SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

EX VIVO ADHERENT CULTURES FOR OCULAR DISEASE MODELING, CHARACTERISATION AND INFLAMMATION STUDIES

Natasha Josifovska, M.Sc.

Supervisor:
Prof. Dr. Goran Petrovski, MD, PhD, Dr. med.habil.

UNIVERSITY OF SZEGED
DOCTORAL SCHOOL OF CLINICAL MEDICINE

DEPARTMENT OF OPHTHALMOLOGY

SZEGED, 2017

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Natasha Josifovska (M.Sc.)

Supervisor: Prof. Dr. Goran Petrovski, MD, PhD, Dr. med.habil.

Doctoral School of Clinical Medicine, Department of Ophthalmology, University of Szeged

Head of the Examination Committee: **Dr. Zsuzsanna Bata, DSc**
(SzTE ÁOK Bőrgyógyászati és Allergológiai Klinika)

Members: **Dr. Miklós Resch, PhD**
(SE ÁOK Szemészeti Klinika)

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Dr. Adrienne Csutak, PhD

(DE KK Szemklinika)

Members: **Dr. Miklós Resch, PhD**
(SE ÁOK Szemészeti Klinika

Dr. Éva Csősz, PhD

(DE ÁOK Biokémiai és Molekuláris Biológiai Intézet)

Introduction

The eye is a sensory organ protected from foreign materials by its curved architecture, compartmental organization, impermeable epithelium, tear secretion, and ocular drainage pathways. It is a slightly asymmetrical sphere with an approximate length of 24-25 mm and a transverse diameter of 24 mm.

It is composed of three layers: 1) external layer or so called connective tissue, which consists of transparent cornea connected to white sclera via the limbus; 2) intermediate layer or vascular tissue composed of the choroid as well as the ciliary body in the middle connected by the iris in front of the globe; 3) internal layer or neural tissue constituted of the retina, which has the function of transmitting the electrical impulse to the brain through the optic nerve. There is also another important transparent entity inside the eye called the lens, located behind the iris and between the ciliary body. The eye consists of two segments, anterior segment which represents a smaller part of the eye and consists of the cornea, conjunctiva, iris–ciliary body (ICB), lens and aqueous humor, and posterior segment representing the major ocular structure consisting of the sclera, choroid, and retina surrounding the vitreous cavity filled with the vitreous humor.

Conjunctiva

The conjunctiva is a transparent mucous membrane lining the inner surface of the eyelids and the anterior surface of the globe, terminating at the corneoscleral rim. It has a dense lymphatic supply and an abundance of immunocompetent cells.

Between the cornea and conjunctiva, there is a junction called limbus which contains epithelial and stem cells - these act like a barrier which prevents outgrowing of the conjunctiva onto the cornea.

Pterygium

Pterygium is a triangular fibrovascular subepithelial ingrowth of degenerative bulbar conjunctival tissue over the limbus and onto the cornea, which is invasive and highly vascularized; it is believed to arise from activated and proliferating limbal epithelia stem cells (LESCs). The pathogenesis of this disorder remains uncertain, although it is thought to be mainly caused by ultraviolet (UV) radiation. Sometimes pterygia can extend upon the pupillary axis and cause vision loss. They usually grow from the nasal side into the interpalbebral area, possibly due to the sun's rays passing laterally through the cornea unobstructed, where they get refracted and become focused on the medial limbal area. When the sun's rays are passing medially, the shadow of the nose can act to reduce the intensity of sunlight focused on the lateral limbal area. If there is progression towards the visual axis or if the lesion is cosmetically unacceptable, then surgical excision is indicated. Recurrence is common after surgery, but it is reduced by complete excision with beta irradiation, Mitomycin C (MMC) in connection with conjunctival autografts or amniotic membrane transplantation.

Lens

In order to accomplish its function of transmitting and focusing light, the crystalline lens of the vertebrate eye has evolved into a unique cellular structure and protein complement. Like the lens in a photo camera, the basic function of the lens of the eye is to transmit and focus light onto the retina. To facilitate this, it contains one of the highest concentrations of proteins of any tissue. As much as 60% of the total mass of the lens can be made up of proteins and water, much higher than almost any other tissue. Apart from that, it contains other

constituents, such as lipids, amino acids, electrolytes, peptides and carbohydrates, that account for only 1% of the lens wet weight. The lens is transparent, devoid of blood supply and innervation at birth. It is composed of epithelial cells that differentiate into long fiber cells reaching the anterior and posterior poles of the lens. The lens contains capsule, which is an acellular and elastic structure that contains the growing mass of developing and differentiating lens cells, an anterior and equatorial layer of epithelium, a peripheral cortical region, and an inner nuclear core. The capsule acts as a barrier to diffusion and takes role in shaping the lens during accommodation. The anterior lens capsule (aLC) remains as a basement membrane supporting the lens epithelial cells (LECs), while the posterior capsule is a thin membrane that is merely adherent to the fiber cells growing along its inner surface. The thickness of the anterior capsule increases with age and the capsule is always thinnest posteriorly. The cells located in the epithelium can divide and diferentiate into lens cell fibers, however, the remaining epithelium which does not normally divide has a role in transporting solutes between the lens and the aqueous humor.

