The Interaction of Indocyanine Green with Human Retinal Pigment Epithelium

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Purpose. This study sought to examine the in vitro interaction of human RPE cells with indocyanine green (ICG). The interaction between ICG and the RPE may have clinical relevance in the interpretation of clinical ICG angiography.

Method. Cultured primary human RPE cells were incubated with ICG. Infrared fluorescence microscopy was used to detect RPE cell ICG fluorescence. The proportions of cells exhibiting ICG infrared fluorescence were quantified. Separate RPE cell populations were incubated with ouabain for 24 and 72 hours, respectively, before addition of the ICG to examine its effect on the uptake of ICG. The effect of ouabain on cell viability was assessed with trypan blue exclusion.

Results. Normal human RPE cells incubated with ICG exhibited strong infrared fluorescence. Exposure to ouabain for 24 hours before incubation with ICG had little effect on cell viability but significantly reduced cellular ICG fluorescence. In contrast, exposure to ouabain for 72 hours reduced cell viability and increased cellular ICG fluorescence.

Conclusions. Cultured human RPE cells take up ICG dye. ICG uptake by RPE cells may involve active transport, as cells incubated with ouabain for 24 hours showed no reduction in cell viability but exhibited reduced infrared fluorescence. The paradoxical increased uptake of ICG into the cells after more prolonged exposure to ouabain may be due to ICG's movement through the damaged cell membrane. Fluorescence due to ICG uptake by RPE has clinical relevance in that it contributes to the fluorescence patterns observed in ICG angiography. (*Invest Ophthalmol Vis Sci.* 2005;46:1463–1467) DOI: 10.1167/iovs.04-0825

Indocyanine green (ICG) angiography provides the ophthalmologist with a means of studying pathologic conditions of the choroidal circulation, including age-related macular degeneration (AMD).[1–5] ICG binds predominantly to serum proteins, reducing extravasation from the fenestrated choriocapillaris. ICG fluorescence properties are in the infrared range, allowing visualization of lesion through blood and pigment, to aid in detection of choroidal neovascularization.

Several studies have shown the binding of ICG to the retinal pigment epithelium. We have shown, in histologic localization studies in the geriatric monkey model and human, that ICG fluorescence is localized to the RPE.[6] We have also demonstrated that ICG is present in intact RPE in surgically excised choroidal neovascularization.[7] It is expected that this uptake of ICG by the RPE is likely to have an effect on the fluorescence patterns that may be observable on clinical ICG angiography.

Despite these clinicopathologic correlation studies, the mechanism of the interaction of ICG with the RPE remains relatively unexplored. In vitro studies of ICG with cultured human RPE cells may yield further information. Flower[8] has reported the apparent binding of ICG to aortic endothelial cells.

The purpose of the present study was to examine the in vitro interaction of ICG with human RPE cells. The possible mechanism of ICG transportation was also explored.

Materials and Methods

Primary Human RPE Culture

Primary cells of adult human RPE was obtained from human donor eyes after informed consent was obtained according to the tenets of the Declaration of Helsinki. Ethical approval was obtained from the Human Ethics Committee, University of Sydney, Australia. RPE cells were isolated and cultured by using a previously published method.[9] Cells were identified using antibodies against cytokeratin, vimentin, and CD31. Confluent RPE cells were passaged by 1:250 trypsin/EDTA, and cells of passages 0 to 3 were used in the experiments.

Incubation of ICG with Cells

The cultured RPE cells were allowed to become confluent to form a monolayer. One milliliter of culture medium containing a final concentration of 25 μg/mL ICG (Pulsion Medical Suppliers, Munich, Germany) was added to the RPE culture and incubated for 6 hours. After this, the culture medium containing ICG was removed, and the cells were washed twice with HBSS to remove the excess ICG. Fresh medium was applied to each Petri dish before infrared fluorescence microscopy.

The pH and osmolarity of the culture medium were tested to ensure that there was no significant change in the pH or osmolarity caused by the addition of ICG.

Infrared Fluorescence Microscopy

The technique of using infrared fluorescence microscopy to detect ICG fluorescence has been published.[6] The cultured cells in the Petri culture dish were placed in a fluorescence microscope (Eclipse; Nikon Corp, Tokyo, Japan). The 810-nm diode laser (Iris Technologies, Mountain View, CA) passing through a custom excitation filter (Chroma Technologies, Brattleboro, VT) was used to stimulate the ICG-stained RPE culture. The emitted infrared fluorescence was detected through a customized barrier filter with peak transmittance at 860 nm placed within the beam splitter of the microscope. A charge-coupled device (CCD) camera (Nikon Corp.) was used to detect and capture the infrared fluorescence emitted from the culture cells. Paired bright-field and corresponding infrared fluorescence images were recorded from each field to allow orientation of the fluorescence image.

