Intracellular Events in Retinal Glial Cells Exposed to ICG and BBG

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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 lower 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

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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 at 37°C 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 stimulated with ICG (0.25 mg/mL and 2.5 mg/mL), BBG (0.25 mg/mL) solution, or vehicle 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-1 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-1 collagen gels in 24-well plates and starved in DMEM containing 3% FBS for 24 hours. Subsequently, the cells were stimulated with ICG (2.5 mg/mL), BBG (0.25 mg/mL), and vehicle control (Opeguard) for 15 minutes at 37°C. Thereafter, the solutions were removed, and the cells were washed with PBS three times. After 150-minute incubation in DMEM 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 veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer. Collagen gels were then postfixed with veronal acetate buffer.
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 and BBG 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 as early as 2 minutes after incubation. In contrast, BBG-treated cells showed constant constitutive levels of p38 MAPK phosphorylation within 60 minutes of incubation (Fig. 5B).
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.3,4 In spite of the benefits of this procedure for MH surgery, the use of ICG has also been associated with unfavorable functional outcome.6–9 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.14,18,39 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.11,40–42 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.12,15,17,21 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.17 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.26 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.13

TB, another dye used for ILM staining, is toxic to cultured RPE cells.19,23 However, TB is thought to be less toxic than ICG.14,20

We recently reported the ability of BBG to effectively stain the ILM and reported its lower toxicity compared with ICG and TB in vivo.28,29 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.28 Lower concentrations of BBG (0.25 mg/mL) have been confirmed to effectively stain the ILM in patients.29 Moreover, patients who underwent BBG-assisted ILM peeling showed better clinical outcomes, including visual acuity, than the population who underwent ICG-assisted surgery.4,8,29 Although neither BBG nor ICG absorbs visible light, ICG is reported to cause light toxicity.13,20,43 We performed ILM peeling using BBG (0.25 mg/mL) for all applicable cases, and no apparent adverse effects have been ob-

![Figure 5](https://example.com/figure5.png)

**Figure 5.** p38 MAPK phosphorylation by ICG and BBG. Subconfluent MIO-M1 cells were starved in DMEM containing 3% FBS for 24 hours. (A) Cells were then stimulated with the indicated concentration of ICG or BBG for 15 minutes or (B) with ICG (2.5 mg/mL) or BBG (0.25 mg/mL) for the indicated time. Whole cell lysates from the treated MIO-M1 cells were subjected to Western blot analysis using an antibody against phospho-p38 MAPK. Membranes were reblotted with an antibody against p38 MAPK.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Cleavage of caspase-9 and caspase-3 by ICG and BBG. Subconfluent MIO-M1 cells were starved in DMEM containing 3% FBS for 24 hours. (A) Cells were stimulated with ICG (2.5 mg/mL) or BBG (0.25 mg/mL) for 15 minutes, washed with PBS, and incubated in DMEM containing 3% FBS at 37°C for the indicated time. The extraction was subjected to Western blot analysis using an antibody against cleaved caspase-3 and cleaved caspase-9. (B) After pretreatment with 100 nM SB203580 for 1 hour, the cells were stimulated with ICG (2.5 mg/mL) for 15 minutes, washed with PBS three times, and incubated in DMEM containing 3% FBS with 100 nM SB203580 at 37°C for 150 minutes. Then cleavage of caspase-9, caspase-3, and GAPDH was evaluated by Western blot analysis in the same manner described in (A).
served during the 2-year follow-up period.\textsuperscript{29} 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 [$^3$H]-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 [$^3$H]-thymidine uptake by the cells, no apparent reduction in cell number or apoptosis was observed. Solution osmolarity is considered to play a part in 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 clinically.

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 Ca\textsuperscript{2+} influx.\textsuperscript{45} The P2X7 receptor is expressed in many different cells,\textsuperscript{46–50} including retinal Müller glial cells, ganglion cells, and horizontal cells.\textsuperscript{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,\textsuperscript{46,49,50} and Bianco et al.\textsuperscript{56} 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 vitrectomy 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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References