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Multiphoton absorption is probably not the primary threshold damage mechanism for femtosecond laser pulse exposures in the retinal pigment epithelium

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ABSTRACT

Laser induced breakdown has the lowest energy threshold in the femtosecond domain, and is responsible for production of threshold ocular lesions. It has been proposed that multiphoton absorption may also contribute to ultrashort-pulse tissue damage, based on the observation that 33 fs, 810 nm pulse laser exposures caused more DNA breakage in cultured, primary RPE cells, compared to CW laser exposures delivering the same average power. Subsequent studies, demonstrating two-photon excitation of fluorescence in isolated RPE melanosomes, appeared to support the role of multiphoton absorption, but mainly at suprathreshold irradiance. Additional experiments have not found a consistent difference in the DNA strand breakage produced by ultrashort and CW threshold exposures. DNA damage appears to be dependent on the amount of melanin pigmentation in the cells, rather than the pulsewidth of the laser; current studies have found that, at threshold, CW and ultrashort pulse laser exposures produce almost identical amounts of DNA breakage. A theoretical analysis suggests that the number of photons delivered to the RPE melanosome during a single 33-fsec pulse at the ED_{50} irradiance is insufficient to produce multiphoton excitation. This result appears to exclude the melanosome as a locus for two- or three-photon excitation; however, a structure with a larger effective absorption cross-section than the melanosome may interact with the laser pulses. One possibility is that the nuclear chromatin acts as a unit absorber of photons resulting in DNA damage, but this does not explain the near equivalence of ultrashort and CW exposures in the comet assay model. This equivalence indicates that multiphoton absorption is not a major contributor to the ultrashort pulse laser damage threshold in the near infrared.

Keywords: comet assay, DNA damage, melanosome, multiphoton absorption, RPE, threshold,, ultrashort pulse laser

1. INTRODUCTION

1.1 Background of multiphoton absorption

The possibility of multiphoton absorption was first hypothesized by Goppert-Mayer in 1931, who proposed a mechanism by which photons absorbed within a sufficiently short time window, i.e. $\leq 10^{-18}$ sec, could cumulatively add their energy to a chromophore, exciting it to an energy level approximately twice as great as that of the individual photons.[1] Due to the two-photon absorption mechanism, relatively low energy photons delivered in a short pulse could produce an effect equivalent to the absorption of a single, more energetic photon. The practical significance of the two-photon excitation mechanism was not realized until ultrashort pulse lasers were available.[2-4]

1.2 Relevance of multiphoton absorption for damage mechanisms involved in ultrashort pulsed

In previous work on the interaction of pulsed, infrared laser sources on retinal pigment epithelial (RPE) cells, we demonstrated that effects normally requiring visible or near UV (UVA) light exposure, such as excitation of melanin fluorescence[5] and DNA breakage,[6,7] could be produced by ultrashort infrared (810 nm) laser pulses. Other authors have also reported two-photon excitation of melanin fluorescence.[8,9] It was proposed that these effects were due to

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Laser Interaction with Tissue and Cells XV, edited by Steven L. Jacques, William P. Roach, Proc. of SPIE Vol. 5319
(SPIE, Bellingham, WA, 2004) · 1605-7422/04/$15 · doi: 10.1117/12.527843

multiphoton absorption within the melanin of the RPE cells. Two-photon excitation effectively upconverted the infrared photons to UVA-VIS wavelengths, which then activated mechanisms that induced single and double strand DNA breaks.[7,10] Although an increased number of DNA strand breaks were demonstrated at threshold radiant exposures, the differences observed between the effects of continuous wave exposures, which would not be expected to induce multiphoton effects, and ultrashort pulse exposures, which might produce bioeffects due to multiphoton absorption, have been difficult to document consistently. Here we present our latest experimental data on the ability of ultrashort laser pulses to break genomic DNA, as well as a theoretical prediction of the number of photons likely to be absorbed in cellular targets during short laser pulses. This analysis indicates that multiphoton absorption in the RPE melanosome is not likely to contribute to threshold damage, although it probably contributes to suprathreshold cellular damage due to induction of photo-oxidative stress, for example, as shown by Denton et al.[11]

