Subthreshold Transpupillary Thermotherapy Reduces Experimental Choroidal Neovascularization in the Mouse without Collateral Damage to the Neural Retina

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PURPOSE. Transpupillary thermotherapy (TTT) is currently being evaluated for treatment of choroidal neovascularization (CNV) in age-related macular degeneration. To optimize TTT for CNV, the effect was analyzed of invisible (subthreshold) or visible (threshold) doses of TTT on the normal mouse retina and on experimental CNV.

METHODS. TTT was delivered to the normal retina of 42 mice with a diode laser at increasing power settings (50, 60, 70, or 80 mW), to obtain thermal lesions ranging from invisible (subthreshold) to visible (threshold) burns. CNV was induced in 53 mice by krypton laser photocoagulation of the fundus, after which the CNV lesions were treated with TTT (50, 60, or 80 mW). Eyes were enucleated 7 days after TTT and prepared for histology; and the CNV complex was evaluated on hematoxylin-eosin stained serial sections by measuring the maximum height of the CNV lesions. Ultrastructural changes were examined by transmission electron microscopy.

RESULTS. Increasing the TTT laser power yielded gradually more visible effects. At 50 mW, which induced subthreshold burns, no damage was seen in the neural retina, retinal pigment epithelium (RPE), or choroid at any time point. By contrast, eyes treated with higher power exhibited progressively more damage to the neural retina, including a complete disruption of the outer nuclear layer. When TTT was applied to the laser-induced CNV lesions, the height of lesions was significantly reduced (P < 0.001) in response to all three power settings at 7 days after treatment. The mean relative thickness of the CNV lesion was 3.29 ± 0.89 in untreated mice, whereas in TTT-treated mice it was 1.69 ± 0.35, 1.69 ± 0.41 and 1.70 ± 0.17 at power settings of 50, 60, and 80 mW, respectively. The overlying neural retina showed no apparent damage with the 50- or 60-mW settings, whereas outer nuclear layer disruption occurred with a power of 80 mW. Electron microscopy confirmed the presence of vascular occlusion at 1 day and a fibrotic scar at 7 days after TTT.

CONCLUSIONS. Subthreshold TTT can effectively occlude newly formed vessels and cause regression of the experimental CNV complex without damaging the neural retina. The results demonstrate the importance of using subthreshold laser power in experimental and clinical evaluation of TTT. (Invest Ophthalmol Vis Sci. 2004;45:1969–1974) DOI:10.1167/iovs.03-1329

In the Western world, age-related macular degeneration (AMD) is a leading cause of central vision loss in patients older than 60 years. AMD can be subgroups into exudative and nonexudative forms, depending on the formation of choroidal neovascularization (CNV). Despite a lower prevalence of exudative AMD compared with nonexudative AMD, approximately 80% of severe vision loss occurs secondary to the formation of CNV. Although laser photocoagulation for some forms of CNV reduces the incidence of severe visual loss, photocoagulation damages the overlying neural retina, resulting in an immediate decline of visual function corresponding to the laser-treated area.

A new therapeutic era in photodynamic therapy (PDT) was started by the use of a photosensitizing dye (verteporfin) and a low laser power setting. PDT can close newly formed subretinal vessels without substantial damage to the neural retina, and its efficacy of occluding subfoveal (predominantly classic) CNV is well documented. Transpupillary thermotherapy (TTT) is a technique in which vascular occlusion can be induced without the use of photosensitizing dye, by delivering radiation at near infrared intensity (810 nm) to the target tissue through the pupil. TTT is successfully used as an adjunctive treatment for choroidal melanoma. However, TTT in this setting usually causes localized retinal destruction, retinal vascular occlusions, and nerve fiber bundle defects. Several reports indicate that TTT may successfully treat CNV in patients with AMD, resulting in a high closure rate and resolution of the CNV complex without apparent retinal complications.

The encouraging results in these pilot studies have led to the initiation of a multicenter prospective randomized clinical trial of patients with subfoveal CNV (TTT4CNV study). In TTT, a low increase in temperature (10°C), maintained for 60 seconds, is used to treat CNV. However, there is controversy about the clinical safety of TTT for CNV. This limits the possibility of applying an optimal dose of TTT, making under- and overtreatment a potential problem. Consequently, reports of neuroretinal and RPE damage after TTT for CNV have been published. Clinical experience with the use of TTT for CNV suggests that it is important to use subthreshold power (i.e., biomicroscopically invisible laser effects), to avoid damage to the overlying neural retina, but the current treatment regimen remains largely empiric. To assess further the feasibility of the clinical TTT management of CNV, the basic mechanisms by which TTT affects the normal retina and CNV should be explored.

In the present study, we analyzed the effect of TTT on the normal mouse retina and on experimental CNV in response to...
different laser power settings that deliver subthreshold or threshold doses.

