Subretinal Posterior Pole Injury Induces Selective Proliferation of RPE Cells in the Periphery in In Vivo Studies in Pigs

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Purpose. To study topographical differences in porcine retinal pigment epithelial (RPE) cell proliferation (1) in vivo, after experimental central surgical subretinal injury, and (2) in vitro.

Methods. Domestic pigs underwent either experimental RPE debridement ($n = 5$), subretinal amniotic membrane transplantation ($n = 4$), or both ($n = 1$) in the left eye. RPE cell proliferation was assayed by injection of the thymidine analogue 5-bromodeoxyuridine (5-BrdU) at postoperative day 0 and 1. RPE cells in S-phase were identified by their incorporation of 5-BrdU, as detected by immunohistochemistry. The in vitro proliferation of primary RPE isolates from the peripheral and central retina was assayed by a colorimetric assay and by [^{3}H]thymidine incorporation.

Results. After subretinal surgery, in vivo incorporation of 5-BrdU was seen in peripheral RPE cells in 8 of 10 surgically treated eyes, but never in central RPE cells. This observation was true of both types of experimental surgery performed. In vitro, RPE isolates from the pre-equatorial region consistently yielded higher cell densities than did RPE cell isolates from more central parts of the epithelium. This was apparent through the three first passages of porcine RPE cells in culture. After 1 and 4 days in culture, pre-equatorial RPE cells had incorporated significantly more [^{3}H]thymidine than had the more central RPE cells.

Conclusions. Experimental subretinal surgical injury of the RPE below the central retina is followed within 48 hours by a peripheral, but not a central, proliferation of RPE cells. In vitro, peripheral RPE cells have a higher proliferative capacity than do central RPE cells. (*Invest Ophthalmol Vis Sci.* 2007;48: 355–360) DOI:10.1167/iovs.05-1565

After rapid cell division during between the fifth and the seventh weeks of embryonic development, the normal retinal pigment epithelial (RPE) cells cease to proliferate and are generally considered to form a postmitotic epithelium in adult individuals.[1,2] In the human embryo, the cessation of rapid cell division occurs between the fifth and the seventh weeks and is most pronounced in the posterior part of the eye. Some cell proliferation continues to occur in the peripheral RPE until a later stage of development.[3] In the fully developed eye, vigorous RPE cell proliferation can occur under pathologic conditions, such as macular puckering and proliferative vitreoretinopathy. In these conditions, the RPE cell proliferation is believed to be stimulated by the direct contact between the RPE and yet unidentified vitreous constituents.[1,4]

After experimental surgical debridement of the central RPE in pigs and monkeys, without damage of the neuroretina, the RPE defect is repopulated with flattened RPE cells after approximately 6 weeks.[5–7] Despite this obvious recruitment of RPE cells, mitoses in the adjacent RPE cell layer were not found.[7] Recruitment of RPE cells without apparent cell division (i.e., mitoses) in the adjacent RPE is also seen after experimental transplantation of basement material into the subretinal space in pigs.[8,9] After transplantation of basement membrane material, RPE cells obtain a cuboidal shape with ample pigment granules.[8,9] This result is in contrast to the flattened, depigmented cells that are present after RPE debridement.

Previous in vitro experiments on adult bovine RPE cells have shown that RPE cells from the periphery have higher proliferative capacity than do RPE cells derived from the posterior pole.[3] Also, backward sliding of peripheral RPE cells has been suggested to compensate for apoptotic posterior RPE cell loss.[10] Hence, the source of the new RPE cells seen after central RPE debridement or subretinal basement membrane transplantation may be the peripheral RPE cell proliferation, with subsequent backward sliding of the new RPE cells.

In the present study, we investigated the hypothesis that central RPE debridement is followed by RPE proliferation in the periphery. We used the 5-bromodeoxyuridine (5-BrdU) labeling technique[11] to identify proliferating RPE cells after surgical RPE debridement and transplantation of basement membrane material into the subretinal space of pigs. We also investigated the in vitro proliferative potential of porcine RPE explants derived from the posterior and peripheral retina.