2. METHODS

2.1 hTERT-RPE cell line and comet assay for DNA breakage

Experiments were performed on a line of human-derived RPE cells provided by the Geron Corp. (Menlo Park, CA). These cells, designated hTERT-RPE, have been transfected with the gene for human telomerase (hTERT) and do not become senescent in culture; however, in other respects the cells exhibit a normal phenotype.[12,13] The hTERT-RPE cells produce melanin pigmentation in culture,[14] and most of the cells used in these experiments exhibited a degree of pigmentation proportional to their time in culture. The hTERT-RPE cells were grown for at least 2 weeks in 24-well, plastic culture plates, in standard Dulbecco's Modified Eagle's Medium (DMEM) containing 10% serum. During the laser exposures, the overlying media was drained from the wells and replaced with Dulbecco's Phosphate Buffered Saline (DBPS). Further details of the procedures for handling the hTERT-RPE cells were described elsewhere.[7]

2.2 Assessment of DNA breakage by comet assay

The use of the comet assay for detecting DNA strand breaks in RPE cells was reported by Hall et al.[6] Following electrophoresis of the lysed RPE cells in agarose gels, DNA fragments were stained with Syber Yellow, a fluorescent nucleic acid stain (Molecular Probes, Eugene, OR). The fluorescent DNA patterns (comets) were imaged with a fluorescence microscope equipped with a CCD imaging system (Optronics DEI-470), and the images processed with a public domain, comet assay analysis macro for the NIH/Scion-Image software[15], in order to extract comet tail length, tail moment (product of length and intensity), and comet area. Statistical analysis of the results was done using the Bonferroni multiple comparison test in the ProStat statistical package (Poly Software International, Pearl River, NY).

A modification was made to the analysis algorithm to address the problem that high-intensity fluorescent figures, such as those from control samples, were scored by the algorithm as having very large comet tail lengths and moments. The algorithm used in our earlier experiments[7] sometimes reported larger comet tails and moments for the cells in the control conditions than for cells in the experimental conditions, although by eye, the control comets appeared to be "rounder" and less comet-like. The original comet analysis algorithm[15] operated by thresholding the comet image, then measuring the length and width of the border of the threshold image. We originally used the same absolute threshold for all comets in a given experiment, to avoid using a subjective setting for the analysis. This introduced an artifact, however, by over-emphasizing the size of bright comets, when the threshold was set to detect the outline of less intense comets. This was due to the fact that the comets in the control conditions tended to be very intense (the DNA did not migrate in the gel, but remained near the location of the cell nucleus), while the comets in experimental conditions tended to be relatively less intense (the DNA with an increased number of breaks migrated through the gel during electrophoresis, and therefore was more dilute). To minimize this problem, the analysis algorithm was modified so that the threshold for a given comet was set to 65% of the maximum white level of that particular image. In effect, the threshold used to determine the comet length was determined by a fixed ratio based on the dynamic range of the comet image, not at an absolute level. The 65% level was derived empirically, i.e. it was the threshold setting that did not over-emphasize the size of the control comets, while still detecting the comet tails in the experimental conditions. This modification was justified because the absolute white level and contrast range varied among different cell samples.

2.3 Melanosome preparation and hTERT-RPE cell melanosome loading

RPE cells were isolated from bovine eyes purchased from AnTech (Tyler, TX). Following sonication to release the cells' contents, the melanosomes were isolated by successive centrifugation using methods previously reported.[16] The

melanosomes were stored frozen in 0.25 M sucrose at -20° C until use. The stock melanosome suspension contained approximately 6 x 10^9 (±20%) granules/ml, as determined by haemocytometer counts. Melanosomes were layered onto confluent cultures of hTERT-RPE 24 h prior to laser exposures, following the method of Denton et al.[11] Excess melanosomes were removed prior to laser exposure by rinsing the cells in normal DMEM.

2.4 Laser sources and exposures

The laser source used in the hTERT-RPE cell exposure experiments was a Coherent (Santa Clara, CA) MIRA 900F Ti:Sapphire regenerative amplifier system, modified from the system originally described by Noojin et al.[17] This system could be operated to produce either CW output at 810 nm, or mode-locked pulses. In mode-locked (ML) operation, it was anticipated that the laser produced an 80 MHz output train of ~48-fsec pulses with a 40 nm bandwidth centered at 810 nm. Subsequent beam analysis, however, revealed that the pulse widths produced were actually between 200 and 300 fsec. This discrepancy may have played a critical role in the determination of the threshold damage mechanisms, as will be discussed later in this paper.