Cataract of the lens

Cataract refers to any opacity of various degree of the crystalline lens, which in normal conditions is almost completely transparent. During aging, the lens increases in overall size and loses the ability to accommodate. Continued growth of the lens fibers with aging causes the nucleus to become compressed and less flexible. Furthermore the proteins of the nuclear lens start to aggregate and produce pigmentation to decrease the transparency causing the lens nucleus to appear yellow or brown where there is excess pigmentation. Cataractous changes can involve any of the structures of the lens like the nucleus and cortex, as well as anterior and posterior subcapsular areas.

Aims of the study

1. To adherently cultivate three types of tissues *ex vivo*: pterygium, aLCs containing LECs and a pathologic fibrovascular tissue/fibrovascular epiretinal membranes (fvERMs).

2. To achieve 2D and 3D outgrowth of cells from the cultivated tissues and to characterize molecularly these cells upon different treatments, as well as measure the secretion of inflammatory/pro-angiogenic cytokines (IL-6 and IL-8) from them.

3 To investigate the surface marker phenotype of the pterygium outgrowing cells using markers for hematopoietic and mesenchymal stem cells (MSCs), as well as cell adhesion molecules (CAMs) in order to determine the possible origin of the cells.

4. To measure intracellular calcium dynamics upon mechanical stimulation, as well as calcium signalling and intercellular communication upon Acetylcholine (Ach) stimulation in aLCs containing LECs.

Materials and methods

Pterygium harvesting and cultivation

All tissue collection complied with the Guidelines of the Helsinki Declaration and was obtained from surgery following a patient signed consent. The tissue harvesting was approved by the Local Committee for Medical Research Ethics at University of Oslo. The removed graft after surgery was cultivated in 24-well cell culture plates using Dulbecco-modified Eagle's medium (DMEM) supplemented with 4.5g glucose/L, 10% fetal bovine serum (FBS), 200mM/mL L-glutamine (Sigma-Aldrich, Germany), and 1% antibiotic/antimycotic solution (PAA, Pasching, Austria). To gravitationally press down the explanted tissue, viscoelastic material (ProVisc, Alcon, Fort Worth, TX, USA) was used as discovered and described for the first time by our group. The viscoelastic was added on top of the explant to allow flattening and adherence of the tissue onto the surface of the well. The goal was to develop stratified or 3D structure of the outgrowing cells from the pterygium over more than three months, with medium change being performed each other day. The 3D cell structure which was formed could easily be lifted from the cell culture plate by Colibri forceps at the end of the cultivation period and before fixation for further analysis.

Cell viability assay

Cell viability of the long-term 3D cultures of the pterygium was determined using Annexin V-Fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) assay (MBL International, Woburn, MA, USA), as well as the trypan blue exclusion test (Sigma Aldrich, MO, USA). For both assays, the cells were collected by trypsinization in the same culture media, then centrifuged and resuspended in Binding Buffer for the Annexin V-FITC/PI assay or phosphate buffered saline (PBS) for the trypan blue test. The cells were stained with Annexin V-FITC, PI, and Annexin V-FITC/PI, then analyzed by Flowing Software 2.5 (PerttuTerho, Turku Centre for Biotechnology, University of Turku, Finland). Alternatively, cells suspended in 50µL PBS were stained by equal part of trypan blue solution and counted in a Hemocytometer (Bürker chamber).

Immunophenotyping of cells

Flow cytometry was performed on the long-term 3D cultures of pterygium containing outgrowing cells to determine their immunophenotype. Three antibodies conjugated with FITC-, R-phycoerythrin (PE)- and allophycocyanin (APC) were used to measure the expression of CD34, CD44, CD90/Thy-1, CD73, CD105 and CD166/ALCAM (all from Biolegend, San Diego, CA, USA); CD47, CD117/c-kit, CD146/MCAM, CD112 (all from R&D Systems, Minneapolis, MN, USA). The prepared samples were measured by FACS Calibur flow cytometer (BD Biosciences Immunocytometry Systems) and data were analysed by Flowing Software 2.5 (PerttuTerho, Turku Centre for Biotechnology, University of Turku, Finland).

Immunofluorescent staining

For the immunofluorescent staining experiment, we wanted to compare expression of different markers on the pterygium obtained right after surgery and that cultivated as long-term 3D cultures. Therefore in both of the cases the tissues were collected and fixed in 4% paraformaldehyde at room temperature, later dehydrated in ascending alcohol series and embedded in paraffin; 3-4µm thick tissue sections were prepared using a rotary microtome,