Materials and Methods

Surgery

We used 3-month-old Danish Landrace pigs. The research was approved by the Danish Animal Experiment Inspectorate and was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A veterinarian supervised animal treatment. Initially, all pigs underwent a standard three-port vitrectomy, and then a subretinal bleb was raised in the visual streak area, and a posterior retinectomy was made in the bleb area. RPE proliferation was stimulated by two different subretinal surgical procedures—a mechanical removal of the RPE (RPE debridement, $n = 5$) or transplantation of porcine amniotic membrane into the subretinal space ($n = 4$)—or a

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combination of the two procedures ($n = 1$). Only left eyes underwent surgery ($n = 10$). Nine right eyes were used as the control.

Pigs were killed and the eyes enucleated at postoperative day 2. Immediately after enucleation, the eyes were fixed in 4% buffered formalin and subsequently sectioned.

Immunohistochemical Identification of Dividing Cells

Dividing cells were identified by their incorporation of the thymidine analogue (+)-5-bromo-2′-deoxyuridine[11] (500 mg 5-BrdU; Sigma-Aldrich, Taastrup, Denmark) dissolved in 150 mL isotonic phosphate-buffered saline and injected intravenously at the end of surgery and on postoperative day 1. The pigs were reanesthetized and clinically examined at postoperative day 2, when both eyes were enucleated and the pigs subsequently killed by intravenous injection of 2 to 4 g pentobarbital (200 mg/mL; KVL, Copenhagen, Denmark). Globes were fixated in 4% buffered formalin, embedded in paraffin, and cut in 5-μm sections in the plane defined by the lesion and the optic nerve (i.e., approximately in the 10 to 4 o'clock meridian). Every 25th section was sampled and used for immunohistochemistry. An anti-5-BrdU antibody (clone BU-33, dilution 1:3000; Sigma-Aldrich) was used to identify cells that incorporated 5-BrdU. Epitopes were retrieved by heating the specimens to 95°C for 5 minutes in a citrate buffer (pH 6.0). A commercial visualization kit based on labeled streptavidin biotin and peroxidase/alkaline phosphatase detection kit was used (LSAB2; Dako, Rødovre, Denmark).

For the histologic examination, the RPE was divided into seven areas, centered around the lesion, as shown in Figure 1. The presence of the reaction product in RPE cell nuclei was assessed by light microscopy (Axioplan 2 imaging; Carl Zeiss Meditec, Göttingen, Germany). Two observers who were masked with regard to the surgical procedure (JUP and MP) performed the histologic evaluation of the sections, which were presented to them in random order. In each section, the observer assessed which areas were included in the section. Each observer then scored the percentage of visible RPE cell nuclei that contained the 5-BrdU reaction product. The scoring was performed semiquantitatively in steps of 5% (i.e., 0%, 5%, 10%, and so forth). At regular intervals the scoring of the two observers were compared, and if discordance was found, the relevant sections were examined again. We examined between 14 and 20 histologic sections from each pig—a total of 185 sections. Only 177 sections included the lesion, which was necessary for proper assessment of the retinal areas studied. Because of difficulties in aligning the sections, one or more areas could not be evaluated in 39 of the sections examined. In eight pigs, all areas were assessable. The lesion area (area L), area II, and area III were included in all pigs studied. Areas I, IV, V, and VI were missing in one pig, and areas IV, V, and VI was missing in another pig. The number of histologic sections, and the number of pigs studied are summarized in Table 1.