An external lens was used to focus the 810 nm beam onto the sample plane. The beam diameter at 1/e was 0.210 mm at the RPE cell monolayer on the culture plate. The laser was delivered to the cells in an exposure of 0.25 s duration, and the average power of both the CW and the train of mode-locked, ultrashort pulses was set to either 80 or 160 W/cm^2 depending on the experiment. These values were estimated as the irradiance equivalent to the corneal irradiance corresponding to 0.5 x ED_{50} and 1 x ED_{50}, respectively, for a retinal lesion in the intact (human) eye. The average power of both laser sources was measured with a Molectron (Portland, OR) EPM 1000 meter equipped with a PowerMax PM-10 thermopile detector. Just prior to the onset of the laser exposures, the culture well was drained of excess culture medium, and replaced with 100 µl of DPBS. To expose as many cells in each culture well as possible, the laser was scanned over the well, delivering the 0.25 s exposures in a spiral pattern. Approximately 1,261 exposures were required to expose each well.

The cells were exposed to the 810 nm CW and ML laser as follows: (1) controls received no laser exposure, but were handled in a similar fashion to the laser-exposed cells; (2) exposed to the 810 nm ML pulses (presumed 48-fsec pulsewidth but actually ~250-fs at 80 MHz in a train of 0.25 sec duration) at the estimated ED_{50} exposure required to produce a retinal lesion (~160 W/cm^2), (3) exposed to the 810 nm ML pulses at 0.5 x ED_{50} (~80 W/cm^2), (4) exposed to 810 nm laser in CW mode at the ED_{50} (0.25 sec at 160 W/cm^2), or (5) exposed to 810 nm CW mode laser at 0.5 x ED_{50} (0.25 sec at 80 W/cm^2).

3. RESULTS

3.1 Evidence for multiphoton absorption in the RPE cells

Evidence for multiphoton absorption produced by the ultrashort pulse laser exposures was provided by the observation of melanin fluorescence excited by 810-nm ML pulses in melanosomes isolated from RPE cells.[5] The fluorescence observed in these experiments exhibited a similar wavelength maximum at approximately 525 nm as that elicited by single-photon excitation with the 406 nm output of a Kr-ion CW laser. The power dependence of the two-photon excited fluorescence, however, closely followed a second order function, as opposed to single-photon excited fluorescence, which was linearly dependent on the excitation power (Figure 1). This observation was consistent with two-photon excitation of melanin fluorescence by the ultrashort pulse, 810 nm laser.

3.2 DNA damage by ultrashort and CW infrared laser exposure

These experiments compared the amount of DNA damage produced in cultured hTERT-RPE cells by exposures to 810 nm CW emissions and to ultrashort mode-locked (ML) laser pulses delivering equivalent average power to the cells. Two exposure conditions were examined: one-half the ED_{50} fluence required for retinal lesion threshold (80 W/cm^2), and threshold (ED_{50}) fluence (160 W/cm^2). The experiments were conducted with hERT-RPE cells not supplemented with melanosomes. These cells will spontaneously produce melanin pigment in their cytoplasm,[14] but were only lightly pigmented at the time these experiments were carried out. The effects of the laser exposures on the cells were assessed by determining single and double DNA strand breaks released by alkaline hydrolysis of the cells, using the comet assay procedure described in section 2.2. The results of the 0.5 x ED_{50} exposures are shown in Figure 2A, and the results of the ED_{50} exposures are shown in Figure 2B. At 0.5 x ED_{50}, cells exposed to ML laser pulses had slightly longer comet

