RPE Damage Thresholds and Mechanisms for Laser Exposure in the Microsecond-to-Millisecond Time Regimen

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PURPOSE. The retinal pigment epithelium (RPE) cells with their strongly absorbant melanosomes form the highest light-absorbing layer of the retina. It is well known that laser-induced retinal damage is caused by thermal denaturation at pulse durations longer than milliseconds and by microbubble formation around the melanosomes at pulses shorter than microseconds. The purpose of this work was to determine the pulse width when both effects merge. Therefore, the RPE damage threshold and mechanism of the damage at single laser pulses of 5-μs to 3-ms duration were investigated.

METHODS. An argon laser beam (λ 514 nm) was externally switched by an acousto-optic modulator to achieve pulses with constant power in the time range of 5 μs up to 3 ms. The pulses were applied to freshly prepared porcine RPE samples serving as a model system. After laser exposure RPE cell damage was proved by the cell-viability stain calceinAM. Microbubble formation was detected by acoustic techniques and by reflectometry.

RESULTS. At a pulse duration of 5 μs, RPE cell damage was always associated with microbubble formation. At pulses of 50 μs, mostly thermal denaturation, but also microbubble formation, was detected. At the longer laser pulses (500 μs, 3 ms), RPE cell damage occurred without any microbubble appearance.

CONCLUSIONS. At threshold irradiance, the transition time from thermal denaturation to thermomechanical damage of RPE cells is slightly below the laser pulse duration of 50 μs. (Invest Ophtalmol Vis Sci. 2005;46:714–719) DOI:10.1167/iovs.04-0136

The interaction of laser radiation with biological tissue is of interest both for medical applications and for the establishment of laser safety standards. Laser treatments of retinal diseases are widely used in ophthalmology. Laser therapies at the fundus range from established continuous wave (cw) photocoagulation to new ophthalmic laser applications, such as selective retinal pigment epithelium (RPE) treatment (SRT), photodynamic therapy (PDT), and transpupillary thermotherapy (TTT). Maximum permissible exposure limits were established for visible and near-IR laser radiation from cw down to femtosecond exposures. The type of damage mechanism depends on the duration of the applied laser pulse. At cw to 10-μs exposure time, a pure thermal denaturation of tissue has been shown to be the primary retinal damage mechanism. In this time frame, the damage can be described as a damage integral based on the Arrhenius law. From microsecond to nanosecond exposure times, there is evidence that RPE damage is induced by intracellular microbubble formation around the strongly absorbant melanosomes inside the RPE cell. The microbubble formation leads to a disintegration of the RPE cell structure and a disruption of the cell membrane. At subnanosecond exposures, other nonlinear damage mechanisms appear, such as shock-waves and laser-induced breakdown. The RPE is the layer that absorbs the highest amount of light in the retina. The ellipsoidal shaped, approximately 1-μm sized melanosomes within these cells are the strongest chromosom for visible light of the fundus. In humans, approximately 60% of the incident light that reaches the retina is absorbed within this cell layer.

Until now, the exact exposure time at which a change of damage mechanism from a pure thermal denaturation to thermomechanical damage occurs is unknown. Until now, the exact exposure time at which a change of damage mechanism from a pure thermal denaturation to thermomechanical damage occurs is unknown. In ANSI-Standard Z-136.1-2000—the maximum allowed exposure—the change of damage mechanism has been defined as occurring at 18 μs. Looking on a plot of the experimental damage threshold data over exposure time from ANSI-Standard Z-136.1-1993 (also shown by Cain et al.), the change of slope at ~50 μs of exposure time can be associated with a change in the damage mechanism. It has been shown that, below this exposure time, the laser-induced retinal temperature increase is limited mostly to the RPE cell layer. The thermal confinement increases the probability that temperatures will be induced that are above the vaporization threshold, which results in microbubble formation.

Acoustic measurements have been used to detect cavitation in water and to monitor laser-induced microbubble formation in RPE. During irradiation with a train of microsecond laser pulses, acoustic transients correlated with the damage of a few RPE cells. In similar experiments, the back-reflected light increase due to the formation of a bubble-water interface was used to confirm the formation of microbubbles in RPE during nano- and microsecond laser pulses.

The purpose of this study was to determine the laser-induced RPE damage mechanism and damage thresholds by using acoustic and reflection measurements as well as cell-viability stains for pulse duration between 5 μs and 3 ms.

