Light-Induced Decomposition of Indocyanine Green

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Purpose. To investigate the light-induced decomposition of indocyanine green (ICG) and to test the cytotoxicity of light-induced ICG decomposition products.

Methods. ICG in solution was irradiated with laser light, solar light, or surgical endolight. The light-induced decomposition of ICG was analyzed by high-performance liquid chromatography (HPLC) and mass spectrometry. Porcine retinal pigment epithelial (RPE) cells were incubated with the light-induced decomposition products of ICG, and cell viability was measured by trypan blue exclusion assay.

Results. Independent of the light source used, singlet oxygen (photodynamic type 2 reaction) is generated by ICG leading to dioxetanes by [2+2]-cycloaddition of singlet oxygen. These dioxetanes thermally decompose into several carbonyl compounds. The decomposition products were identified by mass spectrometry. The decomposition of ICG was inhibited by adding sodium azide, a quencher of singlet oxygen. Incubation with ICG decomposition products significantly reduced the viability of RPE cells in contrast to control cells.

Conclusions. ICG is decomposed by light within a self-sensitized photo oxidation. The decomposition products reduce the viability of RPE cells in vitro. The toxic effects of decomposed ICG should be further investigated under in vivo conditions. (*Invest Ophthalmol Vis Sci.* 2008;49:1777–1783) DOI:10.1167/iovs.07-0911

Indocyanine green (ICG) is a water-soluble tricarbocyanine dye developed in the Kodak Research Laboratories in 1955. The dye was approved by the United States Food and Drug Administration in 1956 and is widely applied in medical diagnosis.[1–3]

Intravenously injected ICG is transported in the blood in two different forms unbound or bound to serum proteins, mainly globulins. Ninety-eight percent of the dye is bound to serum proteins. Unbound ICG is quickly removed from the vascular system by the liver and transported into the bile by protein glutathione S-transferase without modification. ICG is suited for diagnostic purposes because it has no known metabolites. ICG shows a low incidence of adverse reactions,[4] and its pharmacokinetics have been studied intensively.[5]

In ophthalmology, ICG is a commonly used dye with a long history of safety after intravenous administration.[4] ICG has been used to stain and visualize epiretinal membranes and the internal limiting membrane (ILM) to facilitate the delicate surgical maneuver of their removal during epiretinal membrane[6–8] and macular hole surgery.[9–11] Several authors have reported good functional outcomes using ICG-assisted vitrectomy,[9,12–16] whereas some authors have reported less favorable results in visual acuity[6,17,18] and significant visual field defects when intraocular ICG was used.[6,17–19] Because of this controversy, a possible toxic effect of ICG on the retina has become the subject of an ongoing debate.[20,21] Several authors have investigated ICG toxicity using in vitro,[22–26] in vivo,[27–29] and ex vivo models,[30,31] and reports show dose-, light-, and exposure time-dependent toxicity in ganglion cells,[22] Müller cells,[23] and retinal pigment epithelial (RPE) cells.[23–26] After incubation of human colonic cancer cells with ICG and subsequent irradiation with light at 810 nm, the cells were killed by photodynamic mechanisms in which singlet oxygen should play a major role.[32,33] However, the exact mechanism of cytotoxicity of ICG remains unclear.

It was the aim of the present study to elucidate the mechanism of ICG light interaction. We established a method to investigate the light-induced change of ICG using HPLC-DAD technology and mass spectrometry. In a second step, porcine RPE cells were incubated with laser-treated ICG solution to investigate the toxicity of ICG decomposition products.

Materials and Methods

Chemicals

Indocyanine green (ICG, CAS-No. 3599-32-4) is a tricarbocyanine dye purchased from Pulsion Medical Systems (Munich, Germany). The chemical name for ICG is 1H-Benz[e]indolium,2-{7[1,3-dihydro-1,1-dimethyl-3-(-4-sulfobutyl)-2H-benz[e]indo-2-ylidene]-1,3,5-heptatrienyl}-1,1-dimethyl-3-(4-sulfobutyl)-, hydroxide, innersalt, sodium. ICG was dissolved in water (Milli-Q_{185}; Millipore-quality, Eschborn, Germany)/acetonitrile (1:1) (HPLC-Ultra-Gradient-Grade; Mallinckrodt Baker, Griesheim, Germany) with a concentration of 0.054 mg/mL before irradiation. Solution (200 μL) was filled into a precision cell made of special optical glass (type 100-OS; Hellma Optik, Jena, Germany). For cell culture experiments, ICG was dissolved in glucose 5% corresponding to the standard iso-osmolar preparation for the use in ophthalmology. Except for sample preparation and irradiation, the solutions were kept in the dark and analyzed.

Laser Irradiation

Irradiation of the ICG solution was performed using a diode laser emitting light at 810 nm (Asclepion-Meditec, Jena, Germany), for a total light energy of 50 J. The energy was delivered in 20 pulses of 10-ms duration and a radiant exposure of 20 J/cm^2 each. Because the pulse repetition rate was 1 Hz, the irradiation time was finished after 20 seconds, and the total radiant exposure was 400 J/cm^2.

Deterioration Studies

After laser irradiation, the samples were stored in the dark at room temperature and analyzed 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, and 23 hours after laser treatment.

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Exposure to Surgical Endolight

ICG was dissolved in water at a concentration of 0.1 mg/mL and was filled in a quartz cuvette. A xenon light source was used for irradiation (Hexon Illumination System, 1266 XII; Dutch Ophthalmic USA, Kingston, NH). The fiber optic was fixed 1 cm in front of the cuvette, and the light intensity was 5 mW/cm^2 as measured by a power meter (Nova OPHIR, 3A-SH; Optilas, Munich, Germany). The cuvette was irradiated for either 5 or 110 minutes. HPLC measurements were accomplished 1 hour after irradiation.

