Tissue Effects of Subclinical Diode Laser Treatment of the Retina

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**Objective:** To determine whether consistent tissue effects are obtained when laser lesions are produced with a commercially available diode laser that are near the limit of clinical detection at the time of treatment.

**Methods:** Continuous-wave or micropulse diode laser was used to produce clinically undetectable (subthreshold) or barely detectable (threshold) retinal lesions in pigmented rabbits. Tissue effects at intervals after treatment were determined in retinal pigment epithelial (RPE) whole mounts by fluorescence microscopy, and in sections of retina and RPE by light and electron microscopy.

**Results:** Continuous-wave and micropulse laser lesions that were originally clinically undetectable were detectable as zones of pigment mottling after 5 days. By microscopy, compaction and/or swelling was seen in the outer retina, and cells in the RPE monolayer became heterogeneous in size, shape, and pigmentation, but the tissue responses in the outer retina and RPE were variable even within and among lesions in the same eye.

**Conclusions:** Subthreshold energies used to create both continuous-wave and micropulse laser lesions produced variable effects on the RPE and the overlying neurosensory retina. It appears that, near the minimum effective dose of laser irradiation, individual RPE cell heterogeneity becomes detectable as variability in sensitivity to laser injury.

**Clinical Relevance:** As laser energy is reduced to limit collateral tissue damage in clinical applications, it may be difficult to generate reproducible lesions because of heterogeneity among individual cells.


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SINCE Maiman1 first developed a ruby laser, laser design has advanced to the point that lasers are now used to treat many vitreoretinal conditions.2 Argon, krypton, and, more recently, diode lasers have been shown to produce therapeutically useful lesions that have a similar ophthalmoscopic appearance, and that also appear on histological examination of both human and animal eyes to elicit similar tissue effects on the outer retina and retinal pigment epithelium (RPE).3,8 The extent of tissue damage that is required to generate a therapeutic outcome is generally not known. Gray-white burns are used because they can be detected clinically at the time of treatment, and ancillary damage to the retina has been considered acceptable or perhaps unavoidable.

There is now increasing interest in determining whether laser delivery might be refined to obtain therapeutically useful lesions while minimizing unnecessary retinal damage.9-12 To identify a minimal laser treatment for each therapeutic application, it is necessary to know whether subclinical laser lesions limit retinal damage while producing consistent tissue effects. One method that has been developed to spare the retina while ablating the RPE is exposure to repetitive, short-pulse length laser. The use of a laser pulse that is shorter than the thermal relaxation time of the target structure (in this case, RPE melanin) will theoretically produce a temperature rise that is too small to cause prominent collateral damage.12-15 Selective damage to the RPE without damage to the adjacent neurosensory retina in animal eyes has been reported after laser treatment of the retina with repetitive pulse argon green laser and with a micropulse diode laser.13,16,17

It is unclear whether treatment of the retina with lasers, either repetitive short pulse or continuous wave, at subclinical levels generates both reproducible and limited tissue destruction in eyes of variable...
The retinas of mongrel pigmented rabbits were treated by laser at sites inferior to the optic nerve head and medullary ray. Animals were handled in accordance with Association for Research in Vision and Ophthalmology guidelines for the ethical treatment of animals and were anesthetized with ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (6 mg/kg) before all procedures. Eyes were dilated with 1 drop of cyclopentolate hydrochloride and 1 drop of 5% phenylephrine hydrochloride, then a −67-diopter planoconcave lens was placed on the eye.

A micropulse diode laser (810 nm) system (IRIS Oculight SLx MicroPulse, IRIS Medical Instruments Inc, Mountainview, Calif), mounted on a slitlamp, was used to produce 200-µm laser spots. With the micropulse mode enabled, 100 micropulses were delivered during a 200-millisecond pulse envelope, with a 100-microsecond on time and a 1900-microsecond off time. The power was varied as described below to create threshold and subthreshold laser lesions. Continuous-wave laser lesions were also produced by means of a 200-millisecond pulse. For each eye, the energy required to produce threshold and subthreshold lesions was determined by performing test treatments in an area of the inferior retina that was similar in pigmentation to the treatment zone (see below), but outside its boundaries. For micropulse, the energy to produce “threshold” lesions was determined by titrating the energy until a barely detectable gray retinal lesion was obtained. Threshold lesions were not typically visible until approximately 15 to 20 seconds after laser delivery. Clinically undetectable, subthreshold micropulse (mp) laser lesions were produced with laser energies that were 100 mW or 300 mW below threshold (hereafter referred to as mp100 mW and mp300 mW, respectively). For continuous-wave laser treatments, threshold energy was determined by titration in the same way as for micropulse, and subthreshold continuous-wave lesions were produced with laser energy 10 mW below threshold (hereafter referred to as cw10 mW).