then mounted onto histological slides. After heat-induced antigen retrieval with Tris/EDTA or Citrate buffer and blocking, immunofluorescent labelling with primary antibodies was performed. The samples were characterized for markers of pluripotency and stemness (Vimentin (Vim), Tumor protein p63 (ΔNp63), SRY (sex determining region Y)-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), ATP-binding cassette sub-family G member 2 (ABCG-2)), oxidative stress (8-hydroxy-2'-deoxyguanosine (8-OHdG), Hypoxia-inducible factor 1-alpha (HIF-1α)), migration and proliferation- (C-X-C chemokine receptor type 4 (CXCR4) and Ki-67), epithelial- (cytokeratin-19 (CK19) and 8-18) and secretory markers (Mucin (MUC1) and MUC4). In continuation secondary antibody Alexa 488(green) or Cy3(red) was used. Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were taken by a ZEISS Axio Observer.Z1 (ZEISS, Oberkochen, Germany) microscope. The quantification of positive cells was carried out using standard ImageJ software by three independent individuals. The number of positive cells on the full field of view was taken into account with the help of nuclear (DAPI) staining. Multiple pictures were taken of each sample and the results averaged out as mean ± standard deviation (SD).

Secretion of Inflammatory Cytokines by ELISA

For measuring the secretion of inflammatory cytokines we used the same high glucose-containing DMEM medium only this time supplemented with 5% FBS in order to diminish its possible influence on the secretion of the cytokines. The medium was applied to the 3D outgrowing cells and kept for 24h. After the supernatants were collected. In addition, treatment of the cells with 0.5mg/mL MMC (Kyowa, Takeda Belgium-Brussels) was applied for 20 min and supernatant collected. LESC cultures cultivated in high glucose-containing medium with 20% FBS were used as a control. The secreted cytokines, Interleukin 6 (IL-6) and IL-8, were analysed by a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D, Germany) according to the manufacturer's protocol. Three independent experiments were performed on outgrowing cells from four different pterygium donors.

Statistical analysis

Each experiment was performed at least three times and each sample was tested in triplicates. Data are expressed as mean ± standard deviation (SD). Statistically significant differences were determined by student-t tests, a p-value ≤ 0.05 was regarded significant.

Tissue Collection and Processing of aLC-LECs

All tissue collection complied with the guidelines of the Helsinki Declaration and was approved by the National Medical Ethics Committee of Slovenia; following a patient signed consent before surgery which was performed at the Eye Hospital, University Medical Centre (UMC), Ljubljana, Slovenia. Altogether 11 patients were included in this study - 6 cultures were analyzed for mechanical stimulation and 5 cultures for ACh stimulation, with the patients' age ranging from 70 to 92 years.

The aLC explants consisted of a monolayer of LECs attached to the basal lamina and were obtained from uneventful cataract surgeries due to progredient cataract. Lenses were dissected so that the aLCs (i.e., basal lamina and associated LECs) were isolated from the fiber cells that form the bulk of the lens. FvERMs were obtained from patients undergoing vitrectomy due to intravitreal hemorrhage in proliferative diabetic retinopathy (PDR).

All explants obtained were placed in a single dish accordingly. Immediately after isolation, the excised human eye explants were placed in sterile tubes filled with DMEM:F12 (D8437, Sigma-Aldrich, Ayrshire, UK), supplemented with 10% fetal calf serum (FCS) (PAA Laboratories GmbH, Pasching, Austria),. The explants were then transferred to empty cell culture glass bottom Petri dishes (Mattek Corp., Ashland, MA, USA; 3.5 cm in diameter) or tissue culture 12-well plates (TPP, Sigma, Germany) by using microdissecting tweezers (WPI by Dumont, Med.Biologie, Germany). The aLC explants were placed into the culture dish so that the concave side with the LECs was on the top and oriented upwards. The time of culturing ranged from 6 to 48 days.

Tissue Fixation and Adherence by Viscoelastic material

For obtaining adherent conditions, careful removal of the remaining medium from the tissue cultures was performed by a micropipette, and then viscoelastic (HEALON OVD, Abbott Medical Optics, USA) was added on top of the explant to allow for flattening or "ironing" of the tissue onto the surface of the Petri dish (Fig. 12). The next step was carefully applying the DMEM:F12 medium supplemented with 10% FCS not to disturb the already added viscoelastic cover on top of the explants. For that, the micropipette tip was positioned close to the culture dish surface but far away from the explant, so that the medium arrived softly in contact with the viscoelastic and did not move the explant from its location. The culture dishes were then kept in a CO_2 incubator (Innova CO-48; New Brunswick Scientific, Edison, NJ, USA) at 37°C and 5% CO_2. In order to allow the cells to attach and start proliferating out of the explant we were not moving the culture dish from the incubator for 2-3 days. During medium change, the medium was removed gently and a fresh one was added subsequently by a micropipette from the opposite side of the explant in the dish, the pipette tip being close to the surface of the dish all the time. The viscoelastic dissolved over time and got replaced by new medium—time by which the explant was fully attached to the surface of the culture dish.

