Intracellular Events in Retinal Glial Cells Exposed to ICG and BBG

Shubei Kawabara,1 Yasuaki Hata,1 Muneki Miura,1 Takeshi Kita,1 Akibito Sengoku,1 Shintaro Nakao,1,2 Yasutaka Mochizuki,1 Hiroshi Enaida,1 Akifumi Ueno,1 Ali Hafezi-Moghadam,2 and Tatsuro Ishibashi1

PURPOSE. To investigate the intracellular events in retinal glial cells exposed to indocyanine green (ICG) and brilliant blue G (BBG).

METHODS. The human Müller cell line MIO-M1 was exposed to a low dose (0.25 mg/mL) and a clinical dose (2.5 mg/mL) of ICG and a clinical dose (0.25 mg/mL) of BBG for 15 minutes, respectively. To quantify the proliferation and viability of the cells, [3H]-thymidine incorporation was measured and cell numbers were counted 24 hours after treatment. Cell morphology was evaluated using phase-contrast microscopy and transmission electron microscopy. The effects of ICG and BBG on phosphorylation of p38 MAPK and cleavage of caspase-9 and caspase-3 were examined by Western blot.

RESULTS. ICG and BBG significantly reduced [3H]-thymidine incorporation in MIO-M1 cells compared with the vehicle-treated controls (P < 0.01). Cell number significantly decreased after exposure to ICG at 2.5 or 0.25 mg/mL (P < 0.01) but did not decrease after exposure to BBG at 0.25 mg/mL. Transmission electron microscopy revealed apoptotic changes only in the ICG-treated cells. Prominent p38 MAPK phosphorylation was observed in the presence of ICG, even at the low concentration and within a short time exposure; however, no apparent enhancement was observed in the presence of 0.25 mg/mL BBG. Furthermore, ICG, but not BBG, induced the cleavage of caspase-9 and caspase-3, which was inhibited by an inhibitor of p38 MAPK.

CONCLUSIONS. ICG is toxic to retinal glial cells because it induces apoptosis, involving induction of the caspase cascade through p38 MAPK phosphorylation. In contrast, BBG does not cause apoptosis and thus could be a safer adjuvant during vitreoretinal surgery. (Invest Ophthalmol Vis Sci. 2007;48:4426–4432) DOI:10.1167/iovs.07-0358

From the 1Department of Ophthalmology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and the 2Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts.

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Corresponding author: Yasuaki Hata, Department of Ophthalmology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan; hatachan@med.kyushu-u.ac.jp

Removal of the internal limiting membrane (ILM) improves the surgical outcome of vitreoretinal conditions such as macular hole (MH).1,2 Indocyanine green (ICG) is a dye commonly used to selectively stain the ILM, which comes in direct contact with intravitreally injected dyes. The ILM comprises a basement membrane for the Müller glial cells, which are essential for maintenance of the retina’s normal structure and function. Staining of the ILM results in better intraoperative visibility and allows more convenient removal of this structure.3,4 ICG was originally used in cardiac and hepatic tests, and the intravenous application of ICG is viewed as nontoxic.5 However, recent clinical reports suggest that ICG-assisted vitrectomy may occasionally result in unsatisfactory functional outcomes, such as visual field defects or retinal pigment epithelial atrophy.6–9 Furthermore, a number of experimental reports show the toxicity of ICG for retinal pigment epithelial (RPE) and glial cells.10–21 However, the cellular mechanisms underlying the toxicity of ICG during intraocular surgery are not fully understood.

Trypan blue (TB) has better biocompatibility than ICG for ILM staining.13,20,22 Recent reports show that TB is also toxic to retinal cells.19,23–26 Given that dye-assisted ILM visualization substantially improves the surgical process and makes it more reliable, a new and safer dye for staining of the ILM is urgently needed.

