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RPE in Perfusion Tissue Culture and Its Response to Laser Application

Preliminary Report

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Key Words
Eye · Experimental · Pathology · Retinal pigment epithelium · Perfusion tissue culture · Laser photocoagulation · Cell response

Abstract
Purpose: To study the effects of conventional laser application on the retinal pigment epithelium (RPE) in a perfusion tissue culture model of porcine retinal pigment epithelium without overlying neurosensory retina. ***Methods:*** RPE with underlying choroid was prepared from enucleated porcine eyes and fixed in a holding ring (Minusheet®). Specimens were then placed in two-compartment tissue culture containers (MinuCell & Minutissue, Bad Abbach, Germany) and were cultured during continuous perfusion with culture medium at both sides of the entire specimen, the upper RPE and the lower choroid (12 specimens out of 6 eyes). Cultures were kept for 1, 3, 7 and 14 days and were examined histologically. Laser treatment was performed on each tissue ring by application of 3 × 3 laser burns one day after culture began (argon ion laser, wavelength: 514 nm, pulse duration: 100 ms; spot size: 200 μm) using different energy levels (400–1,000 mW); (16 specimens out of 8 eyes). ***Results:*** During laser treatment a marked lightening of the RPE with centrifugal spreading was observed. Using higher levels of energy, a contraction of the RPE towards the center of the laser spot was noticed. One day after laser photocoagulation histology revealed destruction of RPE; within 3–7 days of culture, migration and proliferation of neighboring cells was observed in several lesions. After 7 days the initial defect of the irradiated area was covered with dome shaped RPE cells and after 14 days multilayered RPE cells were showing ongoing proliferation. However, there were also cases without proliferation after laser treatment. The non-treated, continuously perfused RPE showed regular appearance in histological sections: during the first 7 days of culture, light microscopy revealed a normal matrix with a well-differentiated RPE monolayer. Subsequently proliferation even without treatment was observed and after 14 days the RPE became multilayered. ***Conclusion:*** It was possible to study the early healing response to the effect of laser treatment using the permanently perfused tissue culture

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system. A marked proliferation and repair of the laser defect could be observed in several but not all lesions. After 14 days even without laser treatment a proliferative multilayered RPE was present. Although this limits the use of the system for longer than 7 days, it seems to be useful for investigation of RPE-related disorders.

Background

The retinal pigment epithelium (RPE) is a complex cell layer involved in many eye diseases such as age-related macular disease (AMD), diabetic maculopathy (DMP), proliferative diabetic retinopathy (DRP), or central serous retinopathy (CSR). In these diseases laser photocoagulation is known to have a therapeutic effect and is therefore widely used. Despite this widespread clinical use, the exact mechanism of laser photocoagulation is not completely understood. It is known that the therapeutic effect derives not from the laser burn itself, but from the subsequent biological reaction [1–8]. Laser application using 514 nm is absorbed up to 50% by RPE cells resulting in damages with subsequent healing and therapeutic effects, widely used clinically today [9].

The ability to maintain RPE cells in culture has important advantages for the understanding of the pathobiology of several ocular diseases [10–12]. Especially in organ cultures, the interaction of different tissue cells can be studied. The main difficulty encountered when culturing RPE is to retain an intact monolayer over a long period of time, which would be helpful for elucidating complete pathomechanisms of several diseases. However, most previous studies are of limited value because the RPE cells were maintained on artificial substratum (e.g. polystyrene culture disks) although it has been well established that the substratum has pronounced effects on cell behavior [13–15]. To avoid these limitations Del Priore and coworkers first developed an organ culture system to maintain monolayers of RPE still attached to Bruch's membrane which provided the opportunity to study the response of the RPE to external stimuli such as photocoagulation [16]. Using this model it was demonstrated that laser photocoagulation led to an acute disruption of individual RPE cells and a separation of damaged RPE cells from Bruch's membrane. Treated areas were covered with irregular mounds of RPE cells within 7 days, which was supposed to mimic the response of RPE following laser photocoagulation in vivo [17].

