Neuroprotective effect of geranylgeranylacetone against ischemia-induced retinal injury

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Purpose: This study was conducted to assess the effects of geranylgeranylacetone (GGA) on ischemia-induced retinal injury.

Methods: Adult C57BL/6J mice were given oral treatments of GGA at 200 mg/kg daily for seven days. Ischemic retinal injury was carried out, and the extent of retinal cell death was quantitatively examined after 7 days. Immunohistochemistry for single-stranded DNA, phosphorylated form of p38 mitogen-activated protein kinase (p38 MAPK), and cleaved caspase-3 were performed one day after ischemic injury.

Results: In GGA-treated mice, we found the number of surviving retinal neurons was significantly increased compared with vehicle-treated mice. Ischemia-induced phosphorylation of p38 MAPK, which mediates apoptosis of retinal ganglion cells, was suppressed by GGA treatment. In such retinas, cleaved caspase-3- and single-stranded DNA-positive cells were also decreased compared with vehicle-treated mice.

Conclusions: Oral GGA is a useful treatment for various retinal degenerative diseases that involve ischemic injury.

Ischemic retinal injury is implicated in a number of pathological states, such as retinal artery occlusion, glaucoma, and diabetic retinopathy [1-5]. To find a treatment for this condition, it is necessary to understand the molecular regulation following ischemia. Ischemic injury is mainly associated with excessive concentrations of glutamate, which results in overactivation of glutamate receptors such as *N*-methyl-D-aspartate (NMDA) receptor, and initiates a cascade of events that leads to necrosis as well as apoptosis [6]. Several studies have suggested a possibility that retinal neurons can be protected by glutamate receptor antagonists [2,6,7], selective inhibition of N-acetylated-a-linked-acidic dipeptidase (NAALADase) [8], and activation of glutamate transporters [1,7]. In addition to typical glutamate neurotoxicity, reactive oxygen species, such as superoxide and hydrogen peroxide, are important mediators in damage caused by retinal ischemia [6,9,10]. Under stress conditions, several stress proteins, such as heat shock proteins (HSPs) are increased and function as molecular chaperones that protect native proteins from damage or refold defective polypeptides in an attempt to restore their native conformation [11]. For example, induction of HSPs by hyperthermic preconditioning contribute to retinal protection against NMDA-induced neurotoxicity [12] and light-induced photoreceptor degeneration [13].

Recent studies have shown that administration of geranylgeranylacetone (GGA), an acylic isoprenoid compound, upregulates HSP expression and exerts protective effects on a variety of organs, such as eye, brain, and heart [14-19]. In the retina, intraperitoneal injection of GGA increased HSP72 in the ganglion cell layer (GCL) and protected retinal ganglion cells (RGCs) in a rat model of glaucoma [14]. GGA also induced thioredoxin (Trx) in various tissues [20] that renders protection against ischemic injury [21] and glutamate neurotoxicity [22]. In mouse retina, GGA induced both HSP72 and Trx predominantly in the retinal pigment epithelium layer and protected photoreceptors from light damage [15]. GGA has been found to have an extremely low toxicity and has been clinically used for the treatment of gastric ulcers. In the present study, we investigated whether oral administration of GGA induces neuroprotective effect against ischemic retinal injury.

METHODS

Animals: Experiments were performed using eight week-old C57BL/6J mice (CLEA Japan, Tokyo, Japan) in accordance with the ARVO statement for the Use of Animals in Vision Research. Light intensity inside the cages ranged from 100 to 200 lux under 12 light: 12 dark cycle. Food and tap water were given ad libitum.

Oral administration of geranylgeranylacetone: GGA was a gift from Eisai Co., Ltd. (Tokyo, Japan). GGA (200 mg/kg) was used as an emulsion with 5% gum arabic (Kozakai Seiyaku, Tokyo, Japan) and 0.2% tocopherol (Wako, Osaka, Japan). The vehicle contained these reagents except for GGA.

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All medicines were orally administered to animals dairy for seven days using feeding needles.

Ischemic retinal injury: Ischemia was achieved on the day seven, and the animals were treated essentially as previously described [3,23]. Briefly, we instilled sterile saline into the anterior chamber of the left eye at 110 cm H_2O pressure for 20 min while the right eye served as a non-ischemic control. Seven days after reperfusion, animals were deeply anesthetized with diethylether and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.5% picric acid at room temperature. The eyes were removed and postfixed overnight in the same fixative and then embedded in paraffin. The posterior part was sectioned sagittally at 7 μm thickness through the optic nerve. It was then mounted and stained with hematoxylin and eosin.