Effect of Ouabain on the Interaction of ICG with the RPE

Ouabain, an Na^+,K^+-ATPase pump inhibitor, was used to modulate the ICG uptake activity on cultured RPE cells. Ouabain (250 μg /mL;

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Sigma-Aldrich, Australia) was added to the confluent monolayer cell culture for increasing intervals before the addition of ICG then the cells were incubated with the ICG for a further 6 hours.

Experimental Groups

Three experimental groups were formed to examine the interaction of ICG and ouabain with the RPE cells. (1) *Normal group*: RPE cells without prior ouabain incubation. (2) *Ouabain-24 group:* RPE cells exposed to ouabain for 24 hours before the addition of ICG (25μg/mL). (3) *Ouabain-72 group:* RPE cells exposed to ouabain for 72 hours before the addition of ICG (25μg/mL).

Control Groups

Infrared microscopy was performed on each of the three cell groups without ICG, as the control.

Quantification of Infrared Cellular Fluorescence

The infrared ICG fluorescence of the cells in each of the three experimental groups was graded by quantifying the proportion of individual RPE cells that were fluorescent in six randomly chosen standard microscopic fields.

Cell Viability Testing

Trypan blue exclusion was performed to examine the effect of ICG and ouabain on the viability of the RPE cells. Eight assays were made of the number of viable cells in the three experimental groups. The cell viability was correlated with the ICG fluorescence in each of the experimental groups.

Statistical Method

The number of cells that were infrared fluorescent was compared with the total number of cells. The proportions of cells that were fluorescent in the three groups were analyzed with χ^2 analysis to determine whether a statistically significant difference exists between the groups.

The number of cells remaining viable was counted over eight assays in each of the groups. The quantifications in the three experimental groups were analyzed with a χ^2 test. $P < 0.05$ was considered statistically significant.

Results

The results of the interaction of ICG with the cultured RPE cells are presented quantitatively and qualitatively in Table 1 and Figure 1, respectively. In the control group without ICG, the monolayer arrangement of the cells was intact (Fig. 1A). The corresponding infrared fluorescence image showed no detectable fluorescence (Fig. 1a). In the normal RPE group, 97.9% of the healthy cultured cells incubated with ICG were fluorescent on infrared microscopy (Table 1). The ICG fluorescence was localized to the cytoplasm of the cell, with sparing of the nucleus (Figs. 1B, 1b). The monolayer arrangement of the cell culture was not disturbed by the addition of ICG.

In the ouabain-24 group, 14.8% of the cells showed ICG fluorescence. Figures 1C and 1c show the reduced infrared fluorescence qualitatively. Morphologically, the monolayer arrangement remained intact, and the cells showed mild vacuolation. In the ouabain-72 group, 93.3% of the cells were fluorescent (Table 1). After 72 hours of exposure to ouabain, the monolayer of the cell culture became disrupted, with cells floating freely in the culture medium (Figs. 1D, 1d). These cells were intensely fluorescent.

Comparing the proportion of RPE cells exhibiting ICG fluorescence revealed a significant difference in infrared RPE fluorescence in the three groups ($P < 0.001$, Table 1). The lower proportion of the cells that were fluorescent in the ouabain-24 group (14.8%) compared with the normal (97.7%) and ouabain-72 (93.3%) accounted for the difference (Fig. 2A). The difference between the normal and ouabain-72 group was not significant.

Table 1. RPE Cell ICG Infrared Fluorescence Data

	Total Cells (*n*)	Fluorescent Cells	
		n	%
Normal RPE cell group	85	84	98.8
	79	77	97.5
	81	78	96.3
	78	78	100
	72	68	94.4
	88	87	98.9
Total	483	472	97.7
Ouabain-24 group	110	22	20
	90	13	14.4
	98	5	5.1
	120	28	23.3
	80	8	10
	70	8	11.4
Total	568	84	14.8
Ouabain-72 group	40	38	95
	43	43	100
	33	31	93.9
	38	34	89.5
	55	50	90.9
	44	40	90.9
Total	253	236	93.3

The proportions of cells that were infrared fluorescent in six standard fields in each of three experimental groups were tabulated.

A cell viability test was used to evaluate the effect of ICG and ouabain on the health of the cultured cells.