To produce the laser lesions, the treatment zone was first demarcated at its corners with 4 white reference burns, each 200 µm, made with the continuous-wave mode, so that the site of subthreshold laser treatment could be subsequently identified for clinical examination and in the tissue preparations. Experimental threshold and subthreshold laser lesions consisted of approximately 100 laser spots delivered in a 10 × 10 grid pattern with the burns spaced confluent.

Fifty-eight laser grids were produced in the eyes of 28 rabbits. Treatment sites were examined at a slitlamp several times within 4 hours of treatment (designated time zero) and again just before death for animals that were maintained for 5 days. Four animals were maintained for 6 weeks after treatment and were examined again at that time. Animals were killed by intracardiac injection of pentobarbital after induction of anesthesia as given above. Laser-treated eyes and untreated control eyes were immediately enucleated and prepared either as whole mounts for en face examination of the RPE by fluorescence microscopy, or for examination of the RPE and retina by light and electron microscopy.

RPE WHOLE MOUNTS

Whole mounts were prepared for fluorescence microscopy within 2 hours of enucleation by removing the anterior segment, vitreous, and retina to produce an eye cup lined by RPE. Tissue was fixed for 60 to 90 minutes at 4°C by filling the eye cup with 3% phosphate-buffered paraformaldehyde. Full-thickness pieces of the inferior retina were excised and small cuts were made through the sclera in the periphery of the tissue pieces to flatten them. The RPE cells were permeabilized by treatment for 10 minutes with 0.1% Triton X100 in phosphate-buffered saline, then tissue pieces were incubated overnight in phosphate-buffered saline containing 2 µg of rhodamine-conjugated phalloidin per millilitre. Staining with phalloidin was used to visualize the circumferential actin bundles of RPE cells to show the size and shape of individual cells. Three specimens at 5 days were costained with MIB-1 antibodies (Immunotech, Westbrook, Me) by indirect immunofluorescence with the use of secondary antibodies conjugated to fluorescein. MIB-1, which recognizes a nuclear protein expressed by cells in all phases of the cell cycle except G0, was used to identify proliferating cells. After staining, tissue pieces were transferred to glass slides, coverslips were mounted with glycerol–phosphate-buffered saline (1:1 [vol/vol]), and the specimens were examined and photographed with an epifluorescence microscope (Leitz, Wetzlar, Germany).

LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

Specimens to be examined by light and/or electron microscopy were first immersed in phosphate-buffered 2% glutaraldehyde for a minimum of 24 hours. The anterior segment and vitreous were removed, then full-thickness pieces of eye wall were dissected from treated or control regions of inferior retina, postfixed in phosphate-buffered 2% osmium tetroxide, dehydrated in a graded alcohol series, and embedded in epoxy resin. Thick sections (1 µm) for light microscopy were stained with toluidine blue O; thin sections for electron microscopy were stained with uranyl acetate–lead citrate.

To determine whether tissue changes after threshold and subthreshold laser treatment were consistent throughout the grid, tissue blocks were serially sectioned for light microscopy between the laser reference spots that demarcated the treatment zone, and approximately 100 sections from each grid were examined.

Selected specimens at time zero and 5 days were examined by electron microscopy. Additionally, selected whole-mount preparations used for fluorescence microscopy of the RPE at time zero were subsequently reprocessed for transmission electron microscopy.

pigmentation similar to those that would be encountered clinically. In this study we used a commercially available diode laser with micropulse capability to photocoagulate the retinas of mongrel pigmented rabbits. Laser sites were
RESULTS

THRESHOLD AND SUBTHRESHOLD MICROPULSE DIODE LASER LESIONS

Ophthalmoscopy

Threshold micropulse lesions were obtained in most eyes with a power setting of 800 mW (range, 500–1100 mW). Grids of laser spots were examined several times during the time zero interval during the first several minutes after laser application. Although all spots within each grid were produced with the same laser energy, the ophthalmoscopic appearance of the spots within a grid was heterogeneous and the heterogeneity became more prominent during the minutes after treatment. For threshold grids, faint retinal whitening was detected during laser application, but after several minutes some spots became more prominent than others. Subthreshold grids were not clinically detectable at the time of laser application. However, similar to the threshold lesions, there was evidence of increasing tissue effects after laser treatment, seen as a gradual appearance of clinically detectable spots in some mp100-mW and mp300-mW subthreshold grids. Fundus photographs at time zero were not obtained because the contact lens lubricant that remained on the cornea after laser treatment caused image distortion.

