Heat treatment of retinal pigment epithelium induces production of elastic lamina components and antiangiogenic activity

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ABSTRACT Age-related macular degeneration (AMD) is the leading cause of blindness in the Western world. In advanced AMD, new vessels from choriocapillaris (CC) invade through the Bruch’s membrane (BrM) into the retina, forming choroidal neovascularization (CNV). BrM, an elastic lamina that is located between the retinal pigment epithelium (RPE) and CC, is thought to act as a physical and functional barrier against CNV. The BrM of patients with early AMD are characterized by decreased levels of antiangiogenic factors, including endostatin, thrombospondin-1 (TSP-1), and pigment epithelium-derived factor (PEDF), as well as by degeneration of the elastic layer. Motivated by a previous report that heat increases elastin expression in human skin, we examined the effect of heat on human ARPE-19 cell production of BrM components. Heat treatment stimulated the production of BrM components, including TSP-1, PEDF, and tropoelastin in vitro and increased the antiangiogenic activity of RPE measured in a mouse corneal pocket assay. The effect of heat on experimental CNV was investigated by pretreating the retina with heat via infrared diode laser prior to the induction of CNV. Heat treatment blocked the development of experimental CNV in vivo. These findings suggest that heat treatment may restore BrM integrity and barrier function against new vessel growth.—Sekiyama, E., Saint-Geniez, M., Yoneda, K., Hisatomi, T., Nakao, S., Walsh, T. E., Maruyama, K., Hafezi-Moghadam, A., Miller, J. W., Kinoshita, S., D’Amore, P. A. Heat treatment of retinal pigment epithelium induces production of elastic lamina components and anti-angiogenic activity. FASEB J. 26, 567–575 (2012). www.fasebj.org

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Age-related macular degeneration (AMD), the leading cause of blindness in the elderly population in the Western world (1), is classified as either wet or dry type. In contrast to the patients with dry AMD, in whom impairment of vision is gradual, wet AMD has rapid and devastating visual effects. The clinical and histopathologic features of wet AMD involve the dysfunction of retinal pigment epithelium (RPE), Bruch’s membrane (BrM) and the choriocapillaris (CC). In wet AMD, new vessels from the CC invade through the BrM into the retina, resulting in choroidal neovascularization (CNV). Early AMD is distinguished by subretinal deposits and atrophic changes in the RPE, which are not associated with changes in visual acuity. However, once new blood vessels develop and invade the retinal space, vision is lost. Thus, strategies that could prevent the progression to wet AMD would be valuable.

BrM, which is located between the RPE and CC, is composed of 5 distinct layers: a central elastic layer, bounded on both sides by collagenous layers, and bordered externally by the basal laminas of the RPE and CC. The basement membranes underlying the RPE and the CC endothelial cells contain collagen IV, laminin, and decorin. Many of these molecules have reported effects on the proliferation and/or survival of vascular endothelium. Collagen IV α 2 chain has been reported to induce apoptosis of vascular endothelial cells (2), and α 3 chain has been shown to inhibit the vascular endothelial proliferation and block tube formation in vitro (3). The collagenous layers of BrM include collagens I, III, and XVIII, and fibronectin. Collagen I is reported to down-regulate VEGF-mediated VEGFR2 activation (4) and to bind thrombospondin-1 (TSP-1), a major antiangiogenic factor (5). Through

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cleavage by enzymes, including cathepsin B and MMP-7, collagen XVIII produces endostatin, a well-described endogenous antiangiogenic factor, which has been shown to regulate CNV (6). The elastic layer, which is formed by the cross-linking of tropoelastin on microfibrils of fibrillin-1 and -2 molecules, is believed to act as physical barrier against new vessel growth (7, 8). Interestingly, BrM from patients with early AMD has been reported to have decreased levels of antiangiogenic molecules, including endostatin, TSP-1, and pigment epithelium-derived factor (PEDF) (9–11), as well as degeneration of the elastic layer (8). Taken together, these observations indicate that the BrM functions as a physical and functional barrier against the growth of new blood vessel from the CC.