Quantification of retinal damage: Ischemic damage was quantified in three ways. First, the thickness of the inner retinal layer (IRL), located between the internal limiting membrane and the interface of the outer plexiform layer and the outer nuclear layer (ONL), were measured at about 0.5 about 1.0 mm from the optic nerve head. Second, in the same sections, the number of neurons in the GCL was counted from one ora serrata through the optic nerve to the other ora serrata. Third, RGCs were retrogradely labeled from the superior colliculus (SC) with the Fluoro-Gold (FG; fluorochrome, Englewood, CO) soon after ischemic injury [23]. Each mid-

brain was exposed. The skull was removed, using a micro drill, and 2 μl of 4% FG was injected into the SC to mark the RGCs by retrograde axonal transport. Seven days after FG application, the eyes were enucleated and the retinas were detached and prepared as flattened wholemounts in 4% PFA in 0.1 M PBS solution. GCL was examined in whole-mounted retinas with fluorescence microscopy to determine the RGC density. Four standard areas (0.04 mm^2) of each retina at the point of 0.1 mm from the optic disc were randomly chosen. Labeled cells were counted by observers blinded to the identity of the mice, and the average number of RGCs/mm^2 was calculated. For statistical analysis, six animals were used for each vehicle- and GGA-treated group.

Immunohistochemistry: Frozen retinal sections were treated with 0.3% H_2O_2 for blocking endogenous peroxidase. After sections were washed in phosphate-buffered saline (PBS), they were blocked with PBS containing 10% normal goat serum and 0.8% Triton-X 100 for 1 h at room temperature. They were incubated overnight at 4 °C with a rabbit polyclonal antibody single-stranded DNA (ssDNA; 1:100; Dako Kyoto, Japan), phosphorylated form of p38 mitogen-activated protein kinase (p38 MAPK, 1:100; Promega, Madison, WI) or cleaved caspase-3 (R&D system, 1:10000; Minneapolis, MN). The sections were then incubated with goat anti-rabbit immunoglobulins conjugated to peroxidase labeled-dextran polymer (Dako EnVision, Dako) for 30 min and visu-
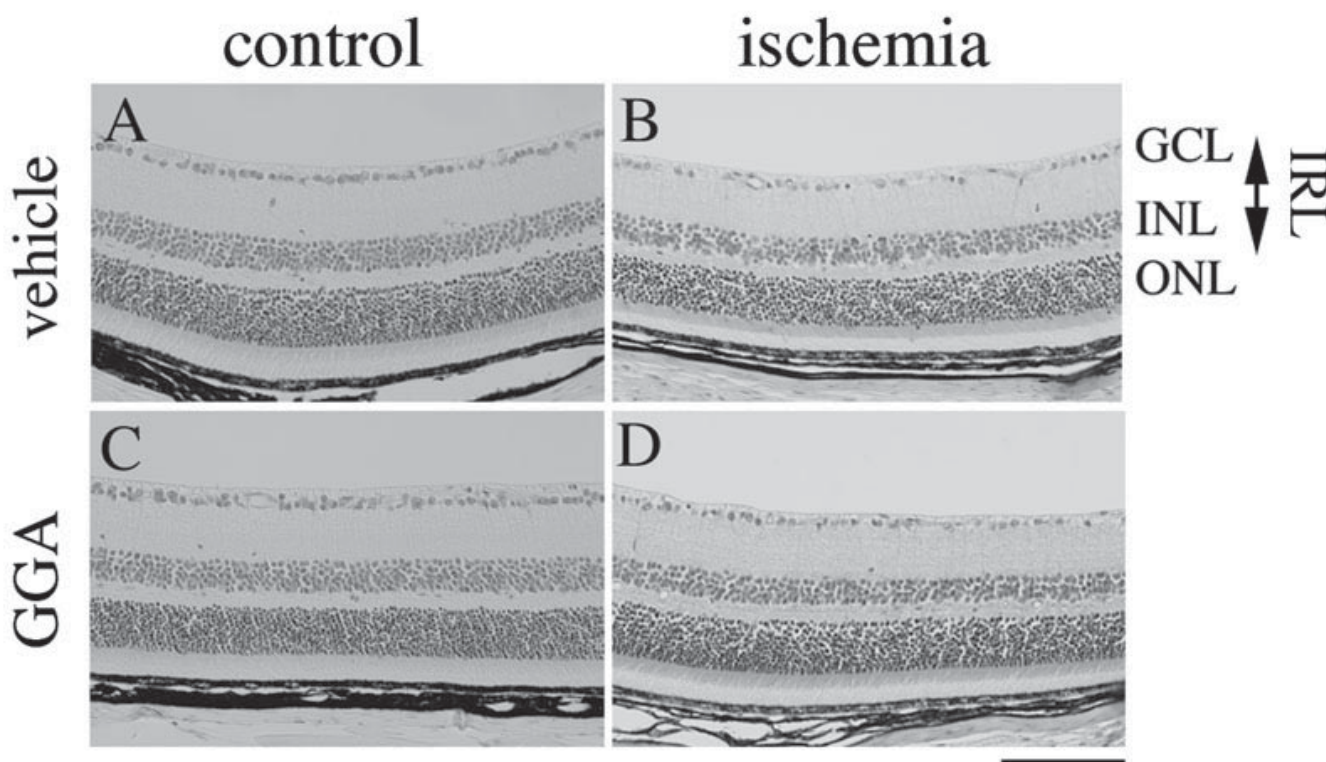