Five days after laser treatment, pigmentary changes were observed in the laser grids. For both threshold and subthreshold grids, the pigmentary changes at 5 days varied from spot to spot and from region to region within the same grid as well as among grids. Some pigmentary changes could be found in at least some spots in all subthreshold grids at 5 days, even in grids that showed no clinically detectable spots several minutes after laser treatment at the time zero interval. Pigmentary changes ranged from subtle mottling to prominent hypopigmented spots. To illustrate the heterogeneity in ophthalmoscopic appearance, 3 mp100-mW subthreshold grids at 5 days are shown (Figure 1). Grids examined after 6 weeks showed similar variations in the ophthalmoscopic appearance of pigmentary changes (not shown).

RPE Whole Mounts

Rhodamine phalloidin treatment of RPE cells stains the dominant circumferential actin bundles at the perimeter of the cells, as well as the lesser amounts of F-actin in the central cytoplasm. In untreated control regions of the monolayer, the cell-to-cell staining was fairly uniform, and the size and shape of the cells was also usually fairly uniform (Figure 2, A). Patches of morphologically variable cells were, however, seen in the untreated monolayer (Figure 2, B). Occasionally, groups of RPE cells formed rosettes consisting of cells oriented centripetally around a central cell (Figure 2, C); the central cell varied in size from large to small or absent.

At time zero after treatment with either threshold or subthreshold laser energy, there was heterogeneity both among and within the laser spots such that not all spots could be found. Some spots within the threshold laser grids could be identified as areas of damage to the monolayer that resulted from removal of the RPE with the retina when the retina was dissected during preparation of the whole mounts (Figure 3, A). Other spots in both threshold and subthreshold (Figure 3, B) laser grids appeared as regions of darker cells with indistinct cell borders. Transmission electron microscopy of the latter regions indicated that the reduced fluorescence staining for actin within and at the borders of some cells was caused by the removal of just the RPE cell apices; immediately adjacent cells differed in whether the cell apex remained (Figure 3, C).

After 5 days, RPE cells varied in size and shape, as well as in amount and distribution of pigment, in both threshold (Figure 4, A) and mp100-mW subthreshold grids (Figure 4, C). Not all spots in the laser grids were uniformly affected, such that by 5 days the treatment zone consisted of both normal-appearing and phenotypically heterogeneous regions. Proliferation was induced in the RPE monolayer within and immediately adjacent to some threshold laser-treated areas, as indicated by the presence of MIB-1–positive nuclei at 5 days (Figure 4, B and E). At 6 weeks the RPE cells at laser sites differed in pigmentation and size, including fields of small, tightly packed cells (Figure 4, F). No distinct lesions could be detected in whole mounts of mp300mW laser grids. There were regions within the treatment zone where cells were phenotypically heterogeneous, but these could not be distinguished from similar regions in untreated controls.

**Figure 1.** Fundus photographs of 3 mp100-mW (micropulse laser energy that was 300 mW below threshold) subthreshold grids 5 days after laser treatment. In each, the 4 reference spots demarcating the corners of the treatment zone can be seen. A, Pigmentary changes are seen in only a few spots and are very faint. B, More spots are detectable and some spots are fairly prominent. C, Spots are even more prominent, with some complete rows of spots that are detectable.

**Figure 2.** Whole mounts of the control, untreated retinal pigment epithelial monolayer stained with rhodamine phalloidin for F-actin. In much of the monolayer, retinal pigment epithelial cells are fairly homogeneous in size, shape, and pigmentation (A), although in some regions cells are less uniform (B). Rosettes of centripetally oriented retinal pigment epithelial cells, with pigment translocated toward the center of the cell cluster, can be found occasionally in the normal retinal pigment epithelial monolayer (C) (A and B, original magnification ×260; C, ×3000).

