Histological Effect and Protein Expression in Subthreshold Transpupillary Thermotherapy in Rabbit Eyes

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Objective: To investigate the histological effect of subthreshold transpupillary thermotherapy (TTT) on the retina.

Methods: We performed TTT in normal pigmented rabbit eyes using an 810-nm diode laser with spot size of 1.2 mm, power of 50 mW, and varying durations of 15, 30, or 60 seconds. Four weeks later, fluorescein angiography was performed, and the enucleated eyes were examined by means of electron microscopy and immunohistochemical staining.

Results: Funduscopy immediately and at 4 weeks showed no discernable changes at TTT sites, and fluorescein angiography at 4 weeks showed no abnormalities. However, electron microscopy showed photoreceptor and retinal pigment epithelium cell disruption, changes more prominent with longer durations of treatment. Immunohistochemical staining was positive for heat shock protein 60, heat shock protein 70, tumor necrosis factor α, and vascular cell adhesion molecule 1 in the photoreceptors and retinal pigment epithelium at TTT sites. Untreated control eyes showed no staining.

Conclusions: Despite the absence of changes evident by funduscopy and fluorescein angiography, TTT resulted in dose-dependent histological changes in photoreceptors and retinal pigment epithelium. The induction of heat shock proteins, cytokines, and cell adhesion molecules may play a role in the tissue response to subthreshold TTT.

Clinical Relevance: Unrecognized damage to the retina and retinal pigment epithelium may contribute to visual loss in eyes that undergo subthreshold TTT.

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SUBTHRESHOLD TRANSPUPILLARY thermotherapy (TTT) using the 810-nm diode laser represents a method for delivering low-energy, large-spot-size hyperthermia to the retina and choroid. Recently, TTT has been used with some success to reduce exudation associated with subfoveal choroidal neovascularization (CNV) in age-related macular degeneration.1,2 It has been estimated that the temperature elevation one can expect with subthreshold TTT is 4°C to 10°C.3,4 However, the exact temperature elevation achieved with such therapy, the influence of various clinical variables on treatment, and dose-dependent histopathological changes that occur with subthreshold TTT are not known. Furthermore, although some researchers have suggested that heat shock protein (HSP) and apoptosis may play a role in the tissue response to TTT,4,5 the mechanism of action of TTT in eyes with CNV remains largely unknown.

METHODS

ANIMALS

We used rabbits weighing approximately 2 kg with normal pigmented eyes. All animals were housed and experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research. General anesthesia was induced by intramuscular injection of 30 mg/kg ketamine hydrochloride and 6 mg/kg xylazine hydro-
chloride. Pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine eye drops.

DETERMINATION OF LASER SETTINGS

We performed TTT using an 810-nm diode laser (OcuLight SLx; IRIS Medical Instruments, Inc, Mountain View, Calif) fitted with a TTT slitlamp adapter (SLALS; IRIS Medical Instruments Inc). In all laser applications, we used a Goldmann laser–coated contact lens (−67 diopters) with a magnification factor of approximately 1.247 in rabbits with 810-nm radiation calculated using values from Murphy and Howland6 (Martin A. Mainster, PhD, MD, e-mail communication, May 27, 2002). First, we investigated the power required to create a funduscopically visible laser burn (threshold application). Using a spot size of 1.2 mm and a power setting of 75 mW or greater, a 60-second TTT application produced whitening of the retina. However, at a power of 50 mW with the same spot size and duration, no funduscopically visible change to the retina was observed (subthreshold application). We confirmed this subthreshold power setting in several different animals. Because 50 mW was the lowest power setting available on the laser system used, duration of treatment was changed to vary the total laser energy dose in the subthreshold applications in this study.

TTT PROCEDURE

Before TTT, 2 strong photocoagulative burns spaced slightly apart were created using the diode laser (size, 50 mm; power, 800 mW; duration, 0.2 seconds) in the posterior pole to serve as markers for use in subsequent histopathological processing. This was followed by 3 subthreshold applications (3 spots) of TTT placed in a slightly overlapping fashion between the 2 marker burns. Laser settings were as follows: diameter of 1.2 mm; power of 50 mW; and duration of 15, 30, or 60 seconds (with a fluence of 71, 143, or 286 J/cm², respectively). Color fundus photographs were taken immediately after TTT (A) or at 4 weeks (B). Fluorescein angiography at 4 weeks did not show any hyperfluorescence or hypofluorescence (C).

