Phototoxicity of Indocyanine Green and Brilliant Blue G under Continuous Fluorescent Illumination on Cultured Human Retinal Pigment Epithelial Cells

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Purpose. We compared the phototoxicity of indocyanine green (ICG) and Brilliant Blue G (BBG) in cultured RPE cells under fluorescent lamp illumination imitating ambient light.

Methods. Cultured human RPE line cells were stained with ICG or BBG solution at concentrations of clinical use, and cultured in a colorless medium for 24 hours in the dark or under illumination from a fluorescent lamp. After culture, cell morphology and TUNEL-positive apoptotic cells were observed. Cell viability and cell death rate were evaluated. Absorption spectral changes of BBG before and after incubation were measured.

Results. ICG-stained cells cultured under illumination changed to an oval morphology with increased number of apoptotic cells, whereas ICG-stained cells cultured in the dark, and BBG-stained cells cultured under illumination and dark conditions maintained a flat morphology without increase in apoptotic cells. Cell viability decreased and cell death rate increased only in cells stained by ICG followed by culture under illumination. Staining cells with ICG at one-tenth concentration of clinical usage induced no cytotoxicity after culture under illumination. Approximately 30% of total BBG retained in the stained cells was released into the culture supernatant after incubation for 24 hours. The absorption spectrum of BBG did not change after fluorescent light irradiation.

Conclusions. Illumination with a fluorescent lamp caused cell death via apoptosis in ICG-exposed, but not in BBG-exposed cultured RPE cells. BBG may be a safer dye than ICG because of low light-induced cytotoxicity and rapid elution from stained cells. (*Invest Ophthalmol Vis Sci.* 2012;53:7389–7394) DOI: 10.1167/iovs.12-10754

Peeling of the internal limiting membrane (ILM) is performed in vitrectomy for macular hole,[1–4] epiretinal membrane,[5,6] and diabetic macular edema[7–9] to improve surgical outcome. Intravitreal indocyanine green (ICG) injection for visualization of the transparent ILM to facilitate complete removal of ILM is a standard procedure in vitreoretinal surgery for macular holes, because ILM peeling is essential for successful anatomic macular hole closure.[3,10,11] Although ICG is used widely as a vital dye in ILM peeling without causing surgical complications, its dose- and exposure time–dependent cytotoxicity as well as phototoxicity have been reported in experimental studies.[12–20] Moreover, a meta-analysis indicates that treatment of macular hole with ICG results in worse functional outcomes than treatment without ICG.[21–23] Thus, intravitreal ICG injection remains controversial.

Use of safer dyes other than ICG for ILM staining and the conditions of clinical ICG administration have been investigated. Recently, Brilliant Blue G (BBG) has been reported to be a useful and safe dye for visualization of ILM.[24–26] Its cytotoxicity was studied in ocular tissues and cells, including RPE cells,[27,28] ganglion cells,[29,30] and Müller cells[30] in vivo and in vitro, and BBG was considered to be safer than ICG. In these reports, the cytotoxicity and phototoxicity of BBG were examined mainly under conditions simulating ocular surgery, including exposure to high concentrations of BBG and intense light irradiation from light pipes for brief illumination time.[17,31–36] To the best of our knowledge, there are no reports on phototoxicity of the BBG retained on the retina after ocular surgery under irradiation by ambient light. We examined the phototoxicity caused by persistent ICG on ICG-stained cultured Müller cells, and found that fluorescent lamp illumination imitating ambient light enhances the cytotoxicity of ICG on Müller cells in a ICG concentration- and exposure time–dependent manner.[13]

In the present study, we used RPE cells because RPE is in direct contact with the vital dye at a high concentration in macular hole surgery with ILM peeling, and RPE injury is considered to affect visual prognosis. We compared phototoxicity of ICG and BBG retained in cultured RPE cells under ambient light illumination. Under visible light irradiation, we found that illumination equivalent to conventional fluorescence lamp light induced cytotoxicity in ICG-stained RPE cells but not in BBG-stained cells.