Deterioration Studies during Solar Light Exposure

ICG was dissolved in water/acetonitrile (1:1) with a concentration of 0.1 mg/mL. Three samples were stored at different conditions: one sample was incubated at 4°C in darkness, the second sample was stored at room temperature in the dark, and the third sample was exposed to solar light at room temperature. HPLC measurements were accomplished after 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, and 14 days. We measured radiant exposure using a power meter (Nova OPHIR, 3A-SH; Optilas) sensitive in the range from 100 to 1200 nm and yielding a light dose up to 210 J/cm^2.

Thermal Studies

One milliliter ICG solution (0.054 mg/mL water/acetonitrile [1:1]) was filled into glass ampoules and kept in an oil bath at temperatures of 21°C, 40°C, 60°C, 70°C, 100°C, 120°C, 140°C, 160°C, 180°C, 200°C, and 220°C for 5 minutes. Finally, the samples were cooled down in an ice bath, filtered using a PTFE-filter, and kept in the dark at 4°C.

Effect of Sodium Azide on Laser Decomposition

A solution of sodium azide was prepared in water/acetonitrile (1:1; 50 mM), and ICG was dissolved with a concentration of 0.054 mg/mL. Laser irradiation was performed as described.

Laser Treatment in Different Solvents

ICG was dissolved in dimethyl sulfoxide (DMSO), water, and blood plasma with the concentration of 0.054 mg/mL. The solutions were irradiated by laser light with the standard laser settings analyzed immediately after irradiation.

High-Performance Liquid Chromatography Analysis

Fifty microliters of each ICG probe was mixed with 50 μL naphthalene (0.03 mg/mL water/acetonitrile [1:1]) as internal standard (ISTD). The solutions were filtered (PTFE-filter), and 6 μL of each probe was analyzed using an HPLC system (HP1050 Quaternary Pump 79852AX, HP1050 Autosampler 79855A, HP 4-Channel-Online-Degasser G1303AX, Agilent 1100 Column Thermostat G1316A, and Agilent 1100 PDA Detector G1315B; Agilent Technologies, Waldbronn, Germany). Integration software (ChemStation, Rev. A.08.03; Agilent Technologies, Palo Alto, CA) was used for data analysis. Analyses were performed at 40°C on a column (ODS; 250 × 4.0 mm ID, 5-μm particle size; Thermo Hypersil Keystone, Bellefonte, PA). Gradient elution was performed with acetic acid/ammonium acetate puffer at pH 5 (solvent A) and acetonitrile (solvent B) at a constant flow rate of 1.5 mL/min. A gradient profile with the following proportions of solvent B was applied ([t (min), %B]: (0,30), (7,75), (8,75), (10,30), (17,30)). The chromatograms were monitored at 290 nm.

Quantitative Analysis

The concentration of ICG in the solutions was determined by the method of internal standard. For the compound (i), the calibration factor (CF_i) was determined in a calibration run (single-level calibration).

$$CF_i = \frac{f_{Tr}}{f_i} = \frac{m_i^K \cdot a_{Tr}^K}{m_{Tr}^K \cdot a_i^K}$$

where f_{Tr} is the response-factor of the internal standard (ISTD), m_i^K is the mass of compound i in solution k, and m_{Tr}^K is the mass of ISTD in solution k. a_{Tr}^K is the area of ISTD in solution k, and a_i^K is the area of compound i in solution k.

LC/MS Online Coupling

HPLC separation coupled with mass spectrometry was performed with a column phase that differed from the conditions used in the analytical studies. The solid phase was a chromatography column (Xterra MS C18; 150 × 2.1 mm, 3.5 μm; Waters, Milford, MA). Mass spectra of the laser-induced decomposition products were acquired on a triple-stage mass spectrometer (TSQ 7000; Thermoquest Finnigan, Toronto, ON, Canada) equipped with a positive electrospray ionization (+ESI) interface.

Cell Viability

RPE cells were harvested from fresh porcine eyes, proliferated to passage 3 in culture flasks (Roux Paris F; Dunn Labortechnik, Asbach, Germany) under standard conditions (DMEM [Gibco, Karlsruhe, Germany] plus FCS 15% [Biochrom, Berlin, Germany], penicillin/streptomycin 1% [Life Technologies, Karlsruhe, Germany], and CO_2 incubator), and seeded in 12-well plates (Corning Costar, Bodenheim, Germany), 25,000 cells per well. ICG was dissolved in glucose 5% at a concentration of 0.025% and irradiated by laser light (810 nm, 50 J) that produced the decomposition products. Then RPE cells were incubated with this decomposed ICG for 60 minutes, rinsed twice with fresh medium, and cultivated for another 4 days before the viability was tested. An equivalent number of RPE cells was kept without ICG (control) or was incubated with untreated ICG for 60 minutes in the dark. Cell viability was assessed by trypan blue (TB) exclusion. Total cell number of viable cells was calculated directly as mean values of viable (unstained) cells using the Neubauer chamber. Each experiment was repeated five times.

Results

Laser Light-Induced Decomposition of ICG

Laser irradiation of the ICG solution (0.054 mg/mL) produced the peak pattern shown in the HPLC chromatogram (see Fig. 2b). In addition to the peak of ICG molecules, the chromatogram shows six additional peaks between retention time (RT) 2 minutes and 4 minutes. Using LC/MS online coupling, the respective mass of the single peaks could be determined (Table 1). Calculation of the molecular weight was not achieved by the exact molecular atomic units but by summation of the empirically obtained data from the mass spectra. Therefore, the values show an experimental accuracy of approximately 0.1%.

