Photo-oxidation from Mode-locked Laser Exposure to hTERT-RPE1 Cells ‡

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ABSTRACT

Human retinal pigment epithelial (RPE) cells (hTERT-RPE1) were used to detect photo-oxidation products generated from chronic NIR (810 nm) laser exposure. Exposure of a discrete area within cell monolayers provided a means of distinguishing fluorescence above background levels. Oxidative stress was detected using the fluorescent dye H_2DCF-DA and its analog CM-H_2DCF-DA. Fluorescence was detected in cells exposed to mode-locked (76 MHz, ~160 femtoseconds) but not CW laser exposure. Detection of photo-oxidation from the mode-locked laser was dependent upon radiant exposure, but only if irradiance was greater than a threshold value. The CM-H_2DCF-DA dye proved a more sensitive indicator of oxidation than H_2DCF-DA, and the radiant exposure threshold for detection was dependent upon dye concentration. No oxidation was detected from CW exposures (using the most sensitive fluorescent dye conditions) when using 3 times the irradiance, and 10 times the radiant exposure needed to detect fluorescence from mode-locked exposure.

Keywords: photochemical oxidation, hTERT-RPE1 cells, mode-locked laser, fluorescence microscopy

INTRODUCTION

Retinal pigment epithelial (RPE) cells perform many functions in the eye. With a dense layer of intracellular melanosomes, the RPE act as a backstop for all light transmitted to the retina. They serve as a barrier between the neural retina and the blood supply of the choriocapillaris, filtering substrates and products of metabolism for the neural retinal cells. By nature of their phagocytic functions the RPE cells chemically recycle the membrane and vision pigment components of the rod and cone outer segments. These *in vivo* functions subject the RPE cells to chronic metabolic and oxidative stress. Through a combination of *in vivo* analysis and *in vitro* studies, knowledge of how this combination of factors leads to increased amounts of intracellular reactive oxygen intermediates (ROI), and how these compounds may lead to pathology,[1] is becoming increasingly clear.

Various *in vitro* models for studying oxidative stress in RPE cells have been established, utilizing either high doses of chemical oxidants,[2-5] chronic mild hyperoxia,[6] or blue-light illumination.[7,8] For other cell types, there are indications that the damaging effects of violet/blue light is mediated by the production of hydrogen peroxide (H_2O_2), as a consequence of the photoreduction of flavins within oxidase enzymes.[9]

‡ Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the United States Air Force.

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We are interested in laser bioeffects. Applications of mode-locked lasers have been increasing of late. For instance, in the field of fluorescence microscopy mode-locked lasers are used to generate multiphoton absorption of fluorescent probes, providing several advantages over single-photon excitation.[10] Because mode-locked lasers generate beams with high peak-irradiances per pulse we have investigated their potential for causing damaging biological effects by comparison to CW lasers of the same average power. Our findings suggest that near-IR (NIR) mode-locked lasers do produce *in situ* production of photochemical oxidation in RPE cells, as detected by a fluorescent dye probe. We characterize the response of the cell line hTERT-RPE1 to laser exposure at 810 nm by measuring threshold irradiances and threshold radiant exposures for various concentrations (preloaded into the cells) of two dichlorofluorescein dyes.

METHODS AND MATERIALS

Cell Culture

All cell cultures were grown at 37°C in an atmosphere containing 5% CO_2. Stock cultures of the human RPE cell line hTERT-RPE1 (ClonTech) were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biochemicals) and were passaged at, or prior to confluence. At 2-9 days prior to exposure cells were plated into 48-well microtiter dishes (~90,000 cells/cm^2).

Fluorescent dyes (Molecular Probes, Portland, OR) used in this study were dichlorodihydrofluorescein diacetate (H_2DCFDA; cat # D-399) and its 5-(and-6)-chloromethyl- derivative CM-H_2DCFDA (cat # C-6827) for detection of oxidation, and the combination of calcein-AM (cat# C-3099) with ethidium homodimer-1 (EthD-1, cat# E-1169) for viability staining.