HISTOPATHOLOGICAL EXAMINATION

Freshly enucleated eyes were fixed in 2.5% glutaraldehyde and 2% formaldehyde for 48 hours. Sections of the posterior pole were stained with toluidine blue and examined by means of light microscopy. Samples postfixed in 1% buffered osmium tetroxide and stained with uranyl were examined by means of transmission electron microscopy (JEM-1010; JEOL, Ltd, Tokyo, Japan). For immunohistochemistry, sections were blocked and incubated with primary antibody against HSP60, HSP70, VCAM-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), or TNF-α (Technne Corp, Minneapolis, Minn). Sections were then washed, incubated with secondary antibody using fluorescein isothiocyanate–conjugated anti–goat IgG (Vector Laboratories Inc, Burlingame, Calif), and viewed with a fluorescence microscope (Model BX50; Olympus Corp, Tokyo). Normal untreated eyes used for control samples were prepared in a similar manner for light and electron microscopy and for immunohistochemical staining.

RESULTS

FUNDUSCOPY AND FLUORESCEN einmal

Ophthalmoscopically, no visible change to the retina was observed for all durations of treatment, immediately or at 4 weeks after TTT application (Figure 1A and B), with the exception of 1 eye with relatively heavy fundus pigmentation that underwent TTT for a duration of 60 seconds. Development of some pigmenitary changes was observed by 4 weeks after TTT, and this eye was not included in the histopathological examination results. Regardless, fluorescein angiography performed at 4 weeks showed no hypofluorescence or hyperfluorescence in the area of TTT application in this eye or in any other eye (Figure 1C).

LIGHT MICROSCOPY

In comparison with control eyes (Figure 2A), disruption of the normal configuration of photoreceptor outer segments was observed at 4 weeks after TTT application, even with the shortest duration of treatment of 15 seconds (Figure 2B). This disruption was increasingly more pronounced at 30 and 60 seconds of treatment (Figure 2C and D), with observation of fewer cell nuclei and overall thinning of the outer nuclear layer. In addition, with progressive TTT duration, decreased density of nuclei in the outer nuclear layer and greater pigmentation in the retinal pigment epithelium (RPE) were noted. No changes were observed in the inner layers of the retina or in the choroid for any duration of treatment.
TRANSMISSION ELECTRON MICROSCOPY

At low magnification, overall thinning of the photoreceptor layer with disruption of the outer and inner segments was observed, even at the shortest TTT duration of 15 seconds compared with controls (Figure 3A and B). With 60 seconds of treatment, the loss and disruption of photoreceptors were more pronounced (Figure 3C). Higher magnification showed vacuolization and disruption of photoreceptor outer segments, with disruption of the lamellar structures of photoreceptors and apical microvilli of RPE cells at 15 (Figure 3D) and 30 seconds (not shown) of TTT duration. The normal basal infoldings of RPE cells appeared to be preserved. No changes were observed in Bruch’s membrane or the anterior choroid for any duration of treatment.

IMMUNOHISTOCHEMISTRY

Results of tests for all antibodies were negative in all untreated controls (Figure 4A, D, F, and H). Staining for HSP60 was observed in the RPE and the inner segments of photoreceptors in eyes that received 30 seconds of TTT (Figure 4B), with the addition of staining for HSP60 observed in the photoreceptor outer segments at 60 seconds of treatment (Figure 4C). Staining for HSP70, TNF-α, and VCAM-1 was observed in the RPE and photoreceptor outer and inner segments for 30 and 60 seconds of TTT, with the staining appearing more pronounced for 60 seconds of TTT (Figure 4E, G, and I; data for 30 seconds of TTT are not shown). No staining for HSP60, HSP70, TNF-α, or VCAM-1 was observed in the inner layers of the retina, the outer nuclear layer, or the choroid for any duration of treatment.

