Effect of Indocyanine Green and Illumination on Gene Expression in Human Retinal Pigment Epithelial Cells

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Purpose. To investigate the biological effects of indocyanine green (ICG) and acute illumination on human retinal pigment epithelial (RPE) cells.

Methods. Three concentrations (0, 0.25, and 2.5 mg/mL) of ICG were applied to ARPE19 cells for 1 minute. After isotonic rinsing, the cells were irradiated with a light beam with a wavelength spectrum from 400 to 800 nm and an output of 1850 lumens for 15 minutes. The cells were collected at timed intervals for the investigation of cell death and expression of stress-response genes by reverse transcription–polymerase chain reaction, immunofluorescence, and Western blot analysis.

Results. After ICG incubation, photoreactive changes were observed in the RPE cells. A reduction in cellular viability and considerable shrinkage of the cells were observed. The expressions of the apoptosis-related genes *p53* and *bax* and the cell cycle arrest protein p21 were upregulated in cells treated with both ICG and light. Of the early-response genes, the expression of c-*fos* was specifically enhanced by light, with additive effects from the presence of ICG. Such stimulatory effects on these gene expressions were greater at 2.5 mg/mL than at 0.25 mg/mL ICG.

Conclusions. ICG in the presence of acute illumination can elicit cell-cycle arrest and even apoptosis in RPE cells. The establishment of a safety level in the application of ICG in the region of 0.25 mg/mL is recommended. (*Invest Ophthalmol Vis Sci.* 2003;44:370–377) DOI:10.1167/iovs.01-1113

Indocyanine green (ICG), a water-soluble, tricarbocyanine dye with a peak spectral absorption at 800 to 810 nm, was first used in hepatic biliary imaging because of its preferential staining in the hepatic lymph that enables observation of the extent of biliary obstruction. In ocular angiography, ICG has been extensively used to image the choroidal circulation.[1,2] It improves the linearization of ocular vasculature and offers a visual basis for guided laser photocoagulation in the treatment of choroidal neovascularization.[3,4] ICG has been shown to be safe for human use.[5–7] It is safe for intravenous injection in an ophthalmic fundus angiogram, despite its known tendency to leak around the choroid and retina.[5] Extravasation of ICG molecules into the choroidal stroma has been found to accumulate in the RPE-Bruch's membrane complex during fundus angiography.[8]

Recently, ICG has been used for selective staining of the transparent retinal inner limiting membrane (ILM), because of its efficient and complete removal during intraocular surgery.[9,10] The procedure is assisted by intraoperative endoillumination with a high level of light intensity. Accordingly, photodynamic damage to retinal cells, including photoreceptors and retinal pigment epithelial (RPE) cells, is possible although not affirmed. The toxicity may cause atrophy of the RPE over the bases of macular holes. Such atrophy is probably related to the amount of ICG used and the scale and duration of endoillumination.[11] After its intraocular administration for macular hole surgery, persistent ICG fluorescence was found after 6 weeks.[12] This suggested possibly incomplete removal of the dye. In cultured human RPE cells, mitochondrial dehydrogenase activity was decreased after exposure to ICG, with and without illumination, indicating possible dye-induced cellular alteration, even though no histologic or ultrastructural effects were evident.[13] Intravitreous injection of ICG into rabbits causes retinal toxicity signaled by consistent morphologic changes in the RPE, formation of vacuoles in ganglion cells, and intraocular inflammation.[14] Meanwhile, phototoxicity has been reported in cultured RPE cells in which apoptosis or necrosis has been induced by blue light in an intensity-dependent manner.[15,16] Because the inclusion of free radical scavengers can alleviate the situation, involvement of a photooxidative mechanism has been suggested.[16,17] In cultured HaCaT keratinocytes, diode laser irradiation in the presence of ICG at concentrations higher than 10 μM is lethal to the cells, with the cell death probably mediated by a photodynamic mechanism.[18] The cell-killing effect is probably photo-oxidative and does not involve thermal damage to cellular ultrastructure, as revealed by electronic microscopy.[19] It is noted that very few of these ICG phototoxicity studies involved human RPE cells, except the study by Sippy et al.[13]

In this study, we sought to investigate the biological effects on human RPE cells of a combination of ICG and acute illumination. We used nonpigmented ARPE19 cells to define the photodynamic reactivity of ICG clearly, eliminating any possible interference caused by endogenous pigmentation. With the reduction of cell viability and upregulation of genes relating to apoptosis and cell-cycle arrest, we hypothesize the induction of cell cycle arrest and apoptosis in RPE cells by such treatment.

Materials and Methods

Cell Culture

Human ARPE19 cells[20] were obtained from American Type Culture Collection (Manassas, VA) and grown in 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's and Ham's F12 medium (DF; Gibco, Rockville, MA) containing 3 mM L-glutamine, 10% fetal bovine serum (Gibco), and antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin sulfate; Gibco). RPE cells within five passages were grown to 70% confluence for ICG treatment and illumination.

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Drug Treatment and Exposure to Light

Indocyanine green (ICG) for injection (Diagnogreen) was obtained from Daiichi Pharmaceutical (Tokyo, Japan) and reconstituted with the provided solvent 3 minutes before use. The cells were washed twice with 0.01 M phosphate-buffered saline (PBS) and incubated in 0.25 and 2.5 mg/mL ICG in PBS (denoted hereafter ICG-0.25 and ICG-2.5, respectively) for 1 minute. The dye-free samples did not contain ICG (denoted ICG-0). After a wash in PBS, the cells were placed in fresh culture medium in the dark and subjected to acute exposure to light with a standard endoillumination probe connected to a light source (Hexon Illumination System with ultraviolet impermeable diffuser, model 1266 XII; Dorc, Phoenix, AZ) with an emission spectrum from 400 to 800 nm, irradiance of 1850 lumens, and temperature maintained at 4700 K. The probe was positioned at 1 cm above the cells, so that they were evenly exposed for 15 minutes at 37°C. After illumination, the cells were washed, culture medium was added, and cultures were maintained at 37°C with 5% CO_2 balanced with air. The culture medium was refreshed every 2 days. Samples receiving no light served as the control.