Laser exposure

CW laser exposures (0.25 sec) at 405 nm were carried out[12] using a Coherent INNOVA-100 krypton laser where irradiance ranged from 2400 W/cm^2 to 15 W/cm.^{2} A Coherent MIRA900F titanium:sapphire laser provided CW and mode-locked beams at 810 nm. The repetition rate and pulse duration for the mode-locked beam were 76 MHz and ~200 fsec, respectively. Maximum irradiance from the MIRA was 27,000 W/cm^2 for the mode-locked beam and 35,000 W/cm^2 for the CW beam.

All laser exposures were performed in 48-well plates. Exposure durations at 810 nm ranged from 2 to 600 sec and beam diameters were set at 70 µm. The CW 405 nm beam was 140 µm in diameter. The physical formation of the laser exposure was a centrally located concentric ring formed by 7 individual laser exposures, creating an overall exposure area (figure 1) with diameters of about 340 µm (405 nm) and 170 µm (810 nm). This provided a defined region of fluorescence above background if the laser-induced oxidation was above the minimum level of detection when observed by microscopy.

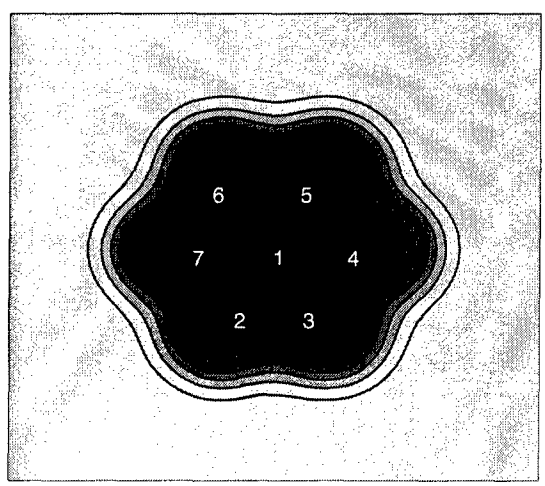

Figure 1. Pattern of individual laser exposures forming the overall exposure area. Beams were separated by a 1/e spacing distribution.

Just prior to exposing, cells in experimental and mock wells were washed twice with Buffer (phosphate buffered saline [PBS] or Hank's balanced salt solution [HBSS]), allowed to pre-load with a fluorescent oxidation-sensitive dye (diluted in buffer) for 20 min in the culture incubator, and finally rinsed with 1 ml

fresh buffer. Control (no treatment) wells were treated in the same fashion except their cells were not subjected to fluorescent dye. Exposures were carried out in 0.1 ml buffer. Exposed cells were viewed directly using fluorescence microscopy. Fluorescent dye was either H_2DCFDA or CM-H_2DCFDA at varying concentrations from 10 μM to 100 μM.

Microscopy

After laser exposure cells were visualized and imaged on an inverted phase-contrast microscope set up for fluorescence (Olympus CK-40F; Leeds Instruments, Inc, Irving, TX). Photography was done with a Hamamatsu ORCA-100 digital camera with the resulting images processed using Simple PCI (C-Imaging Systems). Fluorescence from both DCF-based dyes was detected using a bandpass exciter of 460-490 nm and a bandpass emitter of either 515-555 nm or 490-530 nm.

RESULTS

Detecting laser-induced oxidation in hTERT-RPE1

Using the oxidation-sensitive fluorescent probe dichlorodihydrofluorescein diacetate (H_2DCFDA) we detected oxidative stress *in situ* in cultured RPE cells exposed (0.25 sec) to a CW laser at 405 nm.[12] The use of small exposure areas (see Figure 1) provided graphic representation of where laser-induced oxidation took place, within the detection limits of fluorescence using our dye conditions, microscope, and attached CCD camera. If laser exposure produced oxidation (presumably H_2O_2) within the cells of these targets, the H_2DCF is converted to the excitable species (DCF) that is visualized by excitation with blue light from the microscope. Targets with significant DCF appeared immediately during fluorescence microscopy on a black background. For exposures (0.25 sec) at 405 nm, cells were first preloaded with 10 μM H_2DCFDA. Figure 2 shows a fluorescent "target" in the RPE monolayer corresponding to the laser exposed area. As further proof that the oxidation "target" is laser-induced, figure 2 also provides an image of "background" fluorescence due solely from blue light excitation at the microscope (Panel D). Threshold irradiance required to generate oxidized targets under these conditions (see ref. 12) was 38 W/cm^2 which correlated to 9.5 J/cm^2 delivered. Uncertainty was 8% for measuring both irradiance and radiant exposure.