Figure 1. Effect of geranylgeranylacetone on ischemic retinal injury. Hematoxylin and eosin staining of retinal sections seven days following ischemia-reperfusion (**B**, **D**) and non-ischemic fellow eyes (**A**, **C**). (**A**, **B**) were treated with vehicle and (**C**, **D**) were treated with GGA. The following abbreviations were used: geranylgeranylacetone (GGA), ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), inner retinal layer (IRL). The scale bar equals 100 μm.

alized with DAB substrate kit (Dako). Counting of ssDNA- and phosphorylated p38-positive cells was performed by two masked investigators and averaged.

Statistics: Data are presented as mean±SD except as noted. When statistical analysis was performed, Student's t-test was used to estimate the significance of the results. Statistical significance was accepted at p<0.05.

RESULTS

Effect of geranylgeranylacetone on ischemia-induced retinal cell death: Since GGA may prevent neural cell apoptosis in adult retina, we examined the effect of vehicle and GGA during ischemic retinal injury. The morphology of vehicle- and GGA-treated mice was normal (Figure 1A,C). Histological evaluation demonstrated a significant decrease in ischemic damage in GGA-treated mice (Figure 1D) compared with vehicle-treated mice (Figure 1B). The thickness of the IRL after ischemia was decreased to 65±7 μm (n=6) in vehicle-treated mice, but 79±11 μm (n=6) in GGA-treated mice (Figure 2A). Second, the number of surviving cells in the GCL was also
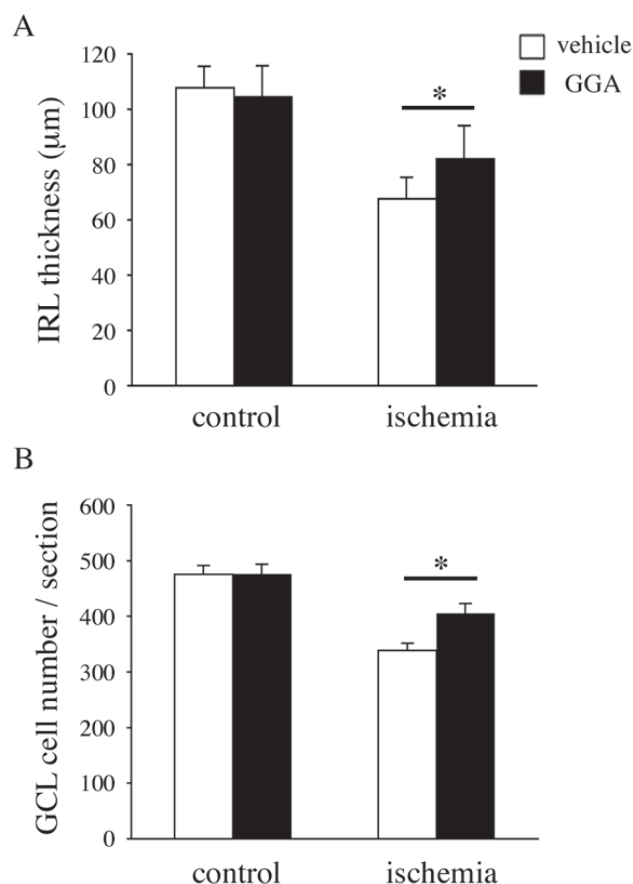

