A millifluidic-based 3D-platform as a useful tool for the efficacy of glaucoma therapeutic strategies

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Abstract

The aim of this present study is to investigate the role of damaged Trabecular Meshwork (TM) in triggering neuron-like cell apoptosis. Preliminary results showed that stressed TM releases harmful signals to neuron-like cells, suggesting its pivotal role in glaucoma cascade.

Introduction

Glaucoma is characterized by optic nerve damage and the slow progressive death of Retinal Ganglion Cells (RGCs). To date, although the main clinical approach is the management of Intraocular Pressure (IOP) by surgery or drugs, these have not turned out to be successful in preventing vision loss.[1] Indeed, RGC death is also driven by different converging molecular pathways, engaged in additional damage more or less closely connected to IOP elevation, which are able to trigger or exacerbate the glaucomatous cascade.[2] For example, defects in the Trabecular Meshwork (TM), one of the main tissues involved in the conventional outflow pathway, are responsible for IOP elevation in high tension glaucoma.[3] Therefore, given that, in the anterior segment of the eye, the TM is the most sensitive tissue to Oxidative Stress (OS), we aimed to verify whether the TM plays an active role in triggering RGC death.[4]

Thus, we used a millifluidic-human based 3D-platform *in vitro*, recently set up by us,[5-7] to analyze the potential role of oxidative stressed-TM cells in affecting the survival of neural-like cells, as a model of RGCs.

Materials and Methods

Cell Cultures

Human Trabecular Meshwork Cells (HTMC) and Trabecular Meshwork Growth Medium (TMGM) were obtained by Cell Application INC (San Diego, CA, USA); human neuroblastoma cells (SH-SY5Y) (American Type Culture Collection; Gaithersburg, MD, USA) were used as a model of retinal cells, since they can differentiate towards the neuronal-like phenotype (N-SH-SY5Y) by being grown in RPMI medium supplemented with 10µM retinoic acid (RA).[8] Both 3D cell cultures and the dynamic culture system were carried out as previously described.[5,7] Briefly, 3D HTMC and N-SH-SY5Y cultures embedded in Matrigel® Matrix, were set up under dynamic conditions by using millifluidic bioreactors connected to a peristaltic pump (Live Box 1 and Live Flow, IV-Tech srl, Massarosa, LU, Italy) with a constant flow rate (70mL/min).

Experimental conditions

Direct effects of prolonged OS were investigated by treating 3D HTMCs up to 72h, by daily exposure to 500 µM H_2O_2 for 2h, with 22-hour recovery phases in between (Figure 1).

Next, the indirect effects of OS were analyzed on differentiated 3D N-SH-SY5Ys using 50% of RPMI, supplemented with RA, and 50% of conditioned medium derived from OS-treated HTMC up to 72h.

Viability Assay

Alamar Blue (AB) assay from Invitrogen™ (Life Technologies, Carlsbad, CA, USA) was performed according to manufacturer's instructions, to analyze the metabolic activity daily of both the 3D-HTMC and N-SH-SY5Y cells. The AB assay was carried out a) on HTMCs to analyze the selective toxicity induced by prolonged H_2O_2 exposure; and b) on N-SH-SY5Y cells to verify the role of stressed-HTMCs in triggering any distress on neural-like cells during their growth in the presence of conditioned medium derived from OS-treated HTMCs.

Human apoptosis array

Analysis of the apoptosis proteomic profile of 3D-HTMC and 3D-N-SH-SY5Y after experimental procedures was carried out by commercial Human Apoptosis Antibody Array C1 (RayBio®; Norcross, GA, USA), a semi-quantitative detector of human proteins. The intensity of protein array signals was analyzed using a BIO-RAD Geldoc 2000 and each protein spot was normalized against Positive Control Spots printed on each membrane. The data analysis was conducted following the Protocol instructions of the Human Apoptosis Array C1.

Statistical analysis

GraphPad Prism 7.0 (GraphPad. Software, San Diego, CA, USA) was used for the statistical analysis. For the inter-group comparison of viability assay, we used the two-way analysis of variance (ANOVA) for single comparisons.

Results

As shown in Figure 2, the prolonged exposure to 500µM H_2O_2, as an oxidative stressor, affected the healthy state of the HTMCs already after 24h, whilst the N-SH-SY5Ys, during their growth in the presence of 50% conditioned medium derived from stressed-HTMCs, displayed a reduction of their metabolic state, only starting from 48h of experimental treatment. Moreover, extrapolated data from the heat map analysis evidenced an increase in apoptotic protein expressions in OS-treated HTMCs, mainly at 48hrs ($p<0.01$). Conversely, the N-SH-SY5Ys increased the levels of pro-

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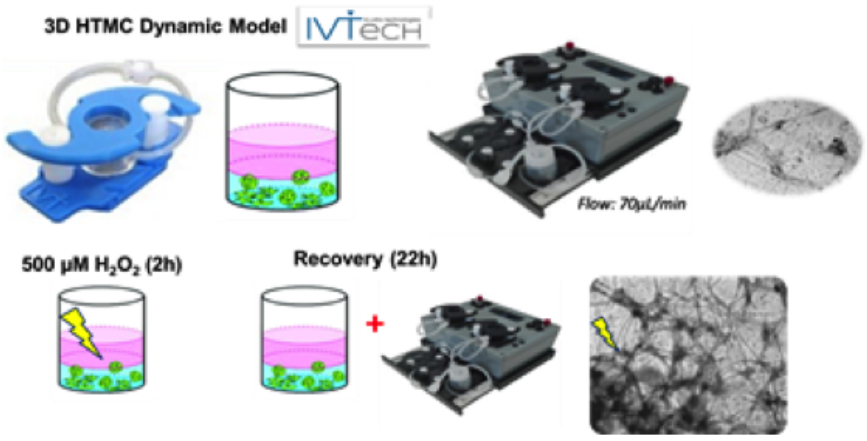

Figure 1. Human 3D HTMC - platform experimental model exposed to oxidative stress conditions. Representative images (on right) at contrast phase microscopy during OS-treatment.

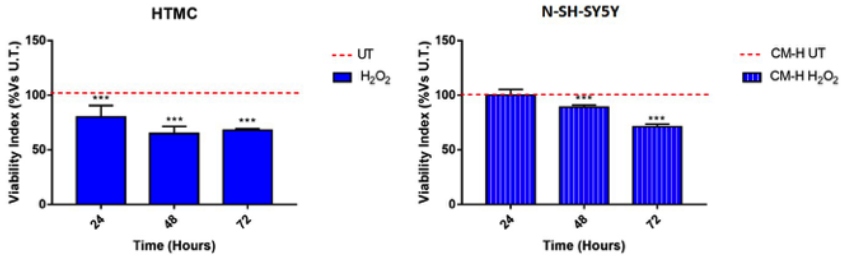

Figure 2. Viability index. Metabolic state of HTMCs and N-SH-SY5Ys during experimental conditions was analyzed by Alamar blue assay. Dotted red line represents viability index of untreated cells. Data are expressed as % vs. untreated cells and each value represents the mean ± SD of 3 independent experiments running in triplicate. UT: untreated cultures; CM-H: conditioned medium derived from HTMC cultures.

apoptotic proteins ($p<0.01$) only after 72h of the above-mentioned experimental conditions.

Discussion and Conclusions

These preliminary results demonstrate that HTMCs, during prolonged exposure to OS are able to release biomolecules which act as pro-apoptotic signals for N-SH-SY5Ys. Therefore, it can be assumed that in glaucoma, RGC cell death could also be promoted by a damaged TM.

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