Release of Different Cell Mediators During Retinal Pigment Epithelium Regeneration Following Selective Retina Therapy

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PURPOSE. To investigate the effect of selective retina therapy (SRT) on the release of AMD-relevant cell mediators, such as matrix metalloproteinases (MMPs), VEGF, and pigment epithelium derived factor (PEDF) using different laser spot sizes and densities.

METHODS. Porcine RPE-choroid explants were treated with a pulsed 532 nm Nd:YAG laser using (1) large spot sizes, (2) small spot sizes with a high-density (hd) treatment, and (3) small spot sizes with a low-density (ld) treatment. Explants were cultivated in modified Ussing chambers. RPE regeneration and RPE cell death were investigated by calcein-AM staining and immunofluorescence. The MMP release was examined via zymography and immunofluorescence. VEGF and PEDF secretion was analyzed by ELISA.

RESULTS. During pigment epithelium regeneration (PER), mitosis and RPE cell migration were observed. Four days after SRT (large spot size) the content of active MMP2 increased significantly (P < 0.01). Hd treatment with small spot sizes resulted also in an increase of active MMP2 (P < 0.05). In immunofluorescence explants showed a localized expression of MMP2 within the healing lesions after irradiation. The PEDF level increased significantly (P = 0.01) after SRT with large spot sizes. VEGF secretion decreased significantly (P < 0.05) following SRT with large spot sizes and with hd treatment of small spot sizes.

CONCLUSIONS. SRT induces a cytokine profile, which may improve the flux across Bruch’s membrane, slows down progression of early AMD by RPE regeneration, and inhibits the formation of choroidal neovascularization. The cytokine release depends on the size and density of applied laser spots.

Keywords: age-related macular degeneration, matrix metalloproteases, pigment epithelium derived factor, retinal pigment epithelium, selective retina therapy, vascular endothelial growth factor

AMD is the main cause for legal blindness of the elderly in Western civilizations.1 The slowly progressing dry form of AMD is characterized by the presence of drusen and macular pigment alterations. Dry AMD may either progress into the exudative form or lead to macular atrophy, in which the RPE degenerates, followed by a loss of photoreceptors, resulting in vision deterioration.2–4 An adequate pathomechanism driven therapy for the dry form of AMD is not available at present.5 One factor in the complex pathophysiology of AMD is the age-dependent alteration of Bruch’s membrane (BrM), which is associated with a reduced ability to exchange nutrients and waste products between retina and choroid.6–8 These alterations are created by a disturbed balance in degradation and synthesis of BrM matrix. In advanced stages, the BrM is thickened and the reduced exchange leads to an undersupply of the neuroretina, which promotes AMD progression.6–12 The degradation and rebuilding pathway of BrM is mediated by the family of matrix metalloproteinases (MMPs) and their antagonists, the tissue inhibitors of metalloproteinases (TIMPs).13 In particular, MMPs 1, 2, 3, and 9 and TIMPs 1, 2, and 3 are part of BrM and secreted by the RPE and choroidal endothelial cells.14–17 Contents of proforms of MMP2 and MMP9 have been shown to increase with age, whereas active forms are reduced or undetectable, which could explain the limited degradation ability of extracellular matrix (ECM) in aged eyes.15,18 The stimulation of the MMP pathway could be a therapeutic option to reduce a thickened BrM, as seen in AMD, and thereby improve the flux across the BrM to slow down or prevent the progression of early AMD.19

Another important factor in AMD pathology is the balance between the expression of proangiogenic and antiangiogenic cell mediators.20 The proangiogenic VEGF is the major factor in the development of choroidal neovascularizations (CNV) in wet form of AMD21 and mainly secreted by the RPE.22 The pigment epithelium derived factor (PEDF), a powerful inhibitor of angiogenesis, is strongly associated with VEGF regulation, development, and survival of photoreceptors, as well as neuroprotection.23–26 Former AMD-related studies have shown that PEDF prevents formation of new blood vessels in murine models with laser-induced CNV.27 Creation of a protein
Release of Cell Mediators after Selective Retina Therapy