Figure 2. Quantitative analysis of ischemic retinal injury in vehicle- and geranylgeranylacetone-treated mice. Shown are the thickness of the inner retinal layer (IRL; **A**) and the number of cells in the ganglion cell layer (GCL; **B**). Results of six independent experiments are presented as the mean±SD. Note the decreased retinal damage in geranylgeranylacetone (GGA)-treated mice. Asterisk (*) indicates p<0.05.

increased in GGA-treated mice (405±18/section; n=6) compared with vehicle-treated mice (339±13/section; n=6; Figure 2B). These results suggest that GGA protects inner retinal neurons in the ischemic retina. Since GCL contains RGCs, displaced amacrine cells, and other minor cell types [24], we next carried out retrograde labeling of RGCs with FG and tried to determine the effect of GGA on RGC survival. Figure 3A-H shows the representative results of RGC labeling in vehicle- and GGA-treated mice. Consistent with the results of GCL counting (Figure 2B), the extent of ischemia-induced RGC death seemed to be mild in GGA-treated mice (Figure 3D,H) compared with vehicle-treated mice (Figure 3B,F). Quantitative analysis revealed that FG-labeled (surviving) RGCs after ischemia was significantly increased in GGA-treated mice (83±3%; n=6) compared with vehicle-treated mice (66±2%; n=6, Figure 4). These results demonstrate that GGA has a protective effect on inner retinal neurons, including RGCs, after ischemic injury.

Effect of geranylgeranylacetone on cell death pathway in the ischemic retina: We next analyzed apoptotic cells in the retina by detection of ssDNA-immunopositive cells one day after ischemic injury [25]. Control retinas showed practically no signals in both vehicle- and GGA-treated mice (data not shown). In ischemic retinas, many ssDNA positive cells were observed in vehicle-treated mice (Figure 5A), but the number was significantly decreased (Figure 6) in GGA-treated mice (Figure 5B). Previous studies have suggested a possibility that activation of p38 MAPK pathway leads to ischemia-induced retinal cell apoptosis [23,26,27]. In control retinas, phosphorylated/activated p38-positive cells were absent (data not shown). In contrast, one day after ischemia, immunohistochemical analysis showed many phosphorylated p38-positive cells in the inner retina in vehicle-treated mice (Figure 5C), but the number was apparently decreased (Figure 6) in GGA-treated mice (Figure 5D). We previously demonstrated that ischemia-induced retinal cell apoptosis in the inner retina is executed mainly by caspase-3 [10,23]. We observed cleaved caspase-3-like immunoreactivity in the inner retina in vehicle-treated mice (Figure 5E), but noted they were sparse in GGA-treated mice (Figure 5F). These results suggest that GGA suppressed the activation of p38 MAPK and caspase-3, and prevented ischemia-induced retinal cell apoptosis.