Materials and Methods

Cells and Culture Medium

The human RPE cell line ARPE-19 (American Type Culture Collection, Manassas, VA) was cultured in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12; Sigma-Aldrich, Poole, UK) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

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The cells were trypsinized and subcultured to confluence in multi-well culture plates.

To eliminate absorption of fluorescent lamp light by culture medium, we used a colorless medium for culture experiments under illumination, which consists of Dulbecco's PBS (Sigma-Aldrich) supplemented with 1% FBS, 1 mg/mL glucose, 1 mg/mL $CaCl_2$, 1 mg/mL $MgCl_2$, and antibiotics.[13]

Dye Exposure and Subsequent Culture under Illumination

Concentrations of ICG or BBG for staining ARPE-19 cells were determined by referring to the clinical usage of ILM staining in vitrectomy.[19,22,24] ICG (Daiichi Sankyo, Tokyo, Japan) first was dissolved in distilled water at a concentration of 25 mg/mL, then diluted with 4 volumes of balanced saline solution (BSS Plus; Alcon, Tokyo, Japan) and further diluted with a 4:1 mixture of BSS and distilled water if necessary.[14,25] BBG was dissolved directly in BSS. The osmolarity of ICG solution was approximately four-fifths of that of BBG solution. A 1:4 mixture of distilled water and BSS was used as ICG staining control, and BSS was used as BBG staining control.

ARPE-19 cells were exposed to ICG or BBG solution for 3 minutes in the dark. After exposure, the cells were rinsed immediately three times with BSS and then cultured in the colorless medium, either in the dark covered with aluminum foil or under 2000 lx illumination from a daylight-colored fluorescent lamp (6504K, Sunline; Hitachi, Tokyo, Japan) at 37°C in humidified air in an incubator fitted with fluorescent lamp equipment (CPO2-171; Hirasawa, Tokyo, Japan). The total light energy on the cells under the illumination was 76.3 J/cm^2/24 h.

Spectrophotometric Measurement of Dye Quantities Absorbed by ARPE Cells

The amount of ICG or BBG that had been retained in the cultured cells after the staining procedure was measured. The cells cultured in a well with 2 cm^2 culture area were exposed to 5.0 mg/mL of ICG or 0.5 mg/mL BBG, washed three times with BSS, and then lysed with 1 mL of 0.1% Triton X-100 in PBS. The absorbance of the lysate was measured by a spectrophotometer at the peak absorbance wavelength of 800 nm for ICG and 619 nm for BBG. Standard solutions were prepared by diluting ICG or BBG to various concentrations in PBS containing 0.1% Triton X-100, and absorbance at the peak wavelength was measured. A calibration curve for each dye was constructed by plotting the absorbance at peak wavelength against dye concentration. The quantity of dye in the stained cell lysate was determined from its absorbance and the calibration curve.

Detection of Apoptosis

Apoptosis was detected by TUNEL labeling using fluorescein-conjugated deoxyUTP as the substrate (In Situ Cell Death Detection kit; Roche, Mannheim, Germany). The cells were fixed with neutralized formalin and labeled according to the manufacturer's protocols. After counterstaining the nuclei with 4′6-diamidino-2- phenylindole (DAPI), the cells were observed and photographed.

Measurement of Cell Viability and Cell Death Rate

Quantitative assessment of cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI), which measures mitochondrial reductase activities in metabolically active cells. Quantitative assessment of cell death rate was evaluated using an LDH assay kit (Roche) that measures the activity of LDH released into the culture supernatant from dead cells. After collecting an aliquot of the culture supernatant containing LDH released from dead cells, the cells were lysed with of 1% Triton X-100 to release intracellular LDH from surviving cells. The LDH activities in the culture supernatant and cell lysate were measured, and the percentage of dead cells in total cells was calculated.

Statistical Analyses

At least four samples were measured under each condition. Data in graphs are expressed as mean with standard deviation. Data were analyzed by nonrepeated measures ANOVA with Dunnett's test for comparison with control. Unpaired two-tailed Student's *t*-test was used to analyze the data of two conditions. $P < 0.01$ was considered to be significant.

