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Effect of benzalkonium chloride on trabecular meshwork cells in a new in vitro 3D trabecular meshwork model for glaucoma

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ABSTRACT

Purpose: To validate a new culture model of primary human trabecular meshwork cells (p-hTMCs) using Matrigel®, in order to mimic in vitro 3D–TM organization, and to investigate the proinflammatory effect of benzalkonium chloride (BAK) in 3D p-hTMC cultures.
Methods: p-hTMCs, seeded onto Matrigel®-coated inserts were stimulated with BAK (10^{-4}%), dexamethasone (DEX) (10^{-6} M) or transforming growth factor-beta 2 (TGF-β2) (5 ng/ml) for 48 h and observed with confocal microscopy. The BAK effect at 10^{-4}% or 5.10^{-3}% on the gene expressions of interleukin-6 (IL-6), interleukin-8 (IL-8) and matrix metalloproteinase (MMP-9) was investigated using qRT-PCR in 2D and 3D p-hTMC cultures.
Results: p-hTMCs seeded in Matrigel® were able to organize themselves in a 3D-spatial conformation in the different conditions tested with cross-linked actin network (CLAN) formation in presence of DEX or TGF-β2 and intercellular space contraction with TGF-β2. IL-6 and IL-8 gene expressions increased in presence of BAK in 2D and in 3D p-hTMC cultures. BAK 10^{-4}% only showed a tendency to stimulate MMP-9 expression in p-hTMCs after 24 h-recovery.
Conclusions: We investigated this new 3D–TM in vitro model in Matrigel® matrix for pathophysiological and toxicological purposes. It appears as a new promising tool for a better understanding of TM behavior in physiological and stress conditions, as well as toxicological evaluations of antiglaucoma eyedrops and preservatives.

1. Introduction

Glaucoma is an optic neuropathy characterized by a progressive loss of optic nerve axons and retinal ganglion cells (RGCs), in response to the major currently identified risk factor, namely intraocular pressure (IOP) increase (Pascale et al., 2012). In the adult human eye, the trabecular meshwork (TM) is the principle drainage structure for aqueous humor (AH). The increased AH outflow resistance contributes to elevated IOP, which in turn causes or at least favors RGCs degeneration. Furthermore, glaucoma is characterized by a marked loss of TM cells (TMCs) (Tamm, 2009; Tektas and Lütjen-Drecoll, 2009). To understand the pathophysiology of glaucoma, experimental tools can contribute to the study of TM behavior. Many studies have therefore been based on animal experimentation. However, experimental data in animal models present many disadvantages due to the differences between animal and human TM structure. In human eyes, compared to non-human mammals, there is an absence of the washout effect, which is a phenomenon whereby the outflow facility of the eye progressively increases as perfusion continues (Scott et al., 2007), which makes interpreting the results of these models and applying them to human diseases very challenging. Moreover, animal experimentation is widely criticized by animal welfare advocates (Grune et al., 2014). Two-dimensional (2D) in vitro TMC cultures are an advantageous alternative model to understand TM behavior (Rhee et al., 2003; Stamer et al., 2000). However, this type of cell culture does not actually reflect the in-vivo conditions of

Abbreviations: p-hTMCs, primary human trabecular meshwork cells; BAK, benzalkonium chloride; 2D, two-dimensional; 3D, three-dimensional; IL-6, interleukin-6; IL-8, interleukin-8; MMP-9, matrix metalloproteinase-9; ECM, extracellular matrix; CLANs, cross-linked actin networks.

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cells due to the absence of differentiation, polarization, cell–cell communication and extracellular matrix contacts (Härmä et al., 2010). Given that the TM's function is to filter AH, its three-dimensional (3D) organization is an important parameter to take into account. 3D-Cell culture presents a good alternative and could therefore mimic the in-vivo properties and functions of cells (Bissell et al., 2002). Many extracellular matrices (ECMs) have been used for such cultures including Matrigel®, which is a basement membrane matrix secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells (Kleinman and Martin, 2005). This matrix, has been shown to allow the growth of a variety of human cells (Ramaiahgari et al., 2014; Rashidian and Luo, 2016). It was used to study tissue engineering and cell behaviors such as cancer cell invasion, angiogenesis, migration and differentiation (Kleinman and Martin, 2005).

Interestingly, 3D-cell culture may present a valuable tool to study drug responses in a more complex system than simple monolayers (Zucco et al., 2004; Hongisto et al., 2013).

Since glaucoma is a chronic disease, it is important to consider the consequences of exposure to medications administered over the long term in glaucoma patients. In particular, benzalkonium chloride (BAK), which is the most commonly used preservative in topical glaucoma medications, has widely and repeatedly shown toxic and proinflammatory effects at the level of the ocular surface and possibly in deeper structures like the TM (Baudouin et al., 2012). Indeed, due to its lipophilic structure, it could penetrate deep in the eye and accumulate in the TM (Brignole-Baudouin et al., 2012).