DISCUSSION

In the present study, we demonstrated that oral administration of GGA exerted anti-apoptotic effects against ischemic retinal injury in vivo. In addition, we found that GGA suppressed both p38 MAPK and caspase-3 activation in the ischemic retina. Our results are consistent with former studies describing that activation of p38 induces RGC apoptosis after axotomy of the optic nerve or mediated by glutamate neurotoxicity [26,27]. In addition to typical glutamate neurotoxicity, reactive oxygen species, such as superoxide and hydrogen peroxide, are important mediators of retinal damage caused by ischemia [6,9,10]. We previously demonstrated that hydrogen peroxide-induced cell death is attenuated in isolated RGCs that lack apoptosis signal-regulating kinase 1 (ASK1)
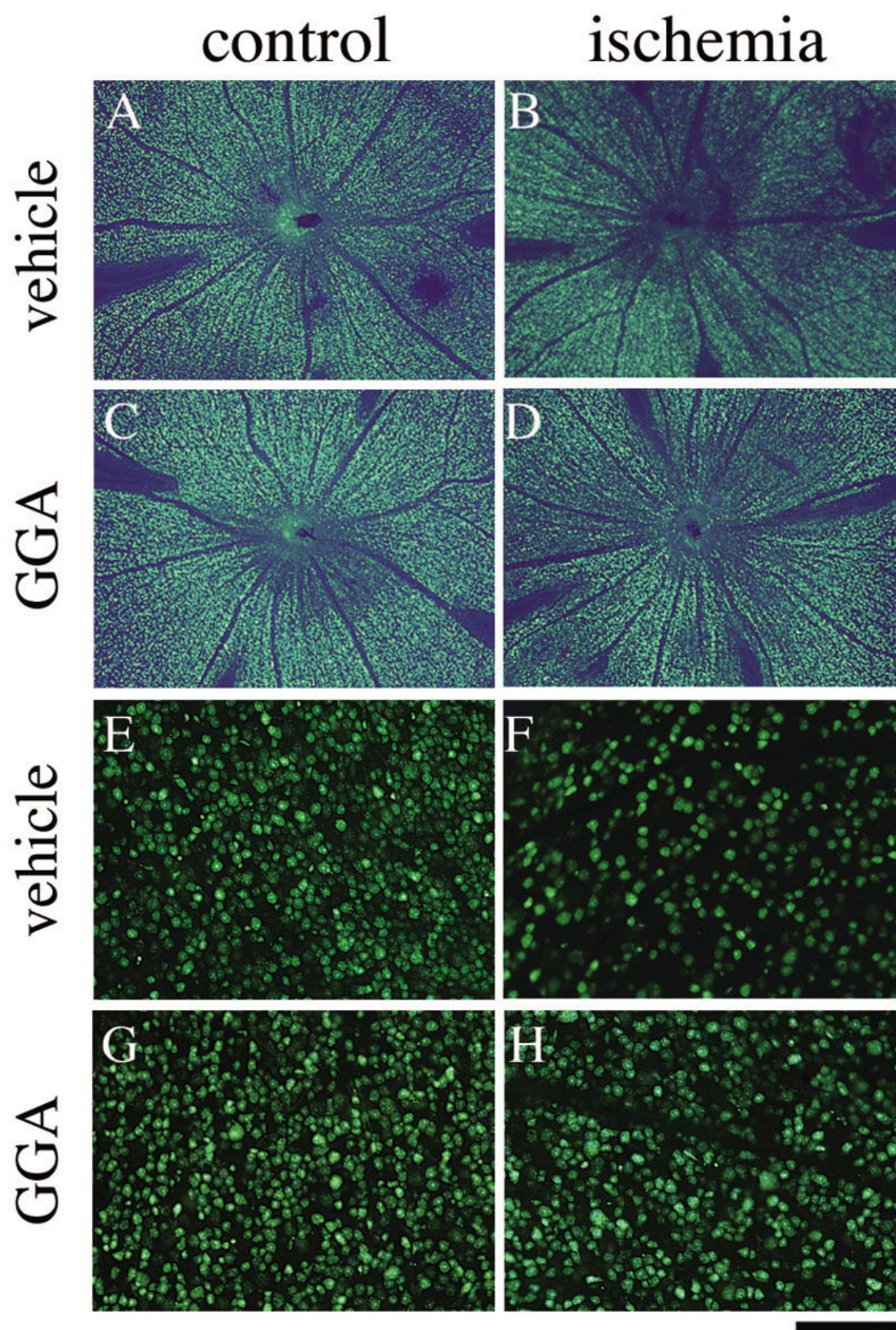
Figure 3. Effect of geranylgeranylacetone on ischemia-induced retinal ganglion cell death. Retrograde labeling of retinal ganglion cells (RGCs) seven days after ischemic injury (**B**, **D**, **F**, **H**) and non-ischemic fellow