Cell Viability Assay

ARPE19 cells in a density of 5×10^4 cells/mL were seeded on 24-well plates (Corning, Acton, MA) and treated as described earlier. A count of viable cells was performed with trypan blue staining every 24 hours. The percentage of viability was calculated as the number of trypan blue–unstained cells against the total number of cells.

Gene Expression Study

Sample Collection. Samples of ARPE19 cells after light and ICG treatment were collected at 0, 10, 20, 30, 40, 50, 90, and 120 minutes for isolation of RNA and at 0, 4, 8, 12, 16, 24, 48, and 96 hours for Western blot analysis. For immunochemistry, the cells were harvested and fixed at appropriate timed intervals after treatment.

RNA Isolation, cDNA Synthesis, Standardization, and PCR. Total RNA was extracted with a kit (RNeasy; Qiagen, Valencia, CA). Cells in lysis buffer containing 1% β-mercaptoethanol (Sigma, St. Louis, MO) were passed through a separation column (QIAShredder; Qiagen), and total RNA was obtained according to the supplier's protocol. Reverse transcription was performed on 500 ng total RNA, with 10 ng/mL random primers and enzyme (SuperScript; Gibco). cDNA corresponding to 5, 10, 15, and 20 ng of total RNA was amplified with primers specific to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) for 25 cycles. The PCR products were separated on 2% agarose gel and quantified with an image analyzer (Gel Doc Quantity One; BioRad, Hercules, CA). The linear range of amplification was determined by plotting the amount of cDNA used in PCR against the optical density of the products. Standardized cDNAs corresponding to 10 ng of total RNA were used, because this amount provides exponential amplification under our PCR conditions. The cDNAs were amplified with the following primer pairs: *GAPDH* forward: 5′-gaa ggt gaa ggt cgg agt-3′, and reverse: 5′-gaa gat ggt gat ggg att tc-3′; *p53* forward: 5′-ttg ccg tcc caa gca atg gat ga-3′, and reverse: 5′-tct ggg aag gga cag aag atg ac-3′; c-*jun* forward: 5′-gtg acg gac tgt tct atg act g-3′, and reverse: 5′-ggg ggt cgg cgt ggt ggt gat g-3′; c-*fos* forward: 5′-aga cag acc aac tag aag atg a-3′, and reverse: 5′-agc tct gtg gcc atg ggc ccc-3′. The amplification products were separated on 2% agarose gel and stained with 0.5 μg/mL ethidium bromide, and band intensity was scanned. The band intensity of each reaction was normalized against that of GAPDH. Each set of experiments was repeated in triplicate to obtain the mean ± SD.

Immunochemistry. ARPE19 cells were grown on glass coverslips precoated with 0.1% bovine serum albumin (Sigma), 10 ng/mL fibronectin (Gibco), and 10 ng/mL laminin (Gibco). After incubation with ICG and illumination, cell samples were collected at 24 hours and fixed with 4% buffered paraformaldehyde (Sigma) at pH 7.40. After retrieval of the antigen by 0.05% trypsin (pH 7.20) and blocking with 5% normal goat serum (Gibco), the cells were incubated with antibodies against human bax, p53, and p21 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Staining was detected by immunofluorescence (secondary antibodies obtained from Amersham, Busks, UK and nuclear counterstaining with 4′,6′-diamino-2-phenylindole [DAPI]; Sigma) or by the avidin-biotin complex method (Vector Laboratories, Burlingame, CA) and observed under fluorescence and light microscopes (Carl Zeiss, Oberkochen, Germany). The staining intensity was graded from 0 to 5+ (low to high). The number of positively stained cells was counted in five optical fields (each 200 μm in diameter) and the frequency was obtained as the ratio of the number of positive cells against the total number of cells.

Western Blot Analysis on Total Soluble Protein. ARPE19 cells after ICG and illumination were counted, and 10^8 cells were lysed in 1 mL buffer containing 50 mM Tris-HCl (pH 7.40), 10 mM $MgSO_4$, 1 mM EDTA.Na_2 [pH 8.0], 1 mM DL-dithiothreitol [Sigma], 1 mM phenylmethylsulfonyl fluoride [Sigma], and protease inhibitor mix [Sigma]) on ice. After sonication, the samples were mixed in equal volume with urea-sodium dodecyl sulfate (SDS) buffer (9 M urea, 125 mM Tris-HCl [pH 6.8], 4% SDS, 15% glycerol, and 0.1% β-mercaptoethanol) and denatured at 95°C for 5 minutes. The samples (equivalent to 5×10^5 cells) were resolved in 10% denaturing SDS-polyacrylamide gel electrophoresis at 200 volt-hours (Vh). The protein profile was transblotted to nitrocellulose membrane (Amersham) at 100 Vh at 4°C. After blocking with 10% skimmed milk in Tris-buffered saline containing 0.05% Tween 20, the membrane was incubated with antibody against human bax (Santa Cruz). The signal was revealed by incubation with Ig-horseradish peroxidase conjugate (Amersham) and detected by enhanced chemiluminescence (Amersham).