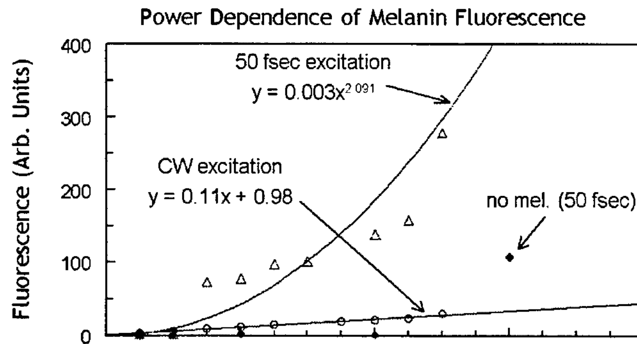

Figure 1: Power dependence of fluorescence in isolated RPE melanosomes

tail lengths than did the CW-exposed cells (Figure 2A). The difference was significant (p<.001, Bonferroni multiple comparison test). The comet tail lengths of the CW-exposed cells were not significantly different from those of the control cells. At the ED_{50} (Figure 2B), the comet tail lengths in both the CW and ML groups were significantly longer than in the control cells (p<.005), and the tail lengths of the ML-exposed cells were longer than those of the CW-exposed cells (p<.02), suggesting that the ML pulses were more effective in producing DNA strand breaks. The data, however, were somewhat inconsistent, because the comet tail moments, a measure of the density of the DNA in the "tails", were not significantly different among any of the exposure groups. The inconsistency of the comet assay rendered the conclusion less compelling, that ML pulses were inducing multiphoton absorption effects in the RPE cells.

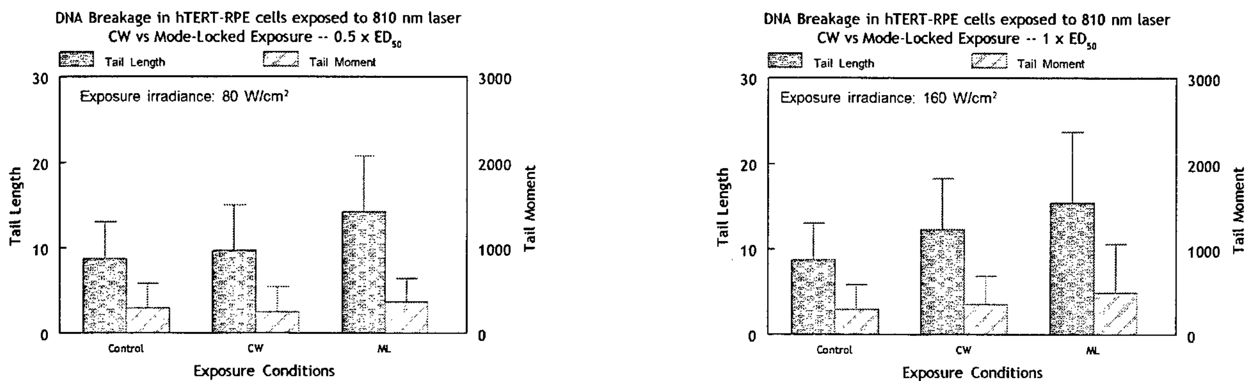

Figure 2: Comet assays of unpigmented hTERT-RPE cells exposed to CW and ML 810-nm laser. Figure 2A (left) shows results from exposures at 80 W/cm^2 (0.5 x ED_{50}). Figure 2B (right) shows results from exposures at 160 W/cm^2 (1 x ED_{50}).

Previous work in this laboratory,[16,18] as well as in that of Denton et al.,[11] indicated that the presence of melanin pigmentation could increase the susceptibility of RPE cells to photo-oxidative stress. Based on these observations, experiments were performed to measure DNA damage in cells with artificially enhanced pigmentation. RPE cell cultures with different pigmentation loads were produced by the melanosome supplementation protocol described in section 2.3, and then exposed at the ED_{50} fluence (160 W/cm^2) to the CW and ML laser protocols.

Under these conditions, only cells with the highest levels of melanin pigmentation, i.e. the cells supplemented with 60 or 180 µl of melanosomes, had significantly greater DNA damage as assessed by comet tail length and moment (p<.03,

Bonferroni multiple comparison test). These data are shown in Figure 3. Interestingly, in the heavily pigmented cells suffering the most DNA strand breaks, there was no significant difference between the comet assays in the CW and ML exposed cells. This finding indicated that multiphoton absorption was not involved – or at least not exclusively involved – in the production of the DNA strand breaks under these laser exposure conditions. These results must be qualified, however, due to the recent discovery that the duration of the ML pulses was in fact approximately 250 fs. Multiphoton absorption obviously becomes less likely for a given irradiance as the pulse width increases and the photon density decreases.

