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BASIC SCIENCE

Transplantation of PEDF-transfected pigment epithelial cells inhibits corneal neovascularization in a rabbit model

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Abstract
Background The purpose of this study was to investigate the effect of recombinant pigment epithelium-derived factor (rPEDF), secreted by ARPE-19 cells transfected with the human PEDF gene and transplanted subconjunctivally in normal and in rabbits in which corneal neovascularization was elicited by a chemical burn.
Methods Twenty grey Chinchilla Bastard rabbits were randomly assigned to four groups; neovascularization was induced in groups A, B, and C by alkali cauterization. Seven days later, group A received no cell implantation, non-transfected ARPE-19 cells were implanted subconjunctivally in group B, and PEDF-transfected ARPE-19 cells were implanted subconjunctivally in groups C and D (non-cauterized). In-vivo rPEDF secretion was analyzed by immunoblotting, and ELISA of extracts of conjunctival tissue samples taken at different time points. Digital photographs acquired on days 7, 14, and 21 after cauterization were evaluated for lead vessel length, vascular invasion area, and overall neovascularization rate.
Results At days 14 and 21 after cauterization, significant differences were observed between groups A, B, and C in lead vessel length (day 21: 5.91±0.45, 5.11±1.22, 3.79±0.59 mm, repectively), vascular invasion area (day 21: 35.5±8.65, 34.86±4.92, 19.2±5.03 mm^2 respectively), and rate of corneal neovascularization. Compared to controls, neovascularization was reduced by 37.5 % on day 14 and 47 % on day 21. Analysis of conjunctival tissue extracts showed that rPEDF was secreted by the transplanted PEDF-transfected cells.
Conclusion Subconjunctivally transplanted, PEDF-transfected ARPE-19 cells secrete rPEDF, which inhibits the corneal neovascularization elicited by alkali cauterization.

Keywords Corneal neovascularizationl · Transfection · Pigment epithelial cells · rPEDF · Transposon · Sleeping Beauty · Rabbit

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Introduction

Under normal physiological conditions, the cornea is devoid of both blood and lymphatic vessels [1]. The lack of vessels is critical to maintain the defined architecture of the corneal stroma, which is essential for transparency and normal vision [2, 3]. Corneal neovascularization (NV) is a harmful process involving the breakdown of the limbal barrier and invasion of the avascular cornea by newly formed blood vessels, leading to corneal opacification, reduction in vision, and possibly blindness. Various diseases, infections, and traumatic injuries to the eye can cause corneal NV [4]. Corneal NV is the main risk factor for corneal graft rejection [1, 5]; in fact, approximately 50 % of patients with normal-risk keratoplasty, who

preoperatively have an avascular recipient bed, develop corneal NV in the first postoperative year [1, 6]. Several drugs have been used to treat corneal NV, e.g., steroids, methotrexate, and thalidomide [7]; however, these non-specific anti-inflammatory drugs are not in wide use because of side-effects and insufficient efficacy [8–11].

The angiogenic factors VEGF and bFGF are expressed during corneal wound healing and up-regulated in inflamed and vascularized corneas in animal models as well as in humans [12–14]. Investigations on the effectiveness of anti-VEGFs have shown that bevacizumab (Avastin®) administered subconjunctivally or topically can inhibit corneal NV in humans [15] and in animal models [6, 16–19]. The binding amino acid sequence and structural architecture of rabbit VEGF-A is equivalent to the human binding sequence [20] and structural architecture, indicating that bevacizumab is capable of binding to rabbit VEGF-A. This assumption is promoted by the findings of van der Flier et al., who reported that bevacizumab does indeed bind to rabbit VEGF-A, although the affinity is 8 times lower [21]. In humans, short-term topical bevacizumab therapy reduces the severity of corneal NV [22, 23], and three subconjunctival injections of bevacizumab appear to reduce recent-onset corneal neovascularization [24]. Reports on the use of bevacizumab in patients with corneal NV have suggested that bevacizumab may be effective and safe for the treatment of ocular surface NV [25–27]. However, there have also been reports of severe complications such as corneal infiltrative keratitis, corneal stromal edema, Descemet folds [28], and ulcerative keratitis [29]. Topical application of bevacizumab has been shown to delay epithelial healing in rabbit corneas [30]. In patients with neurotrophic keratopathy, bevacizumab should be used with care since VEGF possesses neuroprotective effects [31] and blocking VEGF impairs murine corneal nerve fiber regeneration [32]. Although the local safety profile of bevacizumab appears to be good [33], the systemic distribution of bevacizumab following topical or subconjunctival administration should be investigated, since systemic administration of bevacizumab is accompanied by a number of side-effects [34, 35]. Finally, bevacizumab does not seem to be able to completely inhibit corneal NV [16, 36]. A panel of experts on corneal neovascularization meeting at the World Ophthalmology Congress in Berlin in 2010 stated that apart from VEGF other factors are involved in the neovascularization process and therefore a "multi-pronged approach is desirable" [37].