Materials and Methods

Preparation of RPE–Choroid Explants

RPE-choroid explants, consisting of RPE, BrM, and choroid, were prepared as previously described.39 In brief, fresh porcine eyes were opened and anterior segment and vitreous were removed. Eyes were incised on one side and RPE, BrM, and choroid were carefully removed from the sclera. The retina was removed and explants were fixed within two polyacetal fixation rings (inner diameter 7 mm, height 1 mm). The area of the RPE layer was always the same and constantly filled with RPE cells so ensure the integrity of organ cultures. In cases where RPE-choroidal tissues overlapped the outer margin of the rings, these areas were removed with a scalpel, to minimize artifacts of unspecific secondary stress response of RPE cells.

Explants were transferred into 12-well culture plates with 1.5 mL warm culture medium containing equal amounts of Dulbecco's modified Eagle's medium (glucose 4.5 g/L, L-Glutamine, w/o Phenol Red; PAA, Pasching, Austria) and Ham's F12 medium (PAA supplemented with 1% penicillin/streptomycin (Biochrom, Berlin, Germany), 5% to 10% fetal calf serum (Linaris, Wertheim-Bettingen, Germany), 100 μM taurine (Sigma, Steinheim, Germany), and 2 mM calcium (Sigma).

Threshold Titration of Laser-Induced RPE Cell Death

We used a prototype Nd:YAG laser (Carl Zeiss Meditec AG, Jena, Germany) with 532-nm wavelength, which is transmitted by a multimode optical fiber with a square core profile of 70 μm, 100 Hz of repetition rate, and 30 pulses per irradiation, connected with a slit lamp. Depending on the used pulse energy, two forms of pulses arose (Fig. 1).

The spot size on the explants was chosen to 100 or 200 μm by magnification. For laser irradiation, a 12-well plate containing RPE-choroid explants was placed on a metal board on the chin-rest of the slit lamp. RPE integrity was analyzed via slit-lamp examination with a mirror affixed to the slit lamp. Explants were treated with laser radiant exposure between 120 and 220 mJ/cm² per pulse. Irradiation levels were tested with Thermal Power Sensor S302C (Thorlabs, Lübeck, Germany). Four trial lesions with directly visible RPE destruction were set in a square formation to determine the titration region. Three test lesions per irradiance level were set within the titration region. Threshold titration of laser-induced cell death was investigated by calcein-AM vitality staining after a 24-hour incubation. For calcein staining, explants were incubated with calcein-AM (4 μg/mL; AnaSpec, San Jose, CA, USA) for 45 minutes and washed twice with PBS (Serva, Heidelberg, Germany). RPE cell death was investigated by fluorescence microscopy (Axiovert 100; Carl Zeiss, Jena, Germany) with λex/λem = 497/517 nm. Images were taken by Axio-cam (MRc5; Carl Zeiss). Areas of cell death were analyzed with a semiautomatic program in AxioVision V 4.7.2.0 (Carl Zeiss) and calculated as percentage of the irradiated area. Irradiated areas that contained more than 50% dead cells were defined as lesions above the threshold and were used for further experiments.

RPE Regeneration and Examination of Cell Size and Number

RPE regeneration to laser-induced wounds was examined after two different time intervals (1 and 4 days after SRT laser irradiation) via calcein staining. Images were taken as described above. The cell number was determined within the maximally irradiated area (0.04 mm²) using SRT with 200-
μm spot size. Cells were counted with a semiautomatic program in AxioVision in defined areas of RPE.

### Scanning Electron Microscopy

The organ cultures were treated with pulsed laser irradiations (100-μm spot size, 200 mJ/cm², 100 Hz of repetition rate, and 30 pulses per irradiation) in a square formation of four trial lesions as described above. The samples were cultivated for 6 hours in cell culture medium at 37°C. After cultivation, the samples were stored in 2.5% glutaraldehyde until further use. The explants were rinsed in 0.1 M phosphate buffer and treated with 2% osmium. Afterward, they were dehydrated in increasing concentrations of ethanol, dried by a critical point dryer, and sputtered with gold targets. Samples were investigated using an XL20 scanning electron microscope (Philips, Eindhoven, the Netherlands).

