

# Effects of Indocyanine Green on Retinal Ganglion Cells

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**PURPOSE.** Recently, indocyanine green (ICG) has been used to visualize the internal limiting membrane during vitrectomy. After intraocular administration, ICG accumulates at the optic disc. In this study, the effects of ICG on retinal ganglion cells (RGCs) were examined.

**METHODS.** In vitro, rat RGCs were purified by a two-step immunopanning procedure, briefly exposed to ICG ( $2.5 \times 10^3$  mg/L), and irradiated with an endoilluminator for 15 minutes or incubated in the presence of ICG (concentration: 2–250 mg/L) without irradiation. The number of viable RGCs was counted after 3 days in culture. In vivo, after rats received an intravitreal injection of 3  $\mu$ L ICG (0.25 and 2.5 mg/L), the distribution of ICG was observed with a fundus camera, and the number of viable RGCs was examined by a DiI (1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate)-retrolabeling technique.

**RESULTS.** In vitro, a brief exposure to ICG and light did not affect RGC survival. However, ICG reduced the number of viable RGCs in a dose-dependent manner when the cells were exposed for 3 days. In vivo, the dye was initially distributed on the retinal surface and around the optic disc. At day 7, the fluorescence became invisible in the 0.25-mg/L group, whereas the staining of the optic disc contour was evident in the 2.5-mg/L group. The number of viable RGCs decreased significantly in the 2.5-mg/L group 14 days after the injection.

**CONCLUSIONS.** ICG showed an inherent toxicity to RGCs in a dose-dependent manner. Lower concentration and shorter staining time of ICG should be used for dye-assisted vitrectomy. (*Invest Ophthalmol Vis Sci.* 2004;45:943–947) DOI:10.1167/iov.03-1026

Recently, indocyanine green (ICG), an amphiphilic tricyanocyanine dye, has been used to stain and visualize the internal limiting membrane (ILM) during intraocular surgery to facilitate ILM removal.<sup>1–3</sup> However, several investigators have reported adverse effects, such as visual field defects<sup>4–6</sup> and retinal pigment epithelial atrophy,<sup>7</sup> possibly related to the use of ICG during vitrectomy. On the contrary, several groups have demonstrated that good visual function can be attained after ICG-assisted vitrectomy free of such ICG-related adverse ef-

fects.<sup>8–11</sup> However, the underlying cause of ICG-related adverse effects is not fully unraveled.

After the intraocular administration of ICG, the residual dye is distributed around the macular area and accumulates thereafter at the optic disc.<sup>12–14</sup> Using anterograde and retrograde labeling techniques in rabbits and rats, a recent study clearly demonstrated that ICG diffuses into retinal ganglion cells (RGCs) and traces axons.<sup>15</sup> However, the toxic effects of ICG on RGCs has not been studied. In this study, we investigated the effects of ICG on rat RGCs, both in vitro and in vivo.

## MATERIALS AND METHODS

### Animals

Wister rats (6–8 weeks and 8 days old) were purchased from Saitama Laboratory Animal Supply Inc. (Saitama, Japan). The animals were kept under a standard laboratory condition with a 12-hour light–dark cycle. All experiments were conducted in accordance with the Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Preparation of ICG Solution

For in vitro experiments, 25 mg of ICG (Diagnogreen; molecular weight, 774.96; Daiich Pharmaceutical, Tokyo, Japan) was dissolved with 1 mL of distilled water, which was further diluted with distilled water to obtain ICG solutions with concentrations of 0.2, 1.0, 2.5, 5, 10, and  $25 \times 10^3$  mg/L. These solutions were further diluted (at a dilution of 1:100) with B27 complete medium (described later) to give rise to ICG solutions with concentrations of 2, 10, 25, 50, 100, and 250 mg/L. For in vivo experiments, 25 mg of ICG was dissolved with 1 mL of distilled water, which was further diluted with distilled water to obtain ICG solutions with concentrations of  $0.25 \times 10^3$  and  $2.5 \times 10^3$  mg/L. The solutions were further diluted at 1:1000 in physiological saline to obtain 0.25- and 2.5-mg/L ICG solutions. The pH and osmolality of the ICG solutions used in this study are shown in Table 1.