Results

Quantification of Dyes Retained by the Cells after Dye Exposure

Quantification of the dye retained by the cells after the staining procedure is important, because the dye that remains in the culture environment would be the primary cause of cytotoxicity and phototoxicity. We stained ARPE cells with dyes at concentrations used clinically (5.0 mg/mL for ICG and 0.5 mg/mL for BBG), and measured the absorbance of lysed cell preparation soon after dye exposure. The quantity of dye in the cell lysate was obtained from a calibration curve. The quantity of dye retained in the cells plated in a 2-cm^2 culture well was 5 μg for ICG and 0.5 μg for BBG. These results indicated that the proportion of dye retained in the cells was almost the same for ICG and BBG solutions.

Changes of Cell Death Rate in Dye-Exposed Cells Cultured under Illumination

Next, we examined how cellular damages proceeded in the dye-stained cells. Dye-stained cultures were incubated in the dark or under illumination from a fluorescent lamp, and percent cell death at indicated time was determined by measuring LDH activity in culture supernatants and cell lysates (Fig. 1). When cultured in the dark, the cell death rates increased gradually until 18 hours and remained almost unchanged thereafter in ICG- and BBG-stained cultures as well as BBG control (Fig. 1A). When cultured under illumination, the cell death rate of ICG-stained culture accelerated after 18 hours (Fig. 1B), while the cell death rates in BBG-stained and control cultures were almost the same as those cultured in the dark until 24 hours, and then increased slightly. After 30 hours,

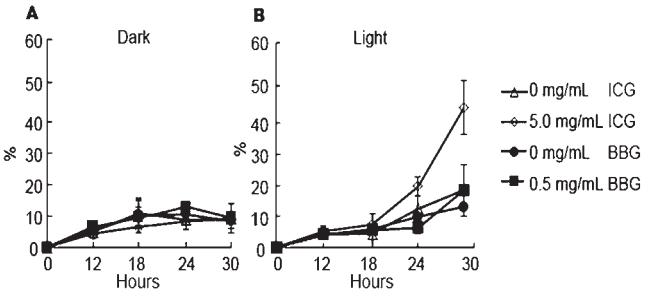

Figure 1. Changes in cell death rate of dye-stained cells cultured in the dark or under illumination. Percentage of dead cells at indicated time was determined by measuring LDH activities in culture supernatants and cell lysates. (**A**) In dark cultures, cell death rates were almost constant after 18 hours in all dye-stained and control cultures. (**B**) In illuminated cultures, cell death rate increased rapidly in 5.0 mg/mL ICG-stained culture after 18 hours, but not in other dye-stained and control cultures.

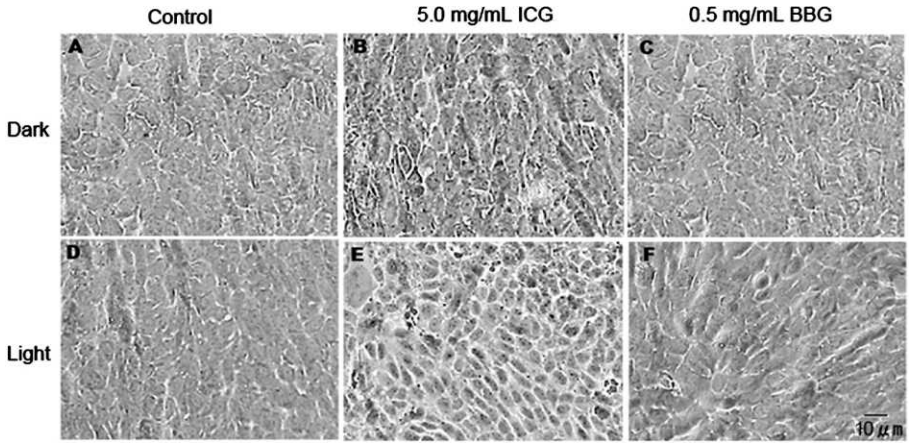

Figure 2. Morphologic changes of dye-stained cells after culture in the dark or under illumination for 24 hours. (**A–C**) When cultured in the dark, cells stained with ICG or BBG and control cells (0 mg/mL BBG) maintained a flatten morphology. When cultured under illumination, ICG-stained cells became oval and showed shrinkage in some cells (**E**), while the morphology of the cells in control (**D**) and BBG-stained cultures (**F**) was similar to that of cells cultured in the dark.

all cell death rates in the illuminated cultures were higher than those cultured in the dark. In 24-hour cultures under illumination, the difference in cell death rate between ICG-stained cells and cells under other staining conditions was evident, while light irradiation–induced cellular damage was still mild. Therefore, we adopted 24-hour culture to evaluate the effects of dye staining and light irradiation in further experiments.