In glaucoma, TM degeneration has been found to associate several features of tissue aging, matrix remodeling, inflammatory cytokine release and oxidative stress. Furthermore, transforming growth factor-beta2 (TGF-β2) and steroids such as dexamethasone (DEX) are known to cause major changes in matrix remodeling. Matrix metalloproteinase-9 (MMP-9), one of the MMP members contributing to ECM turnover, has been detected in TMCs, which supports its role in regulating outflow resistance in the TM (Keller et al., 2009). In addition, inflammation plays a role in the development and progression of glaucoma (Vohra et al., 2013). An increased expression of inflammatory markers including interleukin-6 (IL-6) and interleukin-8 (IL-8) in the conjunctival epithelium of glaucoma patients was found under the influence of preservative accumulation (Baudouin et al., 2004; Guenoun et al., 2005). Moreover, increased levels of these cytokines were reported in glaucomatous AH (Huang et al., 2014), which supports that inflammation may play a role in glaucoma at various levels of the anterior segment.

In the present study, we aimed first at investigating a 3D model of primary human trabecular meshwork cell (p-hTMC) culture using Matrigel® and then evaluating (i) the influence of BAK on the cytoskeleton and matrix structures in these cells in comparison with DEX and TGF-β2 and (ii) the proinflammatory effects of BAK through the analysis of the gene expression of IL-6, IL-8 and MMP-9 and comparing its effects in 2D and 3D p-hTMC cultures.

2. Materials and methods

2.1. Materials and reagents

p-hTMCs, isolated from the juxtacanalicular and corneoscleral regions of human eye (ref 6590-SC) and TMC medium (TMCM) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). Matrigel® Basement Membrane Matrix and Polycarbonate transwell inserts (pore size 0.4 μm) were purchased from Corning Costar (Corning Incorporated; Corning, NY, USA). PBS was purchased from Gibco Invitrogen (Carlsbad, CA, USA). BAK 0.02% solution was provided by Novagali Pharma (Evry, France). DEX and TGF-β2 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Pepro-Tech (Rocky Hill, NJ, USA), respectively. Alexa Fluor 488 phalloidin and 4',6-diamidino-2 phenylindole (DAPI) were obtained from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

p-hTMCs were used in their fifth passage to minimize variations. They were grown at 37 °C in humidified air with a 5% CO_2 incubator and in TMCM, which consists of basal medium, 2% fetal bovine serum (FBS), 1% TMC growth supplement (TMCGS) and 1% penicillin/streptomycin solution (Penicillin 10,000 U/ml, Streptomycin 10,000 μg/ml).

For 3D Matrigel®-embedded culture, Matrigel® (1:3 and 1:2 dilutions) and 5.10^4 cells/ml and 10^5 cells/ml conditions were tested (data not shown). p-hTMCs at a concentration of 10^5 cells/ml were gently mixed with Matrigel® (1:3 dilution) and deposited onto inserts in a 12-well plate. Cell cultures were maintained for 15 days and the culture medium was changed every 2–3 days. At day 16, immunofluorescence analysis and quantitative RT-PCR were performed. Phase-contrast images of randomly chosen fields were taken with a Nikon Eclipse TS-100 inverted microscope (Nikon, Tokyo, Japan). The protocols involving the use of human tissue were consistent with the tenets of the Declaration of Helsinki.

2.3. BAK, DEX and TGF-β2 cell stimulations

p-hTMCs (10^5 cells/ml) seeded in Matrigel® onto transwell inserts were incubated with medium, BAK 10^{-4}% corresponding to 1/5–1/20 dilution of the BAK concentrations found in commercially available eye drops (0.005%–0.02%), DEX (10^{-6} M) or TGF-β2 (5 ng/ml) for 48 h before immunofluorescence staining. To preserve the cells for immunofluorescence analysis, p-hTMCs were treated with a low subtoxic concentration of BAK (10^{-4}%). For quantitative RT-PCR analysis, we used a protocol based on 30 min of stimulation of p-hTMCs (10^5 cells/ml) seeded in Matrigel® onto inserts with two dilutions of BAK (10^{-4}% or 5.10^{-3}%), known to be subtoxic and toxic, respectively, followed by 3, 6 and 24 h of cell recovery. Parallel cultures maintained without treatment served as controls. The similar treatment was conducted in 2D p-hTMC cultures.

2.4. Confocal microscopy

The membranes of the cell inserts were gently cut, detached and mounted on microscope glass slides. p-hTMCs were fixed in 4% paraformaldehyde for 15 min. Then they were permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature and stained for 30 min with Alexa Fluor 488 phalloidin at a 1:200 dilution and for 5 min with DAPI at a 1:2000 dilution. Phalloidin and DAPI were used for fluorescent staining of the actin filaments and the cell nucleus, respectively. Confocal images were captured with an Olympus FV 1000 confocal microscope using a ×200 objective and Olympus Fluoview software (version 4.1). The images were reconstructed to 3D shape using Imaris® software (Bitplane AG, Zurich, Switzerland).

2.5. RNA extraction

Cells were scraped and the mRNA was extracted using Nucleospin RNA XS kits (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The amount of mRNA was measured with the NanoDrop Spectrophotometer 2000 (Thermo Scientific, Waltham, MA, USA).

2.6. Quantitative real-time PCR

To determine the mRNA expressions of IL-6, IL-8 and MMP-9, total RNA extracted was reverse-transcribed using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The steps were: 10 min at 25 °C followed by 120 min at 37 °C and