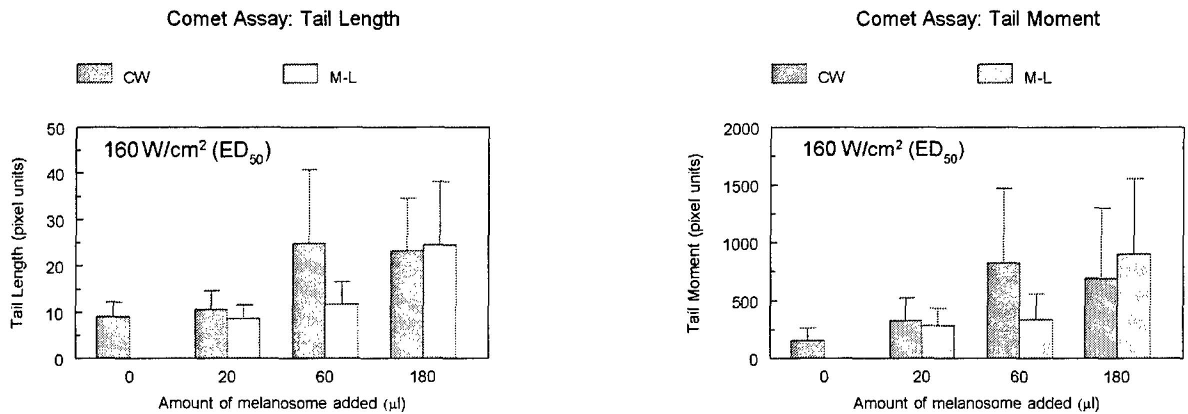

Figure 3: Comet assay results with HTERT-RPE cells pigmented by melanosome supplementation. All exposures were made at the ED_{50} (160 W/cm^2). Figure 3A (left): comet tail length measurements. Figure 3B (right): comet tail moment measurements.

In order to emphasize the effect of cellular pigmentation load, the results from the melanosome-supplemention experiment were replotted in Figure 4. Restricting the comet assay analysis to tail length, the amount of DNA breakage, as a function of the amount of isolated melanosomes added to the cells, is shown in Figure 4. Our previous work clearly showed that cellular pigmentation is directly related to the amount of melanosome supplementation, cf. Figure 2 in Glickman et al., 2003[7]. The trend of the data in Figure 4 indicates that there was little or no increase in DNA damage until at least 60 µl of melanosomes were added to the cells. While the difference in cellular pigmentation was a major determinant in the extent of DNA strand breakage, the laser output mode, i.e. CW or ML, appeared to be less important. This observation motivated us to calculate the number of photons absorbed in a typical RPE cell melanosome during the laser exposures used in these experiments, and, to determine if sufficient photons were being absorbed to make multiphoton absorption a likely (or even possible) outcome. These calculations are developed in the following sections.

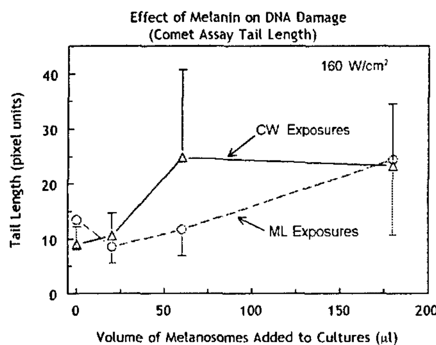

Figure 4: DNA strand breakage following CW or ML laser, as a function of the amount of cellular melanin pigmentation. Exposures were made at the ED_{50} (160 W/cm^2).

3.4 Photon energy available for absorption in melanin

Initially, the energy available in each photon is calculated:[19]

$$E = h\frac{c}{\lambda} \tag{1}$$

This calculation yields 2.44×10^{-19} J as the energy of each 810 nm photon. Next, the number of photons available in a single laser pulse is calculated for the case of a 160 W/cm^2 laser and a 250 fs pulse duration (this was the actual pulsewidth used in the experiments; the planed pulsewidth of 48 fs was not available). The total energy per pulse is thus 4×10^{-11} Joules/cm^2. The number of 810 nm photons available per square centimeter is then 1.6×10^8.