Light Microscopy and Calcium Imaging

After culturing the cells were followed and their proliferation and migration were recorded throughout their continued growth using inverted light microscope (Axiovert S100, Carl Zeiss, AG, Oberkochen, Germany). The same microscope was used for $[Ca^{2+}]_i$ measurements. Image acquisition was carried out by a 12-bit cooled CCD camera SensiCam (PCO Imaging AG, Kelheim, Germany). The software used for the acquisition was WinFluor (written by J. Dempster, University of Strathclyde, Glasgow, UK). Microscope objectives used were 4x/0.10 Achroplan, 10x/0.30 Plan-Neofluar, 40x/0.50 LD A-plan, and 63x/1.25 oil Plan-Neofluar (Zeiss). The excitation filters used were mounted on a Lambda LS-10 filter wheel (Sutter Instruments Co., CA, USA) and had a wavelength of 360 and 380 nm (Chroma Technology Corp., Bellows Falls, VT, USA). Excitation with the 360 nm filter (close to the Fura-2 isosbestic point) allowed observation of the cells' morphology and of the changes in the concentration of the dye, irrespective of the changes in $[Ca^{2+}]_i$, while the 360/380 nm ratio allowed visualization of the $[Ca^{2+}]_i$ changes in the cytoplasm. Image acquisition, timing, and filter wheel operation were all controlled by WinFluor software via a PCI6229 interface card (National Instruments, Austin, TX, USA). The light source used was XBO-75W (Zeiss) Xe arc lamp. The light intensity was attenuated when necessary with grey filters with optical densities 0.5, 1, and 2 (Chroma Technology Corp., Bellows Falls, VT, USA). The criteria for selecting the region for imaging were the presence of adherent cells and good cell morphology both assessed by observation of transilluminated and 360 nm fluorescence

images. Individual image frames were acquired every 500 ms resulting in frame cycles being 1 second long (two wavelengths).

For $[Ca^{2+}]_i$ monitoring, the cell cultures were loaded with the acetoxymethyl (AM) ester of Fura-2 (Fura-2 AM, Invitrogen-Molecular Probes, Carlsbad, CA, USA), intracellular calcium indicator. For loading, Fura-2 AM in dimethyl sulfoxide (DMSO) was suspended in 3 mL of medium (high glucose medium with FBS) or physiological saline with (in mM) NaCl (131.8), KCl (5), $MgCl_2$ (2), NaH_2PO_4 (0.5), $NaHCO_3$ (2), $CaCl_2$ (1.8), HEPES (10), glucose (10), pH 7.24 to the final working concentration of 2 μM (aLC). The loading was done in the incubator at 37°C for 30 min (aLC). After loading, the cell cultures were washed twice for 7 min with the medium or physiological saline. The final working concentration of Fura-2 and the time of incubation/washing were larger for larger eye explants (it depended on the explant size). Fura-2 dye has two excitation (absorption) peaks (340 and 380 nm), an isosbestic point at 360 nm and one emission peak at 510 nm. Its absorption and fluorescent properties change in accordance with Ca^{2+} binding (low $[Ca^{2+}]_i$ —high absorption at 380 nm, high $[Ca^{2+}]_i$ —high absorption at 340 nm while the absorption is not Ca^{2+} dependent at the isosbestic point of 360 nm). The absorptive properties of Fura-2 allow the use of ratio imaging (360/380 ratio), which considerably reduces the effects of uneven dye loading, leakage of the dye, and photobleaching as well as problems associated with measuring $[Ca^{2+}]_i$ in cells of unequal thickness.

Mechanical and Acetylcholine (ACh) Stimulation

Further responses to mechanical stimuli were tested, and for that was used a tip of a glass micropipette mounted on a MP-285 micromanipulator (Sutter, Novato, CA, USA). Prior to use, the tip of the pipette was heat-polished until it rounded up.

The agonist acetylcholine (ACh; Sigma, USA) was applied in 10 μM concentration, which was enough to induce >90% maximal $[Ca^{2+}]_i$ response, according to the data by Collison et al. [12]. The agonist application as well as its washout from the bath was driven simply by the hydrostatic pressure of a 35 cm of water column and controlled manually by a luer-lock stopcock (WPI) and applied through a polyethylene plastic tubing (inner diameter 2 mm), attached to the coarse micromanipulator. The excess bathing solution was removed by a suction line.

Secretion of Inflammatory Cytokines by ELISA

Secretion of inflammatory cytokines IL-6 and IL-8 from fvERM and aLC-LECs upon TNFα was measured. Therefore the expanded fvERM cells and aLC-LECs were plated onto 6-well plates at a density of 2×10^5 cells per well in triplicates and kept until proper cell number was achieved for cytokine measurements. After 24 hrs, the medium was changed, and the cells were treated with 100 ng/mL recombinant human Tumor necrosis factor alpha (TNFα) (Preprotech, Rocky Hill, NJ, USA) for additional 24 hours. The secreted cytokines, IL-6, and IL-8 were analyzed by commercial ELISA kit (R&D, Germany) according to the manufacturer's protocol. Three independent experiments were performed on outgrowing cells from three different donors both fvERM and aLC.

Results

Morphology and viability of pterygium cells

Pterygium consists of mixture of cells and the morphology depends mostly on two types of cells: fibroblastic which are long-shaped, and squamous epithelial cells tightly packed together. After long term cultivation, pterygium formed spontaneous multi-layered structure that was easily tangible and removable from the plate for further analysis. The viability of the outgrowing cells tested by the Annexin V-FITC/PI assay and Trypan blue method was (98.6±1.7% and 82.6±6.9%), respectively showing that the cells were enough viable to continue with further analysis.