Previous reports have demonstrated that heat induces elastin expression in human skin (12) and that the expression of heat-shock protein (HSP) increases the levels of endostatin and TSP-1 in tumors (13). With a goal of identifying a means to restore BrM, we investigated the effect of mild heat treatment on human RPE-production of BrM components. In addition, we tested the effect of heat on the retina in vivo using topical heat treatment with an infrared diode laser (IDL). Results of these studies suggest that heat treatment can induce the expression of components of BrM and thus might be useful in preventing the progression to neovascular AMD.

MATERIALS AND METHODS

ARPE-19 cell cultures

ARPE-19 cells obtained from American Type Culture Collection (Manassas, VA, USA) were used between passages 21 and 25. Transwells (0.4-μm pore size, 12- or 24-mm diameter; Corning/Costar, Corning, NY, USA) were coated with laminin, and ARPE-19 cells (~1.7×10⁵ cells/cm²) were seeded in DMEM/F-12 culture medium, supplemented with 100 U/ml penicillin-streptomycin and 1% FBS. The medium was changed 2×/wk. Cells were cultured for 4 wk to form differentiated monolayers. RNAs were isolated from cells after 1, 2, 3, and 4 wk of culture.

For heat treatment, ARPE-19 cells grown for 4 wk on the transwells were cultured at 43°C for 30 min. RNA was isolated at 15 min and 1, 2, and 4 h after heat treatment, and cell-associated proteins were examined in cell lysates collected at 2 and 4 h after heat treatment. Proteins secreted into the culture medium were collected at 4 h after heat treatment and analyzed.

RNA isolation and real-time PCR analysis

Total RNA was extracted (RNAaqueousTM-4PCR kit; Ambion, Austin, TX, USA), according to the manufacturer’s instructions. Residual DNA was removed by treatment with 1 U DNase I (Ambion) at 37°C for 20 min. RNA (1 μg) was reverse-transcribed, and 1/20 of the total cDNA was used in each amplification reaction. Each gene was quantified [Prism 9700 Sequence Detection System; Applied Biosystems (ABI), Foster City, CA, USA] according to the manufacturer’s instructions (Table 1). Reactions were performed in 25 μl with 0.3 μM primers and master mix (SYBR Green Master mix; ABI). PCR cycles consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. To confirm amplification specificity, PCR products from each primer pair were subjected to a melting curve analysis. Amplification of the GAPDH was performed on each sample as a control for sample loading and to allow normalization between samples. Each sample was run in duplicate, and each experiment was conducted ≥3 times.

Western blot analysis

ARPE-19 cells were collected in lysis buffer (10 mM Tris-HCl, pH 7.4; 5 mM EDTA; 50 mM NaCl; 1% Triton X-100; 50 mM NaF; 1 mM phenylmethylsulfonyl fluoride; 2 mM Na₃VO₄; and 20 mg/ml aprotinin), and protein concentrations were quantified using the NanoDrop (Scrum, Tokyo, Japan). Medium conditioned by ARPE-19 cells was concentrated 10-fold with a centrifugal filter with a molecular size cutoff of 10 kDa (Amicon Ultra; Millipore, Bedford, MA, USA), and equal volumes of samples were analyzed. Proteins from cell lysates and conditioned medium were separated by SDS-PAGE. Cell lysates were probed with rabbit polyclonal anti-human tropoelastin (1:300; Elastin Products Co., Owensville, MO, USA) and mouse monoclonal anti-human TSP-1 (1:400; Abcam, Cambridge, MA, USA). Concentrated medium was probed with mouse monoclonal anti-human endostatin (1:100; Oncogene, San Diego, CA, USA) and mouse monoclonal anti-human PEDF (1:1000; Millipore). Binding was detected with the appropriate HRP-conjugated secondary antibody (1:1000; Amersham Bioscics, Piscataway, NJ, USA) and ECL-Plus Western blotting Detection System (GE Healthcare, Wauke sha, WI, USA). The intensity of Western blot bands was