We screened various dyes, focusing on their toxicity and their ability to stain the lens capsule and the ILM in intraocular surgery. Brilliant blue G (BBG), also known as acid blue 90 and Coomassie BBG, is a blue dye (color index 42655) whose formula is C47H48N3O7S2Na and whose molecular weight is 854 Da. It was originally used for protein staining and quantification because it nonspecifically binds to virtually all proteins. Although the pharmacologic functions of BBG remain to be investigated, we recently reported that BBG-assisted membrane staining, at a concentration of 0.25 mg/mL, provides sufficient tissue visualization in animal eyes to allow continuous curvilinear capsulorrhexis and ILM peeling.27,28 We also investigated the staining pattern of the membranes and the clinical outcome using BBG in patients with MH and epiretinal membrane (ERM). Intravitreal BBG injection showed no acute toxicity or other adverse effects, such as RPE atrophy, during the follow-up period of 2 years.29 Nevertheless, intracellular events in retinal glial cells exposed to these dyes remain to be investigated. In response to numerous cytotoxic stimuli, including inflammatory cytokines (TNF-α, IL-8), hydrogen peroxide, UV light, heat shock, and DNA damage, p38 mitogen-activated protein kinase (MAPK) is activated, resulting in inflammation and apoptosis.30,31 Apoptotic signals are conveyed to the cell through caspases, which are downstream of p38 MAPK.32–35 Caspases are synthesized as inactive zymogens and are activated by proteolytic cleavage. Caspase-9 cleavage is the signature of the mitochondrial pathway. Caspase-3, which is activated by the initiator caspase-9, cleaves a number of...
substrates and activates endonucleases, leading to DNA fragmentation, a hallmark of apoptosis. This study focuses on the intracellular events that occur in retinal glial cells exposed to ICG and BBG in vitro.

METHODS

Cell Culture

The spontaneously immortalized human Müller cell line MIO-M1 was used to carry out the experiments. Cells were cultured in Dulbecco modified Eagle medium (DMEM; Sigma, Poole, UK) containing 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml, penicillin, 100 mg/ml streptomycin) at 37°C under 5% CO₂/95% air atmosphere.

[³H]-Thymidine Uptake

We prepared 0.25 and 2.5 mg/ml ICG (Dai-ichi Seiyaku, Tokyo, Japan) and 0.25 mg/ml BBG (Sigma-Aldrich, St. Louis, MO) solutions with intracocular irrigating solution (Opeguard; Senjyu Pharmaceutical Co., Ltd., Osaka, Japan). MIO-M1 cells were seeded in 24-well plates at a density of 1.0 × 10⁴ cells/well in DMEM containing 10% FBS and were starved in DMEM containing 3% FBS for 24 hours. Subsequently, the cells were exposed to ICG (0.25 and 2.5 mg/ml), BBG (0.25 mg/ml), or vehicle control (Opeguard) for 15 minutes at 37°C. Thereafter, the solutions were removed and the cells were washed three times with PBS. After incubation in DMEM containing 3% FBS for 18 hours, the cells were pulsed with [³H]-thymidine for 6 hours. Cells were then harvested, and [³H]-thymidine incorporation was measured by liquid scintillation counting.

Cell Viability Assay

MIO-M1 cells were seeded in six-well plates in DMEM containing 10% FBS and starved in DMEM containing 3% FBS for 24 hours. After cells were allowed to grow until reaching 70% confluence, they were pretreated with ICG (0.25 mg/ml and 2.5 mg/ml), BBG (0.25 mg/ml), or vehicle control (0.25 mg/ml) for 15 minutes at 37°C. The solutions were withdrawn, and the cells were washed with phosphate-buffered saline (PBS) three times. DMEM containing 3% FBS was added to the cells, and the plates were incubated at 37°C for 24 hours. After washing with PBS, the cells were collected with trypsin and were counted (Coulter Particle Counter Z1; Beckman Coulter, Hialeah, FL).

