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SHORT COMMUNICATION

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Human and porcine anterior lens capsule as support for growing and grafting retinal pigment epithelium and iris pigment epithelium

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Abstract ● Purpose: To establish a method for transplantation of cultured monolayers of RPE and IPE into the subretinal space, anterior lens capsule was evaluated for its suitability to serve as growth support and carrier for transplantation procedures. ● Materials and methods: Twenty-four anterior lens capsules were obtained from porcine eyes. The same number of human lens capsules was obtained during cataract surgery. Six lens capsules of each species were stored at –80°C. Subsequently, the capsules were transferred onto type-I collagen. A second set of six lens capsules was treated identically except for the cryo treatment. A third set of six capsules was initially exposed to 0.05% trypsin for 30 min. Suspended porcine RPE and IPE cells (5×10^4 cells/well) were seeded on the top of each capsule. The remaining six lens capsules served as controls and were incubated in uncoated 12-well dishes without undergoing experimental treatment. The cultures were maintained in a water-saturated atmosphere at 37°C with 5% CO_2. Six days later, viability, morphology, and cell density were determined. The capsules covered by a confluent monolayer of cells were transferred into uncoated wells and cultivated for another 10 days. At the end of the experiment, light and phase-contrast microscopy was performed on all capsules. ● Results: Storage at –80°C and exposure to trypsin resulted in significant reduction of cellular contamination. The highest cell density was found after 5 days when capsules which had undergone cryopreservation or trypsin exposure served as support for RPE and IPE. The pigment cell layer was firmly attached to the capsules and permitted a transfer to other culture flasks without significant cell loss. The IPE cell layer remained confluent after transfer to uncoated culture flasks, while the RPE cell layer ceased to proliferate 10 days after transfer. ● Conclusions: Lens capsules may be suitable for growing and supporting monolayers of pigment epithelial cells. Especially IPE cells formed stable monolayers on anterior lens capsules which could be transferred to secondary culture flasks without inflicting damage on the cells.

Introduction

Retinal pigment epithelium (RPE) is essential for maintaining vision by providing metabolic support for the photoreceptors [3, 4]. Under normal conditions, RPE forms a nonproliferating monolayer of highly differentiated cells [28]. Factors stimulating RPE to proliferate are poorly understood [10, 22]. The clinical picture of proliferative vitreoretinopathy (PVR) is a typical complication of uncontrolled RPE proliferation [11, 19, 21, 25]. Cir-

cumscribed loss of RPE in age-related macular degeneration (ARMD) results in loss of central vision [13]. Besides relocating the macula to an area of healthy looking RPE, replacement of lost RPE by transplantation is considered as an option for treatment of ARMD [8, 18, 29]. Since RPE cells cannot be obtained from living donors without the risk of inflicting severe damage on the retina and choriocapillaris, IPE cells are being investigated as a potential alternative because they can be acquired by simple iridectomy. The two cell types have a common embryogenic origin, and IPE cells have been shown to be able to take over functions of the RPE [26]. If cells are obtained from the future recipient, immunogenic rejection can be expected to be minimized. Some previous studies focused on the transplantation of RPE cells to the subretinal space as cell suspensions or patches [1, 17]. A new approach is to culture RPE cells on suitable carriers and to transplant both the confluent cell layer and the support medium to the subretinal space [2, 20, 30]. Transplanting confluent cell layers may facilitate the covering of areas of RPE atrophy due to ARMD. We evaluated human and porcine anterior lens capsules for their suitability as growth support and carrier for transplantation of RPE and IPE.

Materials and methods

Preparation of human anterior lens capsules

Twenty-four human lens capsules were harvested during cataract surgery. Capsulorrhexis was performed using a capsule forceps. After its removal, the lens capsule was stored in sterile balanced salt solution (BSS) for further processing.

Preparation of porcine anterior lens capsules

Twenty-four porcine eyes were obtained from a local slaughterhouse and prepared according to standard procedures for corneal grafting [23]. Briefly, after exposure to 2% polyvinylpyrrolidone for 3 min, the globes were washed in sterile NaCl. The corneal-scleral disk was then removed with a 16-mm trephine. The iris was gently dilated with two forceps. An anterior capsulectomy was performed using a capsule forceps. The anterior capsule was then removed in one piece and stored in BSS.