**Material and Methods**

**TTF of Normal Retina and Choroid**

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines established by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. C57BL/6j mice (18–20 weeks old, body weight 25–30 g) were anesthetized by intramuscular injection of 0.04 mL of a 5:2 mixture of 50 mg/mL ketamine and 20 mg/mL xylazine. Pupils were dilated with 1% tropicamide. TTT was delivered through a slit lamp (model 30 SL-M; Carl Zeiss Meditec; Oberkochen, Germany) by a trimode infrared diode laser emitting at 810 nm (Iris Medical Instrument, Inc., Mountain View, CA). To avoid variations in output power, the diode laser was calibrated before and after the experimental series. The treatment was performed with a beam diameter of 1.2 mm for 60 seconds with a handheld contact lens and a viscous surface lubricant. With this setup, the contact lens adheres to the corneal surface, making it possible to avoid compression of the eye. To obtain thermal burns ranging from invisible to visible, power settings of 50, 60, 70, and 80 mW were used. For each power setting, series of four laser spots were delivered to the posterior pole of the retina. Two mice died during anesthesia and were excluded. Eyes were enucleated at 1, 3, 7, 14, and 28 days, respectively, and fixed in 4% formaldehyde at room temperature. Forty-two mice were analyzed (two mice were analyzed for each power setting and time point and two mice died).

**Induction of Experimental CNV in Mice**

CNV was generated by krypton laser-induced rupture of Bruch’s membrane, essentially as previously described. Briefly, C57BL/6j mice (18–20 weeks old) were fixed on a rack connected to the slit lamp delivery system. Four krypton laser photocoagulation burns (50-μm spot size, 0.1-second duration, 120-mW power) were induced in each eye, through a handheld contact lens (647 nm; Spectra-Physics 265 exciter; Lasertek, Helsinki, Finland). Only eyes in which a subretinal bubble was formed for each burn were included in the study. Fifty-three mice were used in the CNV experiments, 40 of which were processed for quantitative histologic analysis (14 mice served as control subjects without subsequent TTT treatment, 10 mice received a TTT power of 50 mW, 8 a power of 60 mW, and 8 a power of 80 mW), whereas 13 mice were processed for transmission electron microscopy.

**TTF of the Experimental CNV Complex**

TTF was performed 6 days after krypton laser induction of CNV, using the same procedure as described earlier, at a power setting of 50, 60, or 80 mW. Some of the eyes were enucleated 1 day after TTF, fixed in 4% glutaraldehyde at 4°C overnight, and further processed for transmission electron microscopy. The other eyes were enucleated at 7 days after TTF, fixed either in 4% formaldehyde at room temperature or in 4% glutaraldehyde at 4°C overnight, and further processed for light or transmission electron microscopy. Studies have shown that experimentally induced CNV in mice is at its maximum 10 to 14 days after krypton laser treatment, with a slow, spontaneous regression occurring after this time point. For this reason we chose to limit the experimental end point to 13 days.

**Transmission Electron Microscopy**

Glutaraldehyde fixed eyes were washed in 0.1 M cacodylate buffer (pH 7.4) after removal of the cornea and lens. The TTF-treated area was selected and dissected into 2 x 2-mm pieces. The tissue samples were postfixed in 1% osmium tetroxide, dehydrated in graded alcohol, and embedded in agar (Agar-DDSA-MNA-BDMA; Agar Scientific Ltd, Stansted, UK). For light microscopy, 1-μm sections were stained with 1% toluidine blue in 1% borate buffer. For transmission electron microscopy, 100-nm sections were stained with a saturated aqueous uranyl acetate solution and lead nitrate. The sections were examined with a transmission electron microscope (JEM-1230; JEOL, Tokyo, Japan).

**Quantitative Analysis of Experimental CNV**

Formalin fixed eyes were embedded in paraffin and serial sections (4 μm thick) were cut throughout the entire extent of each laser burn and stained with hematoxylin-eosin. To evaluate the effect of TTF on CNV membranes, hematoxylin-eosin-stained serial sections were examined at 200× magnification with a light microscope (Axioskop; Carl Zeiss Meditec) and a digital color camera (Axiomat; Carl Zeiss Meditec), as previously described. For each section, the observer was masked to the power setting used. The maximum thickness of CNV complex was estimated indirectly by measuring the difference between the thickness from the outer border of the pigmented choroidal layer to the top of the CNV complex (T) and the thickness of the intact, pigmented choroid adjacent to the lesion (C). Five to 10 serial sections from each CNV membrane were measured, and the highest value (representing the top of a given CNV complex) was stored. The stored data from each mouse were pooled, and a mean T and C were calculated. The relative thickness of the CNV membrane complex in each mouse was then calculated using the formula \( T = C/C \).