In vivo, the vasculature is prevented from invading the cornea and other ocular tissues (e.g., the retina) by inhibitors of VEGF. The cornea contains the soluble VEGF receptor-1 (VEGFR-1), which traps soluble VEGF, thereby maintaining corneal avascularity [38], and it also contains pigment epithelial-derived factor (PEDF), a potent inhibitor of VEGF. PEDF is the most potent of the naturally occurring VEGF inhibitors, and a critical inhibitor of VEGF-induced neovascularization [36]. PEDF inhibits the angiogenic activity of VEGF, bFGF, and IL-8 [39]. PEDF binds competitively to the pro–angiogenic VEGF-R2, preventing receptor activation [40]. PEDF also inhibits the phosphorylation of VEGF-R1, and induces VEGF-R1 cleavage [41]. Additionally, PEDF mediates neuroprotective, neurogenic, and antioxidative effects. The neuroprotective activity of PEDF may be beneficial in neurotrophic keratopathy, e.g., a possible cause of corneal NV. In ocular tissues, PEDF mRNA expression has been identified in various ocular cells including corneal epithelial and endothelial cells [42]. High levels of PEDF have also been detected in developing and mature corneas [43]. PEDF appears to be an important anti-angiogenic factor in the cornea. In fact, blocking PEDF via anti-PEDF antibodies in mouse corneas induces corneal neovascularization [36]. A number of studies have also shown that PEDF is a potent inhibitor of neovascularization in various animal models [44–47]. PEDF's short half-life time [48] has prevented its clinical use.

In case of persistent corneal neovascularization, it may be difficult to inhibit neovascularization continuously using either topical application or subconjunctival injection of anti-VEGFs. It appears practical to have PEDF secreted continuously by subconjunctival implantation of cells transfected to stably express PEDF that would continually suppress the neovascular stimuli. To such an end, we have transfected ARPE-19 cells with the *PEDF* gene and transplanted the PEDF-transfected ARPE-19 cells subconjunctivally in rabbits to inhibit corneal neovascularization induced by alkali burn.

Material and methods

Corneal alkali burn

All animal experiments were conducted in accordance with the principles of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals, and were carried out with the permission and supervision of the District Government of Cologne, Germany. In this study, 20 female, 2-4 month-old Chinchilla Bastard grey rabbits weighing between 2.7 and 3.5 kg were used. To ensure a healthy corneal surface, all animals' eyes were carefully examined using an operating microscope (Carl Zeiss, Germany) prior to any experimental procedure.

Rabbits were randomly assigned to four groups: A (n= 4), B (n= 5), C (n= 6), and D (n=5). Rabbits in groups A, B, and C were placed under general anesthesia by intramuscular injection of combined ketamine/xylazine (35 mg/kg and 5 mg/kg respectively) supplemented by topical anesthesia (proparacaine hydrochloride 0.5 %). A corneal burn was produced in anesthetized rabbits by applying to the cornea, 1–

3 mm away from the limbus, a 5 mm diameter filter paper disc soaked for 1 min in 1.0 M NaOH. The disc was removed, and after 2 minutes the corneal surface was rinsed thoroughly with 10 ml of a 0.9 % NaCl solution. To insure maximum reproducibility, the alkali burn to all rabbits was carried out by the same investigator. Rabbits were sacrificed at day 21 by an overdose of phenobarbital.

Cell transfection and transplantation

ARPE-19 cell transfection was carried out following the procedures previously published [49]. ARPE-19 cells were transfected with PEDF gene using the enhanced *Sleeping Beauty* (*SB100X*) transposon system, which integrates the *PEDF* gene into the RPE cell's genome, allowing continuous "constitutive" expression of PEDF.