Transmission Electron Microscopy

Type-I collagen (Koken Co., Ltd., Tokyo, Japan), 10× RPMI 1640 (Sigma-Aldrich), NaHCO₃, and distilled water were mixed on ice at a ratio of 6:6.1 to 1.25:1.1. The resultant mixture (0.5 ml) was added to a 24-well plate and allowed to solidify in an incubator at 37°C. MIO-M1 cells were seeded on this type-I collagen gels in 24-well plates and starved in DMEM containing 3% FBS for 24 hours. Subsequently, the cells were stimulated with ICG (0.25 mg/ml and 2.5 mg/ml), BBG (0.25 mg/ml), or vehicle control (0.25 mg/ml) for 15 minutes at 37°C. Before the experiment, the solutions were removed, and the cells were washed with PBS three times. After 150-minute incubation in TMEM containing 3% FBS at 37°C, collagen gels were washed with PBS and fixed with 4% glutaraldehyde in phosphate buffer. Collagen gels were then postfixed with 1% osmium tetroxide, dehydrated in gradient concentrations of ethanol, and embedded in Epon resin. Ultrathin sections were mounted on copper grids, and the specimens were observed with an electron microscope (JEM 100 CX; JEOL, Tokyo, Japan).

Western Blot Analysis of p38 MAPK Phosphorylation

ICG- and BBG-dependent p38 MAPK phosphorylation was evaluated by Western blot analysis. Subconfluent MIO-M1 cells were starved with DMEM containing 3% FBS for 24 hours. The cells were first incubated with the indicated concentrations of ICG and BBG for 15 minutes or with ICG (2.5 mg/ml) and BBG (0.25 mg/ml) for the indicated duration. Subsequently, the cells were washed once with PBS and lysed in 1x Laemmli buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol) containing protease inhibitors (Complete Mini; Roche Diagnostics, Germany; 1 mM NaF, 0.5 mM Na₃VO₄). Whole cell lysates were subjected to 15% SDS-PAGE and transferred to nitrocellulose filters (New England Biolabs, Beverly, MA). After blocking with 3% skim milk, the blots were incubated overnight at 4°C with an antibody against phospho-p38 (9211, 1:1000; Cell Signaling, Beverly, MA) for 1 hour at room temperature. Visualization was performed using an enhanced chemiluminescence (ECL; Amersham Arlington Heights, IL) detection system according to the manufacturer’s instructions. The membranes were then reblotted with anti-p38 mAb (9212, 1:1000; Cell Signaling).

RESULTS

Impact of ICG and BBG on MIO-M1 Cell Proliferation

To investigate whether ICG and BBG affect glial cell proliferation, we quantified [³H]-thymidine incorporation in MIO-M1 cells. ICG at 0.25 and 2.5 mg/ml significantly reduced [³H]-thymidine incorporation by 64.5% and 69.7%, respectively, compared with vehicle-treated control cells. BBG at 0.25 mg/ml also significantly diminished [³H]-thymidine incorporation by 24.6% compared with vehicle-treated control cells (P < 0.01) (Fig. 1).

Effects of ICG and BBG on MIO-M1 Cell Viability

To examine whether ICG and BBG may have an effect on the viability of glial cells, we exposed MIO-M1 cells with ICG (0.25 and 2.5 mg/ml), BBG (0.25 mg/ml), or vehicle for 15 minutes and counted, 24 hours later, the number of attached cells in each well. Treatment of the cells with 0.25 or 2.5 mg/ml ICG significantly decreased their numbers by 38.1% and 55.6%, respectively, compared with the cell numbers before treatment (P < 0.01) (Fig. 2). In contrast, the number of cells treated with BBG (0.25 mg/ml) did not decrease compared with the number of cells before treatment. However, cell
growth was significantly attenuated compared with control (P < 0.05). Within 60 minutes of the ICG treatment, cells started to shrink and then to detach from the plates. In contrast, the BBG-treated cells appeared unchanged (Fig. 3).

Electron Microscopic Findings of Apoptosis in ICG-Treated MIO-M1 Cells

To further address the morphologic changes after dye exposure, MIO-M1 cells were exposed to ICG (2.5 mg/mL) and BBG (0.25 mg/mL) for 15 minutes and subsequently were examined by transmission electron microscopy. ICG-treated cells showed signs of apoptosis, such as nuclear shrinkage and chromatin condensation, but cell organelles appeared normal (Fig. 4). The condensed chromatin tended to marginate around the nuclear envelope. Some cells also formed apoptotic bodies. These morphologic changes occurred within the first 60 minutes of ICG treatment and coincided with the changes we observed in light microscopy. In contrast, the cells treated with BBG did not show any morphologic signs of apoptosis or necrosis.