| Primer         | Source                        | Catalog number |
|----------------|-------------------------------|----------------|
| Collagen I     | Qiagen (Valencia, CA, USA)    | QT00037793     |
| Collagen IV    | Qiagen                        | QT00035250     |
| Collagen XVIII | SABiosciences (Frederick, MD, USA) | PPH01414E-200 |
| Decorin        | SABiosciences                 | PPH01900A-200  |
| Fibronecin     | SABiosciences                 | PPH00143B-200  |
| Laminin        | SABiosciences                 | PPH02901E-200  |
| Tropoelastin   | SABiosciences                 | PPH06895E-200  |
| Fibrillin-1    | Qiagen                        | QT00025407     |
| Thrombospondin-1 | SABiosciences               | PPH01799E-200  |
| PEDF           | SABiosciences                 | PPH00805A-200  |
| Cathepsin B    | Qiagen                        | QT00088641     |
| MMP-7          | Qiagen                        | QT0001456      |
quantified by densitometric analysis using NIH ImageJ \((n=3;\) U.S. National Institutes of Health, Bethesda, MD, USA).

**TUNEL assay**

ARPE-19 cell apoptosis was evaluated using the in situ cell death detection kit (TMR red, Roche, Mannheim, Germany), according to the manufacturer’s instructions. Briefly, cells were fixed with 4% paraformaldehyde for 1 h at room temperature and then permeabilized with 0.1% TritonX-100 in 0.1% sodium citrate for 2 min on ice. After the incubation with TUNEL reaction mixture and DAPI for 60 min at 37°C in the dark, cells were observed. Three wells were analyzed by counting apoptotic cells in 4 randomly chosen fields.

**Transmission electron microscopy**

Monolayers of ARPE-19 cells cultured on transwells for 3 d and 4 wk were fixed with half-strength Karnovsky’s fixative, followed by 2% osmium tetroxide and stained en block with 0.5% uranyl acetate. After dehydration and embedding, ultrathin sections were visualized using a transmission electron microscope (Model 410; Phillips, Amsterdam, The Netherlands).

**Effect of medium conditioned by heat-treated ARPE-19 cells on endothelial cell wound closure assay and proliferation**

Medium was collected after 4 h of conditioning by heat-treated ARPE-19 cells; unconditioned medium served as a control. Medium was mixed with an equal volume of endothelial basal medium (EBM-2) supplemented with SingleQuots (Lonza, Walkersville, MD, USA), 20% FBS, and 1× glutamine-penicillin-streptomycin and tested for its effect on the closure of scratch wounds by human umbilical vein cells (HUVECs). Monolayers of confluent HUVECs in 24-well plates (Corning/Costar) were scratch wounded using the pipette tips. Two scratches/well were made \((n=4)\). The progress of wound closure was photographed with an inverted microscope equipped with a digital camera (SPOT; Diagnostic Imaging, Sterling Heights, MI, USA) immediately after injury and at 16 h after wounding. The width of the wound was measured using NIH ImageJ software. Three random measurements were taken of each wound, and their average was taken as the width of each wound.

For the assay of proliferation, HUVECs were seeded at \(2 \times 10^4\) cells/well in triplicates onto 12-well plates (Corning/Costar). After 3 d, cell proliferation was evaluated by direct cell count of trypsin-detached cells with a hemocytometer \((n=3)\).

**Corneal micropocket assay**

Rodent studies were approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. BALB/c mice were anesthetized by intraperitoneal injection of ketamine at 100 mg/kg and xylazine at 10 mg/kg. Hydron pellets (0.3 μl) containing 200 ng of human VEGF (201-LB; R&D Systems, Minneapolis, MN, USA) were prepared. Heat-treated \((n=6)\) or control untreated \((n=10)\) ARPE-19 cells cultured on the transwells were dissected into 0.5-μm-square pieces and implanted into the corneas with VEGF-containing pellets. The pellets and tissues were positioned 1 mm from the corneal limbus, as described previously \((14)\). Implanted eyes were treated topically with bacitracin ophthalmic ointment (E. Fougera & Co., Melville, NY, USA). At 6 d after implantation, digital images of the corneal vessels were obtained and recorded using OpenLab 2.2.5 software (Improvision Inc., Waltham, MA, USA) with standardized illumination and contrast. Quantification of neovascularization in the mouse corneas was performed using NIH ImageJ software.