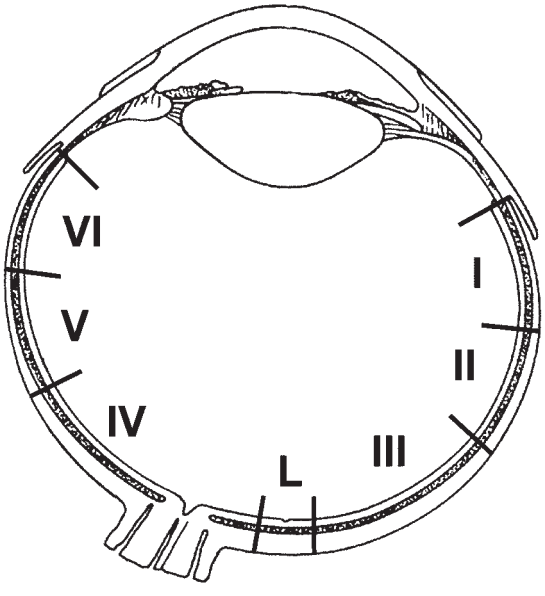

Figure 1. Schematic drawing of the eye showing the different areas used for evaluation of 5-BrdU incorporation. The plane of the section shown in the illustration, as of the histologic sections studied, runs almost horizontally. The *left* part of the figure faces nasally, the *right* part faces temporally, and the lesion area (L) is centered in an area just below the temporal aspect of the visual streak.

Table 1. The Number of Evaluated Sections for Each Area

	RPE Debridement		Amniotic Transplant		Comb. Surgery	
Area	Sections	Pigs	Sections	Pigs	Sections	Pigs
I	62	4	76	4	19	1
II	82	5	76	4	19	1
III	82	5	76	4	19	1
L	82	5	76	4	19	1
IV	43	3	76	4	19	1
V	43	3	76	4	19	1
VI	43	3	76	4	19	1

See Figure 1 for definitions of areas. Data are the number of sections and pigs.

The histologic sections were perpendicular to the plane of the retinal pigment epithelium. Because of the pigmentation of the epithelium, some RPE nuclei were completely or partially obscured by pigment. We did not include such nuclei in the evaluation where it could not be assessed whether they included the 5-BrdU reaction product or not.

The nontreated right eyes were used as the control ($n = 9$). In the left eyes, 5-BrdU incorporation in the limbal area and in the conjunctiva was used as the positive internal control. The lack of 5-BrdU incorporation in corneal endothelium and stroma, neuroretina, choroid, and optic nerve served as the internal negative control.

In Vitro RPE Cell Proliferation Assay

Primary cultures of porcine retinal pigment epithelial (pRPE) cells were isolated from freshly enucleated eyes of specific pathogen-free (SPF) Danish Landrace pigs (3 months old, 30 kg). Eyes were sterilized in 70% ethanol for 25 seconds. The sclera was carefully removed from the eyes, first by sharp incision 3 mm behind the limbus and thereafter by blunt dissection until only the optic nerve was adherent, leaving the uvea intact. Thereafter the eyes were incubated for 60 minutes at 37°C with 2 mg/mL collagenase (Sigma-Aldrich) dissolved in culture medium (DME-H16; Invitrogen-Life Technologies, Taastrup, Denmark). The eyes were place with the cornea downward. The optic nerve head was removed and the uvea-Bruch's membrane-RPE complex was then isolated in three concentric zones numbered 3, 2 and 1, centered around the putative lesion area. The zones were selected to match the areas in Figure 1 (zone 1 corresponds to areas I and IV, zone 2 corresponds to areas II and V, and zone 3 corresponds to areas III and IV). It was then possible to remove the RPE in small sheets using gentle manipulation by the water-jet method. Contamination of choroidal cells was avoided by washing the cells in calcium-magnesium–free phosphate-buffered saline (CMF-PBS). The isolated sheets were seeded directly into 35-mm tissue culture dishes (Invitrogen-Life Technologies). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 300 mg/mL glutamine, 50 mg/mL gentamicin, and 2.5 mg/mL amphotericin B and maintained in humidified 10% CO_2 at 37°C.[12] The medium was changed every second day. Figure 2 shows phase-contrast micrographs of the cultured porcine RPE cells derived from the three anatomic regions.