Table 1. Mass to Charge Ratio and Molecular Weight of Single Peaks as Determined by LC/MS Online Coupling

Peak	Mass to Charge Ratio (m/z)	Molecular Weight (MW)
1	412.0	411.0
2	373.9	372.9
3	364.9	363.9
4	438.0	437.0
5	438.0	437.0
6	785.5	783.5
7	753.4	752.4

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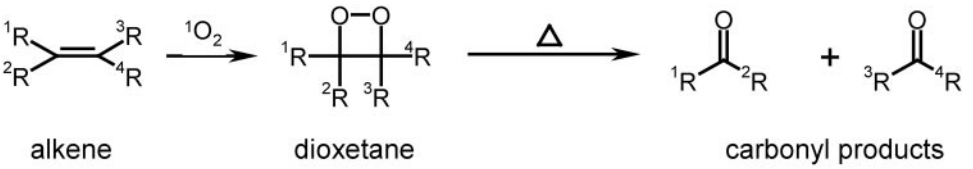

FIGURE 1. Postulated decomposition mechanism of cyanine dyes. A general structure of an alkene (double bond in a polymethine chain) is shown, with ^{1}R to ^{4}R representing different functional groups.

ICG eluted at RT 5.7 minutes (peak 7) with a mass-to-charge ratio (m/z) of 753.4. Given that the detection was accomplished by +ESI, the molecular weight of the uncharged molecule was 752.4 Da. ICG itself is a negatively charged ion with a respective molecular weight of 751.4 Da (see Fig. 2).

For peak 6 (RT 3.7 minutes), the respective mass of 784.5 Da could be obtained for the uncharged molecule. The difference between ICG (752.4 Da for the uncharged molecule) and this molecule was 32 Da, indicating a reaction of ICG with oxygen. Peak 6 can be a dioxetane derived from ICG.[34] It is also negatively charged and shows a mass of 783.5 Da. Because the position of the addition of singlet oxygen was unknown, no possible structure for the dioxetane is shown. Mass spectra of the other four products can be separated into two groups. The first group shows peaks with m/z = 412.0 (peak 1, RT 2.5 minutes) and m/z = 373.9 (peak 2, RT 2.6 minutes). Resultant molecular weights for the uncharged products were 411.0 Da for product 1 and 371.9 Da for product 2. Addition of both these values resulted in the mass of 782.9 Da, the weight of the uncharged product 6. That is a further hint of the reaction mechanism of the polymethine chain with singlet oxygen by dioxetane and finally thermal decomposition into carbonyl compounds (Fig. 1, scheme).

The second group shows peaks with m/z = 348.0 (peak 3, RT 2.8 minutes) and m/z = 438.0 (peak 4, RT 2.9 minutes; peak 5, RT 3.1 minutes). Masses of the uncharged molecules were 346.0 Da (product 3) and 437.0 d (products 4 and 5). The summation also resulted in 783 Da, the mass of product 6. Hence, these compounds were carbonyl products resulting from dioxetane generated by the reaction of singlet oxygen with ICG, but at a position other than that for the generation of products 1 and 2. Finally, this dioxetane was also thermally fragmented. Figure 2b shows the structures for these compounds, whereas products 4 and 5 may be isomers.

To check for the stability of the laser-irradiated solution and to detect any alterations in the peak pattern, a solution of ICG exposed to laser light (standard laser settings) was incubated in darkness at room temperature up to 23 hours. Within 23 hours, product 6 decreased strongly, a further indication of a thermally instable dioxetane (data not shown). In addition, products 1 and 4 decreased with increasing time, whereas products 2, 3, and 5 increased within 23 hours. During the deterioration studies, no other decomposition products were detected, and the area under the ICG peak did not change. After laser treatment, the remaining ICG was stabile and did not further de-