**Effect of elastin on endothelial cell migration**

Transwells (3.0μm pore size, 6.5-mm diameter; Corning/ Costar) were coated overnight at 4°C with soluable elastin (Elastin Products) diluted in PBS (0, 10, 100, or 1000 μg/ml). HUVECs \((\sim 2.5 \times 10^5\) cells/well) were seeded on the elastin-coated transwells in EBM-2-supplemented 20% FBS, 1× glutamine-penicilllin-streptomycin, and SingleQuots. After 2 h, the culture medium was replaced, and the number of unattached cells was counted with a hemocytometer to determine plating efficiency. At 14 h after plating, the cells from the upper side of the filter were removed with a cotton swab, and the cells that had migrated through the pores to the opposite side of the membrane were stained with hematoxylin and eosin. The filter was gently cut from the chamber, and the cells that had migrated were counted in 4 high-power fields/insert. For each migration condition, 3 replicates were performed.

**Heat treatment of mouse retina**

These mouse studies were approved by the Committee on the Ethics of Animal Experiments at the Kyoto Prefectural University of Medicine. C57BL/6J mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Pupils were dilated with 1% tropicamide. Heat from an IDL was delivered through a slit lamp (model 30 SL-M; Carl Zeiss Meditec, Oberkochen, Germany) by a trimode IDL emitting at 810 nm (Iris Medical Instrument, Mountain View, CA, USA) at a power setting of 50 mW and a beam diameter of 1.2 mm for 60 s. A series of 4 laser spots were delivered to the posterior pole of each retina at 2 disc diameters from the optic nerve.

To examine the effect of IDL on retinal structure, one IDL spot was delivered to the posterior pole of the retina at 2 disc diameters from the optic nerve. To be able to locate the irradiated spot, a burn was created by argon laser photocoagulation (PC) directly across the optic nerve from the irradiated spot. At 14 d after heat treatment, eyes were enucleated, and the IDL-irradiated retinas were dissected into 0.8-μm sections that were stained with hematoxylin and eosin, and cut into ultrathin sections, which were visualized using a transmission electron microscope.

**Induction of CNV in heat-treated mouse retina**

At 1 d after the heat treatment, mice were anesthetized as above and fixed on a rack connected to the slit lamp delivery system. To induce CNV, PC burn was placed in the center of the IDL heat treatment area at a power setting of 300 mW and a beam diameter of 50 μm for 0.05 s to induce CNV. Only eyes in which a subretinal bubble was formed following each burn were included in the study. At 7 d after argon photocoagulation, mice were perfused with concanavalin A lectin (20 μg/ml in PBS; Vector Laboratories, Burlingame, CA, USA), then the eyes were enucleated and fixed in 2% paraformaldehyde. The RPE-choroid-sclera complex was flat mounted and was imaged using a Zeiss fluorescence microscope (Universal; Carl Zeiss Meditec). The neovascular area was measured using Scion Image 4.0.2 software (Scion Corp., Frederick, MD, USA).
Statistical analysis

Values are expressed as means ± se; statistical analysis was performed using the Mann-Whitney U test.

RESULTS

ARPE-19 cells secrete a BrM-like matrix

The matrix produced by ARPE-19 cells cultured on transwells for 3 d and 4 wk was examined by transmission electron microscopy. Cells cultured for 3 d had deposited little matrix; however, after culture for 4 wk, a 0.3- to 0.4-μm-thick matrix had accumulated under the basal surface of the cells (Fig. 1A).