### Laser Treatment and Cultivation of RPE–Choroid Preparations in Modified Ussing Chambers

Cultivation of RPE–choroid explants in modified Ussing Chambers allowed a differentiation of basal (choroid facing) and apical (RPE facing) protein secretion.59 Tightness of the system was tested after 3 days of culture in Ussing chambers using Brilliant blue G (Brilliant peel; Fluoron, Ulm, Germany) in basal compartment by measurement of a possible amount in the opposing compartment by photometry at 584 nm (Spectronic Genesys 10 Bio; Thermo Fisher Scientific, Waltham, MA, USA).

A passage of Brilliant blue from basal to apical compartment was not observed (data not shown).

We prepared two organ culture rings from one eye and compared the release of different cell mediators of every SRT-treated organ culture with an untreated control culture, prepared from the same eye.

Explants were treated with a radiant exposure of 200 mJ/cm² per pulse for small 100-μm spots and 140 mJ/cm² per pulse for larger 200-μm spots. Three different types of treatments were applied: large spot size (200 μm, 70 spots), small spot size (100 μm) with a low number of spots (low density; ld, ~280 spots), and small spot size (100 μm) with a high number of spots (high density; hd, ~550 spots). The total area of cell destruction depends on spot size and density. In cases of SRT with 200-μm spot size, the maximal destroyed area was 7% of the whole explant. After SRT with 100 μm in a hd treatment the maximal destroyed area was 14% and 7% in ld treatment.

Preparations were inserted in modified Ussing Chambers and each compartment was filled with 1.1 mL medium and incubated at 37°C and 5% CO₂. Culture medium was replaced every 24 hours. The basal supernatants were collected, centrifuged, and stored at −20°C.

### Detection of Matrix Metalloproteases Via Zymography

Twenty microliters of zymogram sample buffer (Bio-Rad, München, Germany) were added to 10 μL of control medium, basal supernatants of treated explants, or untreated controls, respectively, and was loaded onto 10% zymogram ready-gel (Bio-Rad). Proteinases were separated via electrophoresis (20 mA, 150 V for 30 minutes, and 50 mA, 200 V for 40 minutes). Gels were washed in aqua dest and incubated in renaturation buffer (2 × 30 minutes; Bio-Rad) and developing buffer (Bio-Rad) at 37°C (~18 hours). Gels were stained with Comassie Blue (Bio-Rad) for 3 hours and destained with destaining solution (Bio-Rad) for 1 hour. Densitometric MMP evaluation was performed with MP-Chemiblis 1.6 Imager (Biotek, Jahnshofer, Germany) and 1D analysis (Total Lab TL100; Biosystematica, Mountain Hall, Great Britain). Contents of MMPs were depicted as x-fold of untreated control after subtraction of medium control.

### Detection of MMP2 and Ki-67 by Immunofluorescence

Expression of MMP2 and Ki-67 were observed by immunofluorescence. Explants were fixed with 4% paraformaldehyde (PFA; Carl Roth, Karlsruhe, Germany) on ice, incubated in PFA:PEM (1:1) for 5 minutes and PFA:BF (1:1) for 10 minutes, and washed with PBS. Afterward, explants were treated with Triton X-100 (5 minutes), NaBH₄ (10 minutes), and washed in PBS twice following 1 hour in Immunoblock (Carl Roth). Staining was performed with primary antibodies for MMP-2 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and Ki-67 (Abcam, Cambridge, United Kingdom) at 4°C over night. Explants were washed with PBS three times and stained with Phalloidin Atto-488 (Sigma), Hoechst (Sigma), and Alexa-fluor-647 donkey-2-goat (Invitrogen, Darmstadt, Germany), following wash steps with PBS and aqua dest. Explants were removed from rings and covered with Fluoromount G (EBioScience, San Diego, CA, USA) under cover slips. Fluorescence microscopy was performed at Axio Imager microscope (Carl Zeiss); images were taken with Axio-cam MRc5 and processed with Zen 2012 (Carl Zeiss).