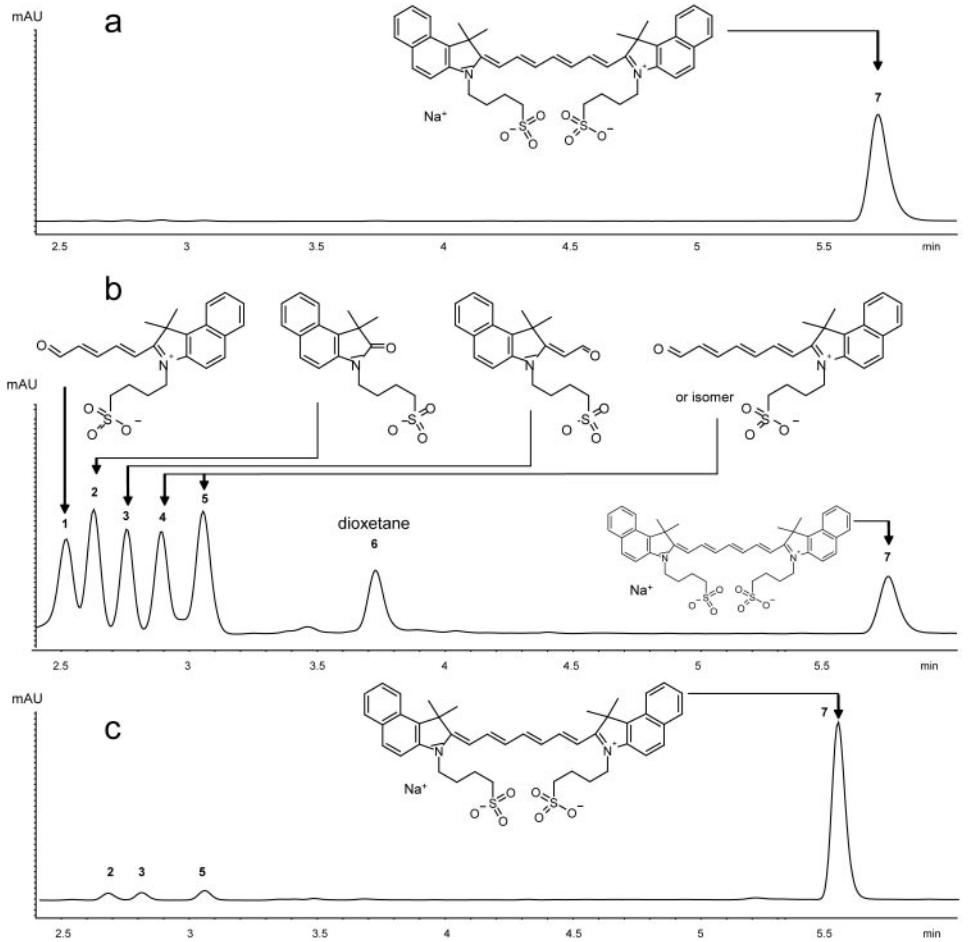

FIGURE 2. Standard HPLC chromatogram of an ICG solution [0.054 mg/mL water/acetonitrile (1:1)] without laser treatment (**a**), laser-treated ICG solution [0.054 mg/mL water/acetonitrile (1:1)] (**b**), and laser-treated solution containing ICG [0.054 mg/mL water/acetonitrile (1:1)] and sodium azide [50 mM]. The chemical structure of ICG (peak 7) and the decomposition products (peaks 1–5) are shown. During laser treatment ICG decomposed, resulting in a dioxetane as intermediate product (peak 6) and carbonyl products as decomposition products (peaks 1–5). By adding sodium azide to the solution of ICG only products 2, 3, and 5 are generated in much lower quantity.

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compose. Thus, decomposition of the dye strongly depended on light.

Effect of Sodium Azide on the Laser-Induced Decomposition of ICG

The azide anion is a strong physical quencher of singlet oxygen. It is often used for the proof of singlet oxygen in oxidation processes.[35] In the presence of sodium azide (50 mM), the laser-induced decomposition of ICG diminished significantly. Only products 2, 3, and 5 could be detected with much smaller areas under the HPLC peak (Fig. 2c). During irradiation, and in contrast to laser irradiation of ICG without quencher, the generation rates of decomposition products were reduced by 82% (peak 2), 75% (peak 3), and 77% (peak 5) in the presence of sodium azide. These results confirm the impact of singlet oxygen for the light-induced decomposition of ICG.

Solar Light-Induced Decomposition of ICG

The laser light used for irradiation matches the absorption maximum of ICG at approximately 800 nm. To check whether polychromatic incoherent light such as solar radiation can induce comparable decomposition products, fresh ICG solution was exposed to ambient solar light for 14 days. One sample was incubated in darkness at 4°C, another was incubated in darkness at room temperature, and a third sample was exposed to solar light at room temperature. After certain intervals, the samples were analyzed by HPLC (Fig. 3). The color of ICG samples stored in darkness at 4°C was still dark green, and the chromatogram showed only the ICG peak (data not shown). In addition, the sample incubated at room temperature in darkness did not show any additional peaks and maintained the green color. The solution stored at room temperature in solar light showed several alterations in the peak pattern, and the color changed from green to yellow. After 14 days of incubation, the chromatogram revealed several decomposition products, and the ICG peak in HPLC disappeared. These results reveal the photosensitivity of ICG and show that it is absolutely required for the storing of untreated and treated solutions in darkness at 4°C.

Exposure to Surgical Endolight

The exposure of ICG to the surgical endolight yielded the same products compared with laser light (data not shown). After 5 minutes of irradiation, the peaks of light-induced decomposition products (peaks 1–5) were small but above the noise level. After 110 minutes of irradiation, peaks 1 to 5 were considerably higher. Decomposition products were exactly the same as they were with laser or solar irradiation.

Heat-Induced Decomposition of ICG

Photothermal effects can damage cells by an increase in intracellular temperature, shown by the use of ICG for photocoagulation or tissue welding.[36,37] Most of the light energy absorbed by the ICG molecule is converted to heat by internal conversion,[38] which could lead to two different decomposition mechanisms. First, the decomposition of ICG may occur because of light absorption and the generation of singlet oxygen in the excited state of the molecule. Second, ICG is cleaved by heat energy generated by other light-absorbing ICG molecules. To elucidate these mechanisms, ICG solutions were exposed to different temperature levels in the dark. Temperatures higher than 180°C resulted in several decomposition products, and its peak areas increased with increasing temperature. At the same time, the concentration of ICG decreased (data not shown).

ICG solution irradiated by laser light or heated to 220°C showed a different peak pattern (data not shown). Only compounds 2 and 3 could be detected in the heated sample. The other compounds were different from those detected in the laser-treated solutions with regard to retention time and UV spectra, confirming two distinct mechanisms in case ICG was exposed to light or heat. However, the heating effects in the ICG molecules should have played a minor role because the addition of sodium azide (singlet oxygen quencher) clearly arrested the decomposition under light exposure.