### Purification and Culture of RGCs

RGCs were purified by a two-step immunopanning procedure, as described previously.<sup>16,17</sup> Briefly, the dissociated cells of retinas from 8-day-old Wister rats were incubated in flasks (Nunc A/S, Roskilde, Denmark) coated with an anti-rat macrophage monoclonal antibody (1:50; Chemicon, Temecula, CA) to exclude macrophages, and then incubated in tubes (Corning, Acton, MA) coated with an anti-rat Thy1.1 monoclonal antibody (1:300; Chemicon). RGCs adherent to the tubes were collected by centrifugation at 600 rpm for 5 minutes and seeded on 13-mm glass coverslips in a 24-well plate that had been coated with 50  $\mu$ g/mL poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and 1  $\mu$ g/mL laminin (Invitrogen, Carlsbad, CA). Purified RGCs were plated at a density of approximately 1000 cells/well. RGCs were cultured in serum-free B27 complete medium containing neurobasal medium<sup>18</sup> (Invitrogen) with 1 mM L-glutamine (Sigma-Aldrich), B27 supplement (Invitrogen), 40 ng/mL human recombinant brain-derived neurotrophic factor (BDNF; Sigma-Aldrich), 40 ng/mL rat recombinant ciliary neurotrophic factor (CNTF; Peprotech, Rocky Hill, NJ), 10  $\mu$ M forskolin (Sigma-Aldrich), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. To examine the effect of acute light exposure, the cells were treated essentially as described previously.<sup>19</sup> Briefly, they were incubated in 1 mL of  $2.5 \times 10^3$  mg/L ICG in PBS for 1 minute, 1 hour after their

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TABLE 1. pH and Osmolarity of ICG Solutions

Concentration (mg/L)	In Vitro Experiments											
	Long Exposure								Brief Exposure		In Vivo Experiments	
	0	2	10	25	50	100	250	0.25	2.5	0	0.25	2.5
PH	7.37	7.39	7.4	7.42	7.48	7.48	7.44	7.4	7.41	6.40	6.45	6.46
Osmolarity (mOsM)	201	202	197	205	192	210	189	204	201	288	286	287

For in vitro experiments, ICG was diluted with distilled water, which was further diluted with the culture medium. For in vivo experiments, ICG was diluted with distilled water, which was further diluted with physiological saline.

isolation, washed three times with PBS, and subjected to an acute exposure to light for 15 minutes with a standard endoillumination probe connected to a light source (Accurus; Alcon, Fort Worth, TX; spectral radiance measured as previously described<sup>20</sup> is shown in Fig. 1). The probe was positioned at 1 cm above the cells, and the cells were irradiated evenly. The purified RGCs were then cultured for 3 days in 400  $\mu$ L of serum-free B27 complete medium. To examine the effect of ICG after long exposure, RGCs were cultured for 3 days in 400  $\mu$ L B27 complete medium containing ICG at a concentration of 2, 10, 25, 50, 100, and 250 mg/L. Plates were incubated in a tissue culture incubator with humidified atmosphere containing 5% CO<sub>2</sub> and 95% air at 37°C.

Three days after experiments, cell viability was determined by using the fluorescence viability agent calcein-acetoxymethyl ester (calcein-AM, 1  $\mu$ M; Molecular Probes, Eugene, OR). In the present study, a surviving RGC was defined as a cell with calcein-AM-stained cell body and a neurite outgrowth of at least two cell diameters from the cell body. All surviving RGCs on each glass coverslip were counted at 200 $\times$  magnification under an inverted fluorescence microscope. In B27 complete medium, approximately 500 cells were counted per well. The number of RGCs after 3 days of culture in B27 complete medium was set at 100%. The fluorescent images of the stained RGCs were captured by a laser scanning microscope (Fluoview; Olympus, Tokyo, Japan).