Results

Cell Culture

ARPE19 cells were arranged in monolayers at 96 hours and were flattened and epithelial in morphology (Fig. 1A). After a 1-minute incubation with 2.5 mg/mL ICG and a 15-minute illumination the cells showed membranous and cytoplasmic retention of ICG traces (Fig. 1C). They were oval and of reduced size, when compared with cells in the control sample (no ICG and without illumination; Fig. 1A). Treatment with ICG but without illumination caused no observable morphologic changes, even at 2.5 mg/mL (Fig. 1B).

Cell Viability

ICG and acute light suppressed ARPE19 cell viability in a dose- and intensity-dependent manner (Fig. 2A). At an ICG concentration of 2.5 mg/mL, there was more reduction in cell viability than at lower ICG concentrations. After illumination, the number of viable ICG-0.25 cells remained at about two thirds ICG-0 cells throughout the whole incubation period, whereas viable ICG-2.5 cells were at a much lower level (Fig. 2A). When the illuminated ICG-2.5 cells were compared with the control sample (ICG-0 and without light), there was a significant decrease in the percentage of viable cells to a level of less than 40% (Fig. 2B). The decrease was much less in illuminated ICG-0.25 cells, which were maintained at a level of approximately 80%. For the cells treated with light only, there was a slight reduction in the percentage of viable cells (Fig. 2B). No obvious changes were found in ICG-2.5 cells with and without illumination.

Gene Expression Study

Expression of the Housekeeping Gene. All cells, with or without ICG and light treatment, showed similar expression of *GAPDH* in 10 ng of total RNA (Fig. 3).

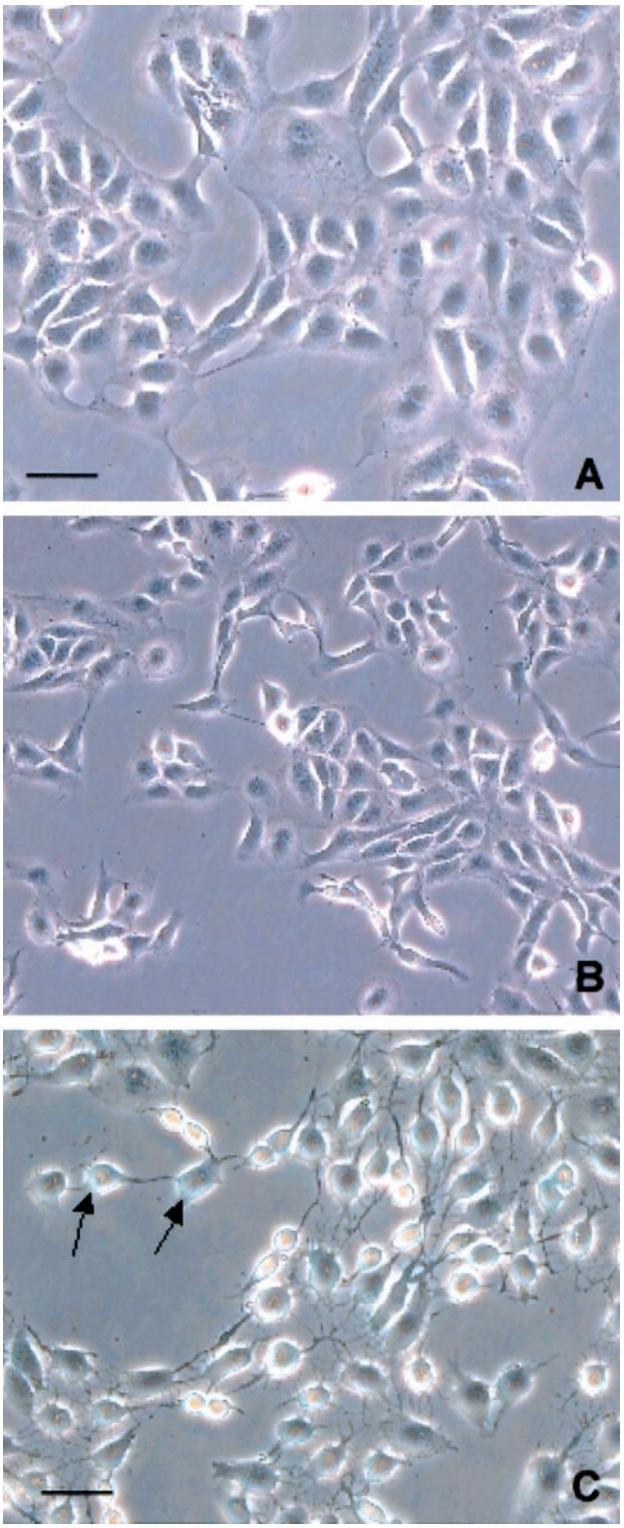

FIGURE 1. Phase contrast micrographs of RPE cells. (**A**) control cells without no ICG and without illumination. (**B**) Cells treated for 1 minute with 2.5 mg/mL ICG without illumination. (**C**) Cells treated for 1 minute with 2.5 mg/mL ICG with 15 minutes of illumination. Comparison of cells in (**A**) and (**B**) shows that treatment with ICG alone without illumination did not cause any morphologic changes in the cultured RPE cells, whereas the cells in (**C**), having both treatment with ICG and illumination, appeared oval in shape with shrinkage. Traces of dye were observed on the cell surface (**C**, *arrows*). Bar, 50 μm.