COMMENT

Despite reports that TTT leads to decreased exudation in patients with CNV in age-related macular degeneration, the mechanism of action of this therapy has yet to be delineated. Because little or no color change or burn is observed in the retina immediately after the subthreshold treatment involved in TTT, it is clear that the tissue effects differ from those of conventional laser photocoagulation. In addition, it is presumed that the higher wavelength of the infrared laser used (810 nm) would result in deeper tissue penetration and, consequently, less damage to the sensory retina when compared with photocoagulation using the argon or the dye laser. Thus, it has been suggested that TTT may be an ideal treatment for lesions involving the center of the fovea.

The present study shows that, even in the absence of funduscopic or angiographic evidence of alterations to the fundus, the outer retina and RPE are affected histologically by subthreshold TTT. Dose-dependent disruption of photoreceptor outer segments with loss of the outer nuclear layer was observed by means of light and electron microscopy. Furthermore, dose-dependent vacuolization of photoreceptor outer segments and RPE cells and disruption of RPE apical microvilli were also observed by means of electron microscopy. No adverse effect on the choroid was noted at the subthreshold energy levels used. Because normal pigmented rabbit eyes were used in these experiments, the results obtained cannot be directly extrapolated to the clinical setting in which TTT is used to treat a CNV lesion under the retina. Such lesions are usually in association with subretinal fluid, and this may serve to insulate the retina to some degree against heat absorption during TTT application. However, in eyes with CNV in which there is little associated subretinal fluid, our findings certainly highlight the need to consider decreasing power settings.

Previous histological studies of TTT have involved threshold settings to photocoagulate choroidal tumors. However, 1 study examined subthreshold TTT to the normal macula in a human eye with choroidal melanoma that subsequently underwent enucleation. That study reported abnormal cytoplasmic lipofuscin and melanofuscin granules in RPE cells and disruption of photoreceptor outer segments, with no changes observed in the choroid. Aside from the RPE granules, these findings are in agreement with what we observed in rabbit eyes.

Interpretation of our results must be tempered by differences in the refractive error and structure of rabbit vs human eyes. The irradiance (power/area) achieved using the same power, spot size, and contact lens will be lower in rabbit eyes than in human eyes. For example, using the Goldmann lens and 810-nm radiation, the magnification factor is 1.247 in rabbits but only 1.08 in human eyes. This results in an approximately 33% higher irradiance in human eyes than in rabbit eyes. There-
Therefore, one would expect the tissue effects observed in rabbits to be more pronounced in human eyes using identical laser settings.

The mechanism of action in the treatment of CNV using TTT is currently unclear. The expression of HSPs are known to be induced by heat and other pathologic stresses, and recent studies have suggested that HSPs may play a major role in the effect of TTT. Heat shock proteins are believed to act as molecular chaperones, theoretically allowing cells to adapt to unfavorable changes in their environment. Heat shock proteins are also known to induce apoptosis, and therefore this may also contribute to the cellular alterations observed after TTT. We examined the expression of HSP60 and HSP70 by immunohistochemistry after TTT in rabbit eyes and found both proteins to have a dose-dependent expression in the RPE and outer retina. In contrast, Desmettre and colleagues reported the presence of HSP70 expression 24 hours after TTT in rabbits in choroidal cells, including capillary endothelial cells, but not in the retina. Expression of other proteins was not examined in that study. The fact that we assessed protein expression at 4 weeks after TTT may account for the difference in results between the study by Desmettre et al and our study. Expression of HSP has been shown to be induced within several hours after hyperthermia and to revert to control levels within 48 hours. Expression of HSP60 and HSP70 at 4 weeks after TTT may therefore be the result of molecular interactions other than those induced by transient hyperthermia.