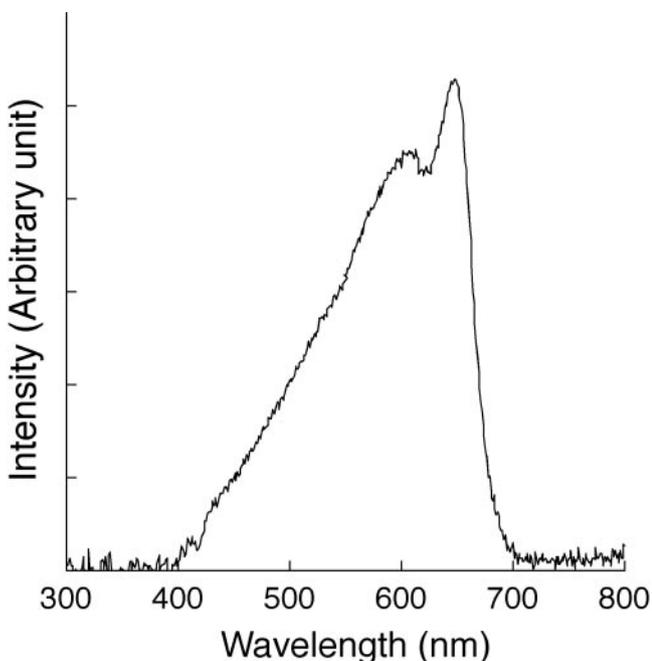


FIGURE 1. Spectral radiance of the endoilluminator used in this study. The spectral distributions are normalized to their integrated radiances.

## Intravitreal Injection

ICG was injected to the vitreous cavity of right globes, as described previously.<sup>21</sup> A general anesthesia was induced with an intraperitoneal injection (1000  $\mu$ L/kg) of a mixture (5:1) of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Celactal; Bayer, Tokyo, Japan). After mydriasis was achieved with a drop of 0.5% tropicamide, a 33-gauge needle was inserted into the midvitreous of the right eye, guided by a stereoscopic microscope, with care taken to avoid lens and retinal injury. A single injection of 3  $\mu$ L ICG at a concentration of 0.25 or 2.5 mg/L ( $n = 5$  in each group) was completed in 1 minute. For control, 3  $\mu$ L of physiological saline was injected intravitreally to the right eyes of other animals ( $n = 5$ ).

## Fundus Epifluorescence Examination

Twelve, 24, and 48 hours and 7 days after the intravitreal injection, the fundus of the rats was observed with a fundus camera (model TRC50IX; Topcon, Tokyo, Japan) equipped with a 780-nm infrared illumination and ICG filter sets. After anesthesia and mydriasis was achieved, the fundus photographs were taken with a slide glass placed on the rat cornea. All fundus images were obtained under the same illumination intensity and transported into a computer system with the aid of a charged-coupled device camera.

## RGC Counting

RGC counting was performed as has been described.<sup>21</sup> Seven days after the intravitreal injection of ICG, anesthetized rats were placed in a stereotaxic frame, two holes were made in the skull, and 0.2  $\mu$ L of 5% DiI (1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate) in dimethyl sulfoxide was injected into the superior colliculi of both sides. Each injection was made over 2 minutes with a syringe (Hamilton, Reno, NV). Seven days thereafter, the globes were enucleated, and cornea, lens, and vitreous were removed. Six radial cuts were made at the peripheral retina to the equator, and the retina was separated and mounted on a slide with vitreous side facing up. The number of cell bodies of RGCs was counted. Counts were taken from six circumferential points 1 mm eccentric from the optic nerve of the retinal flat preparation. The counts were averaged to give the count in one eye. RGC counting was performed with the observer masked as to treatment.