Laser-Induced Decomposition of ICG in Different Solvents

To study the influence of the solvent on the decomposition pattern of ICG, solutions of ICG were prepared in DMSO, water, and blood plasma as physiological medium (0.054 mg/mL). Each experiment was performed in duplicate.

With the use of the solvent DMSO, all products except product 1 could be verified. In water, all six compounds could be detected. Only the amount of induced decomposition products was lower than in solvent water/acetonitrile (1:1). In

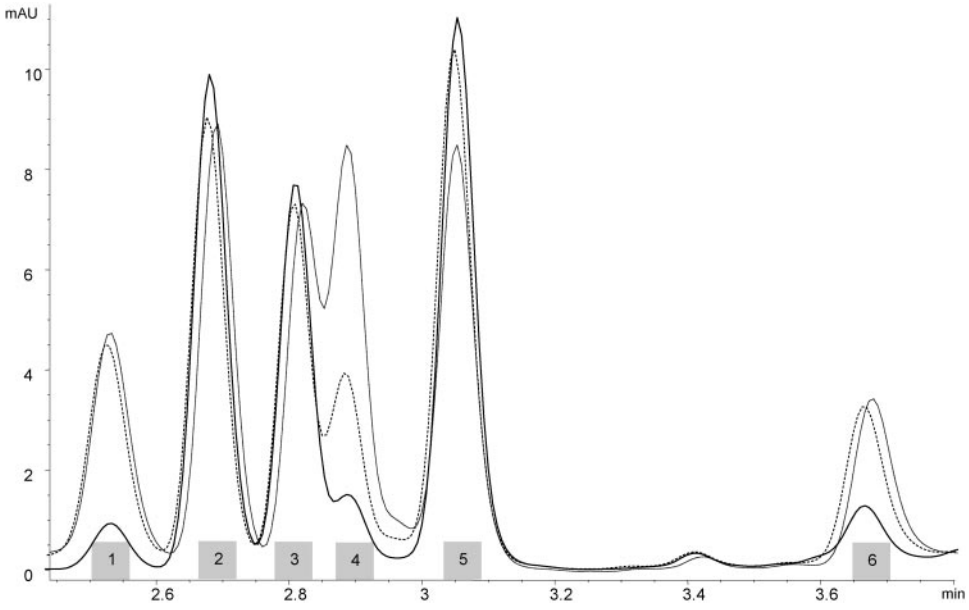

FIGURE 3. Deterioration study of laser-treated sample. At different time points after laser treatment, the content of the ICG solution was analyzed by HPLC. The *blue line* shows the chromatogram after 0.5 hours, the *red line* after 2.5 hours, and the *green line* after 23 hours. Areas of peaks 1, 4, and 6 decreased with increasing deterioration of the laser-treated solution, whereas areas under the curve of peaks 2, 3, and 5 increased.

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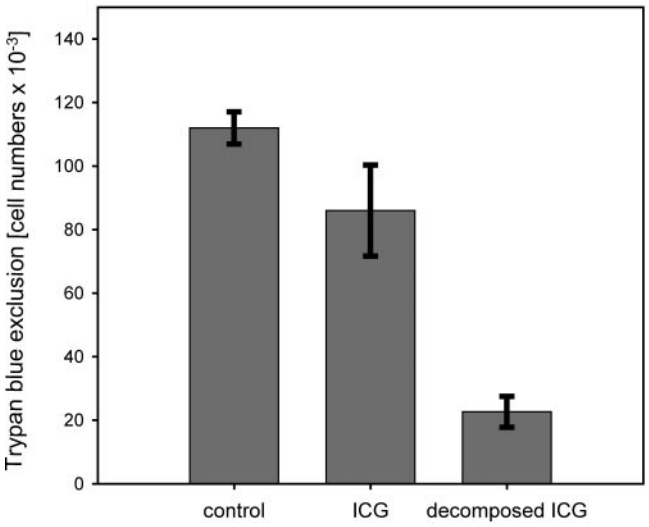

FIGURE 4. The number of RPE cells after incubation with untreated ICG and decomposed ICG using trypan blue exclusion assay. Cells without ICG served as control.

blood plasma, only products 2 and 3 could be detected in low amounts (data not shown). The decomposition products of ICG are reactive species that may quickly react with other molecules such as proteins in the blood plasma. This would lead to a rapid decrease of the concentration of these compounds in the solution. Moreover, plasma proteins such as sodium azide are potent quenchers and may prevent ICG from decomposition by singlet oxygen.

In Vitro Experiments

Using the TB exclusion assay, the number of unstained cells was $112 \pm 5 \times 10^3$ ($n = 5$) without ICG (control). The number of TB-unstained cells decreased to $86 \pm 14 \times 10^3$ ($n = 5$) when the cells had been incubated with untreated ICG for 60 minutes The number of TB-unstained cells decreased to $23 \pm 5 \times 10^3$ ($n = 5$) when the cells had been incubated with decomposed ICG for 60 minutes (Fig. 4).

DISCUSSION

In our previous work, we have shown that ICG kills cells in vitro in a photodynamic reaction.[32,33] Cell killing was inhibited by the addition of sodium azide (quencher of singlet oxygen) but was intensified by the addition of deuterium oxide (D_2O). The use of both sodium azide and D_2O is part of the usual procedure to prove photodynamic action of a photosensitizer that kills cells by oxidative reactions of singlet oxygen.[39] However, direct proof of singlet oxygen by the detection of its luminescence at 1270 nm[40,41] failed in ICG solutions (data not shown). Moreover, it is well known that ICG is chemically fairly unstable once it is dissolved in any solvent, particularly when ICG solutions are exposed to light.[38] The exact mechanism of ICG light interaction was unclear thus far.