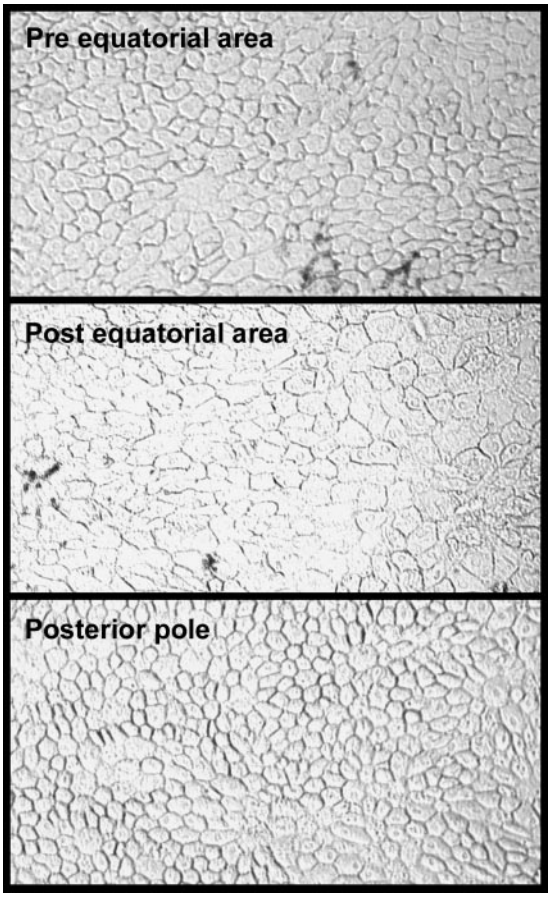

FIGURE 2. Phase-contrast micrographs of primary porcine RPE cultures after 21 days in culture. The pre-equatorial area corresponds to zone 1, the post-equatorial area to zone 2, and the posterior pole to zone 3.

The primary pRPE cultures were harvested on day 7 with 1× trypsin-EDTA (Sigma-Aldrich). These cells were maintained and propagated in three passages. Cells in each of these passages were seeded in 96-well, clear, flat-bottomed, treated tissue culture microplates (model 3595; Corning, Inc., Corning, NY) and assayed for proliferation. We seeded 2000 cells/well, approximately 60 cells/mm^2. Uniform seeding density was obtained by measuring the cell density in the stock solution of cells by triplicate counts of a 10 μL sample in a standard hemocytometer chamber. The stock solution was then diluted to a concentration of 20,000 cells/mL, and 100 μL of this solution was transferred to each of the wells in the 96-well plate by an automated pipette (Response 4850 10–500 μL; Eppendorf, Horsholm, Denmark). This procedure was repeated for each primary isolate each time the 96-well plates were seeded. For both proliferation assays, all 96-well plates were seeded at the same day, and each plate was seeded with isolates from all the three anatomic region in random order, to avoid systematic seeding artifacts.

A colorimetric method was used to estimate the number of viable cells. The assay is based on mitochondrial enzymatic conversion of the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] to a formazan salt (CellTiter 96 AQueous; Promega Corp., Madison WI). We incubated the RPE cells for 2 hours with 20 μL of the tetrazolium compound and then measured the quantity of the formazan reaction product spectrophotometrically as the absorbance at 490 nm. Quadruplicates of each zone from two different pig eyes were obtained at 2, 4, 8, 10, 14, and 21 days for three passages. For each of the investigated anatomic origins, we verified that the measured absorbance was directly proportional to the number of RPE cells. Primary cultures of confluent porcine RPE cells from zones I, II, and III were trypsinized, and the total number of cells was determined as described earlier. Thereafter dilution curves of 100,000, 50,000, 25,000, 12,500, 6,250 and 3,125 cells/well were prepared in triplicate and incubated with the tetrazolium compound, and the absorbance measured (data not shown).

DNA synthesis was measured by incorporation of [^{3}H]thymidine. Cells were incubated with 2 μCi/mL [^{3}H]thymidine for 24 hours, and then the medium was removed and the cells were washed three times with CMF-PBS. Finally, the cells were frozen at −20°C, and subsequently, the lysed with sodium citrate and the [^{3}H]thymidine incorporation was measured in a scintillation counter. Results are expressed as counts per minute (cpm) per well from quadruplicate samples.

Data Analysis

For the in vivo experiments, we calculated the average percentage of 5-BrdU-positive nuclei for each area in each pig. Statistical analysis was performed on these averages, to avoid introducing bias from the different number of histologic sections obtained for each pig. We used factorial ANOVA with Bonferroni-adjusted *t*-tests as a post hoc test to evaluate whether differences in 5-BrdU incorporation existed between the different regions in the two groups of experiments performed. The two experiment types were analyzed separately.