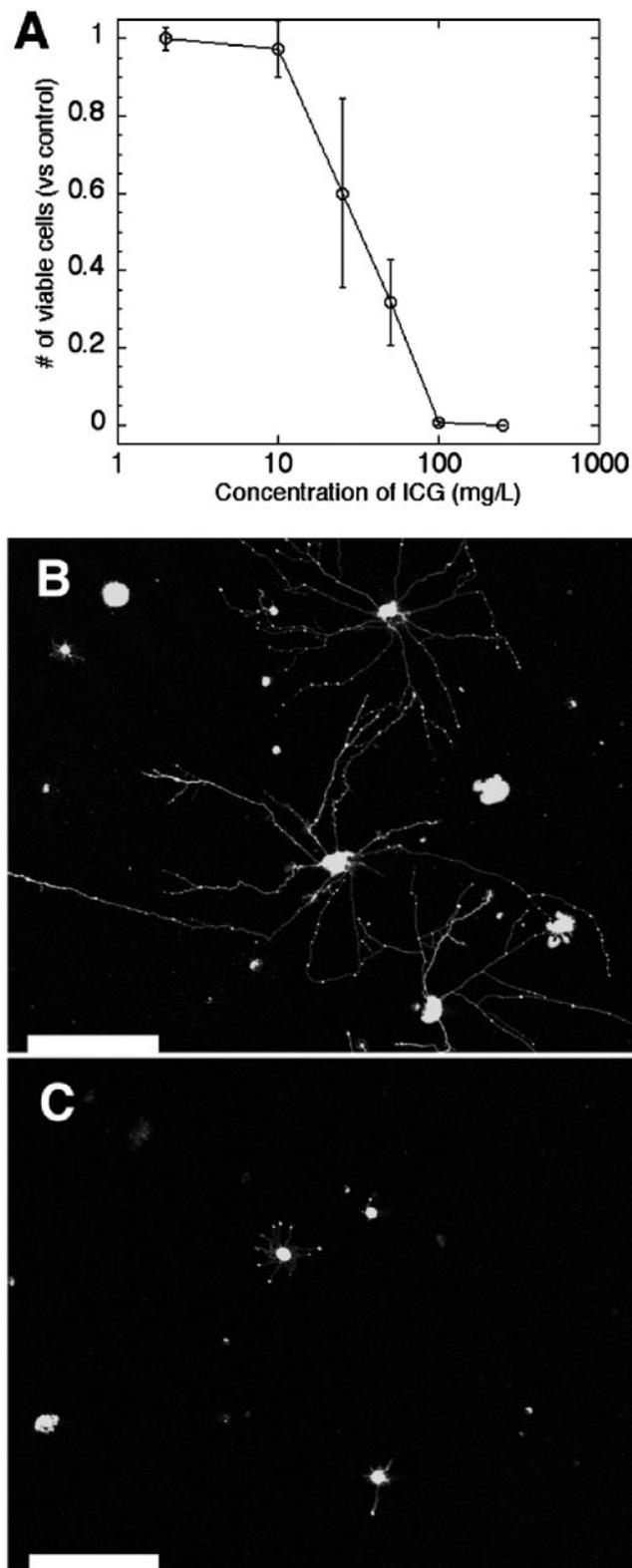
## Statistics

The Mann-Whitney test was used to compare the number of RGCs in vivo analysis.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of ICG on Purified RGCs In Vitro

The number of viable RGCs was not affected after a brief exposure to ICG at a concentration of  $2.5 \times 10^3$  mg/L and exposure to standard endoillumination for 15 minutes (the



**FIGURE 2.** Effect of ICG on purified RGCs in vitro. (A) RGCs were cultured in B27 complete medium containing ICG at concentrations of 2, 10, 25, 50, 100, and 250 mg/L. Surviving RGCs were counted 3 days after culture and the number set at 100%. These experiments were repeated three times in two separate wells. Data are the mean  $\pm$  SD ( $n = 6$ ). Note that the survival of rat RGCs was decreased in the presence of ICG in a dose-dependent manner. (B, C) Fluorescent images of RGCs purified from 8-day-old rats. (B) RGCs in B27 complete medium. (C) RGCs in B27 complete medium+ICG (50  $\mu$ g/L). The cell

number of viable RGCs was  $102\% \pm 3.4\%$  compared with the nontreated control;  $n = 4$ ). In contrast, after a lengthy exposure, ICG showed toxic effects on purified RGCs in a dose-dependent manner (Fig. 2A). In the absence of ICG, RGCs extended long neurites (Fig. 2B). There was no significant effect of ICG below the concentrations of 10 mg/L. A significant toxic effect of ICG was observed at a concentration of 25 mg/L and became more evident at a concentration of 50 mg/L (Fig. 2C and data not shown). No viable RGCs were found when the cells were cultured with ICG at a concentration of 100 or 250 mg/L. The calculated  $IC_{50}$  of ICG to RGCs was  $4.2 \times 10^{-5}$  M (31 mg/L).