eyes (**A**, **C**, **E**, **G**, respectively). (**A**, **B**, **E**, **F**) were treated with vehicle and (**C**, **D**, **G**, **H**) were treated with geranylgeranylacetone (GGA). **E-H** are enlarged images of **A-D** at approximately the same distance (0.1 mm) from the optic disc. Bar represents 50 µm in **A-D** and 100 µm in **E-H**.
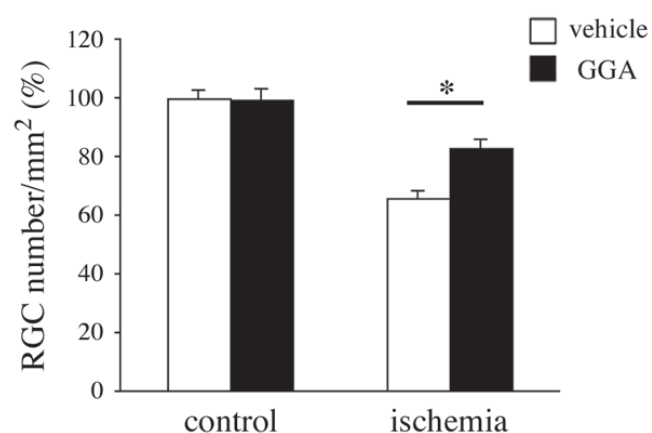
Figure 4. Quantitative analysis of retinal ganglion cell number after ischemic retinal injury in vehicle- and geranylgeranylacetone-treated mice. Retinal ganglion cells (RGC) number is shown as a percentage of eyes from untreated animals. The number of labeled RGCs within four fields of identical size (0.04 mm^2) at approximately the same distance (0.1 mm) from the optic disc were counted, and RGCs/mm^2 were calculated. Results of six independent animals are presented as the mean±SD. Note the increased RGC resistance against ischemic injury in geranylgeranylacetone (GGA)-treated mice. Asterisk (*) indicates p<0.01.
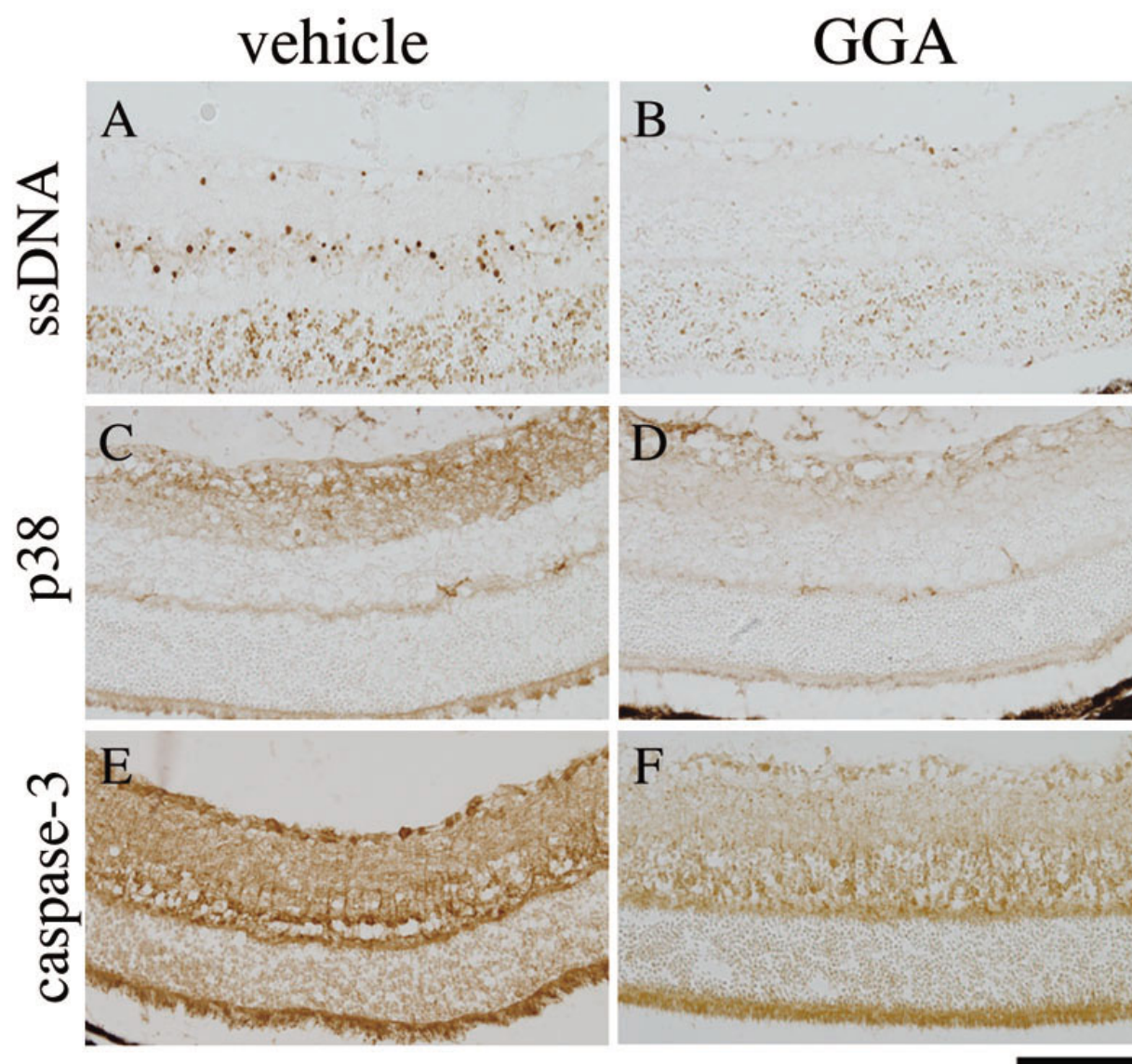