Activation of p38 MAPK

To investigate potential intracellular signaling events that occur with ICG and BBG treatment, we obtained soluble protein samples from treated MIO-M1 cells and examined p38 MAPK phosphorylation using Western blot analysis. Phosphorylation of p38 MAPK was increased in cells exposed to ICG starting at 0.1 mg/mL (Fig. 5A). In contrast, BBG-treated cells showed only a minor increase in p38 MAPK phosphorylation at higher concentrations (1 and 2.5 mg/mL). Notably, at clinically relevant dye concentrations, there was a substantial difference between the levels of p38 MAPK phosphorylation in ICG (2.5 mg/mL)- and BBG (0.25 mg/mL)-treated cells. At these concentrations, p38 MAPK phosphorylation was prominently detectable in cells exposed to ICG, whereas there was no apparent induction in the cells exposed to BBG. Next, we examined time-dependent phosphorylation of p38 MAPK with ICG (2.5 mg/mL) and BBG (0.25 mg/mL) (Fig. 5B). ICG (2.5 mg/mL) induced p38 MAPK phosphorylation in the MIO-M1 cells as early as 2 minutes after incubation. In contrast, BBG-treated cells (0.25 mg/mL) showed constant constitutive levels of p38 MAPK phosphorylation within 60 minutes of incubation (Fig. 5B).

Activation of Caspase-3 and -9

To further investigate potential mediators of dye-induced apoptosis, we examined whether apoptosis of dye-treated cells resulted from the activation of the caspase cascade. Soluble protein samples from treated MIO-M1 cells were used in Western blot and stained with antibodies that recognize cleaved caspase-3 and cleaved caspase-9. ICG (2.5 mg/mL) induced cleavage of caspase-9 and caspase-3 after approximately 150 minutes of incubation. In contrast, treatment of the cells with BBG (0.25 mg/mL) did not result in an increase in caspase-9 and caspase-3 cleavage products during the examined time.
periods (Fig. 6A). To evaluate the biological significance of p38 MAPK activation in ICG-induced activation of the caspase pathway, we examined the effect of the p38 inhibitor SB203580 on ICG-induced cleavage of caspase-9 and caspase-3. SB203580 attenuated the ICG-induced cleavage of caspase-9 and caspase-3, suggesting the p38 MAPK pathway is required for the activation of the caspase cascade by ICG (Fig. 6B).

**FIGURE 3.** Phase-contrast micrographs of MIO-M1 cells 60 minutes after dye treatment. (A) Control cells treated with vehicle solution. (B) Cells exposed to ICG (2.5 mg/mL) for 15 minutes. (C) Cells exposed to BBG (0.25 mg/mL) for 15 minutes (original magnification, ×100).

**FIGURE 4.** Transmission electron micrographs of MIO-M1 cells 150 minutes after dye treatment. MIO-M1 cells were seeded on type 1 collagen gels and were stimulated with ICG (2.5 mg/mL), BBG (0.25 mg/mL), and vehicle for 15 minutes at 37°C. Subsequently, cells were washed with PBS and incubated in DMEM containing 3% FBS for 150 minutes, fixed, and examined by transmission electron microscopy. (A) Control cells treated with vehicle. (B) Cells stimulated with ICG (2.5 mg/mL) for 15 minutes. (C) Cells stimulated with BBG (0.25 mg/mL) for 15 minutes. Bar, 5 μm.
DISCUSSION

Staining of the ILM with ICG is widely accepted by ophthalmic surgeons for safer and more convenient complete removal of the ILM. In spite of the benefits of this procedure for MH surgery, the use of ICG has also been associated with unfavorable functional outcome. Some reports have shown that low concentrations of ICG (0.01–1 mg/mL) for a brief exposure time (0.5–1 minute) cause low cytotoxicity. However, it is known that ICG, even at low concentrations for brief exposure, persists within the eye for several weeks to several months after surgery. Moreover, the ICG concentration clinically applied for effective ILM staining is higher than the concentrations stated in those reports. Additionally, several recent reports indicate that ICG causes apoptotic cell death. For instance, ICG injected into the subretinal space of rabbit eyes causes morphologic damage to the retina and induces apoptosis in the photoreceptor and RPE cells. We examined the effect of ICG by subretinal injection in rats and confirmed typical characteristics of apoptotic cell death, such as chromatin condensation, cell shrinkage, and apoptotic body formation. In vitro, exposure of RPE cells to ICG and light induces the expression of apoptosis-related genes, such as p53, bax, p21, and c-fos.