**Statistical Analysis**

Data were normally distributed and the two-sided t-test for unpaired data was applied for statistical analysis on computer (Medcalc, ver. 1.0; Medcalc, Mariakerke, Belgium). \( P < 0.05 \) was considered statistically significant.

**Results**

**Morphology of TTF of Normal Choroid and Retina**

Increasing the laser power of the TTF resulted in gradually more visible effects on the retina. No apparent color change was seen in any treated area during or immediately after TTF with a power of 50 mW. In contrast, the retinas of all eyes treated with a power of 80 mW showed whitening.

Light microscopy revealed no changes in the areas treated with 50 mW at any time point after TTF (Fig. 1A). A slight edema of the photoreceptor layer was found in the area treated with 60 mW 3 days after TTF (Fig. 1B) and 14 days after TTF the outer segments were slightly shorter than those in the adjacent untreated retina (Fig. 1C). No apparent damage to the RPE and choroid was found at any time point with 60 mW.

When a power of 80 mW was used, pyknotis and vacuolization of cells in the inner and outer nuclear layers of the retina were observed 1 day after TTF (Fig. 1D). The RPE was thickened, and thrombus formation was frequently seen in the lumen of the choroidal capillaries. Macrophage-like cells with pigment granules (presumably melanin) had accumulated in the inner nuclear layer at 7 days after TTF treatment, and the outer nuclear layer had disintegrated (Figs. 1E, 1F). Treatment with 70 mW resulted in similar but less pronounced neural retinal damage than that with 80 mW (not shown).

**Morphology of Untreated CNV**

Krypton laser treatment of the mouse fundus resulted in gross destruction of the RPE and Bruch’s membrane and the formation of a dome-shaped CNV complex containing fibrovascular tissue with vascular endothelial cells and scattered RPE cells (Fig. 2A). Numerous vascular channels that appeared to emerge from the underlying choroid were detected. The sur-
face of the CNV complex was frequently enveloped by an incomplete layer of RPE cells. The neural retina overlying the CNV membranes showed minor damage, mainly confined to a shortening of the outer segments.

Ultrastructural analysis of the CNV complex confirmed the presence of open vascular channels lined with endothelial cells and occasional intraluminal erythrocytes (Fig. 3A). The intervening spaces between the vascular channels were occupied by fibroblasts, proliferating RPE cells, and collagen.

Morphology of CNV Managed by TTT

Histologic examination of CNV membranes at 7 days after TTT with either subthreshold (50 or 60 mW) or threshold (80 mW) doses gave similar results, displaying marked thinning of the treated CNV complex compared with the untreated control subjects (Figs. 2B–D). All membranes managed by TTT showed basically the same overall histology, with pronounced fibrotic scar formation. Occasional patent vascular channels could be detected within the scar tissue. When the dose of TTT was increased from subthreshold to threshold, marked collateral damage occurred in the neural retina, including complete disintegration of the photoreceptors (Fig. 2D). In contrast, when subthreshold doses were used, no apparent damage to the neural retina overlying the CNV complex was seen (Figs. 2B, 2C).

Ultrastructural analysis of CNV membranes managed by TTT confirmed acute closure of the newly formed vascular channels 1 day after treatment when using a power of either 50 or 80 mW (Figs. 3B, 3C). Vacuolization of the endothelial cells, release of fibrin into the extravascular space and thrombus formation within the lumen of the vessels in the CNV complex were present. By 7 days after TTT, a fibrotic scar was present with few cellular components and no apparent vessels (not shown).

Statistical Analysis of TTT-Treated CNV

After TTT, quantitative morphometric analysis showed a reduction in the relative thickness \( \left( \frac{T}{H_{1102C/C}} \right) \) of the CNV complex by approximately 50%, with a power setting of 50, 60, or 80 mW \( (P < 0.001, \text{Fig. } 4) \). The mean relative thickness of the CNV lesion was 3.29 \( \pm \) 0.89 in untreated mice, whereas in TTT-treated mice, the mean relative thicknesses were 1.69 \( \pm \) 0.35, 1.69 \( \pm \) 0.4, 1.70 \( \pm \) 0.17 with power settings of 50, 60 and 80 mW, respectively. In the untreated CNV control group, the combined height of the CNV complex and underlying choroid (T) ranged from 64.4 to 129.1 \( \mu m \) (mean 93.1 \( \mu m \)), whereas the height of the adjacent choroid (C) ranged from 19.9 to 23.7 \( \mu m \) (mean 21.9 \( \mu m \)). The mean thickness of the CNV complex (T – C) was 71.2 \( \mu m \) in the control group.

Discussion

The presented experiments show that there is a correlation between the biomicroscopically visible effects in the fundus,
such as blanching of the retina and morphologic damage to the neural retina in response to TTT. It was further found that TTT induced a statistically significant decrease in the total thickness of the CNV complex. In addition, we observed that the regression of the CNV complex could be induced with a laser power setting that did not cause any apparent damage to the neural retina.