After neovascularization was allowed to develop for 7 days, rabbits in group B were transplanted with an average of 1.1 million (in 5 μl 0.9 % NaCl) non-transfected ARPE-19 cells by injecting the cells subconjunctivally through a 30-gauge needle adjacent to the cauterization side. Rabbits in group C were transplanted subconjunctivally with an average of 1.3 million (in 5 μl 0.9 % NaCl) PEDF-transfected ARPE-19 cells. Rabbits in group A served as injured, non-transplanted controls. Untreated rabbits in group D were transplanted on day 0 with an average of 1.3 million PEDF-transfected ARPE-19 cells, and were used to analyze the expression and secretion of recombinant PEDF (rPEDF) by the transplanted cells. In vitro, the transfected ARPE-19 cells secreted 32 ng PEDF/hour/100,000 cells based on ELISA analysis of culture media.

Conjunctival biopsies and Western Blot analysis

Conjunctival biopsies from group D rabbits were obtained at days 4, 10, 14, and 21. All biopsies were taken approximately 4 mm away from the cell injection site to prevent the inclusion of transplanted cells. The conjunctival tissue was lifted; small samples were cut with a micro scissor and stored on ice. Biopsy weights ranged from 5.0 to 18.5 mg. The biopsy tissue was lysed in 5 μl RIPA buffer (Pierce Biotechnoloy, Rockford, IL, USA) per mg tissue (wet weight), and subjected to SDS–polyacrylamide gel electrophoresis and Western Blot analysis for the detection of rPEDF as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control for gel loading accuracy. Western Blot analysis using anti-Penta-His antibodies was performed as previously described [50]. Briefly, after electrophoresis, proteins were transferred onto a 0.45 μm pore-size nitrocellulose membrane (Whatman, Maidstone, Kent, UK) using the semidry blotting technique, followed by Ponceau S staining to confirm the transfer. Blots blocked overnight at 4 ° C with 3 % bovine serum albumin (BSA) in tris buffered saline (TBS) were incubated at room temperature with anti-Penta-His antibodies (mouse monoclonal, 1:500; Qiagen) diluted in 3 % BSA/TBS for the detection of recombinant PEDF, or with anti-GAPDH antibodies (mouse monoclonal, 1:5000; Novus Biologicals, Littleton, CO, USA) for the detection of GAPDH. After 2 hours, the blots were incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse antibodies (rabbit polyclonal, 1:1000; Dako, Glostrup, Denmark).

To quantitate PEDF in the conjunctiva, biopsies taken approximately 4 mm away from the cell injection site were lysed in 10 μl RIPA buffer/mg tissue (wet weight), and the lysates were analyzed by ELISA utilizing the ELISAquant kit for human PEDF (BioProducts MD, Middletown, MD, USA).

Corneal examination

All eyes were examined under general anesthesia on days 7, 14, and 21, and vascularization recorded using a Nikon digital camera. Digital images were analyzed blindly by two investigators for lead vessel length (mm) and total vascular invasion area (mm^2) using the Image J software. Images were also evaluated using a modification of the corneal neovascularization evaluation model developed by Bahar et al [51] (Table 1).

Statistical analysis

Analysis of variance (1-way ANOVA) and chart creation were performed using the GraphPad Prism software for Windows, Version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). In addition, we also used Tukey's multiple comparison test ("honestly significant difference") to compare multiple samples simultaneously [52]. All significance levels were set at $\alpha=0.05$.

Table 1 Rating of corneal neovascularisation (after Bahar et al, 2008)

Quadrants of corneal opacity displaying distinct neovascularization	1–4
Distance the neovessels extend	1. max 2 mm
	2. 2-4 mm
	3. 4-6 mm
	4. >6 mm
Density	1. very low (one vessel)
	2. low
	3. moderate
	4. high

Maximum = 12 points

Results

Alkali burn

The area of alkali burn was calculated by outlining the central prominent corneal opacity immediately after cauterization. The area of opacity was 38.79±5.07 mm^2 in group A, 38.42 ±3.62 mm^2 in group B, and 38.53±3.62 mm^2 in group C. The difference is not statistically significant, indicating that the alkali burn was replicated accurately in all animals.