Preparation of collagen gels

Flattening and adhesion of the lens capsules were achieved by coating culture wells with type I collagen (Vitrogen 100, Sigma). Vitrogen 100 is a highly purified preparation of pepsin-solubilized bovine dermal collagen type I which polymerizes when the temperature is raised to 37°C. Vitrogen 100 was diluted in Dulbecco's modified Eagle's medium (DMEM) with 10% PBS and 0.225% $NaHCO_3$. A concentration of 1.0 mg/ml of type I collagen was prepared. Each well in a 12-well Falcon tissue culture plate (Becton Dickinson, Denmark) was coated with 500 µl of the diluted collagen, achieving a gel with a thickness of approximately 800 µm. After incubation in CO_2 atmosphere for 1 h, 0.5 ml serum-free culture medium DMEM/F12+0.1% human serum albumin (HSA) was added to each culture well.

Treatment of capsules prior to cell seeding

Twenty-four capsules from porcine and human eyes were assigned to four groups: Six capsules of the first group were transferred into tubes containing 1 ml of a mixture of DMEM and glycerol (1:1). They were stored at –80°C to destroy any adhesive cells. Twenty-four hours later, the tubes were exposed to room temperature. The percentage of dead lens epithelium was estimated applying trypan blue assay. Subsequently, the membranes were washed twice with PBS before being transferred into 12-well flasks coated with type-I collagen. The capsules were flattened on the top of the gel and sterilized with UV radiation for 30 min under a laminar flow hood. They were then covered with regular culture medium. A second set of six lens capsules of each species was treated identically except for the cryo treatment. The third set of six capsules were initially incubated in 0.05% trypsin for 30 min and subsequently washed in PBS for 5 min. Trypan blue assay, flattening on the top of type-I collagen, sterilization and covering with medium was performed following the procedures described for the first set. The final set of six lens capsules served as control and was incubated in uncoated wells without RPE or IPE cells.

Cultures of RPE and IPE

Porcine RPE and IPE cells were obtained from slaughterhouse eyes applying the technique described by Campochiaro et al. [5, 6]. Briefly, pigmented epithelial cells were obtained as following:

IPE cultures

After rinsing, the anterior segment of the eye was removed, leaving the posterior segment intact. The cups were thoroughly rinsed with BSS. The iris was gently dissected from the globe and placed upside-down. The tissue was rinsed with Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline (PBS) and incubated at 37°C with papain solution (7 µl/ml) for 40 min. The separated IPE cells were suspended in culture medium containing 20% fetal calf serum (FCS) and centrifuged at 1400 rpm/min for 10 min. Primary cultures were incubated in neurobasal medium enriched with 20% FCS, 10 µg/ml insulin, 5 µg/ml transferrin, 50 ng/ml selenium, 100 U/ml penicillin sodium, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 2 mM L-glutamine (Sigma). Passage cultures were maintained in culture medium enriched with 10% FCS.

RPE cultures

After dissecting the anterior segment from the globe, the vitreous was removed. The remaining eye cup was thoroughly rinsed with PBS before being filled with papain solution (7 µl/ml) followed by incubation for 40 min at 37°C. The detached RPE cells were suspended in culture medium containing 20% FCS and centrifuged at 1400 rpm/min for 10 min. The cells were resuspended in culture medium DMEM/F12, enriched with 20% FCS, 10 µg/ml insulin, 5 µg/ml transferrin, 50 ng/ml selenium, 20 µg/ml ethanolamine, 5 µg/ml bovine serum albumine, 4.7 µg/ml linoleic acid, 100 U/ml penicillin sodium, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 2 mM L-glutamine (Sigma). Passage cultures were maintained in culture medium enriched with 10% FCS.

Cultivation of RPE and IPE on lens capsules

Cells up to second passage were used in the present study. To each well, 5×10^4 suspended porcine RPE and IPE cells were added. In addition, RPE and IPE cells of the same passage were seeded into uncoated 12-well dishes, serving as control. The cultures were

maintained for 6 days in a water saturated atmosphere at 37°C with 5% CO_2. The culture medium was enriched with 10% FCS and changed twice per week.