ARPE-19 cells cultured for 1, 2, 3, and 4 wk were assessed for the levels of mRNA of the BrM components, including collagen I, collagen IV, collagen XVIII, decorin, fibronectin, laminin, tropoelastin, fibrillin-1, TSP-1, PEDF, MMP-7, and cathepsin B. The expression of collagen IV and I peaked at wk 2, whereas that of decorin began to increase after 2 wk in culture, while that of fibronectin levels were increased at wk 2 and 3. Of the angiogenesis-related proteins, TSP-1 was maximally expressed at wk 3, whereas PEDF and cathepsin B peaked at wk 4. Collagen XVIII, tropoelastin, fibrillin-1, and MMP-7 showed constant expression during the 4 wk of culture (Fig. 1B, C).

Heat treatment increases ARPE-19 expression of endostatin, TSP-1, and PEDF

Heat treatment at 43°C for 30 min did not affect the viability of the ARPE-19 cells, as detected by TMR red TUNEL labeling (Fig. 2). The effect of heat treatment

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on the levels of TSP-1, PEDF, and endostatin mRNA and protein were examined by real-time PCR and Western blot analysis. TSP-1 mRNA levels were increased significantly at 120 min after heat treatment, and PEDF mRNA levels were elevated significantly as early as 60 min after heat treatment. Collagen XVIII mRNA levels were unchanged after heat treatment, but the mRNA expression of MMP-7, which cleaves the C terminus of collagen XVIII to yield endostatin, was increased significantly at 15, 60, and 120 min following heat treatment (Fig. 3A). The levels of cell-associated protein TSP-1 were increased at 240 min after heat treatment, as was the secretion of PEDF and endostatin (Fig. 3B).

**Heat-treated ARPE-19 cells suppress VEGF-induced corneal angiogenesis**

RNA and Western blot analysis indicated that heat treatment of ARPE-19 cells induced an increase in the production of antiangiogenic molecules. To investigate whether heat treatment led ARPE-19 cells to

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**Figure 3.** Heat treatment increases TSP-1, PEDF, and endostatin expression by ARPE-19 cells. A) Expression of TSP-1, PEDF, collagen XVIII, and MMP-7 mRNA by ARPE-19 cells at 15, 60, 120, and 240 min after heat treatment at 43°C for 30 min. B) Expression of TSP-1, PEDF, and endostatin protein at 240 min after heat treatment at 43°C for 30 min. Values are expressed as means ± SE (n=3).
become functionally antiangiogenic, we assayed the effect of heat-treated and control ARPE-19 cells on VEGF-induced angiogenesis using the corneal micropocket assay. The presence of untreated ARPE-19 cells did not affect VEGF-induced corneal angiogenesis; however, the inclusion of heat-treated ARPE-19 cells along with the VEGF pellet in the micropocket led to a nearly 70% reduction in VEGF-induced corneal angiogenesis (Fig. 4A).

In the presence of medium conditioned by heat-treated ARPE-19 cells, HUVEC migration and proliferation were suppressed

Incubation of HUVECs in medium conditioned by untreated ARPE-19 cells led the endothelial cells to close almost 90% of a scratch wound in 16 h. In contrast, HUVECs treated with medium conditioned by heat-treated ARPE-19 cells closed just over 25% of the wound (Fig. 4B). Treatment of HUVECs with medium conditioned by heat-treated ARPE-19 cells led to a modest but statistically significant reduction in HUVEC proliferation relative to cells treated with medium conditioned by untreated ARPE-19 (Fig. 4C).

Heat treatment increased tropoelastin expression by ARPE-19 cells

The effect of heat treatment on the levels of tropoelastin mRNA and protein were examined by real-time PCR and Western blot analysis of cell lysates. Tropoelastin mRNA levels were increased by 180% at 15 min after heat treatment, and the protein levels increased by 170% at 120 min after heat treatment (Fig. 5A, B).

Elastin suppressed HUVEC migration in a dose-dependent manner

Elastin coating did not affect the attachment of HUVECs onto the transwells; however, HUVEC migration was reduced by ~20 and 27% when the transwells were coated using 100 and 1000 μg/ml of elastin, respectively (Fig. 5C).