Conventional laser photocoagulation uses brief 40°C to 60°C temperature increases to produce visible lesions. TTT yields lower (10°C) temperature increases and maintains them for 60 seconds. The infrared irradiation of 810 nm is absorbed mainly in pigmented tissue, such as melanin in the RPE and choroidal melanocytes, and the absorption in the neural retina is comparatively low. Using a noninvasive technique with thermosensitive liposomes, it was recently shown that TTT of the normal rat retina requires a higher power than that for the choroid to achieve a temperature increase to 46°C and 47°C.

It has been assumed that as long as no biomicroscopically detectable retinal whitening occurs, no severe damage to the neural retina develops in response to TTT. Our experiments demonstrate that, indeed, no histologic damage to the neural retina was induced by the laser power that was biomicroscopically assessed to be subthreshold. This is in accordance with findings in other experiments using monoclonal antibodies to detect heat shock proteins, in which subthreshold TTT (no whitening of the retina) induced the expression of heat shock proteins in the choroid but not in neural retina.

A recent report indicates that the focal (f)ERG amplitude decreases transiently during TTT (and recovers after surgery) despite the absence of ophthalmoscopically apparent lesions. These results are also supported by the effect of TTT on the normal human macula in an eye that was scheduled for enucleation due to malignant choroidal melanoma. There was a transient decline of visual acuity immediately after TTT (800 mW, 3.0-mm spot), but visual acuity recovered completely within 5 days, and the RPE and neural retina remained well preserved, showing only minor ultrastructural changes in the photoreceptor outer segments.

Our results, based on serial sections of experimentally induced neovascular membranes, show that TTT resulted in regression of the CNV complex, decreasing the total thickness approximately 50% (P < 0.001) compared with the control eyes at 7 days after treatment. These results were obtained in response to a TTT power setting of 80, 60, and 50 mW, respectively, and indicate closure of newly formed vessels over a reasonably wide range of laser intensities without any appreciable difference in the rate of vascular occlusions with these power settings. The end point was a fibrotic scar with all the power settings used.

In the treatment of CNV, sparing the neural retina from radiation injury is of fundamental importance. High-power TTT (80 mW) induced considerable damage in the neural retina with destruction of the outer nuclear layer in CNV-induced eyes. TTT to normal mouse eyes (without induction of CNV) confirmed the results. When reducing the power to 50 mW, the neural retina was well preserved, the photoreceptor nuclei and inner segments looked normal and the outer segments revealed only a slight edema in circumscribed areas of the specimen.

It appears that the therapeutic window of TTT is quite narrow, confined to the range of power where the newly formed vessels are occluded and the neural retina remains undamaged. The efficacy of TTT to close CNV is dependent on several factors, such as the pigmentation of the tissues of the fundus, the amount of subretinal fluid or hemorrhage, and probably the thickness of the CNV complex, factors that have not been addressed in this study. It has been demonstrated in human eyes that a given laser power induces no histopathologic alterations in a lightly pigmented fundus, whereas the same laser power causes full-thickness retinal alterations along with changes in the RPE and choroids of a more pigmented fundus.
The mechanisms for the TTT-induced vascular occlusion are not fully elucidated. It has been suggested that TTT induces vascular changes mediated by heat shock proteins, leading to apoptosis of endothelial cells and vascular thrombosis.\textsuperscript{15} Immunoreactivity to heat shock proteins was reported to be present in vascular endothelial cells in response to 810-nm irradiation.\textsuperscript{17} In experimental CNV in rat and monkey eyes, PDT with verteporfin induced closure of the CNV, with damage to endothelial cells, thrombus formation, and disruption of the neovascular tissue by 24 hours.\textsuperscript{21,22}

There seems to be some similarity between the pathophysiologic response after TTT and PDT. In human eyes with neovascular AMD, TTT was associated with transiently decreased blood flow in the retinal circulation 24 hours after treatment, but this decrease was not sustained. At 1 month there were no significant differences in the retinal blood flow.\textsuperscript{23} In contrast, increased vascular leakage was demonstrated on angiography within 1 hour after TTT, but absence of leakage was noted at 1 and 2 weeks.\textsuperscript{24} Similarly, PDT also initially caused a breakdown of vascular barrier.\textsuperscript{25}

In conclusion, subthreshold TTT can effectively occlude newly formed vessels and cause experimental CNV to regress, without damaging the neural retina. The results strengthen the theoretical basis for the mode of TTT action and demonstrating the importance of using subthreshold laser power in experimental and clinical evaluation of TTT.

Acknowledgments

The authors thank Berit Spångberg, Anne Winter-Vernersson, Margareta Oskarsson, Monica Aronsson, and Barbro Larsson for excellent technical support.

References