### Distribution of ICG Dye in Rat Eyes after Intravitreal Injection

Twelve hours after rats received intravitreal injection of ICG at a concentration of 2.5 mg/L, the dye diffused to the vitreous cavity (Fig. 3A). Twenty-four hours after the injection, fluorescence was observed throughout the retina, with brightest fluorescence at the contour of the optic disc (Fig. 3C). The staining of the optic disc contour was still visible 7 days after the injection in all animals examined ( $n = 5$ ; Fig. 3D). When the rats received 0.25 mg/L ICG, the ICG diffused to the vitreous cavity 12 hours after the injection (Fig. 3E) and distributed mainly to the optic disc contour 24 hours after the injection (Fig. 3F). Forty-eight hours after the injection, the staining of the optic disc contour was still evident in 60% ( $n = 5$ ) of the animals (Fig. 3G). No fluorescence was observed after 7 days (Fig. 3H).

### Effect of ICG on RGCs In Vivo

When rats received an intravitreal injection of 0.25 mg/L ICG, the number of viable RGCs showed a tendency to decrease, but the difference was not significant (Fig. 4). When rats received an intravitreal injection of 2.5 mg/L ICG, the number of the viable RGCs decreased significantly compared with the number in the saline-treated control group.

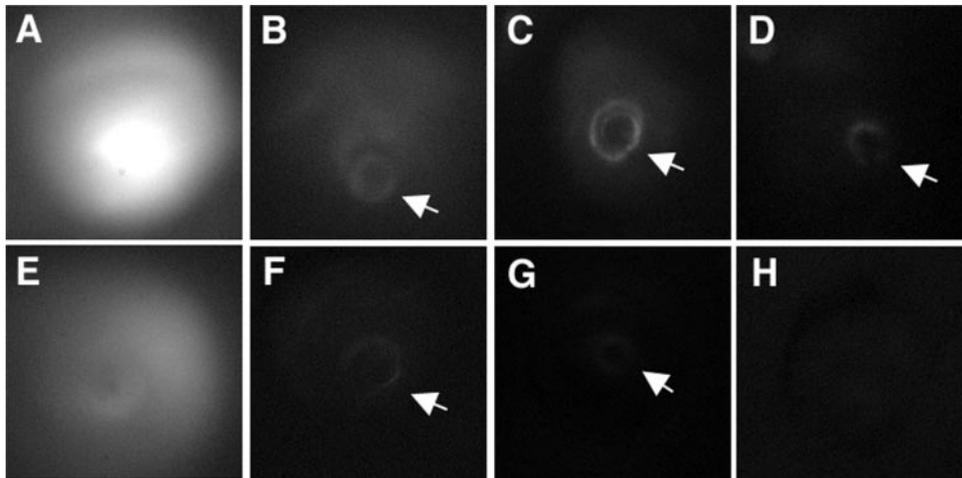
Similar to a previous report,<sup>22</sup> no other histologic abnormalities were apparent on light microscopy (data not shown).

### DISCUSSION

In this study, ICG was toxic to RGCs both in vitro and in vivo. These results suggest that if an excessive amount of residual ICG dye remains in the eye after vitrectomy, ICG-induced RGC damage may occur in a dose-dependent manner.

Our in vitro experiments demonstrated that dose-dependent ICG-induced damage to RGCs can occur without photoirradiation, with an  $IC_{50}$  of  $4.2 \times 10^{-5}$  M (31 mg/L), suggesting that a lengthy exposure to ICG can damage RGCs. The photodynamic effect of ICG, presumably mediated by radical species, is well studied.<sup>23-25</sup> In addition, in vitro viability assays have demonstrated that ICG is toxic to several cells, even in the absence of light.<sup>23,24</sup> After a brief exposure of ICG at a concentration of  $1.3 \times 10^{-3}$  M (0.1%), the viability of RPE cells was reduced by approximately 25%, as demonstrated by WST-1 assay,<sup>23</sup> although this may be related to osmotic effect.<sup>26</sup> Similarly, when HaCat cells were treated by ICG at a concentration of  $5 \times 10^{-5}$  M for 24 hours, cell viability was decreased by 18%, according to MTT assay.<sup>24</sup> On the contrary, another study

bodies and neurites of RGCs were stained by calcein-acetoxymethyl ester (calcein-AM). Similar morphologic changes in RGCs were observed in the presence of 25 mg/L ICG (data not shown). Scale bar, 200  $\mu$ m.



