with vehicle or 100 μg rhTRX, as indicated in the figure. The results are expressed as means ± SD ($n = 6$ and 5 in c and d, respectively). The statistical significance of differences between groups was calculated by one-way ANOVA followed by Scheffe's post-hoc test. $^{*}p < 0.05$ and $^{**}p < 0.001$.

space, TRX directly binds to the apoptosis signal-regulating kinase-1, which is an MKK kinase that activates p38 and JNK pathways, and so inhibits apoptosis signal-regulating kinase-1-dependent apoptosis (Saitoh *et al.* 1998). Our recent data suggested that exogenous TRX might be taken up into the intracellular space (Kondo *et al.*, unpublished data). In view of this, we can speculate that the maintenance of the extracellular redox status and/or the direct regulation of an MAPK cascade molecule might be involved in the cytoprotective effect of exogenously administrated TRX but this remains to be tested. The possible relation between decrease of intracellular glutathione level and glutamate-induced neurotoxicity (Murphy *et al.* 1989), and between increase of extracellular glutathione level and neuroprotection against NMDA-induced neurotoxicity (Levy *et al.* 1991) was reported *in vitro*. However, in contrast to TRX, modulatory effects against the NMDA-induced MAPK-apoptosis pathway and bioavailability of glutathione in retinal tissue are still unclear *in vivo*.

Glutamate receptors are categorized into two main classes: ionotropic and metabotropic. Ionotropic glutamate receptors are further classified into NMDA- and non-NMDA-(α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainic acid) type receptors. RGCs and amacrine cells possess not only NMDA-type receptors but also α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainic acid-type receptors and metabotropic glutamate receptors, and bipolar cells also possess α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainic acid-type receptors and metabotropic glutamate receptors (Thoreson and Witkovsky 1999). As NMDA is not normally present in animal systems, we additionally tested the effect of TRX against neurotoxicity induced by glutamate, a natural agonist of both classes of glutamate receptors. At 12 h after a 2-μL (400 nmol) L-glutamate injection, TUNEL-positive cells were observed in GCL and INL, and the number of TUNEL-positive cells in these retinal layers was significantly less in TRX-treated than in vehicle-treated eyes (59.3 ± 12.4 and 21 ± 3.7 cells/mm, respectively, in GCL, $p = 0.025$; 72.3 ± 12.4 and 34.5 ± 7.1 cells/mm, respectively, in INL, $p = 0.021$). At the same time point after L-glutamate injection, caspase 3 activity in retina was significantly increased compared with that in un-injected retina (2.64-fold and $p < 0.001$), whereas the increase was less remarkable in rhTRX-treated retina (1.35-fold and $p = 0.413$ in comparison with un-injected retina; $p = 0.003$ in comparison with vehicle-treated retina). These results support a previous report that NMDA closely mimicked glutamate-induced neurotoxicity (Schori *et al.* 2001). Taken together, TRX is likely to be protective against glutamate-induced neurotoxicity in natural animal systems.

Expression of TRX is reported in the nerve and photoreceptor cells from developing and mature rat retinas (Hansson *et al.* 1989). We previously reported that endogenous TRX was expressed throughout the retinal layers and was up-regulated by oxidative stresses such as photo-oxidative stress in mouse, indicating that the increase of TRX level in retina is likely to be a defence mechanism against retinal pathology (Tanito *et al.* 2005a). The use of TRX inducers, such as sulforaphane and geranylgeranylacetone, was an effective and safe way to up-regulate TRX *in vivo* (Tanito *et al.* 2005a,b). As NMDA-induced neuronal cell death is well established as a useful model, which is relevant to the pathogenesis of many degenerative diseases, our previous studies and the results presented here suggest novel approaches to the development of new therapies for these diseases.

In conclusion, the intravitreous injection of TRX effectively attenuated NMDA-induced retinal cell damage. The inhibition of oxidative stress and the modulation of signaling cascades, including MKKs, MAPKs and the mitochondrial apoptotic pathway, likely to act downstream of oxidative stress, were all implicated in the cytoprotective mechanism of TRX.

Acknowledgements

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