For our approach to study laser tissue interaction a new model for cultivating RPE cells was employed [18, 19]. It was developed to cultivate tissue in an organotypical environment of a two-compartment system under permanent perfusion with medium [20, 21]. Good results for preservation of adult full-thickness retinal tissue using this model were presented recently [18, 19]. In a second step we used this model to maintain RPE as an organ culture over a longer period of time and to characterize the vitality of this system by irradiating the RPE monolayer with a conventional argon laser and performing light microscopy afterwards.

Material and Methods

Tissue Preparation

Fresh enucleated porcine eyes were prepared with the use of an operation-microscope under sterile conditions according to the method developed by Kobuch et al. using the MinuCell-perfusion system [19]. Thus 14 eyes were cut parallel to the limbus at a distance of 4 mm behind the iris-lens-diaphragm and the whole anterior cap was removed and most of the adherent vitreous. Next the posterior eye cup was separated into two equal parts by dissecting along the median raphe and the optic disc with a sharp knife. Each part was shortened at the edges to become a flat specimen of sclera, choroid, RPE and retina with partly adherent vitreous. The specimen was fixed to a flat surface with pins. Neurosensory retina was peeled off from the RPE with tweezers. Small scissors were used to separate the choroid with the overlying structures from the sclera leaving a layer of choroid and RPE. In order to cultivate this matrix we used a ring holder system, placed into a perfusion container which has been previously described in detail [18, 19, 21]. Briefly, the cell carrier consisted of a black holding ring, in which the cell layer was placed. The internal diameter was 9 mm and the external diameter 13 mm. To fix the support, a white span ring was pressed into the holding ring. Thus the white ring was moved under the choroid and the black ring was put onto the RPE in order to fix the specimen into this ring system thus having choroid at the lower and RPE at the upper side. This was then placed into the container which was designed for one to six ring systems [20, 21]. By placing the ring system into one of the compartments of the container, two compartments were created separated by the tissue and the ring holder system. Both compartments had two openings to be perfused with medium. The use of the gradient container allows permanent perfusion of different media at the upper (retina) and lower (choroid) side of the sheets. In our study the same medium was used at both sides. Over silicone tubes and luer fittings the container was connected to specific screw caps and media bottles allowing the permanent perfusion of the tissue. The system ran with a peristaltic pump with a speed of 1 ml/h on a warming table (40°C) out of the atmosphere of a CO_2-incubator. DMEM (Life Technologies™) with 25 mmol HEPES, 15% porcine serum and 1% penicillin-streptomycin out of 10.000 IU was used. The specimens were cultivated under these conditions for up to 1, 3, 7 and 14 days. Additionally one specimen was cultured up to 28 days. All were examined histologically by light microscopy.