METHODS

Setup

A sketch of the experimental setup is shown in Figure 1. A cw argon laser beam (514 nm; model 2030-15s; Spectra Physics, Mountain View, CA) was externally switched by an acousto-optic modulator (AOM) to achieve temporal rectangular pulse shapes of 5, 50, or 500-μs or 3-ms width. The light was coupled to a 50-μm diameter fiber (50-μm core, numerical aperture [NA] 0.1; Coherent, Palo Alto, CA). The fiber tip was imaged with an ophthalmic slit lamp (Visulas; Carl Zeiss Meditec, Jena, Germany) on the RPE surface with a 50-μm spot diameter on the...
The spatial beam profile was a circular tophat, which was modulated by speckle formation. The beam profile in the sample plane was measured 20 times with a beam analyser (model LBA-300PC; Spiricon Inc., Logan, UT). The average size of the speckle was 4μm and the maximum radiant exposure was 3.80±0.03 (SD) higher than the average. Two physically independent methods were used to detect microbubble formation during exposure. First, the acoustic emission during microbubble formation was detected with a hydrophone (VP-1093, 0–10 MHz, 1.05 V/bar; Valpey-Fisher, Hopkinton, MA; preamplified with model 5676, 40 dB, 50 kHz–20 MHz; Panametrics, Waltham, MA). Second, the increased light reflection from the sample due to the generated bubble–water interface was confocally imaged to a photomultiplier (Typ R1436; Hamamatsu, Hamamatsu City, Japan). All data were recorded by a transient recorder (model RTD710; Sony/Tek, Tokyo, Japan) and transferred to a computer (LabView, ver. 6i; National Instruments, Austin, TX).

Sample Preparation and Vitality Staining

As RPE samples, freshly enucleated porcine eyes from an abattoir were used. After an equatorial opening of the eye globe, the vitreous body was removed, and a 1-cm²-sized sample was prepared. The neural retina including the photoreceptor layer was gently peeled off. The sample with the vital RPE cells in a superficial layer was covered with phosphate-buffered saline (PBS) and fixed in the sample holder. The samples were irradiated with single pulses of 5-μs (Σ = 480 spots), 50-μs (Σ = 270 spots), 500-μs (Σ = 192 spots), or 3-ms (Σ = 546 spots) duration. All laser pulse durations were investigated in at least 10 samples of 10 different eyes. After irradiation, the sample was stained with the cell viability marker calceinAM (Molecular Probes, Eugene, OR). Because of the uncharged structure of calceinAM, it can penetrate the cell membrane. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases. This intracellular released calcein fluoresces when excited with 480-nm light. Living cells fluoresce brightly because of the accumulated calcein, whereas cells without esterases appear dark in the fluorescence microscope image. Figure 2 shows a typical fluorescence microscopic image of a sample with damaged cells after exposure.

Data Analysis

For analysis of the measured acoustic transients P(t) the acoustic energy $E_A$ was calculated by:

$$E_A = \int_{0}^{\infty} (P(t))^2 dt$$

An acoustic energy threshold for microbubble formation could be defined, and the acoustic energy values were sorted in dichotomous values (1 = acoustically detected microbubble formation; 0 = no microbubble).

From the viability-stained fluorescence microscopic images of the RPE samples, cell viability was sorted in dichotomous values (1 = vital cell, 0 = dead cell).

All thresholds of RPE damage and bubble formation were examined by Probit analysis on a logarithmic dose scale (SPSS, 7.0; SPSS, Chicago, IL). In general, the ED84 and the corresponding ED16 describe the width of the adjusted normal distribution with logarithmic covariant basis. The software would calculate only ED84 and ED16 instead of the specified ED84 and ED16, but the deviations are negligible.
RESULTS

To give an overview of the experimental results, some typical measured signals are shown for the shortest (5-μs) and the longest (3-ms) pulse duration.

Measured Acoustic Transients and Reflectance Signals for the 5-μs Pulse Duration

For the 5-μs pulse duration, three acoustic transients and the reflected light signals are shown in Figure 3. At a low-radiance exposure of 256 mJ/cm², which did not damage the RPE, no acoustic transient above the noise level of 50 μbar could be measured (Fig. 3A). Acoustic transients due to thermoelastic expansion were too weak to detect. In the reflected light signal, only the diffuse reflected laser pulse time course could be measured (Fig. 3B). If no acoustic transients were measured, all cells were viable after the exposure.

At the same energy and in the same RPE sample but at a different location, an increased acoustic transient was measured (Fig. 3C), indicating microbubble formation. Although, no significant increase of the reflected laser pulse form was detectable (Fig. 3D), no RPE cells were damaged. This effect of an acoustically detectable microbubble formation without RPE damage was measured 12 times in the 480 applied 5-μs laser pulses.

