Cytotoxicity of Indocyanine Green on Retinal Pigment Epithelium

Implications for Macular Hole Surgery

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Objective: To evaluate the potential cytotoxic effects of indocyanine green (ICG) on cultured human retinal pigment epithelium (RPE) and the resultant implications for macular hole surgery.

Methods: Human RPE cells were exposed to ICG in concentrations from 0.001 to 5 mg/mL. The exposure duration ranged from 5 minutes to 3 hours. Light microscopy, MTS viability assay, and calcein AM–ethidium homodimer 1 staining were used to evaluate the cytotoxic effects of ICG.

Results: The RPE cells incubated with up to 5 mg/mL of ICG for 5 minutes or less exhibited no morphologic change and no significant decrease in dehydrogenase activity. When RPE cells were exposed to 5 mg/mL of ICG for 10 minutes, 1 mg/mL of ICG for 20 minutes, or 0.01 mg/mL of ICG for 3 hours, cell morphologic features were altered, mitochondrial dehydrogenase activity decreased, and some cells were necrotic.

Conclusions: Indocyanine green caused cytotoxicity in cultured human RPE in a dose- and time-dependent manner. Cell death occurred by necrosis.

Clinical Relevance: Exposure of RPE cells to ICG concentrations up to 5 mg/mL for 5 minutes or less was not injurious; prolonged exposure to a low ICG concentration was toxic. Since ICG may be retained in the vitreous cavity for a lengthy period, thorough washout of ICG during macular hole surgery is required.

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RECENTLY, SEVERAL investigators have reported that peeling of the internal limiting membrane (ILM) to eliminate tangential traction force improves the anatomic closure rate and functional outcomes,1-4 although other investigators hold a different opinion.5-7 However, if ILM removal is attempted during macular hole surgery, its visualization may be difficult. The staining of the ILM with indocyanine green (ICG) to enhance its visibility has recently been proposed.8-12 The procedure involves direct application of ICG to the inner surface of the retina in the macular area. No adverse effects of ICG were reported in these studies. The concentration of ICG used in these reports ranged from 0.6 to 5 mg/mL, and ICG was left in the eye for periods ranging from 0.5 to 2.5 minutes. The median preoperative best-corrected visual acuity was 20/200, whereas the median postoperative best-corrected visual acuity was 20/400. The macular hole was closed in 86% of eyes. Gandorfer et al13 held a similar opinion. They used 5 mg/mL of ICG, and the ICG was drained immediately after injection. After a review of the medical records of their patients who had undergone macular hole operations involving removal of ILM viewed with or without the aid of ICG staining, these investigators found a tendency toward less favorable visual outcome in those receiving the ICG-assisted operation than in those receiving the identical operation but without the use of ICG. They assumed that ILM staining with ICG might be responsible for the less favorable functional result, because this was the only surgical step that had been changed.

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In an in vitro study, a significant reduction in enzymatic activity of mitochondrial dehydrogenase was observed in cultured RPE cells exposed to 1 mg/mL of ICG for 20 minutes. That study reported no histologic or ultrastructural differences between the treated and control RPE cultures. To investigate whether ICG is cytotoxic, we evaluated the effects of ICG on cultured human RPE cells. In this study, when RPE cells were exposed to 5 mg/mL of ICG for 10 minutes, 1 mg/mL of ICG for 20 minutes, or 0.01 mg/mL of ICG for 3 hours, cell morphologic features were altered and mitochondrial dehydrogenase activity decreased. Some cells were necrotic as demonstrated by calcein AM and ethidium homodimer 1 and acridine orange–ethidium bromide staining.

**RESULTS**

Cultured RPE cells exhibited no morphologic changes after they were exposed to 5 mg/mL of ICG for up to 5 minutes or 1 mg/mL of ICG for up to 10 minutes. Increasing the duration of ICG exposure induced progressive morphologic changes. After a 10-minute treatment with 5 mg/mL of ICG, some RPE cells swelled, and the cytoplasm was finely vacuolated; other cells appeared to be more shrunken. Similar observations were noted when RPE cells were exposed to 1 mg/mL for 20 minutes (Figure 1A and B). No morphologic change was noted when RPE cells were incubated in BSS for 20 minutes (Figure 1C). For the same exposure duration, the degree of morphologic change was greater as the ICG concentration increased. The morphologic change also increased with ICG exposure time. Membranous and intracytoplasmic retention of fine ICG granules was noted in most cells (even after unbound ICG was washed away). Moreover, with increased exposure duration, ICG began to stain some cells green (including swollen cells and cells with a shrunken appearance) (Figure 1D). Finally, many cells underwent lysis, and the cellular outline became poorly defined with only the nucleus discernible after 3 hours of ICG (1 mg/mL) exposure (Figure 1E). Control RPE cells exposed to BSS for the same period appeared unchanged (Figure 1F). No morphologic changes were evident in RPE cells exposed to diluted BSS (275 mOsm/kg), with an osmolarity identical to that of the 5 mg/mL ICG solution, for as long as 3 hours (data not shown). Therefore, the injury caused by incubation of the cells with the ICG solution could not be attributed to alterations in osmolarity.