In the in vitro experiments, the effects of regional origin on the growth of porcine RPE cells were evaluated by factorial ANOVA. In the colorimetric assay, we performed logarithmic transformation of the absorbances to improve variance homogeneity. Log(absorbances) were analyzed with a model that included the three factors: passage (1 to 3) days in culture, and regional origin, as well as interaction between day in culture and regional origin. The interaction between day in culture and regional origin was found to be nonsignificant (F-test, $P = 0.4$), and the model was subsequently reduced to incorporate only the main effects of passage, days in culture, and regional origins. Since the effect of regional origin thus was independent of days in culture, we could test the effect of regional origin using all data from all regions, days, and passages (F-test). In the thymidine incorporation experiments, counts were analyzed with a model that included three factors: passage (1 to 2), days in culture (1 and 4), and regional origin (zones 1–3). We used an F-test to test for group significance of regional origin and performed post hoc Bonferroni-adjusted *t*-tests to test for significant differences between the individual zones. Throughout the study, a significance level of 0.05 was used (SAS ver. 9.1; SAS Institute Inc., Cary, NC).

RESULTS

In Vivo Experiments

We found 5-BrdU-positive nuclei in the pre-equatorial (areas I and VI) and equatorial (areas II and V) regions in 8 of 10 eyes that were enucleated 2 days after surgery (Fig. 3). This labeling was most pronounced on the same side as the lesion (Fig. 4). In the posterior pole, only a few stained nuclei were found in two pigs (Fig. 4). In the lesion area, we found no stained nuclei in any section (Fig. 4). In control eyes, only a few ($\ll$5%) 5-BrdU-positive RPE cells were seen in the pigmented ciliary epithelium and in the pre-equatorial region of the RPE, but otherwise no positive cells were found in the control eyes. Figure 4 summarizes the experiments and shows the average percentages of stained nuclei in the seven different regions among pigs that underwent either RPE debridement or amniotic membrane transplantation. In one pig, we examined the effects of a combined RPE debridement and amniotic membrane transplantation. In this pig, we found the highest percentage of 5-BrdU–stained nuclei (approximately 90% stained nuclei in regions I, II, and V, and VI). The percentage of 5-BrdU positive cells was different between the seven different areas studied (F-test, $P < 0.001$). This was true for both eyes in which RPE debridement had been performed and eyes that had

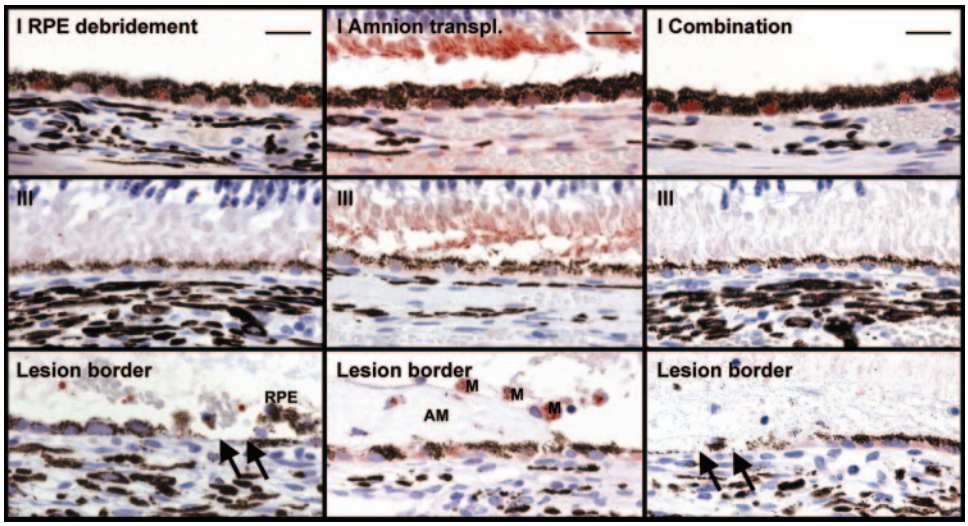

FIGURE 3. Micrograph of the left eye 2 days after removal of the retinal pigment epithelium (RPE, an amniotic membrane transplantation and a combination of the both. *Top*: (I) show RPE from pre-equatorial region or area 1 (see Fig. 1). The *red* color of the nuclei indicates 5-BrdU incorporation. The number of positive cells diminishes as we move posteriorly, and no 5-BrdU-positive cells are seen in the posterior part of the eye as shown in the middle row (III). The third row of panels shows lesion borders. No positive 5-BrdU cells are observed, but diffuse, unspecific staining of the cytoplasma in the RPE and in melanophages (M) in the proximity of the transplanted amniotic membrane (AM) is noted. Bars, 20 μm.