The time for electron de-excitation is assumed to be less than 1×10^{-8} seconds (80 MHz pulse repetition rate), so that the energy from each pulse is assumed to be non-additive. Another simplifying assumption is that only the physical area of a melanin granule is available for interaction with the photons. A melanin granule roughly approximates a rod, 5 microns in length with a 1 micron diameter,[20,21] so the maximum surface area presenting for interaction would be approximately a rectangular area of 5 square microns (1 micron × 5 microns or 5×10^{-8} cm^2). Thus, the maximum number of photons available for primary interaction with a melanin granule would be

$$5 \times 10^{-8}\,\text{cm}^2 \times 1.6 \times 10^8\ \text{photons/cm}^2 = 8\ \text{photons} \tag{2}$$

This does not account for the cylindrical nature of the granule, assumes all photons intersect the granule along the long axis, and should lead to an overestimate of the number of interacting photons, if area of the granule is the primary parameter for absorption. Reducing the pulse irradiance from 160 W/cm^2 or reducing the width of the pulse will only reduce the number of photons available to interact with the granule. A minimum of approximately 200 W/cm^2 would be needed at 48 fs to result in 2 photons intersecting a single granule.

Extrapolating to the near infrared from the visible spectrum absorption coefficient (μ_a) of melanin reported by Sardar et al.[22] yields a value of 0.55 cm^{-1} for μ_a of melanin at 810 nm. If a simplifying assumption is made that the average thickness of the cylinder is 0.5×10^{-4} cm, then using Beer's law, the number of photons penetrating the granule would be

$$8(e^{-0.55\,\text{cm-1} \times 0.5 \times 10\text{-}4}) = 7.99978 \tag{3}$$

This would result in 2.2×10^{-4} primary photons (i.e. 8.0 incident photons minus 7.99978 exiting photons) absorbed per granule. This does not account for potential attenuation of photons in materials that might result in some energy transfer to the granules.

3.5 Photons required to cause breaks

Note that the wavelength for maximum biological effect of DNA in cells is 270 nm, and $3 \times 270 = 810$ nm, implying that three photons could possibly be effective at causing DNA damage. Assuming that the weak hydrogen bonds associated with DNA require approximately 3 eV to break, and each 810 nm photon has an energy of

$$E = 2.44 \times 10^{-19} \frac{\text{J}}{\text{photon}} \cdot \frac{1\,\text{eV}}{1.6 \times 10^{-19}\,\text{J}} = 1.525\ \text{eV per photon.} \tag{4}$$

In this case, a minimum of 2 photons would be required to cause a break. If there is 100% absorption in each granule, and it is assumed to be absorbed on the side (rather than on end), then 8 photons would be available to produce ionization. Scaling this, only 40 W would be needed at 100% absorption to achieve a break. More realistically, if the absorption coefficient for melanin were used in the calculation, then the minimum energy required for the actual

absorption of two photons with total transfer of their energy to the melanosome (using previous simplifying assumptions regarding area and thickness) would be predicted as:

$$\frac{160\,\text{W}}{2.2\times10^{-4}\ \text{photons absorbed}}\cdot\frac{2\ \text{photons}}{5\times10^{-8}\text{cm}^2} = 2.9\times10^{13}\ \text{W/cm}^2 \qquad (5)$$

This calculation assumes that the NIR absorption coefficient for melanin given above, 0.55 cm^{-1}, applies, and only primary photons are present.

3.6 Range of laser exposures predicted to involve multiphoton absorption as a major damage mechanism

The number of photons expected to impact a melanosome as a function of laser radiant exposure can be predicted using the methodology described in sections 3.4 and 3.5. Some representative calculations have been done for a physiologically relevant range of exposures, and are presented graphically below in Figures 5 and 6. Note that these graphs indicate only the number of photons incident on a typical, 1 x 5 μm, RPE melanosome, not the number of photons actually (or realistically) absorbed in the pigment granule.