### Detection of VEGF₁₆₅ and PEDF via ELISA

Secretion of VEGF₁₆₅ and PEDF in culture medium of basal Ussing compartment was analyzed by a human-specific VEGF₁₆₅-ELISA (R&D Systems, Minneapolis, MN, USA), which has the ability to detect porcine VEGF₅₅ and a porcine specific PEDF-ELISA (Novateinbio, Woburn, MA, USA), respectively.

### Statistics

Each experiment was performed with a minimum of three times and statistical analyses were conducted with Student’s t-test or ANOVA, respectively, using SPSS version 23 (IBM Corp., Armonk, NY, USA). A value of $P < 0.05$ was considered significant.

### Results

#### RPE Threshold Titration After SRT Treatment

For a spot size of 100 μm, laser radiant exposures of 150 mJ/cm² did not result in cell death. Laser radiant exposures of 180 mJ/cm² led to lesions above the threshold in 57% of the applied laser spots, whereas 200 mJ/cm² resulted in lesions above the threshold in 87%. An irradiation of 220 mJ/cm² produced 100% lesions above the threshold.

For a laser spot size of 200 μm, radiant exposures of 120 mJ/cm² did not result in cell death. Irradiation with 140 mJ/cm² resulted in lesions above the threshold in 61% of the applied laser spots. Of the applied spots, 180 mJ/cm² led to lesions above the threshold in 100%.

The aim of the PER was to irradiate the cells with energies that produce lesions above the threshold in more than 60% but less than 100% of applied laser spots. For this reason, we used radiant exposures of 200 mJ/cm² for a spot size of 100 μm and 140 mJ/cm² for a spot size of 200 μm in all cultivation experiments.
RPE Cell Morphology, Cell Size, and Cell Proliferation During PER

As a consequence of SRT, the RPE cells in irradiated areas were destroyed. RPE-choroid explants showed vitality and recovery of laser lesions under nutritional conditions. After 24 hours, the full effect of SRT could be observed in the RPE (Fig. 2a). During regeneration the cells at the rim of the SRT-induced RPE lesion began to cover the wound. These cells were irregularly shaped with conspicuous nuclei. Four days after SRT the wound was completely covered (Fig. 2b). The size of cells, which covered the wound increased ($P < 0.01$) at day 4 in comparison to untreated RPE cells (Fig. 3a). Of lesion-covering cells, 5% to 14% showed two nuclei.

During PER, the cell number within the destroyed area increased continuously and was significantly different between the different time points of examination ($P < 0.01$). The RPE cell number of untreated areas was determined as baseline per spot size. Two days after SRT, 8% RPE cells related to RPE baseline were present in the laser treated area. After 3 days of regeneration, cell count analysis showed an increase of cells in irradiated areas up to 37% and 53% after 4 days compared with untreated RPE (Fig. 3b).

### FIGURE 2
Different stages of RPE regeneration after SRT irradiation with 200-μm spot size. One day after irradiation with a radiant exposure, which resulted in lesions above the threshold of cell death (200-μm spot size, 140 mJ/cm² per pulse) calcein vitality staining showed the full effect of SRT (a). Four days after SRT, irregular shaped cells with conspicuous nuclei covered the laser-induced RPE wound. Some of these enlarged cells showed two nuclei (arrow), which suggest mitosis (b).

### FIGURE 3
Variation of cell size and cell number during RPE regeneration. Four days after SRT the mean cell size (874 μm ± SEM 98) within the healing SRT area increased up to 2.4-fold in comparison to untreated RPE cells (360 μm ± SEM 52) (a). The number of cells increased during regeneration at day 2 to 4 within the treated SRT area. Two days after SRT, 8% RPE cells (11 cells ± SEM 2) related to baseline (133 cells ± SEM 6) were present in the irradiated area. Three days after SRT the cell number increased up to 57% (50 cells ± SEM 5). Four days after SRT cell number in irradiated area increased up to 53% (70 cells ± SEM 3) related to baseline of untreated control (b). This suggests both, cell migration and proliferation of cells, which covered the SRT lesion. ($**P < 0.01$).
In addition, cells surrounding the SRT area were positive for the cell proliferation marker Ki-67, whereas untreated RPE showed no signal for Ki-67 (Fig. 4).