Indeed, HSPs have also been implicated in the down-regulation of inflammatory cytokines such as TNF-α, in-
terleukin 1, and interleukin 6, and some of these same inflammatory cytokines have been shown to enhance HSP expression in an apparent cross-regulatory function.\textsuperscript{24-30} In addition, HSP60 and HSP70 have each been shown to induce expression of intracellular adhesion molecule 1 and VCAM-1,\textsuperscript{31,32} and one might speculate that

Figure 4. Results of immunohistochemistry in untreated control eyes showed no staining for heat shock protein 60 (HSP60) (A), HSP70 (D), tumor necrosis factor α (TNF-α) (F), or vascular cell adhesion molecule 1 (VCAM-1) (H). After transpupillary thermotherapy (TTT) duration of 30 seconds, HSP60 staining was present in the retinal pigment epithelium (RPE) (arrowhead) and inner segments of photoreceptor cells (asterisk) (B). After TTT duration of 60 seconds, HSP60 staining was observed in the inner and outer segments of photoreceptor cells (asterisk) and in the RPE (arrowhead) (C). After TTT duration of 60 seconds, staining for HSP70 (E), TNF-α (G), and VCAM-1 (I) were also observed in photoreceptor cells (asterisk) and in the RPE (arrowhead) in a similar dose-dependent manner as for HSP60. No staining for HSP60, HSP70, TNF-α, or VCAM-1 was observed in the inner layers of the retina, the outer nuclear layer, or the choroid for any TTT duration.
the long-term clinical effects of TTT are related to such adhesion molecule expression in the vessels of CNV lesions. In the present study, dose-dependent expression of TNF-α and VCAM-1 were also observed by means of immunohistochemical staining in the RPE and photoreceptors 4 weeks after TTT. Although these data suggest that the tissue effects of TTT involve the late expression of HSPs, TNF-α, and VCAM-1 in normal rabbit eyes, further experiments are clearly required to determine how such protein expression might lead to decreased exudation in eyes with CNV.

**CONCLUSIONS**

Subthreshold TTT in normal pigmented rabbit eyes produced alterations to the photoreceptor outer segments and RPE cells when assessed at 4 weeks after treatment. These alterations were accompanied by expression of HSP60, HSP70, TNF-α, and VCAM-1 within the outer retina and RPE. All changes were dose dependent and suggest that expression of HSPs, cytokines, and cell adhesion molecules may contribute to a delayed tissue response to subthreshold TTT.

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matory infiltrates in and adjacent to many retinal vessels. Immunocytochemical staining revealed macrophages (CD68+) within the lumina of some sclerotic vessels (Figure 2D) and perivascular infiltration of T lymphocytes (CD4+CD8 cells) (Figure 2C).

Comment. Coats disease, usually present in childhood, has been reported to occur in patients in the second to third decade of life (range, 2-52 years).3 Intraocular inflammation, although observed on histopathologic testing, is not a prominent feature in this disease.1,3,4 A previous electron microscopic study has shown the presence of mononuclear and polymorphonuclear cells within the lumina of some vessels and in the perivascular space.5 This case is unique with regard to the prominent inflammatory component in addition to the subretinal and retinal lipid crystal deposits and abnormal retinal vasculature that are characteristic of Coats disease. In this case, the inflammatory component of the disease was partly controlled by prednisolone and cyclosporine, but immunosuppressive therapy did not completely stop the progression of the vascular disease. Immunohistochemical staining revealed that the intraluminal inflammatory cells were macrophages and that the perivascular inflammatory infiltrates were composed largely of T lymphocytes. Immunosuppressive therapy such as cyclosporine, whose action primarily targets the interleukin 2 receptors on the activated T lymphocytes, may be beneficial in the treatment of inflammation encountered in this rare variant of Coats disease.

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Correction
Omission in Byline and Affiliations. In the Laboratory Sciences article by Morimura et al titled “Histological Effect and Protein Expression in Subthreshold Transpupillary Thermotherapy in Rabbit Eyes,” published in the October issue of the ARCHIVES (2004;122:1510-1515), an author’s name was inadvertently omitted from the byline and from the affiliations paragraph on page 1510. The byline should have read as follows: “Yoshihiro Morimura, MD; Annabelle A. Okada, MD; Atsushi Hayashi, MD; Sayuri Fujioka, MD; Noriyasu Hashida, MD; Sumie Kawahara, MD; Tetsuo Hida MD.” The affiliations paragraph should have read as follows: “From the Department of Ophthalmology, Kyorin University School of Medicine, Tokyo, Japan (Drs Morimura, Okada, Kawahara, and Hida); and the Department of Ophthalmology, Osaka University Medical School, Suita, Japan (Drs Hayashi, Fujioka, and Hashida). The authors have no relevant financial interest in this article.” The journal regrets the error.