At higher radiance exposure (440 mJ/cm²) the acoustic transient amplitude (Fig. 3E) increased compared with that in Figure 3C. With this exposure, 100% of the illuminated RPE cells were damaged. Close to the end of the laser pulse, the reflected light signal increased significantly (Fig. 3F). This effect was most likely induced by microbubble formation, as the RPE temperature was highest at the end of the laser pulse.

At all applied radiance exposures, the analyzed acoustic energy correlated with the percentage of RPE cell damage in the irradiated area. Figure 4 shows the acoustic energy over the percentage of damaged RPE cells for one sample. In this plot, there are typically three different areas of interest: (1) region A, without damaged RPE cells, only the acoustic energy of the secondary background noise, such as the electric noise of the amplifier (as shown in the acoustic transient Fig. 3A), was detected; (2) region B, without damaged RPE cells, but increased acoustic energy indicated microbubble formation (as shown in the acoustic transient Fig. 3C); and (3) region C with at least a certain fraction of damaged RPE cells; the acoustic energy was strongly increased, indicating the formation of microbubbles (as shown in the acoustic transient Fig. 3E).

These three kinds of areas appeared in all 10 irradiated RPE samples.

An acoustic energy threshold value for microbubble formation can be defined. After Probit analysis of the data from the 10 RPE samples, the thresholds for microbubble formation were $\text{ED}_{50}^{\text{acoust}} = 223 \text{ mJ/cm}^2$ ($\text{ED}_{15}^{\text{acoust}} = 168 \text{ mJ/cm}^2$; $\text{ED}_{85}^{\text{acoust}} = 277 \text{ mJ/cm}^2$, slope = 8.4) and for RPE damage...
Damage Thresholds and Mechanisms

Exposure thresholds for RPE damage and microbubble formation at different pulse durations are shown in Figure 7 and summarized in Table 1. The error bars correspond to the ED15 and ED85 of the Probit analysis. At a 5-µs laser pulse duration, the RPE damage threshold was slightly above the threshold for microbubble formation. This changed at 50-µs laser pulses, which resulted in a damage threshold slightly below the threshold for microbubble formation. At the longer pulse durations of 500 µs and 3 ms, the threshold for microbubble formation was nearly two and three times more than the RPE damage threshold, respectively. Each single RPE sample showed a very sharp and significant damage threshold. The relatively wide distribution of RPE damage thresholds for laser exposure 717.

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Figure 5. Acoustic transients (A, C, E) and the associated measured reflected light signals (B, D, F) during irradiation of porcine RPE samples with single 3-ms laser pulses: (A, B) 8.4 J/cm²; (C, D) 12 J/cm²; (E, F) 16 J/cm².
tion between $ED_{15}$ and $ED_{85}$ is due to the variation of the sample-specific thresholds.

For determining the primary damage mechanism, the correlation of cell death with bubble formation was calculated for the different laser pulse durations. Only experiments in the RPE damage threshold region $ED_{10}$ to $ED_{90}$ were included. By limiting the analysis to the threshold region $ED_{10}$ to $ED_{90}$, only the primary damage mechanism in the region slightly wider than the width of the normal distribution with logarithmic covariant basis was evaluated. Effects such as bubble formation at threefold $ED_{50}$ exposure as seen with 3-ms laser pulses in Figures 5E and 5F were excluded. At all laser pulse durations, the results were sorted into the frequency of RPE damage, with and without acoustically detectable microbubble formation (Fig. 8). This classification shows, that if RPE cells are damaged, microbubble formation can always be detected with a 5-μs laser pulse duration. At 50-μs and 3-ms pulses, the RPE cells were damaged without microbubble formation in the threshold range of $ED_{10}$ to $ED_{90}$.

**DISCUSSION**

**Detection of Microbubble Formation**

It has been shown by Rögener et al. that the detection of intracellular microbubble formation during laser irradiation of RPE samples is possible by monitoring the back-reflected light. We used two physically independent methods for the detection of microbubble formation during laser exposure of the RPE. The simultaneous detection of microbubble formation by an increase in reflected light signal and also an increase of the acoustic transient clearly indicates microbubble formation (Figs. 3E, 3F). In our experiments, the acoustic detection was more sensitive than the reflected light signal. Microbubble formation was clearly detected by the onset of an acoustic transient without detectable change in the reflected light (Figs. 3C, 3D). Although the reflected-light detection method was less sensitive, it provided additional information over the bubble’s lifetime, as seen in Figures 4C–F. At the short pulse durations of 5 μs, it was not possible to determine the bubble’s lifetime reliably, due to the limited irradiation time.