Figure 5. Localization of apoptotic cells, phosphorylated p38 and cleaved caspase-3 immunoreactivity in ischemic retina in vehicle- and geranylgeranylacetone-treated mice. Immunohistochemical analysis of single-stranded DNA (ssDNA; **A**, **B**), phosphorylated form of p38 mitogen-activated protein kinase (p38; **C**, **D**) and cleaved caspase-3 (**E**, **F**) one day after ischemic injury are shown. In the figure geranylgeranylacetone is abbreviated as GGA. Bar represents 100 μm.

[23]. ASK1 is a MAPK kinase kinase that is activated in response to various stimuli and relays its apoptotic signals to p38 [28,29]. Consistently, ischemia-induced phosphorylation of p38 and RGC death were suppressed in ASK1-deficient mice [23]. Together with previous findings that HSP72 is a negative regulator of ASK1 [30], and GGA has been found to induce this stress protein in various retinal cell types [14,15], ASK1-p38 pathway may be one of the targets of GGA. GGA also has been found to induce Trx, another possible factor that negatively regulates ASK1 activity [31], in the inner retina as well as the retinal pigment epithelial layer [15]. Recent studies have suggested the possibility that Trx is a kind of neurotrophic factor and may play a crucial role in maintaining retinal neurons from ischemic injury [21]. Since other trophic factors such as ciliary neurotrophic factor and glial cell line-derived neurotrophic factor supplied from Müller glial cells rescue neural cells [5,32-35], application of GGA as well as Trx, in combination with these trophic factors, may stimulate multiple cellular targets and activate separate mechanisms in order to rescue RGCs during ischemic injury. Further studies determining the effect of GGA on trophic factor productions in RGCs, Müller glial cells and RPE cells, will be needed.

Ischemic injury is now implicated in a number of retinal diseases including glaucoma [1-3,6,7]. There is evidence that HSPs are involved in the glaucomatous eyes of humans [36] and in experimental models of primary open angle glaucoma (POAG) [37]. Ishii et al. demonstrated that intraperitoneal injection of GGA protected RGCs in a rat model of POAG [14].

In addition, we showed that oral administration of GGA also protected RGCs in response to acute ischemic injury. Although further studies are needed to examine its long-term effect, GGA may be available for the treatment of neurodegenerative diseases including glaucoma.
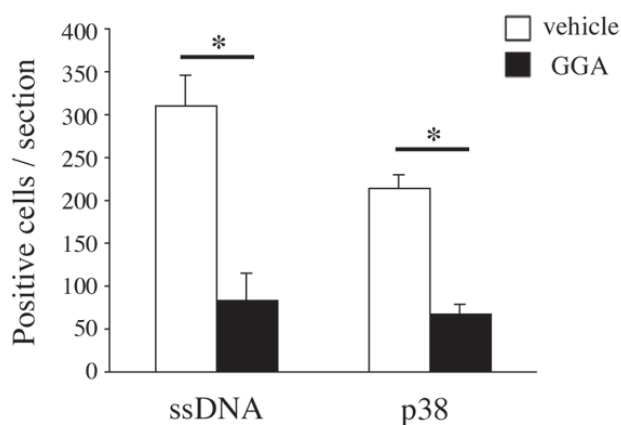

Figure 6. Quantitative analysis of retinal apoptosis after ischemic retinal injury in vehicle- and geranylgeranylacetone-treated mice. The number of single-stranded DNA (ssDNA)- and phosphorylated p38 mitogen-activated protein kinase (p38)-positive cells after ischemic retinal injury is shown. Results of three independent experiments are presented as the mean±SD. In the figure geranylgeranylacetone is abbreviated as GGA. Asterisk (*) indicates $p < 0.05$.

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