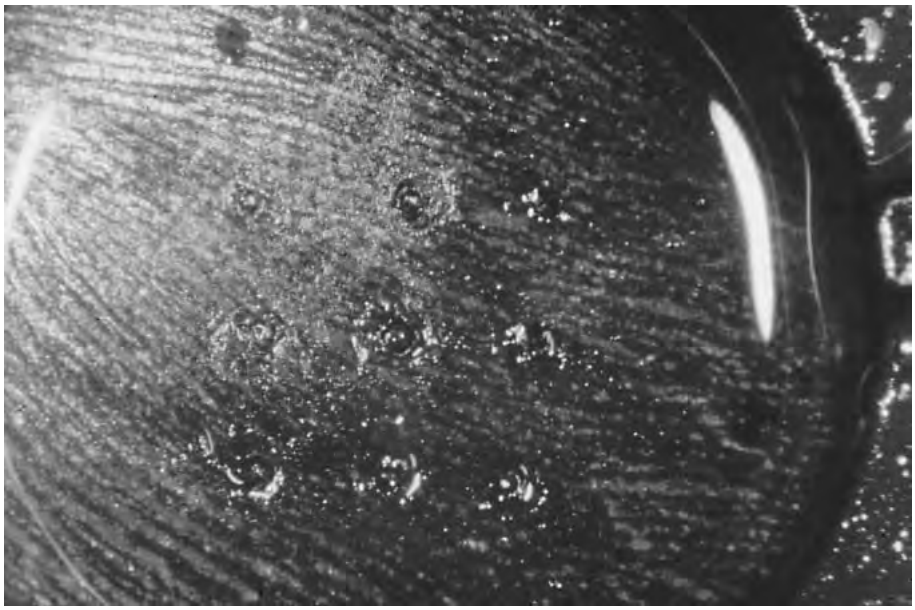

Fig. 1. Tissue holder ring system with coagulated RPE-monolayer on the top. Nine laser lesions can be noticed. ×10.

Table 1. Number of examined specimens during the study differentiated by perfused RPE without laser treatment and perfused RPE with laser treatment: every specimen treated by laser photocoagulation covered nine spots

	1 day	3 days	7 days	14 days
Perfused RPE without laser	3	3	3	3
Perfused RPE with laser	4	4	4	4

Laser Application

Using a special single chamber it was possible to perform laser photocoagulation to the RPE by positioning the chamber perpendicularly to the laser beam. Laser treatment with 3 × 3 spots (distance 200 μm) per tissue ring system (fig. 1) was performed one day after cultivation using a conventional argon laser [wavelength: 514 nm, pulse duration: 100 ms, spot size: 200 μm (even on tissue), power: 400–1,000 mW]. Different energy levels were utilized according to different pigmentation of the tissue. Thus energy was chosen in a way that a laser effect could be recognized ophthalmoscopically as a gray lesion. Also noticed was some pigment which was released from the RPE during the laser application and always a centrifugal enlarging lightening of the RPE could be observed in the first milliseconds after irradiation. The lesion appearance varied according to the laser power, however, the overall variability of the ophthalmoscopic aspect of the lesions was considered to be minor. The irradiated tissue was kept under perfusion conditions for 1, 3, 7 and 14 days and examined histologically afterwards by light microscopy.

Study Design

The total number of all examined specimens is summarized in table 1. The specimens were divided into two subgroups: perfused RPE without laser treatment and perfused RPE with laser treatment. Each laser-treated specimen received nine laser spots having a distance to each other of about 200 μm (fig. 1).

Light Microscopy

Tissue fixation was performed in formalin 10% for paraffin embedding to get a first impression and then in 3% glutaraldehyde (Sigma) in DMEM without porcine serum. Fixation took place after about 24 h. Specimens then were embedded with EPON (EMBED 812 Kit; Electron Mircoscopy Sciences). Sections of 0.8 μm were cut and stained with Fuchsin/Richardson.

Results

Perfused RPE without Laser Application

Under perfusion untreated specimens were well differentiated. Regular RPE formation with apical melanin was seen 1 day (fig. 2a) and 3 days (fig. 2b) after cultivation. The RPE cells were in close apposition and some microvilli at the surface of the RPE layer were recognized. After 7 days the RPE formation changed. Apical melanin was still present but marked alterations of the RPE cells occurred yielding a cuboidal and partly dome shaped morphology. Some vacuoles could be found inside the cell suggesting phagocytosis (fig. 2c). After 14 days of perfusion culture the RPE was multilayered (fig. 2d). After 28 days further growth of the multilayered RPE was noticed in one specimen (fig. 2e). In all histologic sections the choroid showed a good preservation of the matrix with well differentiated vessels having an intact endothelium.