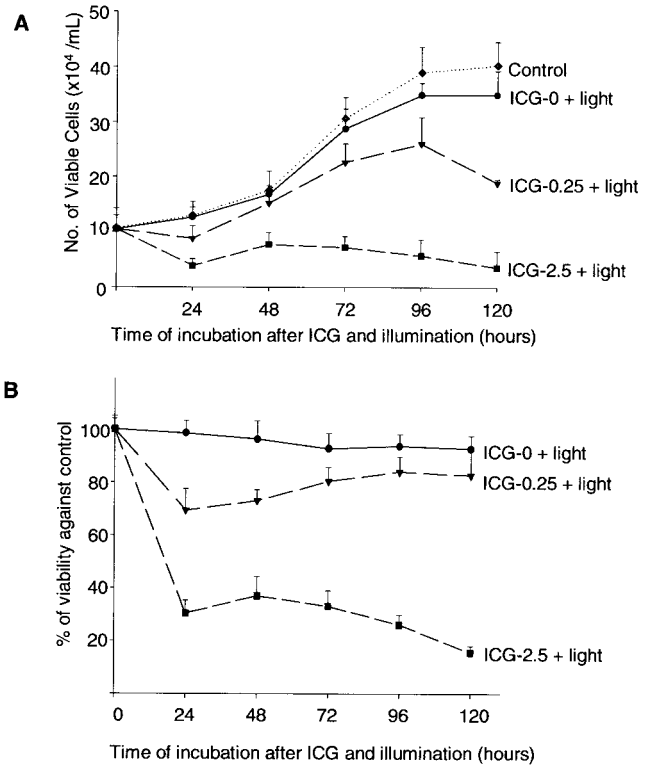

FIGURE 2. Viability assay of RPE cells after ICG and illumination. (**A**) Greater reduction in the number of viable cells was found with higher concentrations of ICG with illumination. (**B**) ICG toxicity was examined by the ratio of number of viable (trypan blue–unstained) cells in samples treated with ICG and illumination against the control sample at the same time point. More decrease in the percentage of viable cells was found in the ICG-2.5 sample with illumination, reaching levels lower than 40% compared with approximately 80% in the illuminated ICG-0.25 cells.

Expression of Apoptosis-Related Genes. RNA of p53 was amplified, and the level of expression varied with ICG dose, increasing more in illuminated ICG-2.5 cells than in ICG-0.25 or ICG-0 cells (Figs. 3 and 4A). It showed upregulation in the first 30 minutes, reaching peak level (approximately 13 times that in the control) at approximately 50 minutes. Without illumination, incubation of cells in 2.5 mg/mL ICG for 1 minute did not cause any alteration in expression of p53. Immunofluorescence showed p53 labeling in RPE cells after ICG and illumination (Figs. 5A–D). Cytoplasmic signal was observed in ICG-0.25 and ICG-2.5 samples (Fig. 5C, arrowheads) whereas no fluorescence was noted in ICG-0 and control cells. Some nuclear p53 labeling was found in illuminated ICG-2.5 cells (Fig. 5B, long arrow). Table 1 summarizes the intensity of p53 staining in different samples. The labeling in illuminated ICG-0.25 and ICG-2.5 cells was scored at +++, whereas dye-free and control had no intensity. ICG-2.5 cells contained more p53-positive nuclei (15.43% ± 3.67%) but less p53 in cytoplasm (1.59% ± 0.14%). In contrast, ICG-0.25 cells after illumination had more cytoplasmic p53 (23.15% ± 5.36%) than nuclear p53 (3.49% ± 1.58%). The difference in nuclear p53 labeling between these illuminated ICG-2.5 and ICG-0.25 cells was significant ($P < 0.05$ by the Fisher exact test). Control and illuminated dye-free samples showed much lower frequencies in both compartments.

Soluble protein samples from 5×10^5 cells after treatment were subjected to Western blot analysis of expression of bax. The optical densities were obtained and normalized