Culture transfer

One capsule of each group, covered with a confluent monolayer of RPE and IPE was transferred into another culture flask which had not been coated with collagen. The cell-capsule sheets floated freely in the medium. The cultures were maintained in a water saturated atmosphere at 37°C with 5% CO_2. The culture medium was enriched with 10% FCS and changed twice per week. This secondary culture period lasted another 10 days.

Viability staining and evaluation of morphology and cell density

Trypan blue staining was performed at the end of all experiments to assess cell viability. Cell morphology was assessed by means of phase-contrast microscopy in the course of the experiment. Finally, light and phase-contrast microscopy were performed samplewise utilizing standard techniques. The density of the cells was evaluated using an ocular grid with an area of 0.378 mm^2 at a magnification ×200. The number of pigment epithelial cells observed in five different areas of each capsule was pooled and the cell density was expressed as cells/ mm^2.

Statistics

Data are given as mean ± SD (n=8). The significance of differences between single data points was tested using a *t*-test (two samples assuming unequal variances). For analysis of variance, the statistics program ANOVA was used. $P<0.05$ was considered to be statistically significant.

Results

After cryo treatment, human and porcine lens epithelium settled as humps on the surface of the anterior lens capsules and failed to cover larger areas (Fig. 1). Trypan blue assays revealed that these cells were nonvital. After trypsin exposure there were also no vital remainders of lens epithelium on the surface of the capsules, as evidenced by vital staining. Untreated lens capsules were covered with proliferating lens epithelium (Fig. 2).

All capsules remained transparent throughout the experiment. No difference was noticed in pigment cell growth between human and porcine lens capsules.

Porcine RPE cultures grown on anterior lens capsules, having undergone cryo or trypsin treatment, exhibited homogeneous monolayers of hexagonal, pigmented epithelial cells after 5 days of cultivation (Fig. 3). In cell morphology and density these cells did not differ from the control cultures. The mean average cell density was 1510 (±280) cells/mm^2. In the control groups, cell density was 1552 (±176) cells/mm^2, which was not significantly different ($P>0.1$).

Porcine IPE grown on anterior lens capsules which had undergone cryo or trypsin treatment showed a confluent monolayer after 6 days of cultivation. The cell density of 1230 (±210) cells/mm^2 was not significantly lower than in the control groups [1345 (±186) cells/mm^2] ($P>0.05$).

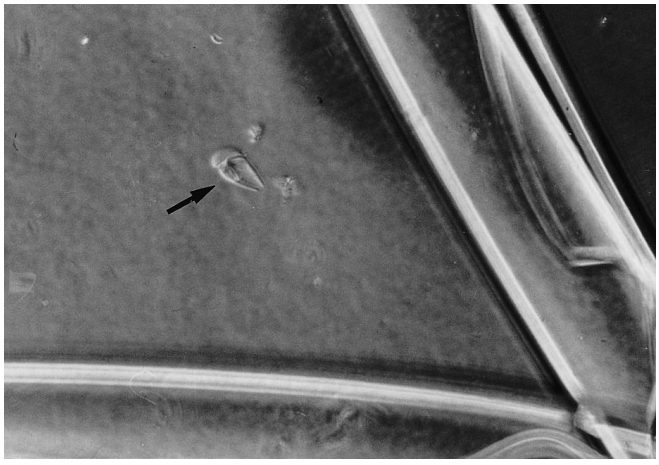


Fig. 1 Non-vital lens epithelial cells settled as humps on the top of a human anterior lens capsule after cryo treatment (*arrow*). Floating capsules tended to curl up at the rims (×200)