Other cyanine dyes are known to decompose on light exposure by self-sensitized photograph oxidation.[42] The decomposition involves singlet oxygen that reacts with double bonds of the polymethine chain under [2+2]-cycloaddition resulting in dioxetanes.[34] These molecules tend to fragment thermally at room temperature, yielding two carbonyl products in which one product is electronically excited (Fig. 1).[43] Based on this knowledge, we investigated the light-induced change of ICG molecules in different experimental setups using chemical analysis.

The light irradiation of ICG yielded the main finding of the present study. ICG in fact produces singlet oxygen under irradiation, but singlet oxygen predominantly oxidizes the ICG molecule itself, causing its decomposition by dioxetane reaction. Here ICG is decomposed when located inside or close to cells; the decomposition product compounds can affect cellular integrity. Therefore, we incubated RPE cells with untreated ICG or decomposed ICG for 60 minutes. The results clearly showed that decomposed ICG decreased cell viability. That is, ICG produces singlet oxygen, ICG is decomposed, and the decomposition products of ICG kill the cells (Fig. 5).

The mechanism of ICG decomposition by light is of clinical relevance mainly because of the widespread use of ICG in macular surgery. ICG toxicity is expected to depend on the concentration of the ICG solution,[22,23] its osmolarity,[26] the time span before ICG is removed from the eye,[27] whether ICG is injected into an air-filled or a fluid-filled eye (with less toxicity in fluid-filled eyes),[29] and on the emission spectrum and the energy of the surgical light source.[32,33] Gandorfer et al.[30] found severe damage of the inner retina of human donor eyes after exposure to ICG and exposure to light from a standard surgical light pipe, whereas Grisanti et al.[31] did not find any disruption of the retinal cytoarchitecture in fresh porcine eyes under otherwise similar experimental conditions. We have previously undertaken experimental safety testing of

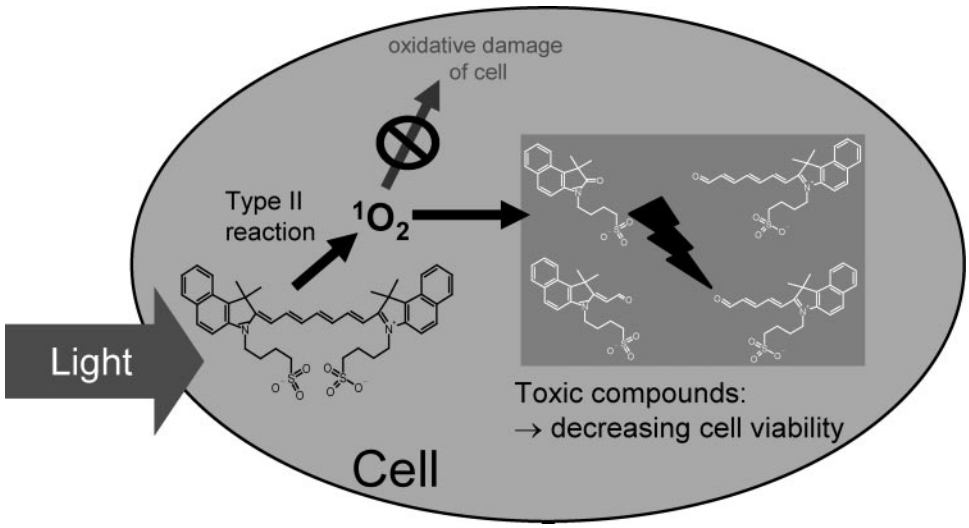

FIGURE 5. Scheme to illustrate the mechanisms of action when RPE or other cells are killed by ICG photodynamically. After light activation, ICG produced singlet oxygen, which did not directly kill cells by oxidative damage of cellular components. ICG was decomposed by singlet oxygen itself, and the decomposition products decreased cell viability.

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ICG using RPE and glial cell cultures[23] and using an ex vivo porcine organ culture retina model.[44] In the latter study, we mimicked macular surgery by applying ICG solutions of different concentrations to the retinal surface and rinsed the surface 1 minute or 3 minutes after application. We then examined the retina for the presence of signs of necrosis and apoptosis. We found that in accordance with our clinical studies,[15,16] a brief application of a low concentrated (0.1%) ICG solution may be safe.[44] However, both experimental studies showed that the safety margin is narrow.[23,44] Furthermore, clinical observations have shown that ICG fluorescence persists after ICG-assisted epiretinal membrane surgery,[45,46] even within the area of internal limiting membrane removal.[45] Fundus fluorescence from residual ICG has been detected for up to 9 months after epiretinal membrane surgery.[46] It is possible that the intraretinal persistence of ICG may cause a low-grade accumulative toxic effect. The results of these investigations indicate that ICG should be used cautiously. Further work aimed at a reduction of its toxicity or the exploration of other, potentially nontoxic, dyes would be of clinical relevance.

In summary, the results of the present study show that RPE cells are not predominantly killed because of singlet oxygen oxidation of cellular constituents but because of the toxicity of the ICG decomposition products. The latter are produced by light absorption of ICG under photodynamic conditions (Fig. 5). ICG light absorption is maximal at approximately 800 nm and decreases for shorter wavelengths. When exposing the eye to solar light, the light is spectrally filtered by the cornea and the lens; however, both are transparent for wavelengths less than 700 nm. Especially from 600 to 700 nm, there is still sufficient light absorption in the ICG molecule. Therefore, once a photon is absorbed by ICG, singlet oxygen can be generated and can decompose the ICG molecule. The spectrally filtering of solar light by cornea and lens could reduce the effect, but the mechanisms of action and the nature of the decomposition products are not affected. This is confirmed by the fact that the decomposition mechanisms and the light-induced products of ICG were independent of the light source used.