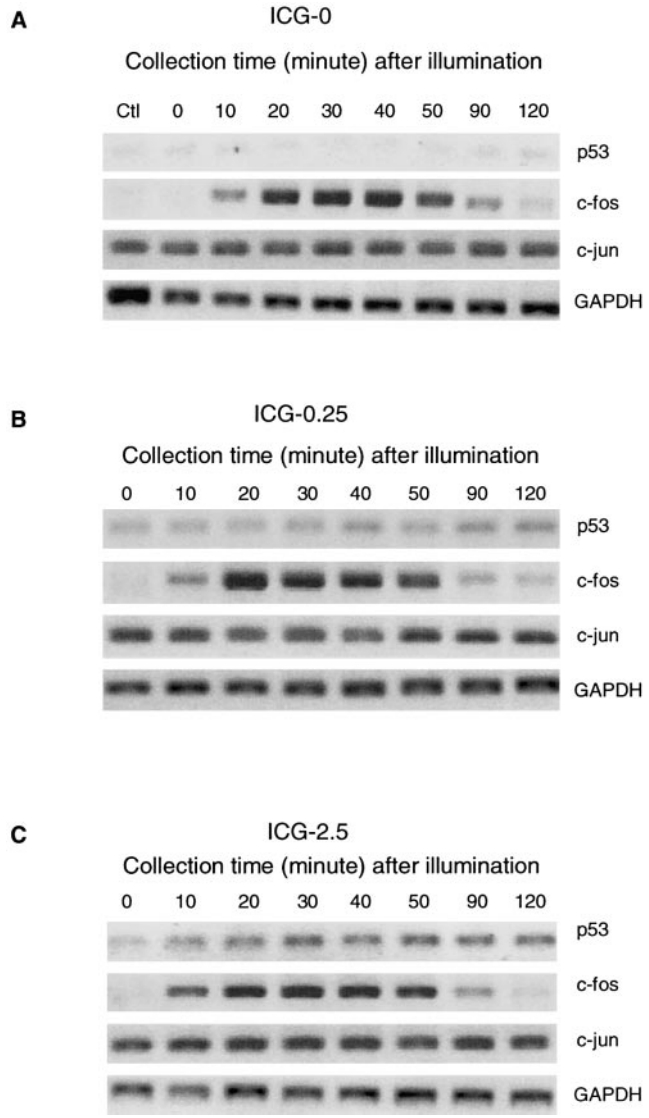

FIGURE 3. Gene expression study of ARPE19 cells with 1-minute incubation of different ICG doses followed by acute exposure to light for 15 minutes: (**A**) 0 mg/mL ICG, (**B**) 0.25 mg/mL ICG, and (**C**) 2.5 mg/mL ICG. Upregulation of p53 appeared in ICG-2.5 samples at approximately 20 minutes after illumination, whereas similar and weak expression was found in other samples. All cells showed transient c-Fos expression after acute exposure to light, and the level was slightly affected by dose of ICG. c-Jun and GAPDH were consistently expressed in all samples, in the presence and absence of illumination and ICG, and GAPDH was used as the internal control.

against the expression of β-actin before comparison. Elevation in expression of bax was observed in ICG-2.5 samples, starting from 12 hours and peaking at 16 to 20 hours after illumination (Fig. 6). The peak was approximately four times that in the control, and the signals gradually decreased. Under the same light intensity and duration, there was no difference in expression of bax between ICG-0.25 and ICG-0 cells. To clarify the effect of light on the expression of bax, ICG-2.5 cells without light treatment were also examined at different time intervals. The levels of bax did not vary much, although they showed slight upregulation at 12 hours and declined after 20 hours (Fig. 6B). The increase was approximately a quarter that in the illuminated samples. Expression was consistent in other samples. There was no significant difference in expression of bax among ICG-0 and ICG-0.25 cells, under the same light intensity and duration (Fig. 6). Time changes in bax localization in ICG-2.5 cells were further examined by immunofluorescence. At 12 hours after illumination, bax protein was dispersed in the cytosol and the perinuclear region (Fig. 5E, arrow). However, when cells were examined at 24 hours, cytoplasmic fluorescence appeared in a dot-shaped pattern (Fig. 5F, arrowheads).

Expression of Cell Cycle–Related Gene. The expression of p21 protein, a downstream effector of p53 on cell cycle arrest, was examined immunologically. The staining was also ICG and light dependent. At 24-hours after illumination, a higher concentration of ICG led to an increase in p21-positive cells and more intense nuclear staining (Figs. 5G–I). ICG-2.5 cells showed more p21 expression at score 3+ (Fig. 5I) than ICG-0.25 cells, which scored + (Fig. 5H). Very faint signal was found in ICG-0 cells (scored ±; Fig. 5G). Most of the cells displayed positive labeling after overnight incubation with 10^{-6} M camptothecin, scored 4+, serving as the positive control (Fig. 5J).

Expression of Early Response Genes Leading to Cell Death. c-Fos and c-Jun are major components of AP-1, which is commonly activated in cell death. In this study, the level of c-Fos RNA in RPE cells increased at 10 minutes after illumina-

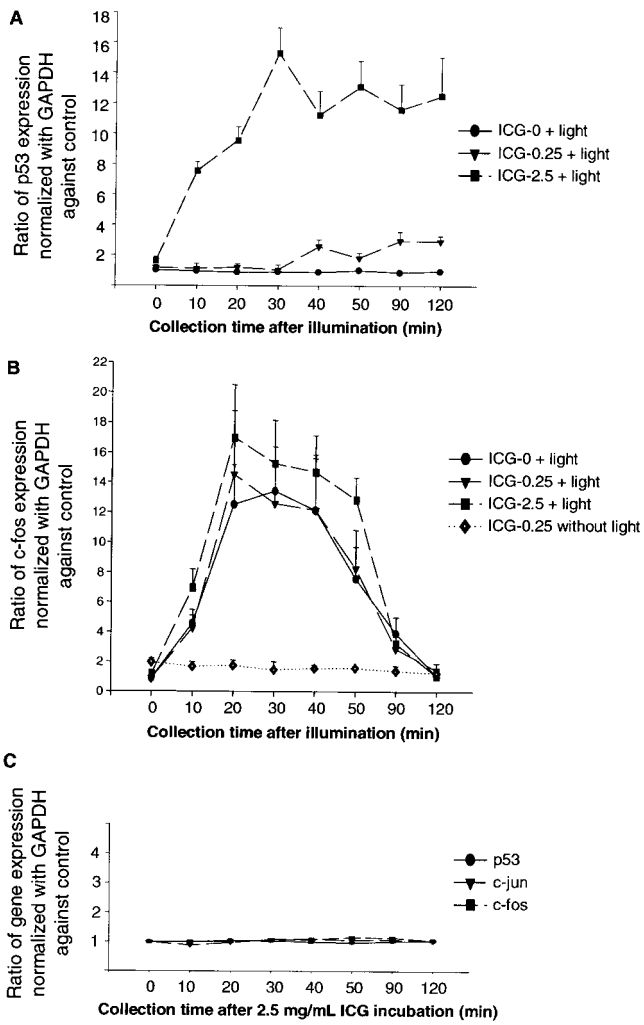

FIGURE 4. Quantification of PCR signal intensities of p53 (**A**) and c-Fos (**B**) in ARPE19 cells after treatment with ICG and illumination. No significant alteration in the expression of p53, c-Jun, and c-Fos in ARPE19 cells after 2.5 mg/mL treatment with ICG (without illumination) was shown (**C**).