**MORPHOLOGIC CHANGES IN RPE CELLS FOLLOWING ICG EXPOSURE**

Phase-contrast microscopy was used to observe the effects of ICG on RPE cell morphologic features. Cell viability was assessed by MTS colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) (Promega Corp, Madison, Wis). This quantitative assay detects living but not dead cells. The absorbance at 490 nm (test wavelength) and at 650 nm (reference wavelength) was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (VERSAmax; Molecular Devices, Sunnyvale, Calif); wells containing culture medium but no cells served as blanks. In our experiments, 10⁴ cells in 100 µL of culture medium were seeded into each well of a 96-well plate. After culturing the cells for 48 hours and achieving 80% to 90% confluence, the culture medium was removed, the cells were rinsed with phosphate-buffered sa-
Enzymatic integrity in cultured human RPE cells was evaluated with an MTS colorimetric assay. The MTS tetrazolium compound is reduced to a colored formazan product by a nicotinamide adenine dinucleotide phosphate- or nicotinamide adenine dinucleotide-dependent dehydrogenase in metabolically active cells. The formazan produced was quantitated with an ELISA microplate reader at 490 nm. Exposure of RPE cells to BSS for 3 hours did not alter the dehydrogenase activity. When RPE cells were exposed to 5 mg/mL of ICG for 10 minutes, dehydrogenase activity was significantly reduced. This threshold concentration of ICG decreased to 1 mg/mL when the exposure time was 20 minutes and to 0.01 mg/mL when the exposure time was extended to 3 hours. As the exposure time increased, the...
threshold ICG concentration that caused a significant reduction in dehydrogenase activity decreased. Indocyanine green induced time- and dose-dependent reductions in RPE cell viability (Figure 2). Exposure of RPE cells to diluted BSS (275 mOsm/kg) for as long as 3 hours did not induce a significant decrease in enzymatic activity (97.5%±8.9% of control value); therefore, the observed effects of ICG on enzymatic activity were not caused by variations in osmolarity.

NECROTIC NATURE OF ICG-INDUCED RPE CYTOTOXICITY

To investigate ICG cytotoxicity, RPE cells were stained with 2-µM calcein AM and 4-µM ethidium homodimer 1. Live cells are identified by the presence of ubiquitous intracellular esterase activity, which converts the virtually nonfluorescent, cell-permeant calcein AM to green-fluorescent calcein, which is retained within live cells. Ethidium homodimer 1 enters the cells with compromised membranes and, upon binding to nucleic acid, provides a bright red fluorescence in dead cells.19

In RPE cells incubated for 5 minutes in 1 mg/mL of ICG, the cytoplasm exhibited intense green fluorescence, and the nuclei were unstained; these cells remained viable (Figure 3A). Some of the RPE cells incubated with 1 mg/mL of ICG for 20 minutes had red-fluorescent nuclei and a small amount of cytoplasmic esterase activity (green fluorescence), indicating that the cells were dead. The intense green fluorescence in the cytoplasm of other cells signified their viability (Figure 3B). Structures of most red fluorescent nuclei were normal. There were no signs of apoptosis, such as condensed and fragmented nuclei or margination of the chromatin to the nuclear membrane. At this concentration, ICG apparently caused cell death by a necrotic mechanism. The number of cells with red fluorescent nuclei and little cytoplasmic green fluorescence increased with longer ICG incubation period (Figure 3C). Almost all cells showed red fluorescent nuclei after 3 hours of exposure to 1 mg/mL of ICG (Figure 3D). The percentage of dead cells (with red-fluorescent nuclei), as observed by means of calcein AM–ethidium homodimer 1 staining, is plotted in Figure 4. The ICG toxicity in RPE cells was dose and time dependent, according to results from the MTS colorimetric assay and the fluorescence viability assays.

ALTERATION IN OSMOLARITY AND ICG-INDUCED RPE CYTOTOXICITY

Exposure of RPE cells to diluted BSS (275 mOsm/kg; identical to osmolarity of 5 mg/mL of ICG solution) for as long as 3 hours caused no cell morphologic changes. Calcein AM–ethidium homodimer 1 staining also supported this conclusion. In addition, we performed acridine orange–ethidium bromide staining,20,21 which confirmed the results obtained with calcein AM–ethidium homodimer 1 staining. During macular hole surgery, ICG is applied for periods ranging from 30 seconds to 5 minutes. Although ICG is removed by washing after the surgery, ICG may persist for a long time (even 8 months after surgery).22-24 Therefore, the exposure times used in this study (ranging from 5 minutes to 3 hours) had clinical relevance. The ICG cytotoxicity was not caused by osmotic variations; exposure of RPE cells to diluted BSSs (with osmolarities comparable to the ICG solutions) did not induce morphologic or functional changes in the cells.