received amniotic membrane transplantation. In RPE debridement eyes, significantly more 5-BrdU-positive cells were found in region I than in the other regions (post hoc testing with Bonferroni-adjusted t-tests, $P \leq 0.01$). In eyes with amniotic membrane transplants, significantly more 5-BrdU-positive cells were found in region I, when compared with regions III, L, and IV (post hoc testing with Bonferroni adjusted *t*-tests, $P < 0.05$).

In Vitro Experiments

We tested the regional differences in proliferative capacity of porcine RPE cells in two different ways.

Figure 5 shows the results of the colorimetric assay of cell proliferation for porcine RPE cells derived from zones 1, 2 and 3 and subcultured in three passages. The assay relies on a mitochondrial enzyme that catalyzes the formation of a formazan product, which can be measured spectrophotometrically by its absorbance peak at 490 nm. This absorbance is linearly dependent on the number of RPE cells. Statistical analysis of the absorbances shown in Figure 5 revealed that the effects of origins, passage, and culture age were all statistically significant (F-test, $P < 0.001$). In all three passages, the cells derived from zone 1 reached higher cell densities than cells from equator and cells from the posterior pole (post hoc Bonferroni-adjusted *t*-tests, $P < 0001$).

Figure 6 shows the incorporation of [^{3}H]thymidine in fast-growing cultures of porcine RPE cells in the first and second passages. Peripheral cells, (zone 1 cells) incorporated significantly more thymidine than did the midperipheral and central cells from zones 2 and 3 (F-test for group difference, $P < 0.001$, post hoc Bonferroni-adjusted *t*-test for paired differences, $P < 0.001$).

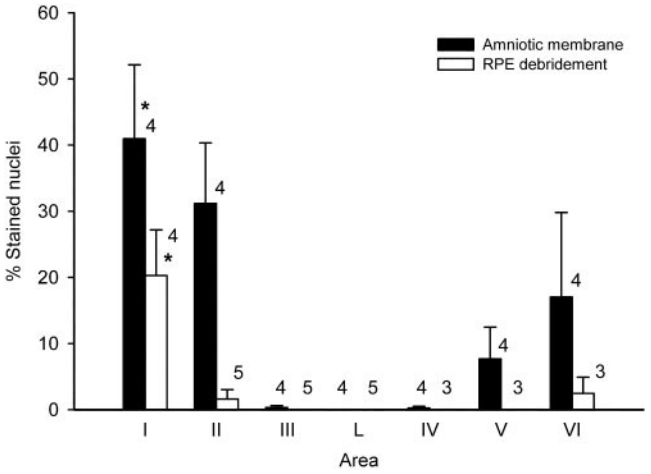

FIGURE 4. Graph showing the mean ± SEM of 5-BrdU positive cells in different areas evaluated in eyes with amnion transplantation and in eyes with RPE removal. Transplantation of the amniotic membrane induces a significantly higher response in the RPE compared to RPE removal. The number of eyes evaluated is given at each bar. $^*P < 0.05$.

DISCUSSION

This is the first study to show that central RPE debridement or transplantation of porcine amniotic membrane to the central subretinal space is followed by peripheral, but not central, RPE cell proliferation in vivo at postoperative day 2 (Figs. 3, 4). We used 5-BrdU incorporation as a well established marker of S-phase detection in cell proliferation in mice, but also in pigs.[11,13] Appropriate internal positive and negative controls validated the method in this study.