Corneal examination

Evaluation of the photographs of corneal neovascularization using the modified model developed by Bahar et al. rated vascularization at days 14 and 21 significantly lower from corneas of rabbits transplanted with PEDF-transfected RPE cells, group C, than from corneas of rabbits transplanted with non-PEDF transfected cells, group B, and from injured, non-transplanted rabbits, group A (1-way ANOVA, Day 14: $p<.014$; day 21: $p<.005$) (Fig. 1). Using Tukey's test, no significant difference was observed in the rating of corneal NV between the two control groups, A and B, at day 14 or day 21; however, there was a statistically significant difference between group C and groups A and B on days 14 and 21.

Lead vessel length

Lead vessel lengths at day 7 after alkali treatment and prior to cell transplantation (Table 2) ranged from an average of 2.40± 0.18 mm to 2.31±0.09 mm. The difference between the three groups is not statistically significant at day 7. The difference between the control groups A (injured, not transplanted) and B (transplanted with non-transfected cells) is not statistically significant at day 14 (unpaired t-test, $p<.022$). However , it is statistically significant at day 21. The difference between group C (transplanted with the PEDF-transfected cells) and groups A and B is statistically significant at days 14 and 21 (1-way ANOVA, $p<.0001$ and $p<.0001$). Group D was not cauterized, and showed healthy corneas without any vascularization.

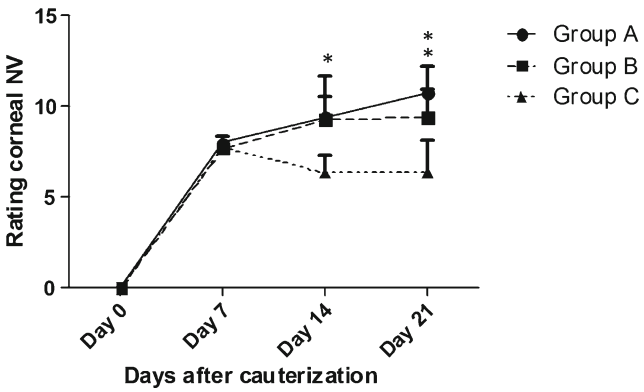

Fig. 1 Rating of corneal neovascularization (after Bahar et al, 2008). Corneal neovascularization was rated significantly lower in group C (PEDF-transfected RPE cells) than in either of the control groups A (non-transplanted) or B (non-transfected RPE cells) at days 14 and 21 (1-way ANOVA $p<.014$ and $p<.005$, respectively). The two control groups did not differ significantly at any time point.

Table 2 Lead vessel length (mean ± SD) on days 7, 14, and 21

Lead vessel length (mm)	Group A (no cells)	Group B (ARPE-19)	Group C ($RPE^{[N\text{-}PEDF\text{-}H\text{-}IV]a}$)	1-way ANOVA
Day 7	2.40±0.18	2.31±0.09	2.32±0.15	$P>.14$
Day 14	4.83±0.7	4.24±0.9	3.19±0.71	$P<.0001$
Day 21	5.91±0.45	5.11±1.22	3.79±0.59	$P<.0001$

Area of vascularization

The development of corneal neovascularization was similar in the three experimental groups until day 7. Dense, short vessels rarely exceeding 2.4 mm, covering up to 25.84 mm^2 of the cornea, developed during the first 7 days. The vessels spread to cover the complete cauterization area, and by day 21 in the control group (group A) and the group transplanted with non-transfected RPE cells (group B), the vessels had spread beyond the area of burn (Fig. 2). On day 7, before cell transplantation, total vascular area was somewhat lower in groups B and C than in group A (Table 3) (1-way ANOVA, $p>.023$). Using post -hoc analysis, the area of neovascularization did not differ significantly between control group B and treatment group C, but both groups B and C did differ significantly from control group A (Tukey's multiple comparison test). On days 14 and 21, the corneas of rabbits transplanted with PEDF-transfected cells (group C) showed fewer, thinner vessels that did not cover much corneal surface. Rarely more than 2 quarters of the burn site were covered and a coherent vessel front did not form. Often few main vessels remained, whereas most of the small vessel branches had regressed (Fig. 2). The difference between control groups A and B and group C on day 14 is statistically significant (1-way ANOVA, $p<.0001$). Figure 3 shows a representative cornea from each group. Note the florid and prominent vasculature with large vessels in the control corneas (groups A and B, left and middle photograph) and the very scant and thin vessels in the cornea transplanted with PEDF-transfected cells (group C, right photograph). In the control groups, little change was observed in the total vascular area from day 14 to day 21, whereas in group C the total area of neovascularization decreased. However, the difference between day 7 and 21 was not statistically significant (paired t-test, $p>0.27$). Further analysis using Tukey's test showed that control groups A and B did not differ significantly at day 14 and 21, but the difference between treatment