Although ICG is a commonly used dye with a long history of safety and low toxicity,25-28 it is most often administered intravenously in humans. After intravenous injection, ICG is taken up exclusively by hepatic parenchymal cells and is rapidly cleared from the circulation via biliary secretion. In earlier publications, the plasma half-life of ICG was estimated to be only approximately 2 to 4 minutes.29,30 More recent spectrophotometric studies31 suggest the clearance of ICG in blood is biphasic, with a rapid initial phase (half-life of 3 to 4 minutes) and a secondary phase (half-life of more than 1 hour) at low concentrations. The pharmacokinetics of intravenous ICG most likely differ markedly from those of ICG injected into the vitreous cavity. Owing to the slow turnover rate in the vitreous cavity, ICG must have a longer half-life in the vitreous cavity compared with its plasma half-life. The reports describing cases with persistent ICG fluorescence after intraocular ICG administration (even as long as 8 months) lend support to our speculation.22,23

Removal of the ILM has been reported to improve both anatomic and visual results.14 However, difficulty in visualizing the ILM may lead to an increase in surgi-
cal time and the risk of phototoxicity. Staining with ICG may improve visualization of the membrane. Some studies reported no adverse effects associated with the use of ICG in macular hole surgery. The concentration of ICG used in these reports ranged from 0.6 to 5 mg/mL, and ICG was left in the vitreous cavity for 30 seconds to 5 minutes. However, toxicity may be associated with ICG use. Gandorfer et al reported that ICG-assisted ILM peeling potentially caused retinal damage and that visual outcomes were poorer when ICG was used during surgery. They used a concentration of 5 mg/mL of ICG, and the ICG was drained immediately after injection. They suggested that the use of ICG was responsible for the less favorable outcome. Engelbrecht et al reported that RPE changes occurred after macular hole surgery when ICG was used as an adjuvant in ILM peeling. They used an ICG concentration of 1 mg/mL, and the ICG was left in the eye for periods ranging from 0.5 to 2.5 minutes.

The cause of the postoperative changes in RPE cells and the poorer visual outcome in these reports is not yet clear. These observations could possibly be attributed to cytotoxic effects of ICG on RPE cells in vivo, similar to those effects observed in RPE cells in vitro. Indocyanine green induced dose- and time-dependent changes in cell morphologic features and cell lysis, decreased enzy-

Figure 3. Retinal pigment epithelium (RPE) cells were stained with calcein AM–ethidium homodimer 1 to assess viability after incubation with indocyanine green (ICG). A, The RPE cells were incubated with 1 mg/mL of ICG for 5 minutes. All cells showed intense green fluorescence, indicating they were alive. B, The RPE cells were incubated in 1 mg/mL of ICG for 20 minutes. Some cells exhibited orange-red fluorescent nuclei with little green fluorescence in the cytoplasm, indicating compromised membrane integrity and reduced esterase activity. Other RPE cells showed green fluorescence in the cytoplasm and remained viable. C, The RPE cells were incubated in 1 mg/mL of ICG for 1 hour. More cells exhibited orange-red fluorescent nuclei with little green fluorescence in the cytoplasm. D, The RPE cells were incubated in 1 mg/mL of ICG for 3 hours. Almost all cells showed orange-red fluorescence and nonfragmented nuclei, indicating they were necrotic. Scale bar=70 µm.

Figure 4. The mean ± SD percentage of dead retinal pigment epithelium cells was determined by counting the cells with red-fluorescent nuclei under calcein AM–ethidium homodimer 1 staining. Data were obtained from at least 4 wells. In each well, at least 250 cells were evaluated. Asterisk indicates statistically significant difference vs control. BSS indicates balanced salt solution; ICG, indocyanine green.
and ICG may be retained in the vitreous cavity for a prolonged period. Therefore, ICG should be used cautiously in the vitreous cavity, and a thorough washout of ICG following vitreal surgery is crucial.

Phototoxicity or photodynamic toxicity may also induce RPE changes after ICG-assisted ILM peeling. In this study, we demonstrated that ICG induced RPE toxicity after prolonged exposure, even at low ICG concentrations. We kept the RPE cells in the dark during and after the ICG exposure period. Therefore, the RPE cytotoxicity demonstrated in this study was due to the toxic effects of ICG itself. However, we did not exclude the possibility that photodynamic toxicity contributed to the RPE changes after ICG-assisted ILM peeling clinically, because ICG is also a photosensitizing agent. Indocyanine green staining of the ILM facilitates its identification and removal during macular hole surgery. Indocyanine green staining can be a valuable tool; however, no standardized procedure that specifies concentration, volume, incubation time, and dilution exists for the intravitreal use of ICG. In this study, ICG caused detrimental effects on cultured human RPE cells in a dose- and time-dependent manner. Further in vivo studies are necessary to establish the ideal parameters for intravitreal use of ICG and to achieve safer staining and efficient ILM dissection.

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Dr Ho had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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