The cell viability of normal RPE culture without ICG incubation was 97.5% and with ICG was 94.3%. A *t*-test statistical analysis showed there was no difference between the viability of the RPE cells, with or without ICG ($P > 0.05$).

Cell viability differed significantly in the three experimental groups ($P < 0.01$, Table 2), diminishing with prolonged exposure to ouabain. It was found that cell viability was significantly reduced in the ouabain-72 group (17.2%) compared with the normal (94.3%) or ouabain-24 (84.6%) groups (Fig. 2B). The difference between the normal and ouabain-24 group was not significant.

Discussion

This study demonstrates the in vitro uptake of ICG by RPE cells. Infrared fluorescence microscopy has shown the uptake of the ICG into the cytoplasm of cultured RPE cells and sparing the nucleus. These findings support previous histologic localization studies in which ICG was shown to be taken up by the RPE in both monkeys and humans.[6,7]

Cell fluorescence detected on infrared fluorescence microscopy is due to the ICG dye that was taken up by the cultured cells. The infrared fluorescence was not due to autofluorescence of the cultured RPE, as indicated by the absence of infrared fluorescence in the control cells. Filter-related crosstalk during infrared fluorescence microscopy is also unlikely, as the spectral characteristics of the excitation and barrier filters were constructed to filter out completely the stimulating diode laser wavelength.[6]

The concentration of ICG used for this study with incubation periods of 6 hours produced fluorescence that could be clearly detected by the infrared fluorescence microscope without detectable toxic changes to the cultured cells. The dose of