Flow cytometry of the long-term 3D cultures of pterygium containing outgrowing cells

The expression of hematopoietic cell surface markers CD34 and CD47 was (10.8±12.7% and 79.1±17%), respectively. CD117/c-kit, a progenitor cell marker was low (3.3±3.2%), while a very high expression of the MSC markers CD90 (87.2±9.5%) and CD73 (76.4±11%) were measured, in contrast to the MSC marker CD105 which was (1.3±1.4%). The expression of ECM attachment proteins, which is important for the maintenance of the cellular growth were tested next: CD146/MCAM (2.6±4.7%), CD166/ALCAM (48.1±18.1%), CD112/Nectin-2 (4.1±4%) and CD44/homing-associated cell adhesion molecule (H-CAM) (41.5±12.3%).

Immunofluorescent staining of pterygium grafts and long-term cultivated 3D outgrowing cells

Immunofluorescent staining of a wide range of markers involved in the different pathways were examined in the pterygium grafts obtained directly from surgery and compared to those from long-term cultivated 3D outgrowths from the pterygia.
The expression of pluripotency markers (Vim and ΔNp63) was (26.5±3.5% and 13.9±0.9%), respectively, in the pterygium graft tissue only, and (43.7±4% and 12.3±1.5%), respectively, in the multi-layered outgrowing cells, while other stemness markers (Sox2, Oct4 and ABCG-2) showed positivity in (22.2±0.9%, 34.2±1.6% and 25±5%) of the cells, respectively, in the pterygium graft itself, and (15.3±0.6%, 39.2±2.5% and 32.7±6.6%), respectively, within the multi-layered outgrowing cells. Oxidative stress markers (8-OHdG and HIF-1α) were determined in the pterygium graft tissue (42.4±3% and 13.8±2.3%), respectively and in the multi-layered outgrowing cells (62.3±8.6% and 26.6±4.8%), respectively. The expression of migration and proliferation markers (CXCR4 and Ki-67), was (27.2±4.5% and 5.1±0.4%), respectively, in the pterygium graft tissue, and (67.3±4.7% and 1.2± 0.3%), respectively, in the multi-layered outgrowing cells. The epithelial cell markers expression (CK19 and CK8-18) was (0% and 24.3±6.5%), respectively, for the pterygium graft tissue, and (11.9±3.7% and 0%), respectively, for the multi-layered outgrowing cells, while that for the secretory markers (MUC1 and MUC4) was (13.1±4.3% and 32.2±3.3%), respectively, for the pterygium graft tissue, and (63.7±5.7% and 55.3±5%), respectively, for the multi-layered outgrowing cells.

ELISA measurement of proinflammatory cytokines IL-6 and 8 secreted from 3D outgrowing pterygium cells

The multi-layered outgrowing cells from the pterygium biopsies secreted moderate and low levels of the proinflammatory cytokine IL-6 and IL-8, respectively, which levels were significantly decreased upon treatment with the antiproliferative agent MMC, in contrast to the level secreted by LESCs (control) for IL-6 ($p<0.05$). The IL-8 secretion upon MMC treatment did not change significantly although was lower than that secreted by LESCs**.** Both, the inflammatory cytokines' release and the treatment by MMC have clinical relevance in case of recurrent pterygia.

Human eye explant tissues adhere to the cell culture dish under a gravitational force of viscoelastic material

Novel, simple, and reproducible method for *ex vivo* cultivation of human explant tissues (aLCs and fvERMs) was established using viscoelastic material. The cells started proliferating out of the explants in 2-3 days. The fvERM cells grew out of the explants within 24 hours and continued proliferating independently throughout the study period (for more than 6 months).

Mechanical stimulation and Ach induce rise in $[Ca^{2+}]$ in the aLC-LECs

The functionality of the aLC-LECs attached under the viscoelastic was examined during mechanical stimulation and application of agonist ACh, both of which induced rise in the $[Ca^{2+}]_i$. Representative examples of 6 explant cultures were analyzed for mechanical stimulation containing 27 cells being stimulated (mostly the cells on the glass surface and some on the aLC); similarly, representative examples of 5 explant cultures were analyzed for ACh stimulation. The calcium signaling upon mechanical stimulation of a single cell of the aLC explant culture showed $[Ca^{2+}]_i$ propagation as well. The $[Ca^{2+}]_i$ dynamics upon mechanical stimulation of fvERMs has been previously described by our group which is a proof of the viability and functionality of these cells.

Measurement of proinflammatory/angiogenic factors secreted by the fvERM and aLC-LECs outgrowing cells upon TNFα treatment

The outgrowing cells from the fvERMs showed basal expression of the proinflammatory cytokine IL-6 *ex vivo*, which was further enhanced by TNFα stimulation. Similar enhancement was noted in the proinflammatory cytokine release of IL-8 upon TNFα stimulation**.** In the case of aLC-LECs, there was only IL-6 TNFα-induced secretion.