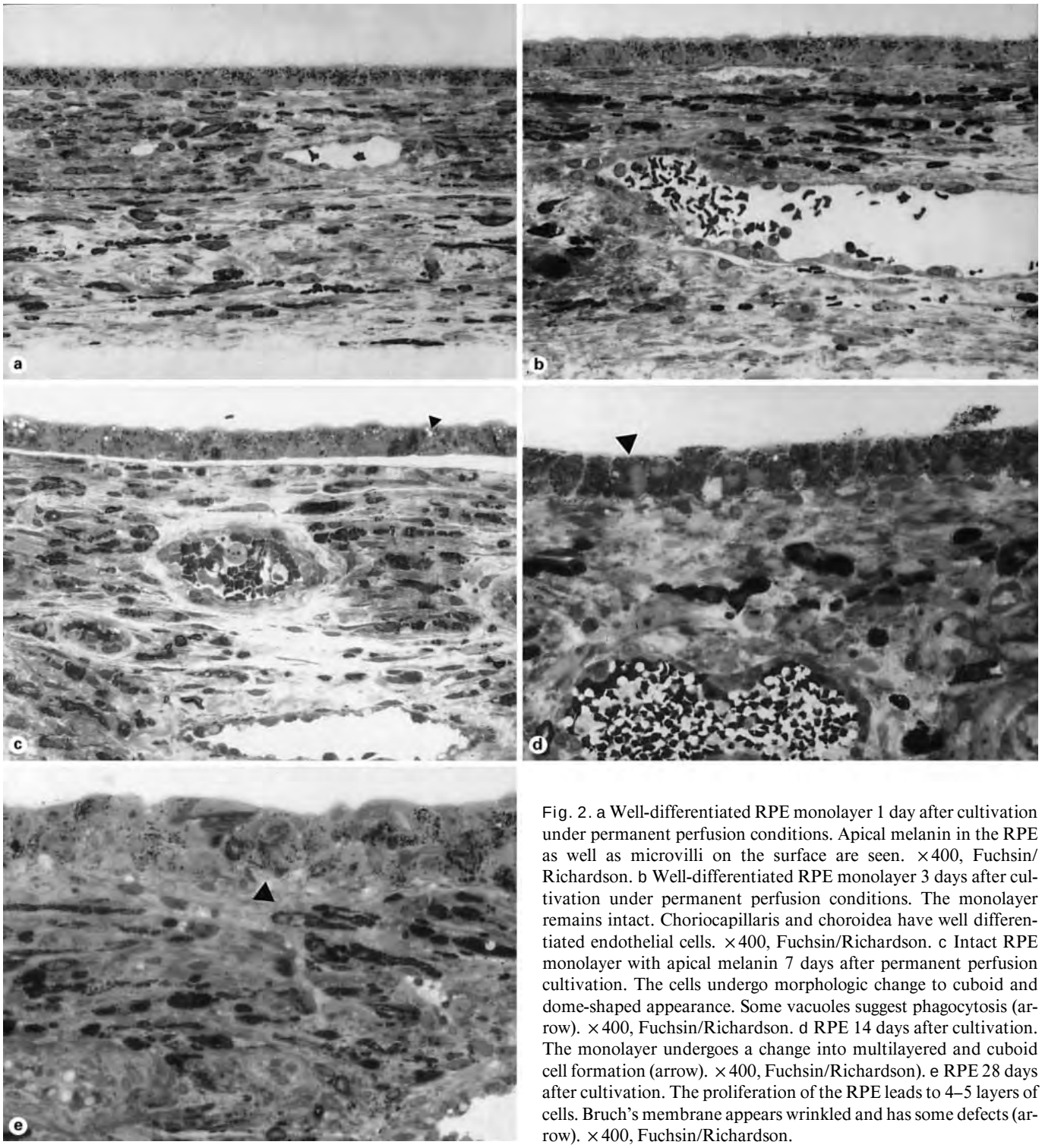

Fig. 2. a Well-differentiated RPE monolayer 1 day after cultivation under permanent perfusion conditions. Apical melanin in the RPE as well as microvilli on the surface are seen. ×400, Fuchsin/Richardson. b Well-differentiated RPE monolayer 3 days after cultivation under permanent perfusion conditions. The monolayer remains intact. Choriocapillaris and choroidea have well differentiated endothelial cells. ×400, Fuchsin/Richardson. c Intact RPE monolayer with apical melanin 7 days after permanent perfusion cultivation. The cells undergo morphologic change to cuboid and dome-shaped appearance. Some vacuoles suggest phagocytosis (arrow). ×400, Fuchsin/Richardson. d RPE 14 days after cultivation. The monolayer undergoes a change into multilayered and cuboid cell formation (arrow). ×400, Fuchsin/Richardson). e RPE 28 days after cultivation. The proliferation of the RPE leads to 4–5 layers of cells. Bruch's membrane appears wrinkled and has some defects (arrow). ×400, Fuchsin/Richardson.