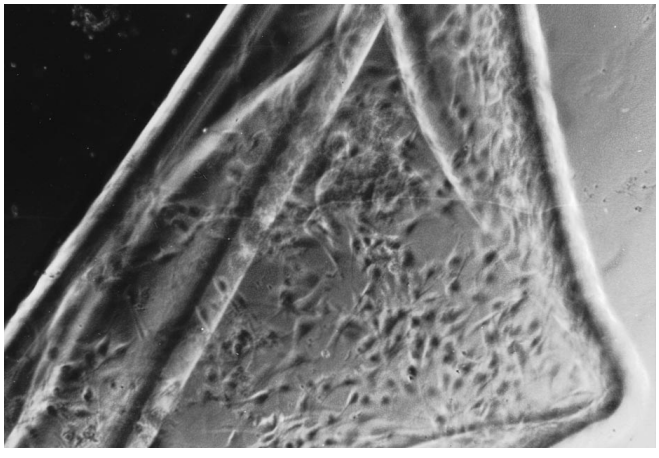
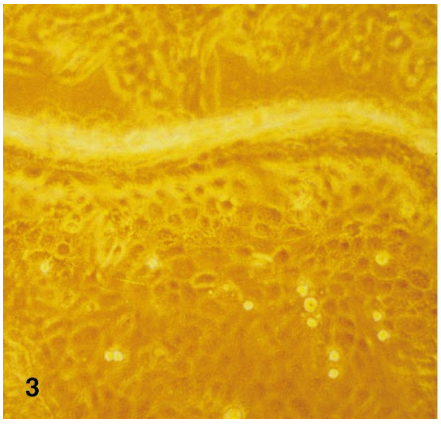


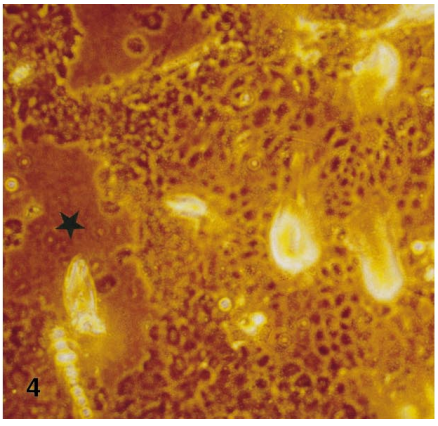
Fig. 2 Anterior lens capsules without cryo or trypsin treatment were still covered with proliferating lens epithelium (×200)

Untreated lens capsules were still covered by lens epithelium which had proliferated during incubation. Only a few pigmented cells had managed to attach to these membranes, forming mixed cell colonies with the lens epithelium.

It was possible to transfer all capsules to other culture flasks without damaging the capsules or the adjacent cell layers. All cells transplanted on cryo- or trypsin-treated lens capsules were firmly attached and formed stable monolayers which could be handled with ease.

RPE cells failed to maintain a confluent monolayer 10 days after transfer to secondary culture flasks. The cells settled partially as humps and exhibited pleomorphic de-





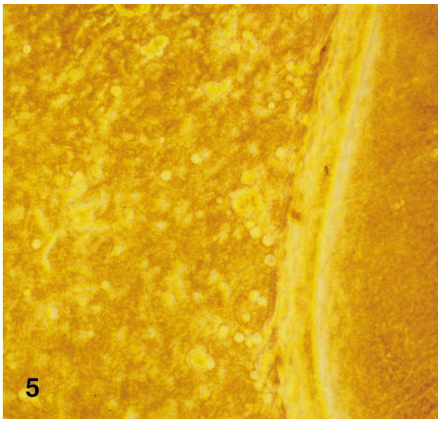


Fig. 3 Porcine RPE cells formed confluent monolayers on top of cryo-treated anterior lens attached to type-I collagen (×200)

Fig 4 RPE cells after 10 days of secondary culture on the top of a cryo-treated human lens capsule: confluency is not maintained. There were cell deaths and areas of bare capsule (*asterisk*) (×200)

Fig. 5 IPE after secondary culture for 10 days on top of a trypsin-treated human lens capsule: the cells maintained confluency and remained firmly attached (×200)

generation and intracellular vacuoles (Fig. 4) instead of their typical epithelioid structure. There were areas without cells and with dead cells where the bare capsule could be observed.

IPE cells remained confluent 10 days after transfer to secondary culture flasks (Fig. 5). The cells maintained normal morphology but became larger than the controls.