Pretreatment with heat reduced the laser-induced CNV

To determine whether pretreatment with heat would affect laser-induced CNV, the retinas of mice were heated by delivering a series of 4 IDL spots to the posterior pole of each retina at 2 disc diameters from the optic nerve.
followed by placement of a photocoagulation burn in the center of the heat treatment. Laser-induced CNV was visualized and measured in choroidal flat mounts. The mean size of neovascular areas in heat-treated mice was only 15% that of control mice (Fig. 6A).

To determine whether heat treatment has any effect on normal retina, IDL-irradiated retinal tissues were dissected and examined. The area that had been irradiated by IDL showed no visible structural abnormalities, including atrophic change or fibrosis of neural retina, or recruitment of inflammatory cells (Fig. 6B). Transmission electron microscopic examination revealed no change in RPE or photoreceptor cell structure (Fig. 6C).

**DISCUSSION**

In mouse, the BrM begins to develop around d 17 of gestation (15), well after the differentiation of the RPE.

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**Figure 5.** Heat treatment increases tropoelastin expression by ARPE-19 cells. 

A) Expression of tropoelastin mRNA by ARPE-19 cells at 15, 120, and 240 min after heat treatment at 43°C for 30 min. B) Expression of tropoelastin protein 120 min after treatment at 43°C for 30 min (n=5). C) Adhesion of HUVECs seeded on the elastin-coated transwells. At 2 h after cell plating, adhesion of HUVECs was determined by counting the number of unattached cells with a hemocytometer (n=5). D) Effect of elastin on migration of HUVECs. At 14 h after cell plating, cells that had not migrated were removed by gentle scraping, and those that had migrated through to the bottom of the transwell were counted (n=3). Values are expressed as means ± se.

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**Figure 6.** Pretreatment of the retina with heat reduced CNV in vivo without causing tissue damage. A) Representative micrographs of PC-induced CNV in retinas with no pretreatment and in retina that had been pretreated with TTT. CNV was visualized in choroidal flat mounts by fluorescein angiography. Hyperfluorescent areas were quantified. Values are means ± se (n=6). B) Untreated and TTT-treated mouse retina. At 14 d after TTT treatment, mouse retina was sectioned and stained with hematoxylin and eosin. C) Transmission electron microscopic examination revealed no change in RPE or photoreceptor cell structure. Scale bars = 30 μm (A); 100 μm (B); 50 μm (C).
and the formation of the CC. During the development of BrM, the basement membranes of the RPE and the CC are laid down first, followed by the collagenous layers and finally the inner elastic lamina (15). Studies of human adult BrM reveal no fibroblasts or other cells, except in the extreme periphery (16). These findings suggest that BrM is produced by RPE and/or CC; however, there have been no studies to assess these possibilities.

In this study, we used ARPE-19 cells to determine whether RPE can synthesize the various components of BrM. ARPE-19 cells are a well-established line of cells that form differentiated and polarized monolayers after prolonged (4 wk) culture on transwells (17). The apical surface of the ARPE-19 cells represents the retinal-facing domain, whereas the basal aspect would be the side that apposes the CC. Using these cells, we have demonstrated that RPE cells produce most of the constituents of BrM and that, after 4 wk in culture, a significant amount of matrix material is deposited basally. Despite the fact that the RPE produces a number of specific BrM components, such as tropoelastin and fibrillin, the matrix that was deposited did not display any structure that would be considered characteristic of pentalaminar BrM. We speculate that the appropriate organization of BrM requires the combined contribution of both the RPE and CC, a notion supported by the recent observation of a lack of a defined BrM in a model of transgenic mice that lack a proper choriocapillaris network (18).

BrM represents both a functional and structural barrier to growth of new blood vessels from the CC into the retinal space. Interestingly, the structure of BrM beneath the macula differs from that under the rest of the retina. Rather than a continuous central elastic layer that is found under most of the retina, the elastic layer under the macula is discontinuous (8). Although this specialization presumably facilitates diffusion of oxygen and nutrients to the metabolically active overlying neural retina, it also renders the macula particularly vulnerable to the development of pathology. The BrM of the patients with early AMD has been documented to contain decreased levels of endostatin, TSP-1, and PEDF (9, 10, 11), as well as fragmentation of the elastic layer (8). A comparison of the BrM proteome profile over the course of AMD revealed decreased levels of collagen I α 1 and fibronectin precursor (19). A breach in the structural integrity of BrM is permissive for the formation of new blood vessels. Patients with hypermyopia who present lacquer cracks (breaks in outer retinal layers of macula) frequently develop CNV, and a break in BrM always precedes the development of neovascularization in wet AMD (20). Thus, restoration of the biochemical and structural integrity of BrM could slow or prevent the progression of CNV.