The results of this study elucidate the mechanism of light-induced decomposition of ICG and demonstrate that ICG decomposition is cytotoxic without quantifying toxicity. We know from our previous experimental work that the safety margin is narrow.[44] Therefore, careless or repeated intraoperative use of ICG should be avoided. The question whether low concentrations of ICG with short intraoperative exposure times can cause clinically detectable damage to retinal function cannot be answered by the present study. Precise safety thresholds for the intraocular application of ICG taking into consideration the different retinal cell types, ICG concentration, ICG exposure time, parameters of the surgical light source, and postoperative exposure to ambient light have yet to be determined. However, the results of the present study provide an understanding of the mechanism of ICG toxicity and thus may lead to ways to reduce ICG-related cytotoxicity, perhaps by adding quenchers of singlet oxygen.

References

1. Fox I, Brooker G, Heseltine D, Essex H, Wood E. New dyes for continuous recording of dilution curves in whole blood independent of variations in blood oxygen saturation. *Am J Physiol*. 1956; 187:599.
2. Flower R, Hochheimer B. Indocyanine green dye fluorescence and infrared absorption choroidal angiography performed simultaneously with fluorescein angiography. *Johns Hopkins Med J*. 1976; 138:33–42.
3. Moneta G, Brülisauer M, Jäger K, Bollinger A. Infrared fluorescence videomicroscopy of skin capillaries with indocyanine green. *Int J Microcirculation Clin Exp* 1987;6:25–34.
4. Hope-Ross M, Yannuzzi L, Gragoudas E, et al. Adverse reactions due to indocyanine green. *Ophthalmology*. 1994;101:529–533.
5. Paumgartner G, Probst P, Kraines R, Leevy C. Kinetics of indocyanine green removal from the blood. *Ann N Y Acad Sci*. 1970;170: 134–170.
6. Haritoglou C, Gandorfer A, Gass CA, Schaumberger M, Ulbig MW, Kampik A. The effect of indocyanine-green on functional outcome of macular pucker surgery. *Am J Ophthalmol*. 2003;135:328–337.
7. Sorcinelli R. Surgical management of epiretinal membrane with indocyanine-green-assisted peeling. *Ophthalmologica*. 2003;217: 107–110.
8. Stalmans P, Parys-Vanginderdeuren R, De Vos R, et al. ICG staining of the inner limiting membrane facilitates its removal during surgery for macular holes and puckers. *Bull Soc Belge Ophtalmol*. 2001;281:21–26.
9. Da Mata AP, Burk SE, Riemann CD, et al. Indocyanine green-assisted peeling of the retinal internal limiting membrane during vitrectomy surgery for macular hole repair. *Ophthalmology*. 2001; 108:1187–1192.
10. Kadonosono K, Itoh N, Uchio E, Nakamura S, Ohno S. Staining of internal limiting membrane in macular hole surgery. *Arch Ophthalmol*. 2000;118:1116–1118.
11. Kwok AK, Li WW, Pang CP, et al. Indocyanine green staining and removal of internal limiting membrane in macular hole surgery: histology and outcome. *Am J Ophthalmol*. 2001;132:178–183.
12. Weinberger AW, Schlossmacher B, Dahlke C, Hermel M, Kirchhof B, Schrage NF. Indocyanine-green-assisted internal limiting membrane peeling in macular hole surgery–a follow-up study. *Graefe's Arch Clin Exp Ophthalmol*. 2002;240:913–917.
13. Wolf S, Reichel MB, Wiedemann P. Clinical findings in macular hole surgery with indocyanine-green assisted peeling of the internal limiting membrane. *Graefe's Arch Clin Exp Ophthalmol*. 2003; 241:589–592.
14. Kwok AK, Lai TY, Man-Chan W. Indocyanine-green assisted retinal internal limiting membrane removal in stage 3 or 4 macular hole surgery. *Br J Ophthalmol*. 2003;87:71–74.
15. Hillenkamp J, Saikia P, Gora F, et al. Macular function and morphology after peeling of idiopathic epiretinal membrane with and without the assistance of indocyanine green. *Br J Ophthalmol*. 2005;89:437–443.
16. Hillenkamp J, Saikia P, Herrmann WA, Framme C, Gabel VP, Sachs HG. Surgical removal of idiopathic epiretinal membrane with or without the assistance of indocyanine green: a randomised controlled clinical trial. *Graefes Arch Clin Exp Ophthalmol*. 2007;245: 973–979.
17. Haritoglou C, Gandorfer A, Gass CA, Schaumberger M, Ulbig MW, Kampik A. Indocyanine green-assisted peeling of the internal limiting membrane in macular hole surgery affects visual outcome: a clinicopathologic correlation. *Am J Ophthalmol*. 2002;134:836–841.
18. Ando F, Sasano K, Ohba N, Hirose H, Yasui O. Anatomic and visual outcomes after indocyanine green-assisted peeling of the retinal internal limiting membrane in idiopathic macular hole surgery. *Am J Ophthalmol*. 2004;137:609–614.
19. Uemura A, Kanda S, Sakamoto Y, Kita H. Visual field defects after uneventful vitrectomy for epiretinal membrane with indocyanine green-assisted internal limiting membrane peeling. *Am J Ophthalmol*. 2003;136:252–257.
20. Jackson TL. Indocyanine green accused. *Br J Ophthalmol*. 2005; 89:395–396.
21. Da Mata AP, Riemann CD, Nehemy MB, Foster RE, Petersen MR, Burk SE. Indocyanine green-assisted internal limiting membrane peeling for macular holes to stain or not to stain? *Retina*. 2005; 25:395–404.
22. Iriyama A, Uchida S, Yanagi Y, et al. Effects of indocyanine green on retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2004;45:943–947.
23. Jackson TL, Hillenkamp J, Knight BC, et al. Safety testing of indocyanine green and trypan blue using retinal pigment epithelium and glial cell cultures. *Invest Ophthalmol Vis Sci*. 2004;45:2778–2785.