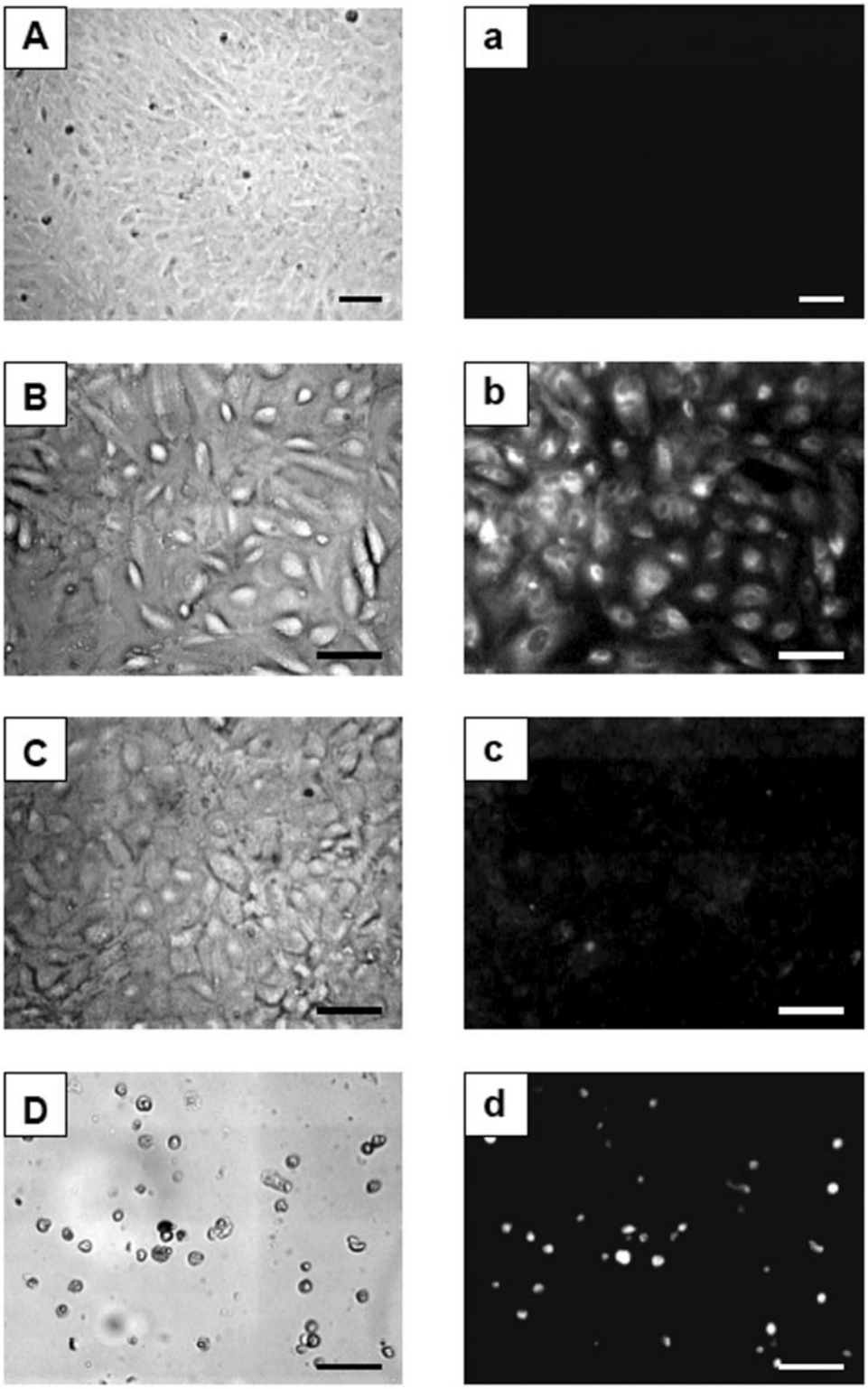

FIGURE 1. Effect of ouabain on ICG fluorescence. Paired bright-field (**A–D**) and corresponding infrared (**a–d**) fluorescence images are presented for the control and experimental groups. (**A**) Control section. No ICG was added to the confluent cell culture. The infrared fluorescence micrograph shows no fluorescence. (**B**) ICG was added to the RPE cells and incubated for 6 hours. The cells had a healthy spindle cell arrangement. The infrared fluorescence micrograph shows the uniform fluorescence due to the uptake of ICG. (**C**) Ouabain was added to the cultured cells for 24 hours followed by incubation with ICG for a further 6 hours. The bright-field micrograph shows that the RPE cells still formed a confluent layer. However, the cells had a vacuolated appearance. The infrared fluorescence was very reduced. (**D**) Ouabain was added to the cultured cells for 72 hours before incubation with ICG for a further 6 hours. The bright-field micrograph shows the disruption to the cell monolayer. Many of the cells were rounded and free floating within the culture medium. The infrared fluorescence micrograph shows the increased ICG fluorescence of the cells. Scale bars, 25 μm

25 μg/mL is comparable to the concentration used by Flower[8] in studying the binding of ICG to aortic endothelial cells.

Ouabain modulated the uptake of the ICG to the RPE cells in an exposure-dependent manner. It has been shown that in liver cells, the uptake of ICG involves a cytoplasmic and canalicular ATP-dependent transport.[10,11] Therefore ouabain was chosen to examine the role of the Na^+,K^+-ATPase pump in the uptake of ICG into RPE cells. In this study, exposure of RPE cells to ouabain for 24 hours significantly reduced the proportion of cells that produced ICG fluorescence, which suggests that ICG uptake by the RPE is likely to involve the Na^+,K^+-ATPase pump. It is noted that ouabain exposure for 24 hours had relatively little effect on cell viability and had a minor effect on the cell architecture, although the trypan blue assay is only a crude assessment of cell viability.

The paradoxical increase in the ICG cellular fluorescence after 72 hours of exposure to ouabain may be explained by an increase in permeability of the damaged RPE cell membranes

to ICG. The severe damage to these cells correlates with the significantly reduced cell viability and morphologic changes.

The effect of ICG on the RPE has generated some interest recently, with ICG being used in vitrectomy surgery to stain the internal limiting membrane[12–14] and the possibility of RPE toxicity.[15] Our present study did not specifically examine the toxicity of ICG to the RPE. However, ICG did not appear to produce significant toxicity in the in vitro RPE cell culture at the concentration used for the incubation time frame. Although, the concentration of ICG used in this study of 25 μg/mL (0.0025%) is lower than that generally used intraoperatively during macular surgery (0.05%–0.5%), Sippy et al.[16] found that RPE exposed to ICG demonstrates a decrease in mitochondrial enzyme activity, with no significant effect on cellular morphology or ultrastructure.

The mechanism by which ICG gains access to the RPE during macula surgery is unclear. Histologic ICG localization studies in monkeys and humans showed that ICG injected intravenously did not pass into the neurosensory retina.[6] If direct contact of intravitreal ICG with the RPE is necessary to produce the toxic effects, this barrier to ICG movement must be disrupted. A study examining RPE cell viability after exposure to ICG concluded that the toxicity to ICG is more likely related to the osmolarity of the solvent than to the ICG itself.[17] We could not detect any osmolarity or pH changes for the ICG incubation, as only 25 μg of ICG was added into each 1 mL of culture medium.