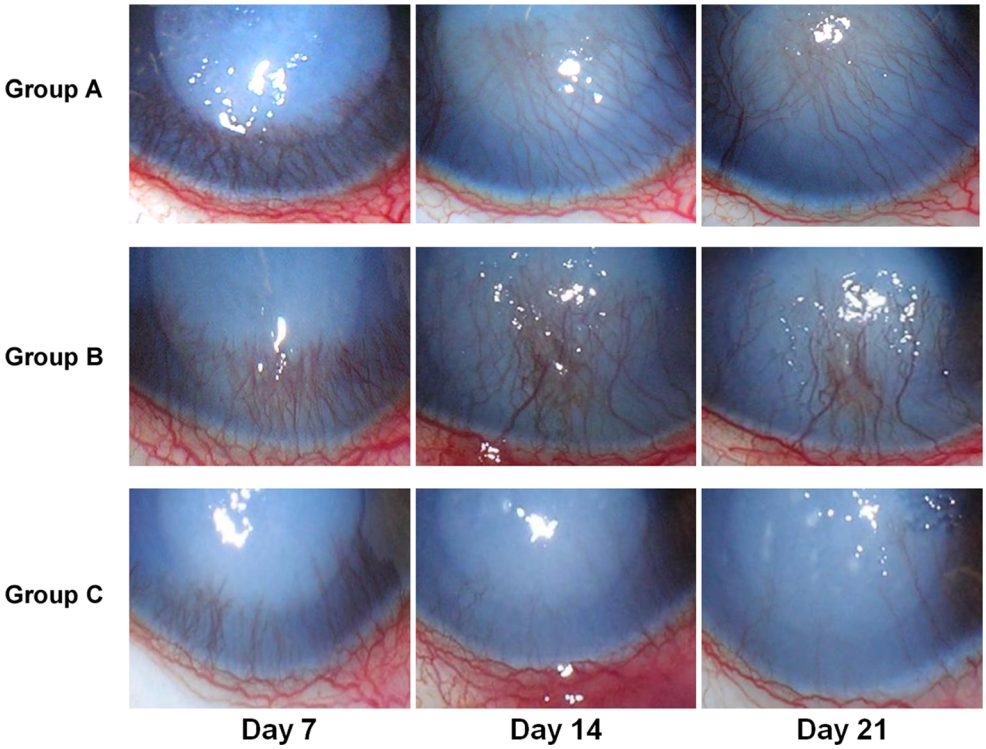

Fig. 2 Photographs of corneal neovascularization at 7, 14, and 21 days from group A (alkali burn, no cell transplantation), group B (alkali burn, transplantation with non-transfected cells), and group C (alkali burn, transplantation with PEDF-transfected cells). Group A: peripheral corneal neovascularization has developed by day 7 after alkali burn (*top left panel*), and by day 14 (*top center panel*) had progressed and infiltrated most of the burn area. By day 21 (*top right panel*) prominent major vessels were established and all 4 quadrants of the alkali burn were covered with new vessels. Group B: by day 7 (*middle left panel*) corneas had developed neovascularization that followed the same progression as the corneas from group A; day 14, *center middle panel*; day 21, *right middle panel*. Group C: by day 7 (*bottom left panel*) peripheral neovascularization has developed before cell transplantation. The *center bottom panel* shows that at day 14, 7 days after transfected cell transplantation, only a few major vessels are still present and many small vessels have regressed. The *right bottom panel* shows that only three major and only a few minor vessels are remaining by day 21

group C and all other groups was statistically significant on both days.