Morphologic Changes in Dye-Stained Cells Cultured under Illuminated and Dark Conditions

The morphologic changes of dye-stained cells were observed by phase contrast microscopy after 24-hour culture in the dark or under illumination (Fig. 2). When cultured in the dark, the cells stained either with 5.0 mg/mL ICG or 0.5 mg/mL BBG maintained a flat morphology, similar to the control (BBG 0 mg/mL, Figs. 2A–2C). When cultured under illumination, the cells stained with BBG maintained a flat morphology (Figs. 2D, 2F), while many ICG-stained cells became oval showing shrinkage in some cells (Fig. 2E). Lower osmolarity in the ICG control did not affect cellular morphology (data not shown).

Detection of Apoptotic Cells after Culture under Illuminated and Dark Conditions

We detected apoptotic cells by TUNEL method to examine whether the cell death was related to apoptosis (Fig. 3). When cultured in the dark, only a few TUNEL-positive cells were observed in ICG- and BBG-stained cultures and in control (BBG 0 mg/mL) culture (Figs. 3A–3C). When cultured under illumination, TUNEL-positive cells increased predominantly in culture stained with ICG (Fig. 3E), while the rates of TUNEL positivity in control or BBG-stained cultures (Figs. 3D, 3F) were the same as cultures in the dark. The difference in osmolarity in BBG and ICG controls did not affect the rate of TUNEL-positive cells.

Concentration Effects of Dyes on Cell Viability and Cell Death Rate under Illumination

We next examined the effects of dye concentration on cell viability and cell death rate (Fig. 4). RPE cells were stained with the dyes at clinical use concentrations (5.0 mg/mL for ICG and 0.5 mg/mL BBG) and one-tenth of the clinical use concentrations, and then cultured in the dark or under illumination. When cultured in the dark, there were no significant differences in cell viability among all cultures at both

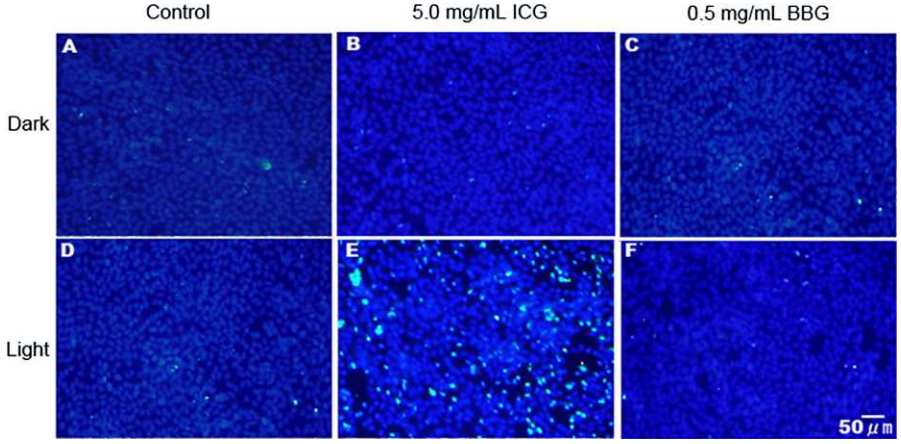

Figure 3. Detection of apoptosis by TUNEL in dye-stained cells after culture in the dark or under illumination. Only few TUNEL-positive cells were observed in the all cultures incubated in dark (**A–C**). TUNEL-positive cells increased in ICG-stained cells cultured under illumination (**E**). The frequencies of TUNEL-positive cells in control and BBG-stained cells cultured under illumination (**D**, **F**) were similar to those in cells cultured in the dark.