We also confirmed earlier findings in bovine eyes[3] that the in vitro proliferative capacity of porcine RPE cells is higher for peripheral than central RPE cells (Fig. 5). Two in vitro proliferation assays were used, both of which have been widely used and validated.[3,14,15] The [^{3}H]thymidine assay is critically dependent on a stable nuclear DNA content in the RPE cells.[12] The colorimetric assay relies on the mitochondrial metabolism of the dividing cells and is therefore critically dependent on this metabolism's being similar between tested groups of cells. Because both methods showed increased proliferation in peripheral cells when compared to central cells, we consider it likely that the peripheral porcine RPE incorporates cells with higher proliferative capacity than does the posterior RPE.

The peripheral RPE proliferation was less pronounced after RPE debridement than after transplantation of porcine amniotic membrane to the subretinal space (Fig. 4). The cause of this difference remains elusive, but the closure of the choriocapillaris caused by the RPE debridement may play a role.[16,17]

The lack of RPE cell proliferation in the vicinity of the experimental RPE debridement is a confirmation of earlier work, where mitoses were looked for, but not found.[7,8] In a previous study of RPE debridement in rabbits, local cell proliferation at the edge of the lesion and within the lesion was detected by [^{3}H]thymidine autoradiography at the seventh postoperative day.[18] However, in this study the neuroretina overlying the lesion was removed, and it is well known that a

retinal detachment or a retinal break stimulates vigorous local proliferation in the underlying RPE.[19] It is likely that RPE stimulation by the retinectomy at least contributed to the cell proliferation observed by Heriot and Machemer.[18] Local cell proliferation after laser photocoagulation has also been demonstrated by ^{3}H thymidine autoradiography.[20] However, the origin of the proliferating cells was elusive, and the damage to the retina was not confined to the RPE.

Immunohistochemistry is at best semiquantitative.[21] In our in vivo experiments we also used a semiquantitative approach, with arbitrarily chosen 5% intervals and masked observers. One could argue that other counting protocols might have been more accurate. However, the preferential localization of stained nuclei to pre-equatorial areas (I and VI) was very robust and not dependent on the choice of interval or data sampling method. We analyzed sections that included the lesion and that were oriented almost horizontally. Accordingly, no information was obtained about peripheral RPE cell proliferation in the ventrodorsal meridian, and it is a limitation of the study that sagittal sections were not studied.