Western blot analysis and ELISA of conjunctivae

Extracts of conjunctival biopsies of rabbits transplanted with PEDF-transfected cells (Fig. 4) showed that rPEDF was present in conjunctival tissue at days 4, 10, 14, and 21 (lanes 1-4) but not from biopsies of non-injured, non-transplanted rabbits (lane 5). Bands of approximately the same density for GAPDH indicate that the amount of sample loaded was similar for each sample. Analysis of conjunctival extracts 7 days after transplantation from control rabbits transplanted with non-transfected ARPE-19 cells (group B) was 0.0046 ng/ml, whereas the amount of rPEDF in the conjunctiva of rabbits transplanted with PEDF-transfected cells (group C) was 3.81 ±0.86 ng/ml, 828-fold higher.

Table 3 Vascular invasion area (mean ± SD) on days 7, 14 and 21

Vascular invasion area(mm^2)	Group A (no cells)	Group B (ARPE-19)	Group C (RPE[N-PEDF-H-IV]a)	1-way ANOVA
Day 7	25.84±1.83	23.57±2.85	23.03±1.37	$P>.023$
Day 14	34.11±13.21	35.52±6.44	21.63±5.59	$P<.0001$
Day 21	35.5±8.65	34.86±4.92	19.2±5.03	$P<.0001$

Discussion

In our studies, ARPE-19 cells were transfected with the *PEDF* gene using the enhanced *Sleeping Beauty* (*SB100X*) transposon system, which integrates the *PEDF* gene into the RPE cell's genome, allowing continuous "constitutive" expression of PEDF. In fact, our study shows that the level of rPEDF in the conjunctival tissue remains constant for the 21 days of the experiment. Corneal NV, which had already developed, regressed and further vessel maturation was inhibited by the PEDF secreted by the transplanted cells. Compared to the corneas from non-transplanted rabbits, at day 14 (7 days of treatment) the vascular invasion area was reduced by 37.5± 2.12 % and by 47±4.24 % at day 21 (14 days of treatment) after transplantation of 1.3×10^6 cells. The results of our study

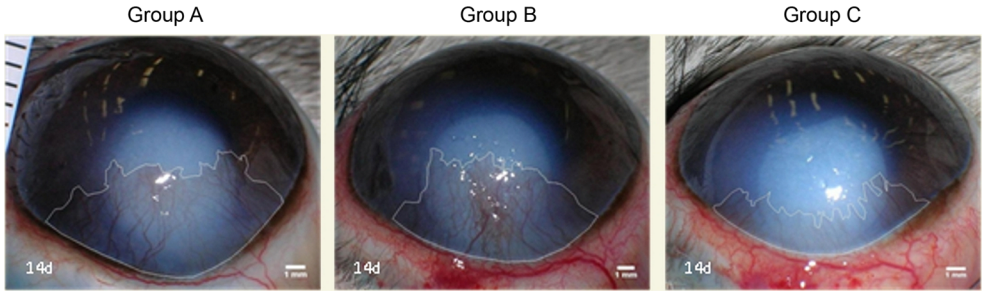

Fig. 3 Representative photographs of the vascular invasion area on day 14 (7 days of treatment). *Left panel* and *center panel*: control groups A and B show distinct neovascularization. *Right panel*: treatment group C shows a statistically significant lower vascular invasion area than the control groups A and B (1-way ANOVA, $p<.0001$))

are comparable to those of Papanthanassiou et al. [17], who reported a reduction of 42.17 % 14 days after subconjunctival administration of bevacizumab. The authors speculate that bevacizumab has more effect on developing vessels than larger ones. We can report similar findings for PEDF, as individual larger vessels often remained in treatment group C (see Figs. 2 and 3 for reference).

Corneal neovascularization, and neovascularisation in general, is driven by multiple angiogenic factors [53]. Therefore, treatment using PEDF has the advantage of inhibiting not only VEGF, but also the angiogenic activity of bFGF and IL-8, whereas bevacizumab and other anti-VEGF antibodies only inhibit VEGF [39]. In fact, sunitinib, a combination of anti-VEGF and anti-PDGF, is a 3-fold more effective inhibitor of neovascularization than bevacizumab at a 10-fold lower concentration [16].