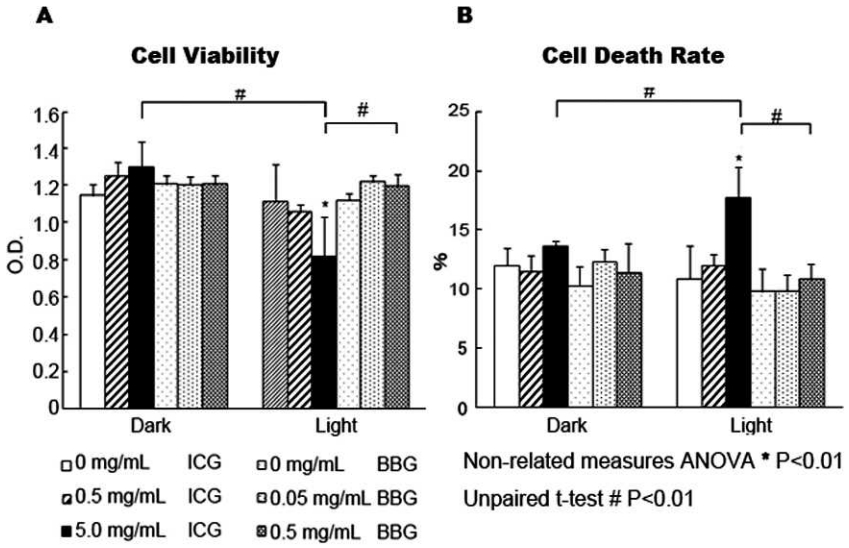

Figure 4. Effects of dye concentration on cell viability and cell death rate. The cells were stained with dyes at concentrations of 0.5 and 5.0 mg/mL for ICG, and 0.05 and 0.5 mg/mL for BBG, and then cultured in the dark or under illumination. Cell viability (**A**) and cell death rate (**B**) were measured. (**A**) When cultured in the dark, there were no significant differences in cell viability among all dye-stained and control cultures. When cultured under illumination, cell viability decreased in 5.0 mg/mL ICG-stained culture, but not in other dye-stained and control cultures. In 5.0 mg/mL ICG-stained cultures, cell viability was lower under illumination than in the dark. Cell viability was significantly lower in 5.0 mg/mL ICG-stained culture than in 0.5 mg/mL BBG-stained culture under illumination, but not in the dark. (**B**) When cultured under illumination, cell death rate was significantly elevated in 5.0 mg/mL ICG-stained culture. Cell death rate was significantly higher in the 5.0 mg/mL ICG-stained culture than in the 0.5 mg/mL BBG-stained culture under illumination.

concentrations of ICG and BBG (Fig. 4A). When cultured under illumination, however, significantly lower cell viability was observed only in the culture stained with 5.0 mg/mL ICG compared to control culture (0 mg/mL ICG), while no significant differences were detected in cultures stained with 0.5 mg/mL ICG, and in those stained with 0.05 and 0.5 mg/mL BBG compared to the controls. Comparing ICG and BBG concentrations at clinical usage, cell viability was significantly lower in ICG-stained culture than in BBG-stained culture under illumination.

Consistent with the results of cell viability, a significantly elevated cell death rate was observed in cells stained with 5.0 mg/mL ICG cultured under illumination (Fig. 4B). Cell death rate in cultures stained with BBG showed no remarkable changes when cultured in the dark or under illumination. At the clinical use concentrations, cell death rate was significantly higher in ICG-stained culture than in BBG-stained culture under illumination.

These data indicated that when RPE cells had been stained with dyes at clinical use concentrations, subsequent fluorescent lamp illumination would damage ICG-stained cells, but not BBG-stained cells.