Motivated by observations that heat treatment of skin can induce the production of elastin (12), we investigated the possibility that heat treatment of RPE cells might stimulate their production of BrM components. Incubation of ARPE-19 cells at 43°C for 30 min led to the increased expression of endostatin, TSP-1, PEDF, and tropoelastin mRNA and protein, as well as mRNA levels of collagen I α 1 and fibronectin (data not shown). Cells exposed to high temperatures (44°C for 4 h) develop a transient thermal resistance that protects them by inducing or enhancing the synthesis of a set of HSPs (21). HSP70 and HSP27 have been detected in the RPE, and HSP70 is reported to have an important regulatory role in the protein turnover of human RPE cells (22, 23). Thus, induction of HSP may mediate some of the observed effects of heat on ARPE-19 cells.

Induction of ARPE-19 cells that had been treated with heat blocked VEGF-induced angiogenesis in the corneal pocket assay, whereas the presence of untreated ARPE-19 cells had no effect. Similarly, medium conditioned by heat-treated ARPE-19 cells significantly blocked endothelial cell migration in a wound closure assay. It has been reported that heat-sensitive TRPV channels in RPE increase VEGF secretion (ref). Certainly, incubation of ARPE-19 at 43°C for 30 min led to the increased expression of not only endostatin, TSP-1, and PEDF but also VEGF (data not shown). Although heat treatment of RPE appears to lead to increased expression of both angiogenic- and antiangiogenic factors, the net effect is antiangiogenic. Among the antiangiogenic agents examined, endostatin showed the most prominent heat-induced increase. This observation is consistent with a report that the antiangiogenic effects of endostatin are likely due to its potent inhibition of migration (24).

Motivated by the results of our in vitro studies, we tested the effect of heat treatment on new vessel growth in the retina by subjecting the retina to topical heat treatment with IDL. IDL has been previously examined for the treatment of AMD by targeting new vessels as a transpupillary thermotherapy (TTT). Although its safety was proven, and there were some reports of positive effect, results of a multicenter TTT4CNV trial did not show benefit (25). TTT has also been previously used in early AMD with the goal of activating RPE phagocytosis of drusen. However, this approach did not reduce progression to wet AMD (26). The treatment regime used in these prior studies includes 48 shots of TTT with a beam diameter of 125 μm and with power settings of ≥50 mW for 0.1 s. The treatment utilized here consisted of 4 applications of laser light (modified TTT) with a beam diameter of 1.2 mm and with power setting of 50 mW for 60 s. Though the previous studies used a similar power level, the spot diameter was 1/10 the size. With a smaller diameter, the energy per unit area increases, very likely destroying the RPE and causing acute inflammation. Such tissue damage does not occur with the treatment protocol employed in our study. In contrast to these early applications where new vessels themselves were targeted and/or very high levels of treatment were applied, our goal was to use pretreatment with heat to restore or increase BrM barrier functions by using mild heat to stimulate RPE cells to produce components of BrM. Our results revealed that
pretreatment with heat, or modified TTT, blocked the formation of laser-induced CNV.

Though it is clear that the normal BrM provides a biochemical and physical barrier against CNV, there have been no reports regarding the regeneration of BrM as an approach for increasing its barrier function targeting the management for AMD. Results of our in vitro and in vivo observations demonstrate that heat treatment may provide a means to restore BrM integrity and its barrier functions against CNV and suggest that a form of TTT may be used to prevent the development of CNV in patients who are at high risk, such as those with neovascular AMD in a fellow eye.

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