Overall neovascularization progressed unabated in the control groups during the first 14 days, and slowed during the next 7 days, whereas in animals transplanted with PEDF-transfected cells, neovascularization did not proceed beyond day 7, when the cells were transplanted. In fact, in the animals transplanted with PEDF-transfected RPE cells, the total area of vascularization decreased, lead blood vessel length increased only slightly from day 7, and the majority of vessels remained shorter and thinner (Figs. 2 and 3). These results indicate that a constant source of PEDF is not only able to prevent further neovascularization, but can eradicate the majority of existing neovessels.

The analysis of the tissue surrounding the area of transplantation of the PEDF-transfected cells clearly showed rPEDF secretion, as evidenced by the reaction with anti-penta-His antibodies. We did not analyze for the presence of native PEDF in these blots. However, ELISA quantitation of PEDF in conjunctival extracts transplanted with non-transfected ARPE-19 cells was 0.0046 ng/ml, whereas it was 3.81± 0.86 ng/ml in conjunctival extracts transplanted with PEDF-transfected cells. Apparently, the amount of PEDF in the conjunctiva even in the presence of transplanted, non-transfected cells was not sufficient to inhibit neovascularization significantly. An immune response reaction around the injection side was apparent at day 7 after cell transplantation. However, the reaction receded and was only slightly evident at day 14 after cell transplantation. The immune response we observed was not as intense as described by Grisanti et al. [54] after subconjunctival implantation of RPE cells in mice , even though we transplanted more than twice as many cells. The difference in immune response may be the result of species difference and of the type of cells used. Grisanti used newborn homologous cells whereas we used ARPE19, a cell line that may have lost antigenic properties.

Kuo et al [44, 47] injected subconjunctivally synthetic amphiphile INTeraction-18 (SAINT-18) vector carrying the PEDF gene, and showed inhibition of corneal neovascularization in a rat model. Delivery of a gene by a vector in vivo has significant limitations, because the vector carrying the gene can disseminate away from the cell of interest. The gene is delivered episomally, and thus duration of expression cannot be certain and the gene may be introduced into inappropriate cells. We have chosen a novel strategy to inhibit corneal neovascularization, namely implantation of cells that synthesize and secrete PEDF as a continuous drug delivery system. One advantage of our approach is safety, since the number of cells transplanted can be adjusted to meet the requirement of a specific condition. A priori, being able to determine and choose the level of expression, being able to localize the expression of the gene of interest, and having the gene integrated

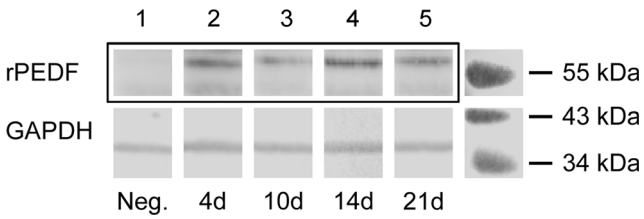

Fig. 4 Western Blot analysis of conjunctival biopsies obtained from non-injured rabbits transplanted with PEDF-transfected cells (group D) show that the transplanted cells secrete rPEDF in vivo at all times analyzed (lane 2 to 5). Lane *1* shows the extract from the biopsy of a conjunctiva of a non-treated, non-transplanted rabbit; as expected, no rPEDF was detected with anti-His antibodies. Lane *2* = day 4, lane *3* = day 7, lane *4* = day 14, lane *5* = day 21

into the cells' genome to continuously express the protein of interest are other advantages of our approach. In addition, this approach offers the possibility of using autologous cells from a patient's biopsy that can be transfected and transplanted within one surgical session.

The results reported here indicate that subconjunctival transplantation of PEDF-transfected cells offers a promising option for the treatment of corneal neovascularization. Further studies will be necessary to define the approach to eventually devise a protocol that can be translated to the clinic.

Conclusion

In conclusion, we have shown in our previous work that using the transposon SB100X it is possible to introduce the *PEDF* gene into the genome of ARPE-19 cells. In this study, we show that these cells can then be transplanted to deliver PEDF continuously to inhibit corneal neovascularization. The therapeutic outcome appears to be promising for controlling corneal neovascularization; in more general terms, using the *Sleeping Beauty* transposon system to safely deliver and integrate any gene into the genome of any cell, which can then be transplanted to continuously deliver a therapeutic protein, may find application in other areas of ophthalmology and medicine .

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Conflict of Interest All authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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