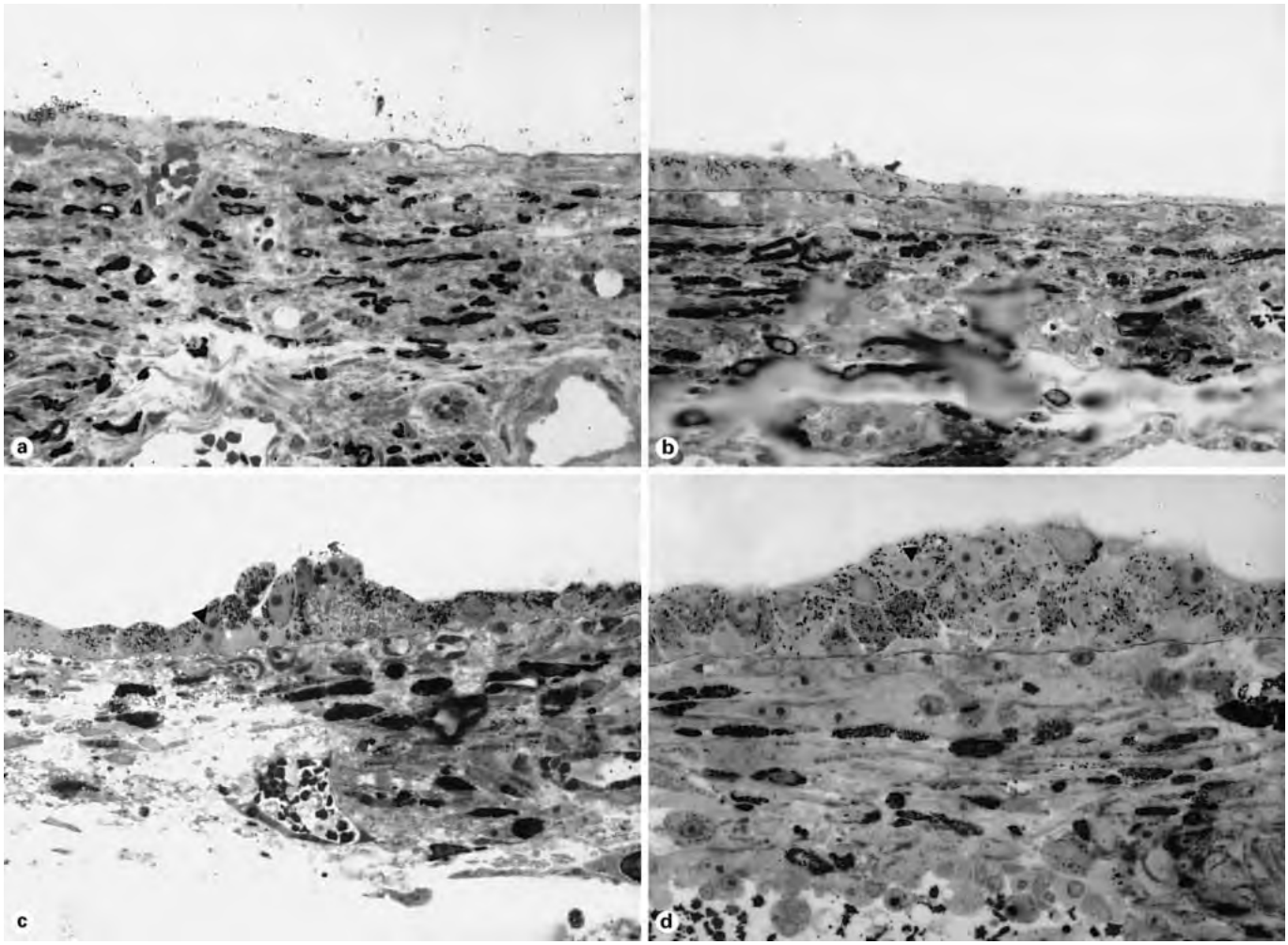

Fig. 3. a The RPE monolayer has a defect and debris 1 day after laser photocoagulation under permanent perfusion conditions. Shown is the left transient from untreated RPE to denude Bruch's membrane (BM), the whole diameter of the lesion (nude BM) is about 380 µm. The choroid has closed vessels, extracellular pigment and cell debris. ×400, Fuchsin/Richardson. b RPE 3 days after laser photocoagulation. The laser defect is covered by a layer of thin cells. ×400, Fuchsin/Richardson. Shown is the left transient from untreated RPE to BM, which is started to get covered again by the thin cells, whole diameter of lesion is about 180 µm. c RPE 7 days after laser photocoagulation. The laser defect is covered by a cell layer presenting localized nodular proliferative multilayered RPE with some pleomorphic cell nuclei (arrow). ×400, Fuchsin/Richardson. Shown is the right transient from untreated RPE (right) to treated RPE (left). The localized proliferation is assigned to the edge of the former lesion. The whole lesion is estimated to be approximately 340 µm in diameter. d RPE 14 days after laser photocoagulation. Multilayered cells are present in the area of irradiation forming a markable prominence. Also some pleomorphic cell nuclei are present (arrow); ×400, Fuchsin/Richardson. Localization of the proliferation and length of the lesion was not determined.

Perfused RPE with Laser Application

It was difficult to identify suspected areas as proper laser lesions. In all specimens examined only a minority of the laser spots applied were found by sectioning the tissue. Thus many areas which were suspected to be laser lesions did not show cell proliferation a few days after irradiation; however, in these cases differentiation from artifacts by preparation was difficult.

Thus for all given times of examination just six laser lesions could be clearly identified as areas treated by laser having following appearances:

One day after laser treatment the irradiated area could be identified as an area devoid of RPE cells. Bruch's membrane remained intact judged by light microscopy but appeared wrinkled over the whole length of the laser burn. The underlying choroid had closed vessels, extracel-

lular pigment and cell debris (figure 3a, showing left edge of the laser lesion; the whole diameter of the lesion was about 380 μm).

After 3 days, migration and proliferation of cells derived from the RPE next to the lesion could be noticed. These cells were flat and revealed some melanin granules. Migration took place towards the defect in the RPE layer. At the edge of the lesion Bruch's membrane was wrinkled and choriocapillary vessels were closed (figure 3b, showing one edge of the laser lesion; the whole diameter of the lesion was about 180 μm).

After 7 days, proliferation could be clearly demonstrated. The whole defect in the RPE layer due to the laser impact was already covered by a mound of focally nodular, multilayered RPE. Maximal proliferation was found predominantly at the edge of the lesion. In this area the melanin granules lost their apical orientation. Bruch's membrane was strongly wrinkled in that area. The choriocapillary vessels were open at the edge of the burn (figure 3c, showing right edge of the laser lesion). The whole lesion was estimated to be 340 μm in diameter, but proliferation made it difficult to determine the exact dimension of the lesion. Hence due to the cell proliferation of untreated cultured RPE it was also difficult to determine the diameter of the laser lesions 7 days after cultivation.

After 14 days, cells with broadly distributed melanin granules were present. Multilayered cells showed some prominent proliferations (fig. 3d). The diameter of the lesion was not determinable because of the general cell proliferation but Bruch's membrane which was focally wrinkled again, was indicating the presence of a lesion.