**FIGURE 3.** Distribution of ICG after intravitreal injection. Infrared fundus photograph showing the distribution of ICG dye observed with a fundus camera. Infrared fundus photographs were taken from the right eyes of rats that received a 3- $\mu$ L injection of ICG at concentrations of 2.5 (A–D) or 0.25 mg/L (E–H) at 12 (A, E), 24 (B, F) and 72 (C, G) hours and 7 days (D, H). (B, C, D, F, G, arrows) Staining of the optic disc contour.

demonstrated that a 24-hour exposure of ICG at a dose up to  $1 \times 10^{-4}$  M did not show a toxic effect on U937 cells.<sup>25</sup> Taken together, these results suggest that ICG possess an inherent toxicity (dark toxicity) that is cell-type-specific. In accordance with these findings<sup>23–25</sup> our results imply that RGCs are highly susceptible to ICG.

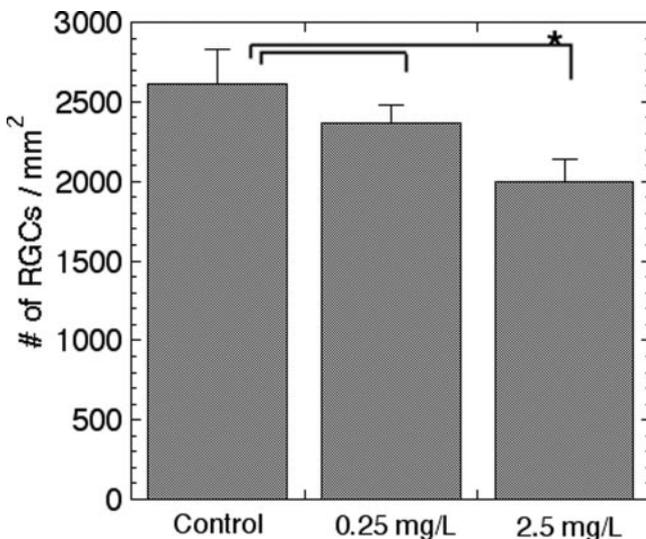
In vivo, we have shown that ICG is toxic to RGCs at a high dose (2.5 mg/L). Under this condition, the number of RGCs was reduced by 23%. If the dye is distributed evenly in the vitreous cavity (60  $\mu$ L in rats), the intravitreal concentration of ICG would be  $1.7 \times 10^{-7}$  M. In light of our in vitro experiments, 23% reduction of RGCs occurs in the presence of ICG at a concentration of  $2.1 \times 10^{-5}$  M. Apparent higher toxicity of ICG in vivo compared with that in vitro may be explained as follows. First, our in vivo observation showed that the dye initially diffuses throughout the retina and accumulates to the optic disc. Thus, RGCs may have been exposed to much higher concentrations of ICG than  $1.7 \times 10^{-7}$  M. Second, previous laboratory studies have demonstrated that exposure

to ICG alone induces ILM detachment and disruption of the ILM in cadaveric eyes.<sup>27</sup> Although we found no apparent light microscopic abnormalities in vivo experiments under the present conditions, yet undetermined damage to the retinal structure may have caused secondary degeneration of the RGCs. Finally, it is possible that ICG exerted a photodynamic effect.