Changes of Absorption Spectra of BBG Solution after Light Irradiation

A previous study has reported that light irradiation decomposes ICG by self-sensitized photo-oxidation and that the colors of decomposed products are changed from dark green of ICG.[12] On the other hand, there are no reports on the influence of light irradiation on the color properties of BBG. We examined whether light irradiation affects the absorption spectrum of BBG under our culture conditions. First, we examined light-induced changes of BBG solution in the absence of cells after incubation under illumination for 24 hours (Fig. 5A). The absorption spectrum of 1.25 μg/mL BBG solution was measured before and after 24-hour incubation in the dark or under illumination. The concentration of BBG solution was determined from the quantity of BBG (0.5 μg) retained on cells after BBG staining and the volume of medium (0.4 mL) in the culture well. Illuminated and dark incubation conditions decreased BBG absorbance at wavelengths ranging from approximately 400 to 520 nm, while the spectrum of the BBG solution incubated in the dark was almost identical to that incubated under illumination. Thus, light irradiation itself did not affect the absorption spectrum of BBG.

Next, we measured the absorption spectrum of BBG in culture supernatant of BBG-stained cells incubated for 24 hours with or without illumination (Fig. 5B). Absorption spectrum of BBG from approximately 520 to 700 nm was detected in the supernatant, indicating that BBG that had stained the cells was released into the culture medium. There was no difference in the absorption spectrum between culture supernatant incubated in the dark and that incubated under illumination. Comparing the peak wavelength absorbance of the supernatants before and after incubation, 30% of the total BBG quantity retained by the cells was released into the culture medium in 24 hours.

Discussion

Our study was designed to compare the toxicity of retained ICG and BBG in ocular cells after dye-assisted ocular surgery under ambient light irradiation in daily life.[34,36] In our study, we used cultured ARPE-19 cells derived from human RPE and fluorescent lamp light as an ambient light source. We showed that BBG exerted lower cytotoxicity than ICG at the clinical use concentration.

To evaluate the effects of light on cultured cells, it is important that the irradiating light reaches the cells without being absorbed by the culture medium. In some previous reports, DMEM with 10% to 20% FBS was used as the medium for light-irradiated culture of RPE cells.[37] However, phenol red and FBS in the medium absorb visual light. Kernt et al.[12] and Tokuda et al.[20] reported phototoxicity of ICG under conditions simulating irradiation by surgical light during intraocular

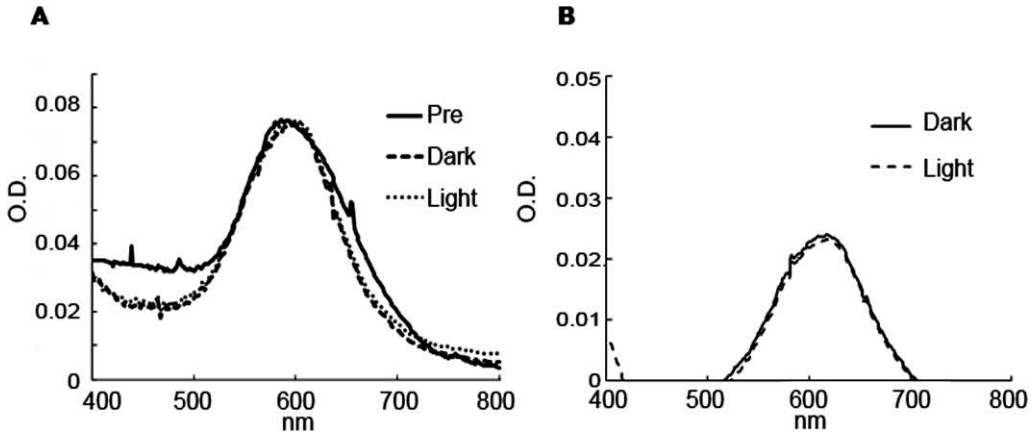

Figure 5. Absorption spectra of BBG after incubation under illumination. (**A**) BBG dissolved in the PBS-based medium was incubated in the dark or under illumination for 24 hours, and absorption spectra were measured. Absorption spectra were almost identical after incubation in the dark and under illumination, although incubation lowers the absorbance at some wavelength ranges. (**B**) BBG-stained cells were cultured in the dark or under illumination, and absorption spectra of the culture supernatants were measured. The spectra of the supernatants were almost identical under both culture conditions.

surgery. Instead of culture medium, Kernt et al. used PBS in which they maintained the cells during light irradiation for a duration of 10 minutes or shorter.[12] PBS is colorless and is considered to have no effect on cellular functions during the short irradiation time. In our study, the light irradiation time was much longer (24 hours), and we had designed a PBS-based colorless medium supplemented with 1% FBS and 1 mg/mL glucose for culture experiments under illumination.[13] Although our PBS-based colorless medium contained inadequate nutrients such as amino acids and vitamins for prolonged culture, it can support survival of approximately 90% of ARPE-19 cells in the dark for at least 30 hours, which is long enough to evaluate the cytotoxicity of dyes.