TB, another dye used for ILM staining, is toxic to cultured RPE cells. However, TB is thought to be less toxic than ICG.

We recently reported the ability of BBG to effectively stain the ILM and reported its lower toxicity compared with ICG and TB in vivo. Subretinal injection of ICG or TB in rats causes apoptotic cell death mainly in the photoreceptors. In contrast, in BBG-treated eyes, a lack of apoptotic cells and a well-preserved retina were observed. Lower concentrations of BBG (0.25 mg/mL) have been confirmed to effectively stain the ILM in patients. Although neither BBG nor ICG absorbs visible light, ICG is reported to cause light toxicity. We performed ILM peeling using BBG (0.25 mg/mL) for all applicable cases, and no apparent adverse effects have been ob-
erved during the 2-year follow-up period. Further examination, however, is necessary to evaluate BBG light toxicity.

In the present study, we compared the effects of ICG and BBG on a human Müller cell line in vitro. We used specific concentrations of ICG and BBG (2.5 mg/mL ICG; 0.25 mg/mL BBG) in the clinic to stain the ILM. Exposure to ICG resulted in marked downregulation of [3H]-thymidine incorporation by the cells, reduction of the cell number, and morphologic changes in the TEM, all indicative of apoptosis. These results suggest that ICG toxicity may in part be caused by the apoptosis of retinal glial cells. In contrast, though exposure to BBG resulted in the suppression of [3H]-thymidine uptake by the cells, no apparent reduction in cell number or apoptosis was observed. Solution osmolarity is considered to play a part in this.44 Our previous report demonstrates that the osmolarity of 0.25 mg/mL BBG (289 mOsm, pH 7.41) was similar to that of intracocular irrigating solution (Opeguard; 289 mOsm, pH 7.33).26 These data suggest that BBG may have a cytostatic effect, but it exhibits no apparent cellular toxicity at clinically relevant concentrations.

To our knowledge, this is the first study pinpointing the phosphorylation of p38 MAPK and caspase cascade involvement in ICG-induced apoptotic cell death. In the present study, SB203580 inhibited the cleavage of caspase-9 and caspase-3, indicating that ICG-induced p38 MAPK phosphorylation subsequently causes activation of these caspases. It is feasible that other apoptotic molecules and pathways are also involved in ICG-induced apoptotic cell death.

BBG is a selective antagonist of the P2X7 receptor, an ATP-gated nonselective cation channel, which allows significant Ca2+ influx.5 The P2X7 receptor is expressed in many different cells,46-50 including retinal Müller glial cells, ganglion cells, and horizontal cells.51-55 We confirmed the expression of P2X7 receptor mRNA in MIO-M1 cells (data not shown). Stimulation of the P2X7 receptor induces cell proliferation,46,49,50 and Bianco et al.49 showed that BBG, as a P2X7 receptor antagonist, decreases cell growth. In the present study, we also showed that BBG inhibits the growth of Müller cells, possibly from blockade of the P2X7 receptor. However, the exact mechanistic details remain to be investigated. Given that 0.25 mg/mL BBG in addition to ILM staining also inhibits cell proliferation, it might also have postoperative benefits by reducing fibrous formation.

In the present study, we demonstrate that BBG is less toxic for retinal glial cells than ICG, and we provide new insight into the mechanisms of ICG-induced apoptotic cell death. BBG, at clinically relevant concentrations, appears to have tolerable toxicity on retinal glial cells and could thus be a safer adjuvant during vitreoretinal surgery. Furthermore, the antiproliferative properties of BBG on retinal glial cells may provide additional benefits in minimizing the level of postoperative scar formation.

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