Detecting NIR laser-induced oxidation in hTERT-RPE1

No definitive oxidation targets were generated from 810 nm exposures using the same conditions giving rise to the CW 405 nm oxidation results described above. By increasing both time of exposure and the concentration of dye during preloading of the cells we were able to detect oxidation products at 810 nm, but only for mode-locked beams. We again used a small exposure area to allow detection of specific laser-induced oxidation above background levels during fluorescence microscopy. Our analysis utilized data collected from several different experimental days. Prior to exposures each day, the laser was aligned to the center of wells to be exposed to facilitate quick identification of even faint regions of oxidation. Damage was scored as either a plus (initial fluorescence) or minus (no initial fluorescence) when observed on the fluorescent microscope. For scoring wells exposed to near threshold energies an experimentally "blind" approach was used. Figure 3 provides an example of an oxidation "target" generated by mode-locked NIR laser exposure (21,800 W/cm^2 for 100 sec) using 100 μM H_2DCFDA. In addition to the H_2DCFDA, we successfully detected oxidation targets using its 5-(and-6)-chloromethyl- derivative CM-H_2DCFDA.

Although the damage targets from NIR mode-locked laser exposure were genuine (fluorescence above background in the shape of the expected target) they were not as sharply demarked as those generated by the (positive control) 405 nm laser.

The sensitivity of our system was clearly dependent upon the choice of dye and its concentration applied to the cells (Table 1). Due to its cellular retention properties (see Handbook for Molecular Probes; Molecular Probes, Portland, OR), CM-H_2DCFDA provided a much more sensitive detection system than the

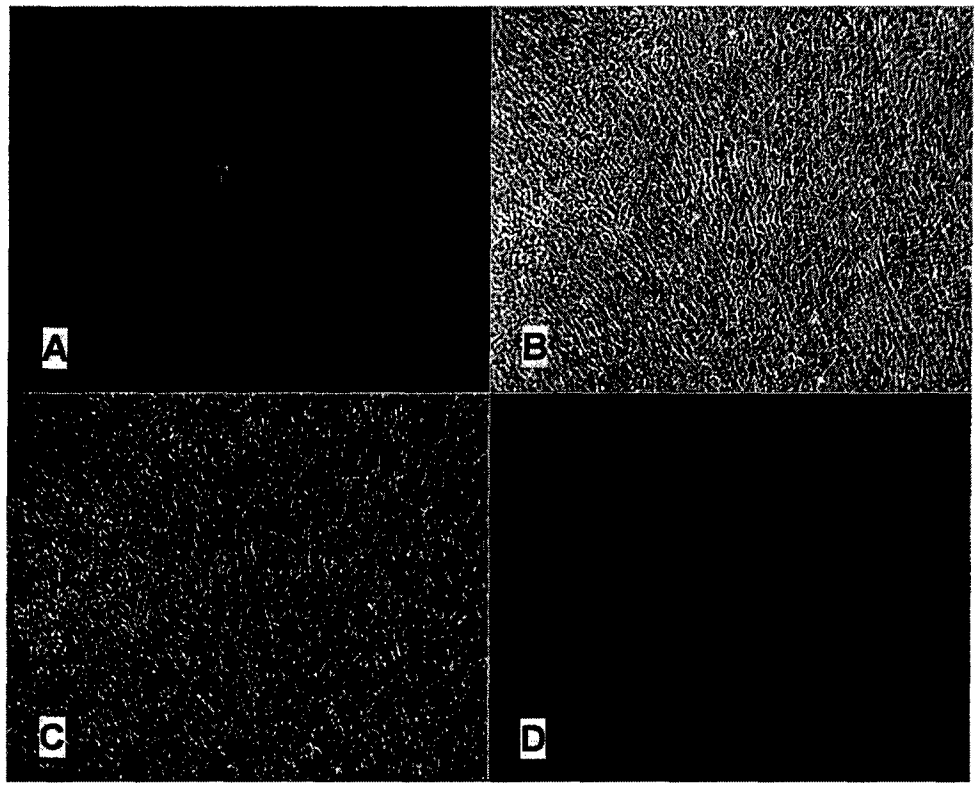

Figure 2. 405 nm CW laser-induced DCF fluorescence in hTERT RPE cells.[12] A: DCF fluorescence image; B: phase-contrast image; C: image of DCF fluorescence overlaying phase-contrast; D: image of DCF fluorescence in unexposed cells upon prolonged excitation on microscope.