Discussion

The anterior segment of the eye ball, which serves as the barrier to the external stimuli includes the transparent cornea - a "window" for the light sensor, conjunctiva, which covers the sclera - the main part of the eyeshell, and the lens. Our aim was to adherently cultivate *ex vivo* cultures from three different types of tissue from the eye, including pterygium, aLCs containing LECs and fvERMs, and to let them form 2D and 3D structures. Furthermore, we aimed to characterize the obtained cells and treat them accordingly so that different markers can be measured, including IL-6 and IL-8 (markers for inflammation/angiogenesis).

Pterygium is a disease of the ocular surface that is associated with chronic UV exposure and is characterized by proliferation, inflammatory infiltrates, fibrosis, angiogenesis and extracellular matrix breakdown. Centripetal growth connected with inflammation and neovascularization is characteristic for the condition. Pterygium is not fully understood and significant progress has been made toward understanding the mechanisms involved in its pathogenesis. Many factors are included in the etiology of the disease, thus making it not just a degenerative, but also a proliferative disorder of the ocular surface. The environmental factors such as exposure to sunlight and UV B light seem to be among the most important risk factors, although genetics may also contribute to the pterygium development. Based on some studies, limbal stem cell deficiency has also been involved in pterygium development. Pterygium as a structure consists of a mixture of epithelial and fibroblastic cells, proliferation of which closely determines its recurrence and severity. The stratified outgrowing cell model for pterygium expansion *ex vivo* shown by our group has the advantage of giving immediate adherence of the graft to the cell culture plate (e.g. using viscoelastic material), therefore, providing both cell types an equal chance to expand, without the use of any growth factors.

Recently, it has been shown that pterygium growth and development are connected with proliferation of the epithelium as well as the fibrovascular layer. It is thus believed that pterygium is formed from an uncontrolled cell proliferation. Two markers: for proliferation cell nuclear antigen (PCNA), and Ki-67, have been studied previously and found to be more highly expressed in pterygium cells compared to normal conjunctiva. Ki-67 is also expressed in conjunctival and eyelid tumours, the positivity of which appeared to be low in our pterygium graft-containing cultures, and was additionally five times less expressed in the multi-layered outgrowing tissue. The SDF-1/CXCR4 signalling pathway has been described in the developmental processes and adult angiogenesis to have a role in vascular endothelial cell migration and proliferation. The pterygium graft-containing cultures showed 2.5 times less positivity for CXCR4 compared to the multi-layered outgrowing cell cultures.

Vimentin is a cytoskeletal intermediate filament and part of the Epithelial-Mesenchymal Transition (EMT), which converts epithelial cells into mesenchymal-like derivatives. Some evidence suggests that Vim filaments take a role during cell motility, spreading and signalling. Expression of Vim has been found in epithelial cells at the edges of healing wounds in corneal epithelium and in cells at the limbal epithelium. Thus, Vim can be considered important for migration and proliferation of stem cells and epithelial progenitors during their centripetal movement into central cornea and through wound closure. Vim was indeed found to be highly expressed by our pterygium-graft containing and the multi-layered/stratified outgrowing cells. Similarly, ΔNp63 was expressed at a steady and similar extent in both cell cultures, the protein being known for its role in activation or inhibition of apoptosis in a cell- and tissue-type specific manner. ΔNp63α is the most present isoform in

the central corneal epithelium, which seems to be lost during apoptotic cell death in the corneal epithelium.

UV-B radiation is known to cause oxidative DNA damage and is thought to be a major factor implicated in the pathogenesis of pterygium, playing an important role in its reccurence as well. Because of that, it is important to maintain proper antioxidant defense in patients after surgery of primary pterygium. The molecule 8-OHdG - a ubiquitous marker of oxidative stress which possesses high mutagenic potential, has been established to be a sensitive marker of oxidative DNA damage. It has been found to be expressed more in the epithelium of the head of primary pterygium compared to healthy conjunctiva. While pterygium is considered to be a non-metastatic lesion with limited local invasion, the presence of 8-OHdG could confirm visible genetic instability, which is in contrast to its benign clinical course. In our model, high expression of 8-OHdG was found in the pterygium graft itseld, which was further increased in the outgrowing cells.

Hypoxia inducible factor HIF-1α plays an important role in the modulation of cell metabolism and survival pathways, as well as couples with stem-cell-like properties in cells containing elevated expression of this marker. There is a coordinated activation of HIF-1α and heat shock proteins in pterygium and the upregulation of the same may represent adaptive process for the survival of cells under stressful conditions.

A healthy cornea is maintained by self-renewing, lineage-specific stem cells (SCs) that reside in the limbus. This regenerative capacity is regulated by proliferation, migration, and differentiation of such cells. Failure to maintain a normal microenvironment as a result of extrinsic (e.g. UV radiation) or intrinsic (e.g. cytokines) signals can cause development of ocular disorders such as pterygium. The epithelial cells in pterygium can keep their potential to divide, which supports the view of pterygium as a disease of altered limbal stem cells. There is a possibility that pterygium cells have stem cell origin, which is confirmed by the presence of telomerase activity in pterygial epithelium. Pluripotent stem cell markers Sox2 and Oct4 has been described as being expressed in many epithelial cell types, including conjunctival epithelial cells while ABCG-2 has a role in the maintenance of retinal stem cells under the regulation of Notch signalling. The *ex vivo* 3D tissue engineered pterygium outgrowths and the graft-containing cultures expressed all of these stem cell markers (Sox2, Oct4 and ABCG-2).