A previous study demonstrated that the toxic effects of an ICG solution on RPE is related to osmotic effects of the solvent.<sup>26</sup> Our in vitro experiments used ICG solutions with osmolarities ranging from 189 to 210 mOsm. These osmolarities were low compared with those used in former studies, in which the osmolarity of the ICG solutions was approximately 275 to 290 mOsm. This is presumably because the osmolarity of the solvent medium (i.e., neurobasal medium<sup>18</sup>: 201 mOsm in this study) is lower compared with the solvent medium used in previous studies ( $\sim$ 290–300 mOsm).<sup>28</sup> Under the current in vitro conditions, the toxic effect on RGCs was observed in a concentration-dependent, but not osmolarity-dependent manner. In addition, osmolarity of the ICG solutions used in vivo experiments was not reduced compared with the physiological saline. Taken together, it is likely that the toxic effect of ICG solution to RGCs is dose-dependent and cannot be related to hypo-osmolarity.

Our results raise the possibility that ICG-induced RGC damage can occur after ICG-assisted vitrectomy. Although our in vivo experiments do not mimic the clinical situation, the dye initially was distributed on the retinal surface and transported toward the optic disc. Although the dye is immediately washed after ILM staining during vitrectomy, persistent retinal staining is observed,<sup>12–14</sup> suggesting that a significant amount of the dye resides in the eye. It is impossible to know the actual concentration of the residual dye; however, the concentration of the retinal ICG may be higher in a patient's eye than was observed our in vitro experiments, because the fluorescence is apparently brighter in patients after ICG-assisted vitrectomy compared with those in rats in the present experiments.

Another important question is whether ICG exerts a photodynamic effect on RGCs during ICG-assisted vitrectomy. In vitro, we showed that the number of viable RGCs was not decreased after a brief exposure to ICG at a concentration of  $2.5 \times 10^3$  mg/L and to a standard endoillumination for 15 minutes. It should be noted that a previous study demonstrated that ICG can induce apoptosis in human RPE cells under an irradiation condition identical with ours.<sup>19</sup> Because the ICG concentration used herein ( $2.5 \times 10^3$  mg/L) is comparable to the clinically used concentrations (0.04%–0.5%)<sup>1–6</sup> and the



**FIGURE 4.** Effect of ICG on RGCs in vivo. The number of RGCs was counted 14 days after intravitreal injection of 0.25 or 2.5 mg/L ICG. Seven days after DiI was injected into the superior colliculi, the number of viable RGCs was counted from six circumferential points 1 mm eccentric from the optic nerve of the retinal flat preparation. Note that the number of RGCs was significantly decreased in the 2.5-mg/L group. The data represent the mean of results from five eyes; error bars, SEM. \* $P < 0.05$ , Mann-Whitney test.

light dose was maximal, we believe that an ICG-induced photodynamic effect on RGCs is unlikely to happen during surgery.

In conclusion, our results demonstrate that ICG was toxic in long exposure of 72 hours to RGCs both in vivo and in vitro at concentrations lower than clinically used and that ICG-induced RGC damage can occur theoretically during and after ICG-assisted vitrectomy. One thing that deserves special mention is that some investigators have reported ICG-related adverse effects.<sup>4-6</sup> Previous investigators have reported a possible toxicity of ICG on the retina. However, ICG was not toxic in brief exposure of 20 minutes in our experimental model. Using human donor eyes, previous investigators have demonstrated that the inner retina is severely damaged when ILM is stained with ICG and illuminated by a light source with the emission beyond 620 nm,<sup>27</sup> a wavelength overlapping the absorption band of ICG.<sup>28</sup> In addition, several laboratory studies have demonstrated that ICG can cause retinal pigment epithelial damage in vitro.<sup>19,23</sup> Together with the present study, these results suggest that the damages can occur in retina when higher concentration of ICG is injected into the vitreous cavity. However, it is uncertain whether ICG-induced RGC damage occurs in every clinical situation. It is particularly noteworthy that several investigators have reported a favorable visual functional outcome free of ICG-related adverse effects after ICG-assisted vitrectomy.<sup>8-11</sup> In vitro, a long exposure to ICG did not affect the RGC survival when the dye was applied at concentrations lower than 10 mg/L (0.001%). Thus, it seems important to reduce the residual dye in patients' eyes. Lower concentration and shorter staining time should be used in ICG-assisted vitrectomy.

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