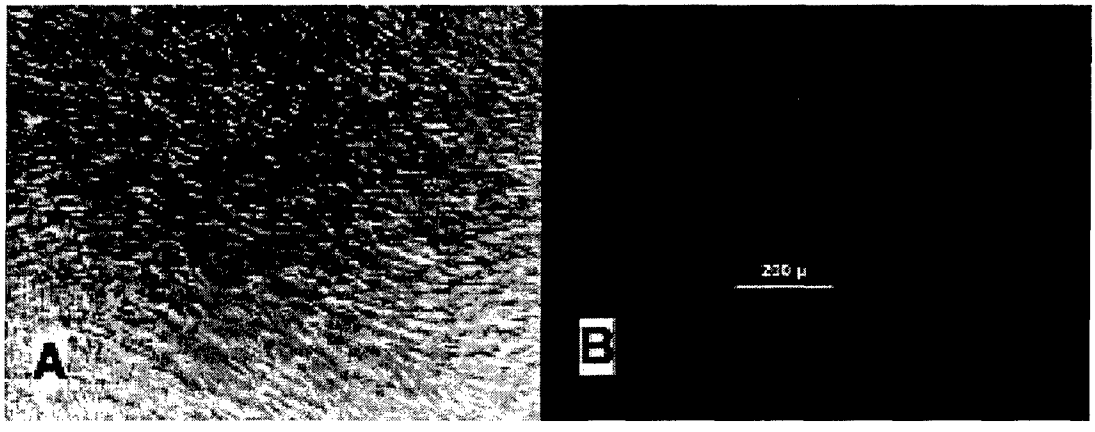

Figure 3. NIR (810 nm) mode-locked laser-induced DCF fluorescence in hTERT-RPE1 cells. A: image of DCF fluorescence overlaying phase-contrast; B: DCF fluorescence image.

H_2DCFDA. Using our most sensitive conditions for detecting cellular oxidation (cells preloaded with 100 μM CM-H_2DCFDA) we have determined the approximate thresholds for irradiance (10,000 W/cm^2) generating the oxidation event and the radiant exposure required for detecting the oxidation in our hTERT-RPE1 cells (200,000 J/cm^2). Again, no targets of oxidation were observed from exposures of CW 810 nm beams, even at our most sensitive conditions (Table 1).

Table 1. Effect of dye and dye concentration on detection of laser-induced oxidation.

Dye preload into cells using HBSS unless otherwise specified	ML or CW	lowest E w/target (W/cm^2)	lowest H w/target (J/cm^2)
H_2DCFDA (50 μM, PBS)	ML	20,500	820,000
H_2DCFDA (100 μM)	ML	14,600	441,000
H_2DCFDA (100 μM)	CW	no target	no target
CM-H_2DCFDA (12.5 μM)	ML	no target	no target
CM-H_2DCFDA (25 μM)	ML	no target	no target
CM-H_2DCFDA (50 μM)	ML	19,900	662,400
CM-H_2DCFDA (100 μM)	ML	9,920	180,900
CM-H_2DCFDA (100 μM)	CW	no target	no target

Exposures of dye-imbedded agarose

To test whether the oxidation observed in our mode-locked laser-exposed hTERT-RPE1 cells was due to an intracellular metabolic (oxidation) reaction, and not a consequence of direct interaction of the dye with the laser, we performed exposures in dye-imbedded agarose samples. Here we chemically cleaved the diacetate group from the CM-H_2DCFDA using the manufacturers recommendations (NaOH treatment followed by neutralization) and incubated (at least 30 min at 37°C) the resulting reagent (163 μM CM-H_2DCF) to wells of a 96-well microtiter plate that had 50 μl solidified 4% agarose. We exposed the wells containing the dye-imbedded agarose to mode-locked 810 nm at irradiances of greater than 19,500 W/cm^2 and for durations leading to over 1,200,000 J/cm^2 delivered. In all cases, no laser-specific "target" area was observed above background, represented in Figure 4. The blue exciter light of the fluorescence microscope (at extended excitation; Figure 4 Panel B) provided proof that the CM-H_2DCFDA was cleaved to CM-H_2DCF and that this chemical form was capable of being oxidized (by blue light) to CM-DCF.