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Light and Electron Microscopy

Within 4 hours of laser treatment (the designated time zero interval), the most prominent change in threshold laser grids observed by light microscopy of the retina was in the photoreceptor layer. Some laser spots exhibited compact and densely stained photoreceptor outer segments, disorganized inner segments, and densely stained or occasionally pyknotic nuclei in the outer nuclear layer (Figure 5, A). The compaction of the outer retina distorted the inner retina, giving the entire tissue a scalloped appearance. The photoreceptor outer segments appeared to adhere to the RPE, and by electron microscopy, RPE cells in these sites were thinned with dense cytoplasm (Figure 6, A) or the cytoplasm was cavitated with loss of integrity of the apical membrane (Figure 6, B). Some mp100-mW subthreshold laser lesions at time zero (Figure 5, B) had a similar appearance to threshold spots, whereas other spots produced by the same laser energy showed milder tissue effects (Figure 5, C). Although the most prominent effects were in the outer retina, Müller glial cells in some spots showed changes in staining intensity throughout their length, including at the vitreoretinal interface (Figure 5, B). The mp300-mW spots were difficult to identify in histological section, although infrequently some compaction of outer segments overlying the RPE could be seen, similar to the milder tissue effects produced by mp100-mW treatment, as shown in Figure 5, C. Within grids produced with either threshold or subthreshold laser energies, many treated sites produced no tissue effects, since serial sections of the treated zones did not demonstrate all the laser spots. The variable tissue effects could result from differing uptake of laser energy by RPE cells, which have a normal variation in pigmentation (Figure 7).

Five days after threshold laser treatment (Figure 8, A), the retina continued to be elevated at laser sites, nuclear density was reduced in the outer nuclear layer, and there was occasionally a gliotic response in the outer retina. Macrophages or vitreous cells were frequently seen on the internal limiting membrane directly overlying laser lesion sites. Retinal pigment epithelial cells were found...
in the subretinal space and within the retina; these cells appeared intact by electron microscopy (Figure 9). Similar, though often milder, changes in the retina and RPE were found in mp100-mW subthreshold laser lesions at 5 days (Figure 8, B). Few mp300-mW laser spots were detected at 5 days, and, when present, they appeared as regions of scalloped outer retina with mildly compacted photoreceptor outer segments (Figure 8, C), similar to lesions at time zero. The microscopic appearance of threshold and mp100-mW subthreshold laser lesions at 6 weeks was similar to that at 5 days (not shown). In a few mp300-mW subthreshold lesion sites, there appeared to be recovery of the normal architecture of photoreceptor outer segments (Figure 8, D) as compared with the disorganization seen at earlier intervals (Figure 8, C).

THRESHOLD AND SUBTHRESHOLD CONTINUOUS-WAVE DIODE LASER LESIONS

Threshold continuous-wave lesions were obtained with a power setting of 60 or 70 mW. Tissue effects produced by continuous-wave laser treatment at threshold and subthreshold energy levels were similar to those produced by micropulse laser. Like the micropulse laser lesions, retinal lesions resulting from low-energy continuous-wave laser also tended to be heterogeneous in appearance both among spots in a grid and within individual spots. At time zero, tissue effects included distortion of the retina, increased staining intensity for nuclei in the outer nuclear layer, and compaction of photoreceptor outer segments. Simi-
Receptor outer segments adjacent to the RPE (Figure 10, A). At 5 days, RPE cells in laser-treated sites varied in size, shape, and pigmentation, as seen in whole mounts (Figure 10, B). In tissue section, the number of nuclei was reduced in the outer nuclear layer, a gliotic response was seen in the outer retina, and RPE cells were seen in the subretinal space and within the retina (Figure 10, C).

**COMMENT**

The goal of this study was to determine whether a commercially available diode laser could produce consistent tissue effects when the laser lesions at the time of treatment were clinically barely detectable or undetectable. We selected the diode laser, because it is a commercially available instrument that can be used in either continuous-wave or micropulse mode, and it has been used in both modes in a human clinical trial. In the micropulse mode it has been reported to produce lesions that are restricted to the RPE while sparing the overlying neurosensory retina.

We developed a protocol in which the laser energy was titrated for each eye to produce a lesion that was barely visible as a faint gray spot to an experienced operator. Then, with the use of this threshold energy and defined subthreshold energies, grids of lesions were produced in relatively small retinal regions of grossly similar pigmentation. Despite our efforts to control the laser treatment, the most striking feature of the results was the variability in the tissue effects among laser spots within a grid. Threshold laser treatments differed from the subthreshold treatments not so much in the extent of tissue damage, but rather in the number of treatment spots within a grid that sustained any detectable damage. The low energies used in this study appear to be at the borderline of levels needed to induce any tissue injury, resulting in considerable morphologic heterogeneity. If, as might be expected, differences in laser focus contribute to some of the variability between spots, then focus is having a significant impact at low laser energies. Even in immediately adjacent spots, 1 spot can be microscopically undetectable while the neighboring spot has tissue changes involving the entire outer retina. As discussed below, differences were observed even within a single spot between adjacent RPE cells. These cannot be attributed to laser delivery and are likely to result from a biological microheterogeneity among individual cells.