**Integrity of RPE–BrM Complex After SRT**

We investigated the effect of SRT on RPE cells and underlying extracellular matrix by scanning electron microscopy. We observed the discrete selective destruction of RPE after 6 hours without damage of underlying RPE basal lamina and extracellular matrix (Fig. 5a). Damage of cell membranes might be the effect of SRT induced microbubbles after SRT as described by others (Fig. 5b). Apical microvilli are absent after SRT compared with untreated cells (Fig. 5c).

**Release of MMPs and Expression of MMP2 After SRT**

SRT spots of 200-μm size are known to induce MMP secretion and activation. To compare the effect of different spot sizes and densities on MMP release, we analyzed the basolateral MMP secretion. A representative zymogram, which is showing the release of different MMPs in irradiated and untreated explants, is shown in Figure 6. Pro-MMP9 (98 kDa) was always detectable in basal compartments of modified Ussing chambers, while active MMP9 was not detectable. In medium control (mc) some pro-MMP2 was detectable because of the background activity of 5% fetal calf serum. The zymogram is showing the moderate increase of pro-MMP9 and pro-MMP2 as well as the significant increase of active MMP2 in SRT-treated explants (200-μm spot size) related to control. Four days after SRT with 200-μm spots, pro-MMP9 and pro-
MMP2 levels showed no difference to control. Active MMP2 increased significantly after 4 days of cultivation to 1.75-fold (\( P < 0.01 \); Fig. 7).

Detection of MMP2 Expression by Immunofluorescence

The localization of SRT-induced MMP2 expression after SRT with 200-\( \mu \)m spots was detected by immunofluorescence using RPE–choroid flat mounts. The irradiated explants showed a localized expression of MMP2 in cell nuclei within the healing lesions while the surrounding cells showed no positive signal for MMP2 (Fig. 8).

Secretion of VEGF165 After SRT

At day 4 the basolateral release of VEGF of explants, which were treated with a ld of small 100-\( \mu \)m spot, was not altered in comparison to untreated control. In the hd group with 100-\( \mu \)m spots, we observed a moderate but significant VEGF decrease to 0.91-fold (\( P < 0.05 \)). The content of irradiated explants with 200-\( \mu \)m spot size decreased significantly after 4 days to 0.88-fold (\( P < 0.05 \)) in comparison to untreated control (Fig. 9).

Influence of SRT on PEDF Secretion

PEDF levels showed no difference, neither in explants with 100-\( \mu \)m ld treatment nor in 100-\( \mu \)m hd treatment, compared with control. In contrast, the amount of PEDF increased significantly to 1.25-fold (\( P < 0.05 \)) after 4 days in the choroid facing compartments of explants treated with 200-\( \mu \)m spots (Fig. 10).

DISCUSSION

Regeneration and Threshold Titration of RPE Cells

To minimize unspecific variability in cytokine release we chose a maximum observation time of 4 days, also referring to the transient effect of cellular dynamics after SRT as Treumer et al.\(^3^9\) have shown. A maximum spontaneous cell death of 10% of the RPE cells in calcein staining was considered acceptable. The increase of cell size, appearance of prominent nuclei, presence of two nuclei in some RPE cells and the positive signal of mitosis marker Ki-67 after SRT indicate RPE migration and mitosis as part of PER, and therefore possible rejuvenation has also been demonstrated by others.\(^3^9\),\(^4^6\) Four days after SRT, cell count analysis showed 53% recovering RPE cells in the irradiated area, compared with baseline (100%) of untreated RPE. We assume that a longer period of observation would show a further increase of cell number in the irradiated area. Differences in threshold of cell death between laser spots with 100- and 200-\( \mu \)m spot size were observed in this study, although unexpected compared with further studies.\(^3^8\) Individual differences in pigmentation of porcine explants, which could influence the formation of microbubbles and cell disruption as well as variation in pulse durations between different energies, which were used for cultivation experiments, might be reasons for differences in threshold of SRT-induced cell death between different spot sizes.

Release of Cell Mediators After SRT

The exchange of nutrients and waste products through BrM plays an important role in maintenance of the neuroretina. In AMD, the BrM is thickened and the supply of the neuroretina is...
reduced, which represents an important factor in development of multifactorial AMD. Therefore, we focused in this study on the basolateral release of AMD-relevant cell mediators during PER to identify the SRT effect on the BrM-facing site. AMD-relevant mediators include MMPs with influence of BrM remodeling as well as VEGF and PEDF due to their pro- and antiangiogenic properties.