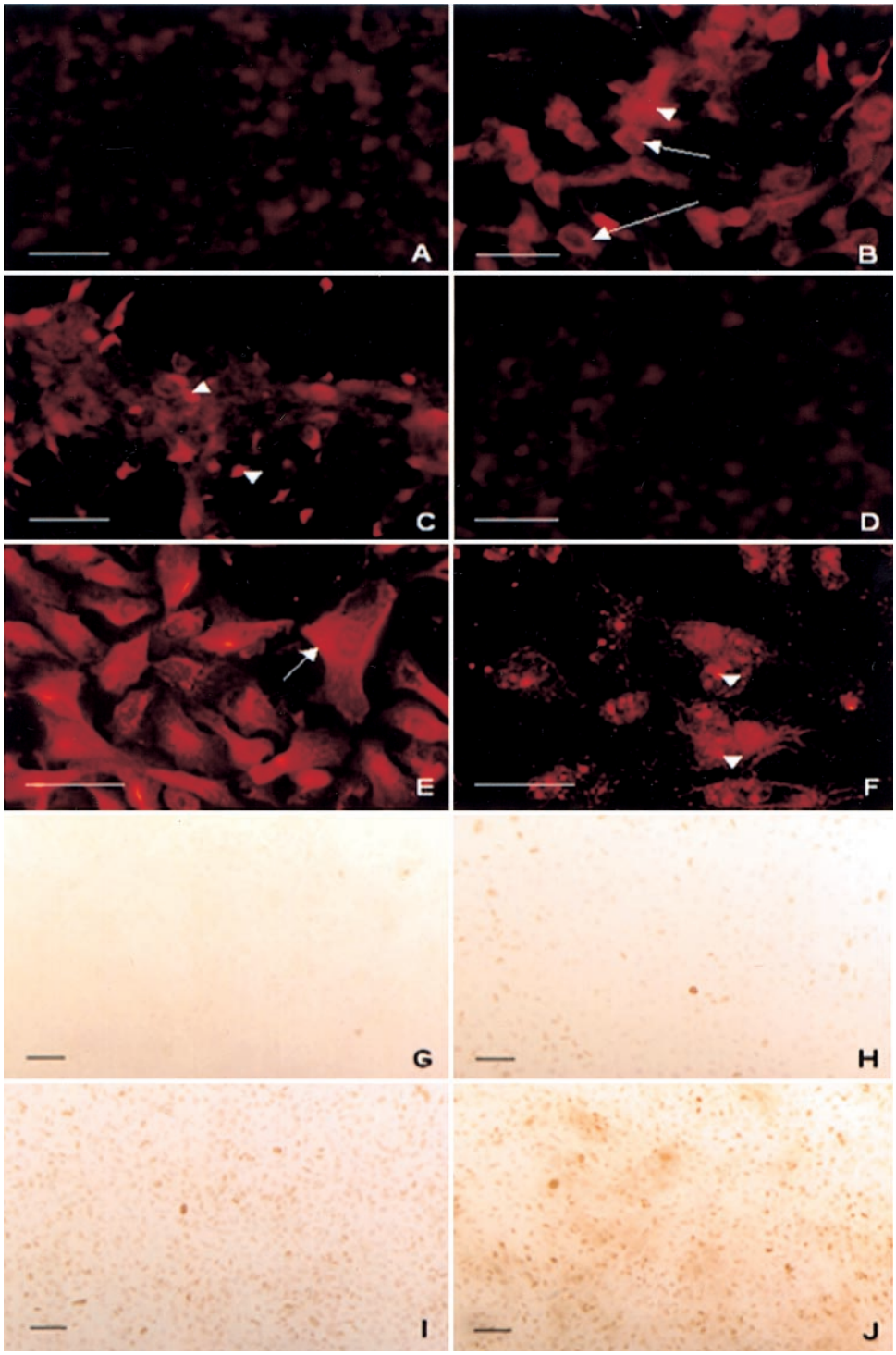

FIGURE 5. Immunofluorescence of p53 and bax in ARPE19 cells. (**A**–**D**) Staining of p53 in RPE cells 24 hours after treatment with ICG and illumination: (**A**) 0 mg/mL, (**B**) 0.25 mg/mL, and (**C**) 2.5 mg/mL ICG and (**D**) control cells (without illumination or ICG). An increasing number of p53-positive cells (*arrows*) was found with higher concentrations of ICG (**B**, *arrows*; **C**, *arrowheads*). Illumination only did not induce any expression of p53 (**A**). (**E**, **F**) Staining of bax protein in RPE cells collected at 12 (**E**) and 24 (**F**) hours after illumination subsequent to a 2.5-mg/mL treatment with ICG. The staining pattern of bax was found to change from a dispersed to a speckled arrangement in the cytosol (**E**, *arrow*; **F**, *arrowheads*). (**G**–**J**) Immunostaining of p21 protein in ARPE19 cells treated with ICG and light: (**G**) 0 mg/mL, (**H**) 0.25 mg/mL, and (**I**) 2.5 mg/mL ICG. More p21expression was found in cells treated with the higher concentration of ICG (**G**, **H**). Camptothecin (10^{-6} M)-treated cells served as the positive control (**J**). Bars, 50 μm.

tion (Fig. 3). The level at 20 to 40 minutes was approximately 12 times that in the cells without illumination (quantitative data shown in Figs. 4B, 4C). In the illuminated ICG-2.5 cells, the expression was increased to approximately 16-fold at 20 minutes, and gradually returned to basal level after 2 hours. In ICG-0.25 cells, the light reaction induced approximately a 14-fold increase, whereas there was also a 12-fold upregulation in dye-free samples. Without light, c-*fos* expression was greatly inhibited (Figs. 4B, 4C). In contrast to c-Fos, the RNA levels of c-Jun were constant in all samples, indicating that expression of c-Jun was not affected by the concentration of ICG and exposure to light (Fig. 3).