**RPE Damage Thresholds**

The measured porcine RPE damage threshold of $ED_{50}^{\text{damage}} = 252 \text{ ml/cm}^2$ for single 5-μs laser pulses are in good agreement with data of single 3-μs laser pulses of 232 ml/cm² at 527 nm of Rögener et al. In a more recent study they reported a damage threshold of 412 ml/cm² at 532 nm for single 6-μs laser pulses in a similar experimental system. At all other pulse durations, no RPE or retinal damage exposure thresholds are accessible.

**RPE Damage Mechanism**

In our study, at a 5-μs laser pulse duration, RPE damage always coincided with the formation of microbubbles. It is remarkable that in some cases microbubble formation was detected without RPE cell damage at the 5-μs laser pulse duration (Figs. 3C, 3D). Therefore RPE cells were able to survive the formation of small or few microbubbles. It can be assumed, that this caused a volume increase too small to disrupt the cellular membranes. These rare cases with microbubble formations without cell damage lead to a threshold of microbubble formation below the RPE damage threshold. This is in contrast to the results of

**Table 1. RPE Cell Damage and Microbubble Formation Threshold Data, Confidence Intervals and Probit Slopes from Figure 7**

<table>
<thead>
<tr>
<th>Laser Pulse Duration</th>
<th>Number of Applied Laser Pulses</th>
<th>$ED_{50}$</th>
<th>$ED_{15}$</th>
<th>$ED_{85}$</th>
<th>Probit Slope</th>
<th>$ED_{50}$</th>
<th>$ED_{15}$</th>
<th>$ED_{85}$</th>
<th>Probit Slope</th>
</tr>
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<tbody>
<tr>
<td>5μs</td>
<td>480</td>
<td>252</td>
<td>166</td>
<td>359</td>
<td>8.2</td>
<td>222</td>
<td>168</td>
<td>277</td>
<td>8.4</td>
</tr>
<tr>
<td>50μs</td>
<td>270</td>
<td>439</td>
<td>340</td>
<td>567</td>
<td>9.4</td>
<td>483</td>
<td>396</td>
<td>570</td>
<td>12</td>
</tr>
<tr>
<td>500μs</td>
<td>192</td>
<td>1278</td>
<td>1075</td>
<td>1519</td>
<td>13.8</td>
<td>2242</td>
<td>1871</td>
<td>2615</td>
<td>13.2</td>
</tr>
<tr>
<td>3ms</td>
<td>546</td>
<td>4346</td>
<td>3488</td>
<td>5414</td>
<td>10.2</td>
<td>12106</td>
<td>9397</td>
<td>14815</td>
<td>9.4</td>
</tr>
</tbody>
</table>

ED data are in microjoules per square centimeter.
Rögener et al. 15 with single 6-μs laser pulses. It appears that their reflected light-based detection setup was not sensitive enough to monitor small microbubbles in a manner similar to our setup.

In a recent study, 29 we also demonstrated that the acoustic detection of microbubble formation during patient treatment with a train of 1.7-μs laser pulses for the selective treatment of the RPE (SRT) 2 coincides with the angiographic retinal leakage of fluorescein after treatment. Angiographic leakage in the retina indicates damaged RPE cells or at least damaged tight junctions between the RPE cells, which act as the blood-retina barrier. Because the induced angiographic lesions on the patients retina were ophthalmoscopically invisible, and the overlaying photoreceptors in the treated spot were still functioning, microbubble formation seems to be the primary damage mechanism of the retina in humans at this pulse duration.

At the 50-μs laser pulse duration, our data show that the death of RPE cells without microbubble formation was dominant (Fig. 8). However, damage with microbubble formation was observed in 16% of the irradiated spots with radiant exposure in the range of ED10 to ED90. At the longer laser pulse durations of 500 μs and 3 ms, all cell death occurred without bubble formation (Fig. 8). This is in good correspondence with the data from other studies. 6–9 At these pulse durations, microbubble formation occurred at the twofold (500 μs) to threefold (3 ms) RPE damage threshold and can be clearly stated not to be the primary mechanism of RPE damage.

Our results show that both damage mechanisms merge at laser pulse durations only slightly shorter than 50 μs. This result is in good agreement with the change of damage threshold at the twofold (500 μs) and 3 ms RPE damage threshold, which can be associated with a change in damage mechanism.

CONCLUSION

Acoustic measurements allow a detailed insight into laser-induced RPE damage mechanisms. This technique is extremely sensitive and even allows the detection of microbubble formation inside the RPE cell if no RPE damage occurs. At a 5-μs laser pulse duration, microbubble formation has been shown to be the primary RPE damage mechanism. The point of change from thermomechanical microbubble-induced RPE cell damage to pure thermal RPE denaturation is ~50-μs exposure time. At longer pulse durations, the primary damage mechanism is purely thermal.

References