Oxidative stress caused by sunlight can induce growth factor production, angiogenesis, chronic inflammation and collagenolysis. After sunlight triggers pterygium onset, the proliferating fibroblasts can generate reactive oxygen species (ROS), through which sustained inflammation can occur. UV light can activate epithelial cells which are near the limbus to produce cytokines such as IL-6 and IL-8, so they can mediate inflammation, proliferation, angiogenesis and anti-apoptosis. Interleukins 1, 6 and 8 are all increased in pterygia after UV-B exposure, which contributes to upregulation of other inflammatory mediators and matrix metalloproteinases and potentially to pathogenesis of the disorder. Indeed, an abundant immunoreactivity for IL-6 and IL-8 has been found in pterygial epithelium, while our 3D pterygium outgrowing cells showed only a high expression of IL-6.

Conjunctival autografts, amniotic membrane transplantation and treatment with radiation or chemotherapeutic agents, usually MMC, are often used to reduce recurrence of pterygium. The optimal dosage of MMC for pterygium surgery is still unknown, however, extreme caution in its use is required due to its toxicity. The lowest possible concentration should be applied for the shortest time period to avoid complications. In our experiments, MMC reduced

the level of secreted cytokines (in particular IL-6), which provides a combined anti-inflammatory and anti-proliferative therapy for pterygium at a cellular and molecular level.

A positive clinical correlation has been found between pterygium and dry eye in some studies, and an unstable tear film has been proposed to contribute to the pathogenesis of pterygium. It is possible that the presence of an elevated tissue like pterygium poses a problem for tears to cover the ocular surface, resulting in localized areas of dryness. Mucins are large glycoproteins expressed by epithelial cells of both conjunctiva and cornea, and are thought to play an important role in determining the interactions between the cornea/conjunctiva and the overlying tear film. Mucins form a hydrophilic barrier for protection and lubrication of the eye. This barrier, the glycocalyx, is formed by MUC1, MUC4, and MUC16. The expression of MUC1 and 4 in our pterygium graft-containing cultures and the stratified outgrowing cells was clearly high, probably making our *ex vivo* model for pterygium being close to the model of a goblet cell hyperplasia.

The pterygium outgrowing cells were still viable after having undergone more than three months cultivation time, which was further confirmed by the high expression of CD47 at the ending check point. The surface marker profile of *ex vivo* cultivated outgrowing cells showed CD44, a protein expressed actively on dividing cells, to be moderately expressed in the stratified outgrowing cells. The expression of ECM attachment proteins, which is vital for the maintenance of cellular growth within the pterygium, was low (CD146, CD112) and moderate (CD166, CD44). Another important cell source for the formation and recurrence of pterygium, can be the bone marrow-derived multipotential stem and progenitor cells. Both hematopoietic and mesenchymal stem cells have been proposed to contribute to pterygial fibrovascular stroma supported by the increase in $CD34^+$ progenitor cells in the blood of such patients. It has been proposed, that a pterygium grading system should be based upon the infiltration of stem cells to better predict the risk of recurrence clinically. The expression of such hematopoietic cell surface markers CD34 and 'don't eat me' signal CD47 in our 3D outgrowing cell from pterygium cells proved to be low and high, respectively. The population of 3D outgrowing cells from the pterygium contained a low level of the early progenitor cell marker CD117/c-kit as well.

In the next part of our study, we established a novel method for *ex vivo* cultivation of pathologic tissues obtained from human eyes during cataract surgery and PDR. Viscoelastic material was used for *ex vivo* expansion of the outgrowing cells. After forming 2D structures, the cells were exposed to mechanical and ACh stimulation, depicting calcium signaling and presence of ACh receptors on the cells as well, as studies on inflammation. The outgrowing cells, over time, migrated out of the explants and grew adherently onto the surface of the cell culture dish, showing signs of continuous proliferation.

There are alternative methods for achieving adherence of tissue explants, however, there is an advantage of using a viscoelastic material, since it can avoid use of extreme conditions such as dryness of the cell culture and serum stimulants, yet preserving natural architecture of the tissue and standard nutritional state of the cells. We used two tissue types to establish adherent *ex vivo* explant cultures: aLCs containing LECs and fvERMs. Tissue and cell adherence allowed measurement of the $[Ca^{2+}]_I$ upon mechanical or pharmacological stimulation, giving advantage of having less noise from cellular movement within the cell culture dish.