Discussion

Transplantation of pigment epithelial cells appears to be a logical approach to replace RPE lost in the course of ARMD [1, 2, 20]. Many investigators share the opinion that injecting suspensions of pigmented cells into the subretinal space might not result in covering RPE defects [24, 27, 30]. In addition, the correct orientation of pigment epithelial cells, which is supposed to be a prerequisite for the integrity and proper function of the retina, may be achieved more easily in a confluent cell layer grown on some kind of matrix [7, 24]. Transplanting confluent cell layers in the subretinal space may be a feasible approach to treatment of certain stages of ARMD. The technique may avoid diffuse dispersion of cells in the vitreous cavity as seen when using cell suspensions. Furthermore, we feel that growing confluent cell layers on natural or artificial extracellular matrices might allow manipulation of cellular monolayers without inflicting excessive mechanical stress or damage on the well-oriented cells. A variety of support matrices have been considered by different authors [2, 20, 30]. Most of these matrices either turned out to be toxic to the cells or disintegrated on exposure to liquid media.

We demonstrated that porcine and human anterior lens capsule can provide growth support for pigment epithelial cells. Anterior lens capsule is a basement membrane which can easily be separated from the lens. It is available simply and in abundance during cataract surgery. Anterior lens capsules permitted growth to confluency of both RPE and IPE, both of which exhibited confluent monolayers with normal morphology. We found that monolayers of IPE cells continued to grow after transfer to culture flasks even if not previously coated with collagen. RPE cells appeared to respond to absence of collagen coating with some degree of deterioration. This difference in sensitivity to culture conditions might be due to specific components of the anterior lens capsule, to effects caused by the underlying collagen type I, or both.

The major component of the human lens capsule is collagen type IV. Additional matrix components are collagen type I and III, laminin, and fibronectin. It is speculated that IPE is better adapted to lens components than RPE. RPE is supposedly used to being in contact with components of Bruch's membrane, such as hyaluronic acid, chondroitin sulfate, and other components of extracellular matrix [9, 14]. After transfer to uncoated culture flasks, RPE exhibited profound structural changes. Since these changes were not seen in primary cultures, it is likely that the cultured RPE suffered accelerated accumulation of metabolic endproducts [13, 14]. We found no degeneration as long as the cells were grown on capsules attached to collagen. The adhesion between pigment epithelial cells, lens capsule, and collagen is thought to be constituted by a major conduit of specific cell functions. Recently it was demonstrated that RPE cells exhibit a preference for fibronectin and to a lesser degree for collagen type I [31]. In PVR, proliferation, contraction, and migration of RPE cells are known to be regulated by integrin-fibronectin interactions [5, 12, 15]. Future histochemical analyses of anterior lens capsules and Bruch's membrane of different ages might reveal de-

tailed information about the presence and proportion of growth-promoting factors or supplements.

Thickness and permeability of the growth supports are thought to play a major role on cell metabolism. The anterior capsule of the lens is the thickest basement membrane in the eye (8–14 µm). Electron-microscopic studies showed a composition of multiple filamentous lamellae. Lens capsule is much thicker than Bruch's membrane (2–4 µm). The latter is formed by the basement membrane of the RPE and the slightly thicker basement membrane of the choriocapillaris.

When using lens capsule as growth support, one should consider the potential impact on diffusion of nutritional components and metabolic endproducts. The diffusional pathways into the lens are divers. Diffusion into the lens from the epithelial surface is slower than from the other side, partly because of the tight junctions on the apical side of the epithelial cells. It appears logical to destroy the epithelial layer prior to seeding pigmented cells on the top of the isolated capsule. The majority of the transport mechanisms used in the lens are not specific to the lens but are used almost universally.

When contemplating potential graft rejection, it appears advantageous to use autologous material. Previous studies focused on evaluation of Descemet's membrane to serve as growth support [30]. The pursuit of this concept has ceased, since only a few donors would be available to obtain Descemet's membrane for RPE growth. In addition, only homologous donors could be used as sources. Kiilggard et al., who described confluent growth of RPE on the surface of anterior lens capsules in vitro, failed to observe any signs of inflammation histopathologically in porcine eyes [16].

In summary, lens capsule could provide a support for growth and transplantation of confluent pigment epithelial cells, particularly IPE cells, into the subretinal space.

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