Discussion

Control Experiment

The permanent perfused culture model offers the possibility to cultivate retinal tissue in an organotypical environment having a perfusion line from both sides, the upper RPE and the lower choroid side. Using this RPE organ model in perfusion culture, it was possible to examine the behavior of the RPE after laser photocoagulation and the behavior of the organ culture without treatment.

The proliferative behavior of the RPE is associated with the substratum on which the cells are cultured. We used the perfusion culture system with the RPE monolayer still attached to Bruch's membrane. In previous static culture approaches it was difficult to maintain the RPE as an intact monolayer. Numerous workers developed culture models but had difficulties finding a solid support for the exoplants of human RPE and choroid [22–24]. Tso et al. used filter paper (Millipore) as support for the isolated uveal tissue and noted a markedly wrinkled Bruch's membrane and hypopigmented RPE cells after only 3 days in organ culture and frequent focal nodular proliferation within 10 days of culture [24]. In contrast Del Priore and coworkers [16] did not observe these alterations using another model. They were able to maintain the RPE as a monolayer for some weeks and explained this by the fact that the RPE and choroid remained attached to the structural support of the underlying sclera in their organ culture system. In their experiments the RPE monolayer remained intact after 2 weeks but they also noticed hypertrophic RPE cells and areas of RPE hyperplasia [16].

Our approach using the permanent perfused culture system supports the findings of other authors, who recognized proliferation of the cells. It was speculated that with this new culture model, RPE without neurosensory retina should be maintained as an intact monolayer for longer than 2 weeks. After 3 days in the perfusion culture the RPE monolayer was intact, after 1 week the cells were also intact as a complete monolayer, though there were some morphologic changes. However, after 2 weeks a marked proliferation of the cells and multilayered RPE was observed. Proliferation of the cells as seen beyond 7 days could be interpreted as a sign supporting the vitality of the organ culture. However, the morphologic findings after 14 days of culture may also suggest a phenotypical change of the RPE cells into other cells such as macrophages. Alternatively an overgrowth of the cell layer due to choroidal cells seems to be unlikely since Bruch's membrane stayed intact [24].

In our study these cell alterations of untreated RPE limits the use of the culture system for RPE organ culture over a period longer than 2 weeks. It is not clear why this cell change occurred in the RPE culture. When full thickness adult retina is cultured in the perfusion system, no signs of cell changes are observed, and the RPE remains intact as a monolayer over a period of 4 weeks [18, 19]. Therefore it could be speculated that with the loss of neurosensory retina the RPE is no longer inhibited, and thus reacts with cell proliferation and subsequently further scarring.

In comparison to the static culture approach as described by Del Priore the perfusion culture system revealed no advantage for RPE cultures since proliferation of untreated cells was evident after 7 days of culture, whereas Del Priore was able to maintain the RPE for 28 days.

Laser Application

The effects of laser treatment of the fundus have been studied by several groups. In vivo it has been observed that argon laser photocoagulation of monkey and human fundus causes necrosis of the RPE and a lifting of the RPE from Bruch's membrane [25–27], budding of individual RPE cells [28, 29] and multilayered RPE formation in the area of laser irradiation by 7 days after treatment [26–32]. Histologic sections revealed that by irradiating the RPE with a conventional argon laser the whole area of the cells was destroyed and the choriocapillaris as well as the choroidal vessels were damaged [17]. After laser photocoagulation RPE cells migrate and proliferate to cover the defect [7]. In vivo after mild photocoagulation (just light whitening of the irradiated area), as usually performed in macular coagulation, the RPE barrier becomes intact again [7]. Histological examinations of rabbit retina showed also a proliferation of the RPE after coagulation with short pulsed repetitive laser systems without destruction of the neurosensory retinal layer [33]. So RPE proliferation can always be observed as a healing mechanism to repair any defects in the RPE monolayer or adjacent structures.