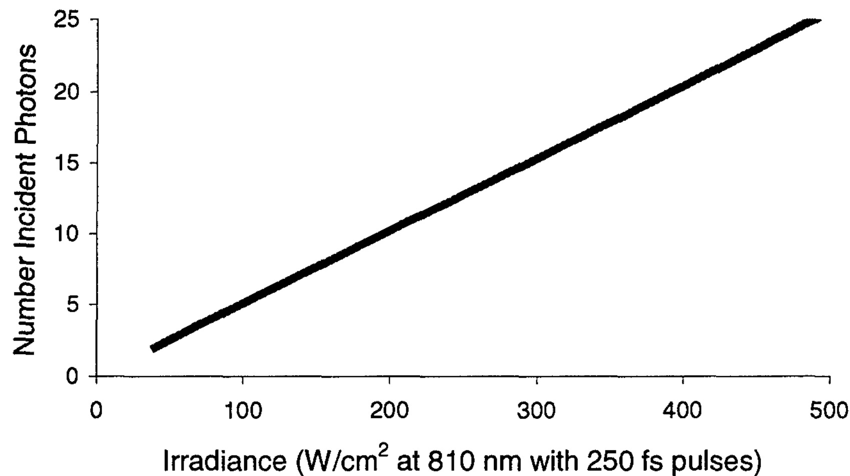

Figure 5: Number of photons incident on a RPE melanosome as a function of irradiance

4. DISCUSSION

4.1 Implication of results

Recent investigation of the principal damage mechanisms induced in ocular tissue by ultrashort pulse (<100 fs) lasers, have shown that laser-induced breakdown has the lowest energy threshold, and therefore is probably the predominant damage mechanism at fluences near the ED_{50} for retinal and RPE lesions.[17,23,24] At suprathreshold fluence, other mechanisms are activated, including photochemical reactions producing oxidative endpoints.[25,26] After our initial observation of increased DNA strand breaks following exposure of cultured RPE cells to ultrashort pulse laser, compared to CW exposures delivering the equivalent average power,[6] we proposed that a damage mechanism activated by multiphoton absorption was responsible for the difference. We reported that two-photon excitation of fluorescence in isolated RPE melanosomes was due to multiphoton absorption, but the fluorescence was observed at suprathreshold irradiance.[5] The present investigation of DNA strand breaks in RPE cells at subthreshold and threshold irradiance

indicated that cellular melanin plays a role in mediating the DNA damage, but it was not clear that multiphoton absorption was responsible for the damage near the presumptive threshold fluence. This conclusion was primarily based on the finding of similar amounts of DNA damage produced by both CW and ML laser exposures at threshold irradiance. The theoretical analysis developed in this paper also supported the conclusion that, at threshold, an insufficient number of photons are absorbed in a RPE melanosome to result in multiphoton absorption.

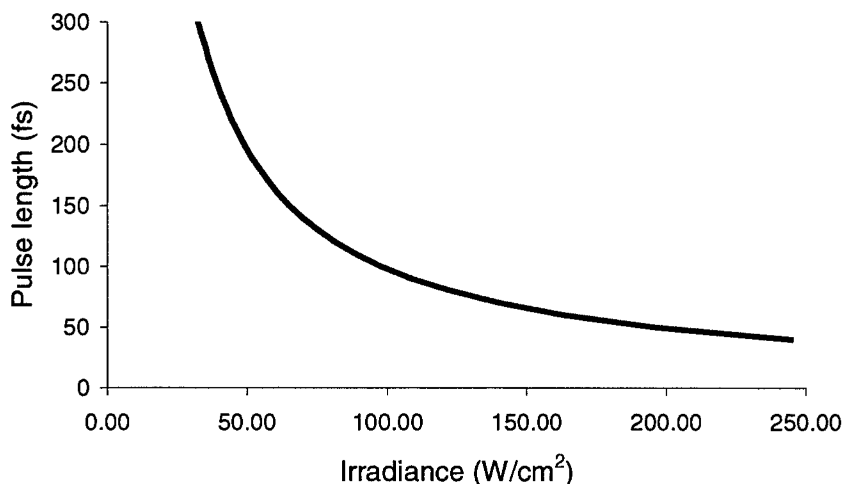

Figure 6: Pulse duration required to produce sufficient irradiance for at least two 810-nm photons to be incident upon a RPE melanosome.