The study was conducted first to use RPE cells freshly isolated from porcine eyes. However, since culture with our PBS-based colorless medium still is not an optimal condition for primary RPE cells, most of RPE cells isolated from porcine eyes died in culture for 24 hours. Therefore, ARPE-19 cells, which are more resistant in an inadequate environment than primary RPE cells, were used for this series of experiments.

ICG staining followed by culture under illumination changed cellular morphology and increased the number of apoptotic cells. These changes were not observed in BBG-stained cells or ICG-stained cells cultured in the dark. Ho et al. suggested that ICG exerts cytotoxicity on cultured RPE cells accompanied by morphological changes.[14] Yam et al. examined the effects of ICG in combination with acute illumination on ARPE-19 cells.[38] In their report, a combination of ICG treatment and light irradiation induced apoptosis and cell cycle arrest with elevated expression of p53 gene coding, a transcription factor, and its target gene products related to apoptotic induction and cell cycle regulation. Similar signs of ICG-induced apoptosis were also observed in other tissues, such as colon cancer and leukemia cells.[39,40] In our study, increased cell death rate and apoptotic induction were also observed in cultures subjected to ICG staining followed by light irradiation. In contrast to the low incidence of TUNEL-positive cells and cell death rate of approximately 30%, almost all ICG-stained cells showed morphologic alteration after culture under illumination, indicating altered intercellular junctions and cytoskeleton organization. Although it is unclear whether the morphologic alteration preceded apoptosis, RPE cells with altered morphology might have impaired barrier functions and phagocytotic ability. On the other hand, no signs of cellular damage or morphologic change were observed in BBG-stained cells cultured with or without illumination. We considered that BBG-stained cells maintained normal functions related to morphology as well as cellular metabolic activities.

When ARPE-19 cells were stained with 5.0 mg/mL of ICG, illumination significantly decreased cell viability and increased cell death rate. However, staining with 0.5 mg/mL of ICG or any concentration of BBG had no remarkable phototoxic effect. Although a previous report suggested that 10 mg/mL of BBG was cytotoxic for RPE cells,[31] this concentration is 20 times higher than that used in vitreous surgery. From our finding, it is conceivable that visual field outcome after ICG-assisted ILM peeling might not be worsened by the use of ICG at an adequately low concentration. However, as the concentrations of ICG used for ILM staining in surgery ranged from 2.5 to 5.0 mg/mL, 0.5 mg/mL of ICG is not a practical concentration.

We reported that ICG that stained cultured Müller cells faded after illuminated culture.[13] Previous reports have shown that light-irradiated ICG produces singlet oxygen, which is a reactive oxygen species, and singlet oxygen decomposes ICG to cytotoxic materials accompanied by color change.[12,17]

In contrast, the absorption spectrum of BBG was not altered after light irradiation. We considered that BBG is stable to light irradiation, and therefore singlet oxygen and decomposed products that can cause cell damage are not produced from light-irradiated BBG. Moreover, BBG dissolves in ionic solution, whereas ICG is soluble in water but not in ionic solution. Approximately 30% of BBG that was retained in stained cells was released into culture supernatant after 24-hour cultivation. We presumed that BBG that stains tissues during intraocular surgery would be cleared readily by circulating intraocular fluid, which is an ionic solution. On the other hand, ICG persists in the eye for a long time after ICG-assisted intraocular surgery,[41,42] perhaps due to its insolubility in intraocular fluid.

BBG is more stable to light irradiation and, therefore, is less likely to produce cytotoxic products by light exposure than ICG. BBG is also expected to be cleared rapidly by intraocular fluid circulation due to its solubility in ionic solution. Although further in vivo and clinical investigations are required, our study supports the notion that BBG is a safer dye than ICG for ILM staining.

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