Previous studies demonstrated that treatment of human BrM with activated MMPs leads to an enhancement of transport capacity. In our study SRT treatment with 200- and 100-μm (only hd treatment) spots resulted in a basolateral increase of active MMP2. This might lead to a reduction of a thickened BrM, which might improve the flux across the BrM to slow down or prevent the progression of early AMD.

The observed increase of active MMP2 was not an effect of cell death, but part of the regeneration process. A cell death–induced increase of MMPs would also affect pro-MMP2 and MMP9. The influence of pulsed laser irradiation on, for example, choroidal endothelial cells could not be tested separately in our system, while endothelial cells would also secrete cell mediators into basal compartments of modified Ussing chambers, like RPE cells do. In accordance with Treumer et al., we consider choroidal endothelial cells as a source for MMP release as unlikely, because Ahir et al. have not observed the release of MMPs in human BrM-choroid explants.

In this study, the content of pro-MMP9 was not affected by SRT and active MMP9 was not detectable. Ahir et al. have shown a cell cycle–dependent release of active MMP9 in RPE cell cultures up to 24 hours after subcultivation. In this synchronized system, active MMP-9 release was terminated at half confluence of proliferating RPE. This suggests that MMP9 activation is taking place in a very early stage of cell proliferation and could explain the absence of active MMP9 in our study as analysis were performed at day 4, when the RPE layer was nearly restored. However, an excessive expression of active MMP9 may contribute to the development of CNV.

The balance between the expression of proangiogenic cell mediators, such as VEGF and antiangiogenic cell mediators is highly regulated in the choroid–RPE–retina complex. A dysfunction of this balance is a crucial factor in the progression of AMD. For prevention of AMD development as well as progression of dry AMD into wet AMD, a therapy with a moderate influence on pro- and antiangiogenic cytokines is needed to generate a protein profile with nonneovascular properties.

The results of our study showed an increase of antiangiogenic PEDF and a decrease of proangiogenic VEGF following SRT treatment, corresponding to the indirect antiangiogenic effect of MMP2. This suggests that SRT may reduce the VEGF/PEDF ratio and generate an antiangiogenic cytokine profile.
Release of Cell Mediators after Selective Retina Therapy

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FIGURE 10. PEDF secretion of RPE–choroid explants after SRT using different laser spot sizes. Explants were irradiated with radiant exposures, which resulted in lesions above the threshold of cell death or cultivated as untreated controls. Basolateral PEDF content in culture medium of modified Ussing chambers is shown as fold of untreated controls (mean ± SEM). PEDF secretion of ld as well as PEDF content of hd treatment group with 100-μm spots (200 mJ/cm² per pulse) was not affected after 4 days of cultivation. After SRT with 200-μm spot size (140 mJ/cm² per pulse) PEDF secretion increased significantly in comparison to control. This indicates a density and lesion size dependent secretion of PEDF after SRT (**P < 0.01).

Influence of Varying Spot Sizes and Densities

The pathology in AMD is limited to the macular region, and a SRT treatment with small spot sizes would enable a precise and safe treatment close to the fovea. The SRT had no effect on cytokines after ld treatment with small 100-μm spots, a moderate effect after hd treatment with 100-μm spots and a clear effect after treatment with large 200-μm spots. This indicates that SRT laser spot size and the density of lesions play an important role in secretion of cell mediators, which is an important finding for further in vivo studies.

CONCLUSIONS

We found an increase in the basolateral release of MMPs triggered by SRT dependent on spot size and density. This could lead to a reduction of a thickened BrM, and thereby improve the flux across BrM and reverse age-related changes of BrM. The VEGF secretion was decreased and antagonistic PEDF secretion was increased by SRT. This could possibly prevent pathologic angiogenesis, such as CNV development. The SRT-induced cytokine milieu might lead to a rejuvenation of the RPE–BrM complex, and therefore represents a possible treatment for early AMD.

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