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24. Ikagawa H, Yoneda M, Iwaki M, et al. Chemical toxicity of indocyanine green damages retinal pigment epithelium. *Invest Ophthalmol Vis Sci*. 2005;46:2531–2539.
25. Rezai KA, Farrokh-Siar L, Ernest JT, van Seventer GA. Indocyanine green induces apoptosis in human retinal pigment epithelial cells. *Am J Ophthalmol*. 2004;137:931–933.
26. Stalmans P, Van Aken EH, Veckeneer M, Feron EJ, Stalmans I. Toxic effect of indocyanine green on retinal pigment epithelium related to osmotic effects of the solvent. *Am J Ophthalmol*. 2002; 134:282–285.
27. Enaida H, Sakamoto T, Hisatomi T, Goto Y, Ishibashi T. Morphological and functional damage of the retina caused by intravitreous indocyanine green in rat eyes. *Graefes Arch Clin Exp Ophthalmol*. 2002;240:209–213.
28. Czajka MP, McCuen BW 2nd, Cummings TJ, Nguyen H, Stinnett S, Wong F. Effects of indocyanine green on the retina and retinal pigment epithelium in a porcine model of retinal hole. *Retina*. 2004;24:275–282.
29. Maia M, Kellner L, de Juan E Jr, et al. Effects of indocyanine green injection on the retinal surface and into the subretinal space in rabbits. *Retina*. 2004;24:80–91.
30. Gandorfer A, Haritoglou C, Gandorfer A, Kampik A. Retinal damage from indocyanine green in experimental macular surgery. *Invest Ophthalmol Vis Sci*. 2003;44:316–323.
31. Grisanti S, Szurman P, Gelisken F, Aisenbrey S, Oficjalska-Mlynczak J, Bartz-Schmidt KU. Histological findings in experimental macular surgery with indocyanine green. *Invest Ophthalmol Vis Sci*. 2004; 45:282–286.
32. Fickweiler S, Szeimies R-M, Baumler W, et al. Indocyanine green: intracellular uptake and phototherapeutic effects in vitro. *J Photochem Photobiol B Biol*. 1997;38:178–183.
33. Bäumler W, Abels C, Karrer S, et al. Photo-oxidative killing of human colonic cancer cells using indocyanine green and infrared light. *Br J Cancer*. 1999;80:360–363.
34. Klessinger W, Michl J. *Lichtabsorption und Photochemie organischer Moleküle*. Weinheim: VCH; 1989.
35. Li MY, Cline CS, Koker EB, Carmichael HH, Chignell CF, Bilski P. Quenching of singlet molecular oxygen ($1O_2$) by azide anion in solvent mixtures. *Photochem Photobiol*. 2001;74:760–764.
36. DeCoste SD, Farinelli W, Flotte T, Anderson RR. Dye-enhanced laser welding for skin closure. *Lasers Surg Med*. 1992;12:25–32.
37. Reichel E, Puliafito CA, Duker JS, Guyer DR. Indocyanine green dye-enhanced diode laser photocoagulation of poorly defined subfoveal choroidal neovascularization. *Ophthalmic Surg*. 1994;25: 195–201.
38. Holzer W, Mauerer M, Penzkofer A, et al. Photostability and thermal stability of indocyanine green. *J Photochem Photobiol B*. 1998;47:155–164.
39. Runnels JM, Chen N, Ortel B, Kato D, Hasan T. BPD-MA-mediated photosensitization in vitro and in vivo: cellular adhesion and beta1 integrin expression in ovarian cancer cells. *Br J Cancer*. 1999;80: 946–953.
40. Maisch T, Baier J, Franz B, et al. The role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria. *Proc Natl Acad Sci U S A*. 2007;104:7223–7228.
41. Baier J, Maisch T, Maier M, Landthaler M, Baumler W. Direct detection of singlet oxygen generated by UVA irradiation in human cells and skin. *J Invest Dermatol* 2007.
42. Chen P, Li J, Quain Z, Zheng D, Okasaki T, Hayami M. Study on the photooxidation of a near-infrared-absorbing benzothiazolone cyanine dye. *Dyes Pigments*. 1998;37:213–222.
43. Saha-Moller CR, Adam W. *Four-Membered Rings with Two Oxygen Atoms*. Oxford: Elsevier; 1996:1041–1082.
44. Saikia P, Maisch T, Kobuch K, et al. Safety testing of idocyanine green in an ex vivo porcine retina model. *Invest Ophthalmol Vis Sci*. 2006;47:4998–5003.
45. Ashikari M, Ozeki H, Tomida K, Sakurai E, Tamai K, Ogura Y. Retention of dye after indocyanine green-assisted internal limiting membrane peeling. *Am J Ophthalmol*. 2003;136:172–174.
46. Tadayoni R, Paques M, Girmens JF, Massin P, Gaudric A. Persistence of fundus fluorescence after use of indocyanine green for macular surgery. *Ophthalmology*. 2003;110:604–608.

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