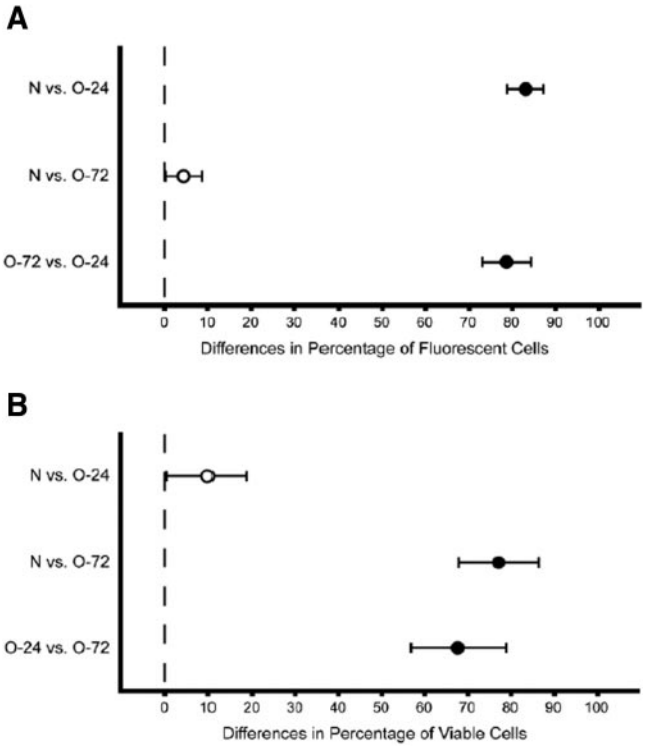

FIGURE 2. (**A**) Differences in the proportion of ICG-fluorescent cells in the experimental groups. The differences in the proportion of fluorescent cells between N (Normal group) and O-24 (Ouabain-24 group) and between O-72 (Ouabain-72 group) and O-24 (Ouabain-24 group) are significant. The difference between N (Normal group) and O-72 (Ouabain-72 group) is not significant. (**B**) Differences in cell viability in the experimental groups. The difference between N (normal group) and O-72 (Ouabain-72 group) and between O-24 (Ouabain-24 group) and O-72 (Ouabain-72 group) is significant. The difference between N (normal group) and O-24 (Ouabain-24 group) is not significant.

TABLE 2. Cell Viability Quantification Data

	Total Cells (*n*)	Viable Cells	
		n	%
Normal RPE cell group	16	16	100
	20	20	100
	12	12	100
	16	12	75
	12	12	100
	24	20	83.3
	20	20	100
	20	20	100
Total	140	132	94.3
Ouabain-24 group	27	22	81.5
	15	12	80
	20	15	75
	28	24	85.7
	10	10	100
	17	15	88.2
	19	16	84.2
	13	12	92.3
Total	149	126	84.6
Ouabain-72 group	14	0	0
	14	4	25
	14	0	0
	46	0	0
	14	7	50
	7	0	0
	28	4	12.5
	14	11	75
Total	151	26	17.2

Data are the proportion of viable cells in eight assays in each of three experimental groups.

This study has implications in the interpretation of clinical ICG angiography. Little significance has been placed on the role of the RPE fluorescence during ICG angiography. We believe that the RPE does not simply behave as a transparent layer during clinical angiography but contributes to the fluorescence patterns observed. Confocal scanning layer ophthalmoscopes are able to focus specifically on this layer to detect the uptake of ICG into the RPE. This RPE fluorescence contributes to the background fluorescence, which should become more prominent in the later phases of the angiogram as more ICG accumulates in the RPE. Conversely, ICG would be expected to wash out from the choroidal circulation during this time. The dynamics of ICG transit through the choroid and RPE have been shown in a histologic localization study.[6]

It is postulated that the variation in background fluorescence pattern, particularly in the late-phase ICG angiogram, may be due to the differential uptake of ICG by altered RPE cells. Reduced RPE uptake of the ICG results in the hypofluorescence observed on an ICG angiogram. When the RPE cells are more severely damaged, then the ICG may enter the damaged cell membrane, more readily producing hyperfluorescence observed on ICG angiography. This damage to the RPE may be primary or secondary to changes in the choriocapillaris. The function and health of the RPE and the choriocapillaris are known to be interdependent.[18–20]

This study has demonstrated the in vitro binding of ICG dye to RPE cells. This interaction in the healthy situation may involve active transport. In pathologic situations, the uptake is altered, and this is reflected in the variation in the fluorescence patterns observed on clinical ICG angiography.

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