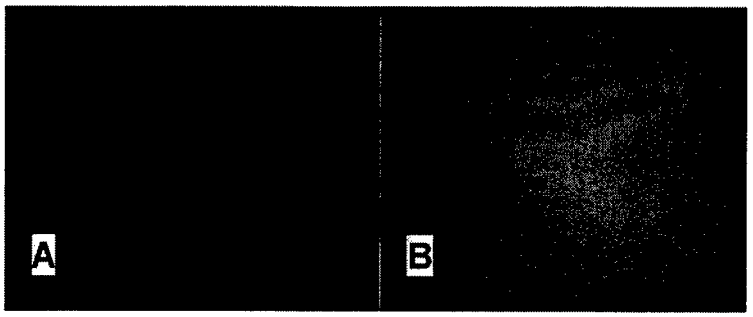

Figure 4. Direct oxidation of CM-H_2DCFDA by blue light (B) but not mode-locked 810 nm light (A).

DISCUSSION

We show that mode-locked NIR lasers can generate a multiphoton photochemical reaction at relatively low average fluences when exposed to living cells. Production of photo-oxidation at 810 nm as a linear event (single photon) is highly unlikely. We were unable to obtain evidence of oxidation when exposing hTERT-RPE1 cell to CW 810 nm laser under our most sensitive conditions, even at maximum output from the Mira laser, and at more than 10 fold greater radiant exposure than that leading to detection with mode-locked exposures.

With the new sensitive detection scheme (preloading cells with 100 μM CM-H_2DCFDA) damage targets from mode-locked 810 nm exposure was easily identifiable as being the result of laser exposures. As compared to the CW 405 nm laser, the oxidation efficiency for mode-locked 810 nm laser exposure is very low, even with the more sensitive conditions for detection. The oxidation from the CW 405 nm laser was detected from a 0.25 sec exposure using 10 μM H_2DCFDA while that from the mode-locked 810 nm laser was detected from a 20 sec (or more) exposure using 100 μM of the more sensitive dye CM-H_2DCFDA. However, the approximate peak irradiance ($8x10^8$ W/cm^2) for the threshold oxidation by mode-locked 810 nm laser exposure seems modest for a multiphoton mechanism. From the current results we now attribute the peculiar fluorescence staining reported previously[12] as an artifact of staining as we were unable to repeat any photo-oxidation at 810 nm using the prior conditions.

As judged by thresholds for radiant exposure required for effect in our hTERT-RPE1 cells, we found the general trend in detection efficiency by the fluorescent dyes to be 100 μM CM-H_2DCFDA as the most sensitive, followed by 100 μM H_2DCFDA (~2 fold lower), 50 μM CM-H_2DCFDA (~3 fold lower), and finally the 50 μM H_2DCFDA (~4 fold lower).

We examined potential cytotoxic effects from the mode-locked laser-induced photo-oxidation (results not shown). Upon assaying for viability post-exposure to near threshold fluences, we found that our cells were able to recover from the oxidative stress generated from the mode-locked 810 nm laser. This was not an unexpected result because our system for detection was very sensitive.

Finally, we tested for the possibility that the mode-locked laser was interacting directly with the fluorescent dyes, leading to the detected oxidation targets. Our exposure to agarose-embedded CM-H_2DCF yielded no targets at maximum irradiance and a total radiant exposure delivered that was 6 times above threshold.

In conclusion, we would caution the use of mode-locked lasers in applications for which continued survival of cells, such as extended fluorescent studies of cell cultures or the real-time detection of fluorescence in tissues *in vivo*, until safety issues can be resolved.

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