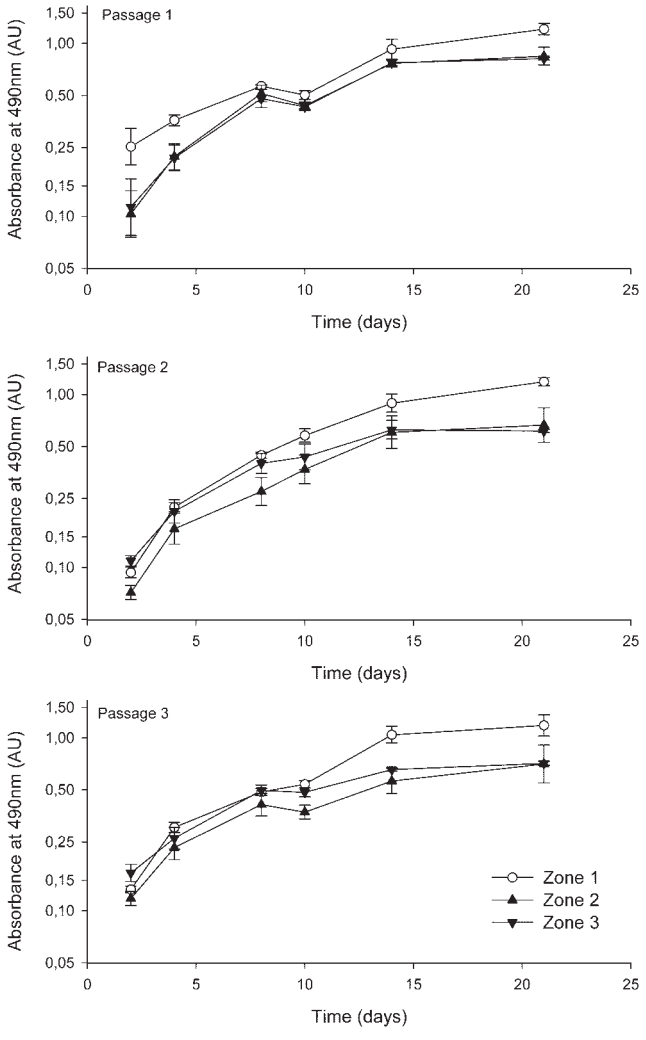

FIGURE 5. Graphs showing the growth of retinal pigment epithelium (RPE) isolated from different regions of the porcine eye. Zone 1 corresponds to areas I and VI in Figure 1; zone 2 to areas II and V; and zone 3 to areas III and IV. The number of cells was determined by a colorimetric assay and measured spectrophotometrically as the absorbance at 490 nm in arbitrary units (AU). The data are expressed as the mean ± SEM. Each data point represents eight experiments, with cells derived from two different pigs. In all three passages, the cells derived from zone 1 reached higher cell densities than did cells from the equator or the posterior pole (F-test, $P < 0.001$).

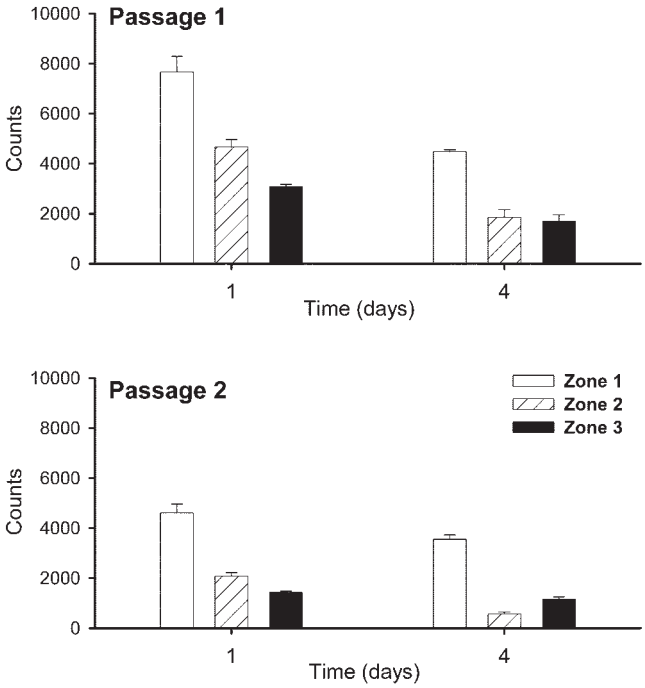

FIGURE 6. The initial growth of cultured porcine RPE cells assayed by the incorporation of [^{3}H]thymidine. RPE cells were isolated from different regions of the porcine eye. The zones are as described in Figure 5. The data are expressed as the mean ± SEM. Each bar represents four experiments. Cells from zone 1 incorporated significantly more thymidine than did cells from zones 2 and 3 (F-test for group difference, $P < 0.001$, post hoc Bonferroni-adjusted *t*-tests, $P < 0.001$).

The RPE has been thought of as a stationary, or postmitotic, epithelium.[22] Recently, it has become clear that in the aging human eye there is a continuous loss of central RPE cells through apoptosis.[10] It has also been found that despite the continuous loss of central RPE cells, the RPE cell density in the macular area remains constant in normal aging eyes, whereas this density declines with age in the peripheral RPE.[10] These findings led Del Priore et al.[10] to suggest that there is a continuous posterior migration of peripheral RPE cells. This hypothesis might explain our previous findings that a single layer of well-differentiated RPE cells are present as early as postoperative day 14 on the surfaces of basement membrane material transplanted into the subretinal space in pigs.[8,9] It is tempting to speculate that there might be a population of RPE progenitor cells in the peripheral epithelium, or in the ciliary body, analogous to recent findings in mammalian neuroretinal cells.[23,24]

The finding that posterior RPE debridement elicits RPE cell proliferation in the peripheral RPE and not in the epithelium adjacent to the lesion is, to the best of our knowledge, new. It raises the intriguing possibility of an endogenous humoral factor capable of controlling the recruitment of RPE cells. It also suggests that we might be able to pharmacologically control in vivo RPE cell proliferation.

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