When a tissue effect of laser treatment was seen, the type of tissue injury was similar for both threshold and subthreshold laser energies, and for both micropulse or continuous-wave modes. The outer sensory retina and RPE were most prominently affected, where compaction of photoreceptors appeared to cause tenting of the retina. Effects on the inner retinal layers were limited to changes in the staining properties of some Müller glial cells. Despite the restricted nature of the gross tissue injury, a focal inflammatory response was induced that lasted for at least 6 weeks, as indicated by the presence of clusters of macrophages at the inner limiting membrane of laser sites.

For the lowest energy treatment used here (mp300 mW), only a few affected sites could be found on microscopic examination. In these, photoreceptor damage was minor and may have allowed some outer segment recovery over time. At 6 weeks, when retinal tenting could still be used to identify laser sites, rare mp300-mW sites showed photoreceptor outer segments of normal orientation and length.

The variable tissue response to low laser energies even within laser spots suggests that heterogeneity among individual RPE cells contributed to the variable outcome, since RPE pigment is the primary structure to absorb laser energy. Retinal pigment epithelial cells are considered homogeneous in phenotype, but in whole-mount preparations of the type used here, variations in RPE cell size, shape, and pigmentation can be seen in animal as well as human eyes. This phenotypic variation is difficult to appreciate in microscopic cross sections, but it is apparent in whole mounts where entire cells rather than parts of cells can be seen, and broad expanses of the tissue can be viewed simultaneously. An incidental finding in this study was the presence of occasional rosettes of cells in monolayers of adult rabbit RPE. Similar cell groupings were reported in developing chick RPE, where they were interpreted as a response of adjacent RPE cells to the death of the centrally located cell. The presence of rosettes in eyes of normal adult animals suggests that there may be ongoing turnover of cells within the RPE monolayer beyond the early developmental period.

Retinal pigment epithelial cell-cell heterogeneity in the response to laser treatment was detectable in stained whole mounts, where it was manifest at time zero as variable reductions in actin fluorescence in cells within laser spots. Transmission electron microscopy of the whole-mount preparations showed that reduced fluorescence was caused by the absence of RPE cell apices in affected cells at the laser sites. We interpret this result to indicate that the apices of some but not all RPE cells became adherent to the compacted photoreceptor outer segments after laser treatment, causing the tops of the RPE cells, including the circumferential actin bundles that define the cell borders, to be removed when the retina is separated from the RPE cells during preparation of the tissue pieces.
At 5 days after laser treatment, RPE cells in laser-treated zones showed a variety of responses to the earlier treatment. In microscopic sections, some cells were swollen and others flattened, and still others had detached from the Bruch membrane and appeared to be intact within the subretinal space or sensory retina. In the whole mounts, cells attached to the Bruch membrane were seen to vary in size and shape. These cells appear to have migrated to restore contiguity to the RPE monolayer. MIB-1 staining indicated that proliferation contributed to RPE cell repair and confirmed that laser treatment, even with the low levels used here, induces RPE cell growth.

The variability in tissue effects when laser treatment is applied at low, subclinical energy levels makes it difficult to define a protocol that will ensure a minimum effective dose of laser energy to produce a particular clinical outcome. We were unable to identify affected RPE cells adjacent to unaffected retinal outer segments with the use of the diode laser in the micropulse mode, although restriction of damage to the RPE cells with micropulse treatment has been previously reported for this laser. It appears that when the energy used is adequate to affect the RPE cells even minimally, the sensory retina also sustains some damage. The effectiveness of threshold burns with this laser in the treatment of diabetic macular edema should probably not, it does not explain the cell-to-cell variation in the effect of the laser on the RPE cells themselves. It appears that near the minimum effective dose of laser irradiation, individual RPE cell variation becomes detectable as a heterogeneity in sensitivity to laser injury. Even with shorter-duration micropulses it may be difficult to generate reproducible lesions because equivalent amounts of laser energy are likely to damage some RPE cells while leaving immediately adjacent cells unaffected.

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