DISCUSSION

Mammalian cells exhibit complex cellular responses (e.g., DNA repair, cell cycle checkpoint, and apoptosis) to stresses or stimuli, including growth factor deprivation, presence of cytokines, and exposure to light.[21] Inactivation of these responses results in genomic instability, which leads to cell transformation with de novo expression and activation of genes.[22,23] Transcription factors are involved in the regulation of such stress-inducible or apoptosis-associated genes, including p53, Oct-1, c-*myc* and AP-1 (c-*fos* and c-*jun*).[24–26]

TABLE 1. Semiquantitative Analysis of p53 Immunostaining in RPE Cells Subjected to Treatment with ICG and Illumination

	ICG-0+Light	ICG-0.25+Light	ICG-2.5+Light	Control
p53 Intensity	0	+++	+++	0
Frequency of p53-Stained Nuclei	0.23 ± 0.02	3.49 ± 1.58	15.43 ± 3.67	0.14 ± 0.02
Frequency of p53-Stained Cytoplasm	0.09 ± 0.05	23.15 ± 5.36	1.59 ± 0.14	0.11 ± 0.02

The frequencies of nuclear and cytoplasmic staining of p53-positive cells were compared. Data are the mean percentage of total cells ± SD.

Our experiments showed altered gene expression in ARPE19 cells after treatment with ICG and illumination. ICG has been used in staining the ILM for easy removal, which contributes to high closure rates in macular hole surgery.[8,10] At times, repeat applications are needed to ensure adequate ILM staining.[8,27] This has been an established ophthalmic surgical technique for some years, but there are only a few studies examining the direct effects on retinal cells of ICG combined with acute illumination.[13] A decrease in mitochondrial dehydrogenase indicates the possible risk of RPE cell damage when the cells are directly exposed to ICG at the base of macular holes. Phototoxicity of ICG had been shown in other cell systems, such as HaCaT[18] and colon cancer cells,[28] where ICG was lethal to cells when combined with diode laser irradiation. ICG's cytotoxic effects included cytoplasmic vesiculation, dilation of rough endoplasmic reticulum, Golgi apparatus, perinuclear cisternae, and chromatin condensation in the nucleus.[19]

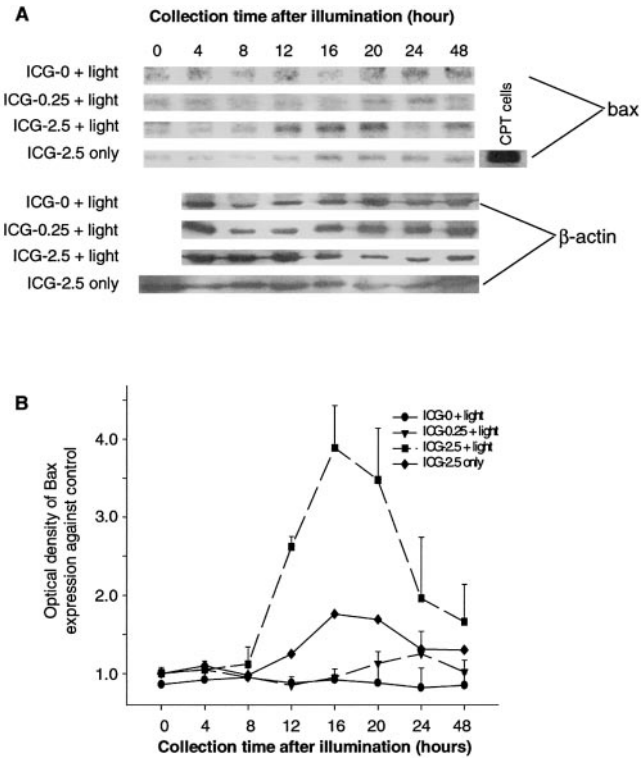

FIGURE 6. Expression of bax in ARPE19 cells, determined by Western blot analysis, after ICG and illumination. (**A**) A significant increase in bax was detected from 12 hours onward in ICG-2.5 samples. The expression was mild and similar between the ICG-0 and ICG-0.25 groups. Without illumination, only moderate staining was found in the ICG-2.5 cells at 12 to 48 hours. Camptothecin-treated cells served as the positive control for bax protein staining. β-Actin staining was used as the internal control. (**B**) The band intensities of expression of bax were normalized against that of β-actin and plotted against the collection time.

Cumulative cellular ICG uptake involved the organic anion transporting polypeptide and cytoplasmic binding of dye molecules to glutathione *S*-transferase.[19] Increase of bax and noticeable abatement of bcl-XL expression indicating apoptosis in human leukemia cells resulted after infrared diode laser photostimulation of ICG.[29] To help assess the safety level of ICG, we used two frequently applied intravitreous concentrations of ICG (0.25 and 2.5 mg/mL) in our experiments. The ARPE19 cells in our study showed dose-dependent responses to ICG combined with illumination, the reduction of cell viability and the shrinkage of cells, indicating the possibility of cell death. Meanwhile, after incubation with ICG, we found traces of dye present in RPE cytosol and cell membrane, even after extensive washing. For macular hole surgery, ICG fluorescence remained present 6 weeks after administration.[12] Although ICG is said to be safe for use in humans, it could be a risk factor for retinal damage, dependent on its concentration, change in osmolarity, pH, and duration of contact.[30] Reduced activity of the mitochondrial dehydrogenase in cultured RPE cells also potentiates the toxic effect of ICG.[13]