The effects described could also be demonstrated in the laser lesions of our approach investigating mild RPE laser photocoagulation in vitro. In these examples, as in vivo, RPE cells started to cover the defect by migration and proliferation. After one week the defect was completely covered by multilayered RPE cells showing still an ongoing proliferation after 14 days. Similar to the findings of Del Priore, one day after argon laser photocoagulation we could also observe a release of the RPE from Bruch's membrane and a total destruction of the irradiated area. It was interesting to note that Bruch's membrane itself was not destroyed but appeared wrinkled.

Thus it was possible to show in vitro the migration and proliferation of the neighboring RPE cells to cover the laser defect up to 7 days. This is consistent with the in vivo findings of the RPE selective laser treatment showing that the RPE is replacing the irradiated area [33]. These healing mechanisms occur even without overlying neurosensory retina. This is an important observation because the neurosensory retina contains potent growth factors that could affect the morphologic response of the RPE to laser photocoagulation [30]. However, since this is not an audio-radiographic study, it should be noted that the 'proliferation' might just be a metaplasia rather than a mitosis of the cells. On the other hand results of previous studies as mentioned above suggested proliferation.

Limitations of the Study

Most authors did not give information about the number of laser spots examined, sizes of lesions in histology and percentage of proliferations [7, 17, 25]. In our preliminary experiments the described effects of laser-tissue interaction could be observed in a minority of all applied laser spots. This could be mainly explained by the difficulties in identifying the laser burns when trimming the tissue due to the fact that no thermal necrosis could be seen because of the lack of neurosensory tissue. Thus major damage is only confined to the RPE and in spite of the macroscopically greyish appearance of the RPE in irradiated areas histological alterations are hard to identify.

Concerning acute laser lesions Bruch's membrane is denuded and void of RPE cells. In our study several areas without RPE were found – also 3 and more days after irradiation – but differentiation from artifacts due to tissue preparation was difficult and therefore no fraction of proliferating lesions could be mentioned. In contrast to this, localized proliferations in treated specimens were a clear sign for laser lesions.

Another explanation for the irregularity of present proliferations could be the possible variation in laser energy, since it turned out to be difficult to judge experimentally an endpoint of laser treatment, when neural retina is absent. However, in all treated specimens laser burns were slightly visible indicating a destruction of the RPE which should be independent from the delivered energy [33]. Also the variation in spot size due to different focussing could be a contributing factor for the different proliferation behavior of the RPE. Interestingly the described laser lesions showed variable sizes of destruction. Although the chosen diameter of irradiation was about 200 µm, the diameter of the histologically observed laser lesions varied. There are several explanations for these findings. First of all, as described above, the focus during laser application through the microscopic culture chamber could change the diameter and additionally, each burn was adjusted and focused individually with a certain variation. Secondly, RPE cells responded variably to laser coagulation depending on their melanin content. This would lead to a variation in heat conduction. Third, each tissue shows individual proliferation behavior, so that for example nodular proliferation as seen in figure 3c could be smaller than the whole lesion. After 7 days it was nearly impossible to determine the exact size of the lesion because of the overall proliferation.

A major limitation for studying laser-tissue interaction for periods longer than 14 days seems to be the perfusion culture system itself because both treated and untreated

tissue revealed proliferation and thus differentiation and demarcation of treatment results are difficult. Therefore, as explained above, further research should consider improving the perfusion system, e.g. by changing the buffer solution or the perfusion time.

Conclusion

In summary this model is useful to study laser effects of the RPE in vitro although preparation and histological documentation of laser spots can be difficult. However a healing process of the RPE could be observed associated with migration and proliferation of the cells. On the other hand, untreated RPE also showed a proliferation after 7 days in the perfusion culture. After 2 weeks a general alteration of RPE cells to multilayered cells occurred. This morphogenesis of cultured RPE cells has been already shown by several authors [16, 34–36]. As a consequence of these RPE alterations our system seems not to be useful after a period of 7 days to examine laser tissue interaction. In contrast, it might be suitable – because of short-term changes – to perform e.g. toxicity tests with intraoperatively used balanced solutions or investigations on growth factor production or RPE transplantation in the early period.

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