Some qualifications must be considered in reaching this conclusion. In the analysis reported here, only primary photons, that is, photons that travel directly from the source to the target are considered. Scattered photons are not included, but are not expected to contribute significantly to the multi-photon effect. Another consideration is that there may be a specific "resonance" effect that causes a significant change in the absorption properties of a melanin granule that has yet to be described. For that matter, the optical properties of RPE melanin in the near infrared optical radiation band have not been precisely measured. The values used in our analysis represent a reasonable extrapolation from the optical properties of melanin measured in the visible spectrum. Additionally, the area of interaction is assumed to be at the most favorable presentation for absorption, and the cylindrical nature of the granule is not included, so the physical interaction area used in these calculations is greater than the actual physical size of the granule. Physical size is not always directly proportional to absorption coefficients, and it is known that resonance reactions exist for many photon interactions.[19]

Other mechanisms besides direct photon absorption may also play an important role in determining the damage to melanin granules. If melanin has undiscovered or unusual properties, such as existing as a stable free radical,[27] then the melanosome may be able to maintain electrons in an excited state such that high repetition rate photon pulses can produce additive (pulse to pulse) effects. In such a case, multiphoton effects would then be possible at lower peak power exposures than indicated by our analysis. In the absence of such properties, however, individual pulses would require supra-threshold energies on the order of megawatts to initiate multi-photon effects by primary photons.

4.2 Contribution of non-linear interactions to possible multiphoton effects

Any of the non-linear interactions known to occur with ultrashort laser pulses[28-30] could possibly cause multiphoton interactions to occur. The photon concentrations produced could be sufficient to cause localized ionization in each case. The concentration of photons would be altered by the non-linear reaction, for example through the effects of self-focusing, which might result in sufficient energy deposition leading to alteration of absorption coefficients with

resulting concentration of energy. Such phenomena could manifest themselves as plasma formation, thermal denaturation (probably highly localized), and/or oxidative damage.

4.3 Is multiphoton absorption mediated by other possible cellular targets?

Finally, the possibility exists that other intracellular targets may be involved in the interaction of ultrashort, infrared laser pulses. In the case of the RPE cell, we have concentrated on the melansome because of its prominent cytoplasmic location, its broadband optical absorption characteristics,[31] and its known involvement in ocular light[32] and laser[33] damage. The RPE melanin is clearly an important factor in mediating CW and ultrashort pulse damage, as indicated by the present results, and it appears to mediate photo-oxidative stress produced by suprathreshold, ultrashort pulse exposures.[26] Nevertheless, at threshold, other cellular structures may play a role in cellular responses to ultrashort laser pulses. Mitochondria are possible targets, but – as is also the case with the melanosomes - it is unclear how, in the short term, damage to these cytoplasmic organelles could affect the nuclear DNA. Potential targets for laser damage exist in the nucleus. As noted above, the absorption maximum for DNA damage occurs near 270 nm; therefore, a suitable absorber to mediate multiphoton absorption of near infrared photons has to be identified. The genomic DNA in eukaryotic cells itself exists in nucleosomes, repeating complexes of DNA and histone proteins. The diameter of these structures is on the order of 30 nm,[34] which is a much smaller cross-section compared to the RPE melanosomes, and presumably would absorb incident NIR photons at much lower efficiency. The chain of nucleosomes comprising the DNA, however, forms an assemblage that is much larger than the individual subunits, and if the entire complex functioned as a single, absorbing unit, then the effective absorption cross-section would be correspondingly larger. Whether the entire nuclear DNA-histone complex functions as a single absorber to promote multiphoton absorption at suprathreshold laser exposures remains to be determined. In addition, better measurements of the near infrared optical properties of cellular organelles are required in order to evaluate hypothetical non-linear laser bioeffects.

ACKNOWLEDGMENTS

This research was supported by the U.S. Air Force Office of Scientific Research Grant F49620-01-1-0211 (to RDG), the U.S. Army Medical Research Materiel Command Grant DAMD17-03-0032 (to TEJ), and an unrestricted grant from Research to Prevent Blindness, Inc., to the Department of Ophthalmology at the University of Texas Health Science Center at San Antonio.

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