To follow and coordinate the physiological processes occurring among cells in different tissues and organs, studying the intercellular communication is very important. Precise regulation of intracellular calcium levels is crucial to maintain normal cellular function, therefore, fluctuations in the cytosolic calcium can act as a signal for numerous physiological

processess. As calcium plays a role in the cell signaling of the lens epithelium, accurate regulation is substantial, indicating that imbalanced levels of it may be a leading cause to the development of cataract in the lens. In experimental conditions, applying a mechanical stimulus to a single cell can elicite intercellular Ca^{2+}-waves. Such stimulation of a single cell within a confluent layer was shown to initiate cell-to-cell calcium signalling. Our results show an increase in the $[Ca^{2+}]_i$ upon mechanical stimulation and application of ACh to aLC-LECs, thus revealing an unique frequency of oscillation by each cell.

The ocular tissues can be exposed to various cytokines and other proinflammatory markers that are released because of injury, infection or disease processes. The lens can also be exposed to various inflammatory mediators associated with bacterial infections present in the aqueous humor. It has been shown that incubation of human LECs with cytokines such as TNFα increases the activation of NF-κB (a well known inducible transcription factor that can cause inflammatory response) and causes cytotoxicity and apoptosis therefore preventing NF-κB activation by Aldose reductase (AR) inhibitors would be expected to rescue human LECs from cell death. IL-1, IL-6, and basic fibroblast growth factor (b-FGF) may be produced *in vivo* by residual LECs after cataract surgery, which can cause postoperative inflammation and LEC proliferation.

The [Ca2+]i dynamics upon mechanical stimulation of fvERMs has been described previously by our group. FvERMs in PDR can raise tractional force upon the retina and eventually lead to retinal detachment. Mechanical stimulation of the cells in the membranes or the retina can result in [Ca2+]i changes in a manner of intercellular waves. Generation of calcium occurs by its release from internal stores. The waves do not evoke changes in the cell membrane potential, but may constitute a pathway for extraneuronal signalling.

IL-6, 8 and TNFα are well known proinflammatory cytokines secreted by macrophages and neutrophils increased in the vitreous of PDR patients giving support to the role of inflammatory cytokines in angiogenesis in PDR. Increased secretion of IL-6 and IL-8 was also measured in our fvERM outgrowing cells upon TNFα stimulation. Understanding their role can provide important diagnostic and therapeutic targets for the treatment and prevention of PDR.

Overall we adherently cultivated and expanded cells from different tissues using viscoelastic material as a novel and simple method. Human pterygium explants have been demonstrated to give rise to 3D outgrowing viable cells that can proliferate and migrate out of the grafts and form a stratified structure very much like the one *in vivo*. The expanding cells carried proteins related to an undifferentiated state, but also a commitment towards epithelial lineage. Manipulating the cell sheets was similar to handling primary human tissues, presuming a possibility for use in tissue engineering and drug discovery. Intracellular calcium dynamics upon mechanical stimulation, calcium signaling, and intercellular communication upon ACh stimulation was measured in aLCs containing LECs, as well as inflammatory cytokines-release studies were performed. Future studies on cell functionality and homeostasis using calcium imaging and inflammation screening open possibilities for development of pharmacological and cell-based therapies that are attractive approach for treating eye diseases.

Publications related to the thesis:

1. **Josifovska N***, Szabó DJ, Nagymihály R, Veréb Z, Facskó A, Eriksen K, Moe MC, Petrovski G. **Cultivation and characterization of pterygium as an *ex vivo* study model for disease and therapy.** Cont Lens Anterior Eye. pii: S1367-0484(16)30117-5. doi: 10.1016/j.clae.2017.04.002 IF:1.783

2. Andjelic S, Lumi X, Veréb Z, **Josifovska N*,** Facskó A, Hawlina M, Petrovski G. **A simple method for establishing adherent ex vivo explant cultures from human eye pathologies for use in subsequent calcium imaging and inflammatory studies.** J Immunol Res. 2014;2014:232659.
IF:2.810

IF of publications related to the thesis: 4.59

Acknowledgement

I would like to gratefully acknowledge the guidence, support and encouragement to the many people who so generoulsy contributed to my work presented in this doctoral thesis.

First and foremost, I wish to thank my highly enthusiastic and motivating supervisor, Prof. Goran Petrovski, for the tireless support and many hours spent helping me, and for the very fruitful and insightful discussions during my work. I was very much privileged to learn many things from him; he helped me complete this PhD work and empowered me with enourmous values towards building my future career.

I take the time to express my gratitude to Professor Andrea Facskó, the Head of the Department of Ophthalmology (University of Szeged) for giving me opportunity to be part of her department of scientific excellence.

The thesis would not have come to successful completion without the help I received from all the past and current members of the department and the Stem Cells and Eye Research Laboratory. Special thanks to Dr. Zoltán Veréb, Dr. Dora Szabó, Richard Nagymihály, Dr. Luna Djirackor, Dr. Mária Szatmári Tóth, Dora Eszes and Dr. Reka Albert who have been very kind enough to extend their help at various phases of this research.

Thanks also goes to Dr. Áron Szabó and Dr. Péter Balázs Kocsis for their friendly advices and precious time spent together during my stay in Hungary.

At the end, I owe much my family far away in Macedonia, who encouraged me and helped at every stage of my personal and academic life.