In the current study, we examined the expression of p53 and its downstream proteins, bax and p21, to investigate the occurrence of cell death and cycle arrest after the treatment.[31–33] The expression of p53 was enhanced in ICG-2.5 cells within the first 30 minutes after illumination. p53 restricts cell proliferation by inducing G_1 arrest and controlling entry into mitosis.[34] The mechanism involves the inhibition of cyclin-dependent Cdc2 kinase that is required to enter mitosis. Cdc2 kinase is simultaneously inhibited by gadd45, p21, and 14-3-3σ, which are also transcriptional targets of p53.[25,35,36] The upregulation in p53 RNA can be due to its promoted transcription or increase in the half-life of RNA, resulting in an accumulation of p53 protein.[25,37] Nuclear translocation of p53 protein also indicates its reactivity in DNA damage (Figs. 5B, 5C). A significant increase in the number of cells positive for nuclear p53 was found in the illuminated ICG-2.5 cells. The stress-induced phosphorylation of p53 protein has been coordinated for its retention inside the nucleus where it is able to function as a master transcription factor.[33] The predominant cytoplasmic localization of p53 in the illuminated ICG-0.25 cells suggests its sequestration and likely inactivation.[38,39] Thus, in terms of apoptosis-related gene expression, ICG at the low level of 0.25 mg/mL should be considered safe for use.

The pathway of p53-mediated apoptosis is linear, involving bax transactivation.[40,41] Bax conveys p53 downstream signal to the release of cytochrome *c* from mitochondria to activate caspases, which proteolytically splice proteins involved in important cellular functions.[42,43] Our blot analysis result showed a time-dependent increase in bax protein after treatment with 2.5 mg/mL ICG. This effect was amplified in the presence of light (Fig. 6A). Without light, reduced intensity of bax was observed in ICG-2.5 samples after normalization with the expression of the control β-actin (Fig. 6B). This suggests that light can have an additive effect on the ICG-induced expression of bax. Overexpression of bax protein promotes RPE apoptotic cell death.[32,44] Thus, upregulation of bax in the present study

suggests committed entry into apoptosis. Also, immunostaining indicated bax protein migration from a pattern of cytosolic dispersion to aggregation (Figs. 5E, 5F). The speckled distribution may be due to the predominant localization of bax protein to the cytoplasmic organelles, such as mitochondria. Because bax and other proteins in the bcl-2 family contain transmembrane domains, they reside in the outer mitochondrial membrane and are involved in the regulation of mitochondrial permeability, which controls the passage of such proapoptotic factors as cytochrome *c* into the cytoplasm.[42,43] Also, the cytotoxicity of ICG in RPE cells has been shown to be linked to reduction in mitochondrial enzymes.[13]

Our study presents evidence of RPE cell arrest by the increase in expression of p21, indicating the concordance of p53 and its transcriptional activity on the regulation of p21.[34] p21 protein is an inhibitor capable of silencing the cyclin-dependent kinases (Cdks) and arresting the cells in the G_0–G_1 phase.[33,45] It also promotes assembly of cyclin-Cdk .[31] Binding of p21 to proliferating cell nuclear antigen blocks its association with DNA polymerases, thus inhibiting DNA synthesis.[46,47] The elevation of p21 in ARPE19 cells treated with ICG and light could suggest the constraint of cell cycle progression or induction of transient arrest in G_1, making room for repair of DNA. As a result, cell growth could be retarded.

Another mechanism of the stress response is through activation of AP-1. AP-1 regulates the expression of its components c-jun or Fra-1, CD44 or E-cadherin, and p53.[48,49] c-Fos, a nuclear proto-oncogene protein, forms linkages with c-Jun/AP-1 transcriptional factor.[50,51] After translation, these proteins regulate gene transcription by binding to the AP-1 sites of target genes. Our results illustrate an immediate upregulation in c-Fos expression after illumination, reaching a 10-fold increase when compared with the control. As in other cell systems, this immediate induction is regulated at the transcriptional level and the transient feature is due to the autocatalyzed shutoff of transcription and the rapid turnover of c-Fos mRNA.[52] We showed the consistent pattern of c-Fos expression after illumination, which was not affected by varying the ICG doses (Fig. 4B). Without ilumination, there was no detectable alteration (Fig. 4C). In contrast, expression of c-Jun remained at a consistent level and was not affected by either light or ICG. This suggests a critical role played by c-Fos as an early-response molecule in initiating the inter- or intracellular signaling cascade.

Although it has been reported that light alone is detrimental to the survival of RPE cells in culture,[17] we showed the phototoxicity of ICG residue in RPE cells, in that the effects of combined light and ICG treatment were greater. Without ICG, light caused only a minor fluctuation in gene expression (except c-*fos*). However, light exposure with a high dose of ICG led to more reduction in cell viability and a tendency toward cell death. The ICG molecule appears to act as a photoinitiator or transducer. The dye can assist the visualization of vitreomacular structures in intraocular surgery. However, ICG molecules can access the RPE cell layer during vitrectomy, thus increasing the risk of surrounding cell damages. Our work illustrates ICG's dose-dependent phototoxicity causing cell cycle arrest and apoptosis. We suggest that the safest dose of ICG is as low as 0.25 mg/mL. Moreover, complete removal of dye at the end of an operative procedure is recommended to prevent any detrimental changes in RPE cells and possibly in other cell types.

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