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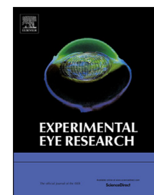

Unravelling the stromal-nerve interactions in the human diabetic cornea

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ABSTRACT

Corneal defects due to diabetes mellitus (DM) may cause severe vision impairments. Current studies focus on the corneal epithelium and nerve defects neglecting the corneal stroma. The aim of this study was to develop a 3D *in vitro* model to examine the interactions between corneal stroma and nerves in the context of DM. Primary human corneal stromal fibroblasts isolated from healthy (HCFs), Type 1 (T1DM) and Type 2 (T2DM) patients were stimulated with stable ascorbic acid to secrete and assemble an extracellular matrix (ECM). Human neuronal cells were then seeded on top and differentiated to create the 3D co-cultures. Our data revealed successful co-culture of stromal fibroblasts and neuronal cells with large elongated neuron extensions. T2DM showed significant upregulation of Collagen III and IGF1 when compared to T1DM. Interestingly, upon nerve addition, those markers returned to HCF levels. Neuronal markers were also differentially modulated with T2DM co-cultures expressing high levels of βIII tubulin where T1DM co-cultures expressed Substance P. . Overall, our unique 3D co-culture model provides us with a tool that can be utilized for both molecular and therapeutic studies for diabetic keratopathy.

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1. Introduction

Diabetes mellitus (DM) is primarily characterized by an elevated blood glucose level, and it is the most common metabolic disorder of the present times. (Schultz et al., 1981; Schultz et al., 1983; Priyadarsini et al., 2016a,b). Prevalence of DM has increased over the years and is expected to affect over 500 million people worldwide by 2030(Priyadarsini et al., 2016a,b). DM presents with a significantly high clinical burden with major ramifications on the quality of life. Several complications associated with DM can lead to severe loss of visual acuity or even blindness. DM is also associated with several structural and functional alterations of the human cornea, such as corneal epithelial changes, morphological changes to the corneal nerves, decreased endothelial cell density, reduced corneal sensitivity and altered central corneal thickness(Schultz et al., 1981; Schultz et al., 1983; Herse, 1988; Rosenberg et al., 2000; Bardsley and Want, 2004; Rother, 2007; Skarbez et al., 2010; Priyadarsini et al., 2016a,b).

The cornea, the outermost layer of the eye responsible for focusing our vision, is a clear, highly organized tissue devoid of any blood vessels. (Busted et al., 1981; Rosenberg et al., 2000; Holmes et al., 2001; Robert et al., 2001; Gekka et al., 2004; Eghrari et al., 2015; Meek and Knupp, 2015). The cornea has five layers consisting of the epithelium, Bowman's membrane, stroma, Descemet's membrane, and the endothelium. The thickest layer of the cornea is the stroma which primarily consists of collagen and the resident cells termed keratocytes(Busted et al., 1981; Robert et al., 2001; Gekka et al., 2004; Eghrari et al., 2015; Meek and Knupp, 2015). The architecture of the stroma layer is responsible for the majority of the corneal transparency, structure and integrity.

The cornea is the most densely innervated tissue of the human body(Shaheen et al., 2014; Eghrari et al., 2015) and several studies agree that corneal nerve dysfunction is one of the common pathobiological features that's commonly seen with DM(Shaheen et al., 2014). The healthy and diseased state of the cornea is closely related to neuronal innervation, as the distribution of innervation is critical in maintaining corneal transparency and structure(Marfurt

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et al., 2010; Bikbova et al., 2016; Wang et al., 2017). Diabetic changes in corneal nerves have primarily been associated with morphological alterations of the subbasal nerve plexus in both retinopathy and peripheral neuropathy(Tavakoli et al., 2011; Nitoda et al., 2012).

There have been various animal models for both T1DM (Nerup et al., 1994; Lenzen et al., 2001; Mathews et al., 2002; Yang and Santamaria, 2006; Zhou et al., 2011) and T2DM (Hummel et al., 1966; Surwit et al., 1988; Ikeda, 1994; Phillips et al., 1996; Leiter and Reifsnyder, 2004; Clee and Attie, 2007; Yoshida et al., 2010; Gault et al., 2011; Park et al., 2011) as reviewed by King(King, 2012). Although animal models that mimick diabetic corneal changes provide a good representation of DM, they have had limitations when translating these findings to human treatments (Schwartz, 1974; Hyndiuk et al., 1977; Schultz et al., 1981; Rosenberg et al., 2000; Dogru et al., 2001; Yoon et al., 2004; Xu et al., 2009). Despite the large number of studies reported to-date, the interactions and specific mechanisms involved in the complications associated with DM including diabetic keratopathy and diabetic neuropathy are not well understood. Furthermore, there have been very little studies involving the interactions between the corneal stroma and the stromal nerves. One of the reasons for this is the lack of human *in vitro* models that can successfully mimic what is seen *in vivo*. One of the most advanced *in vitro* models perhaps is the organotypic cultures, developed by Ljubimov and co-authors (Kabosova et al., 2003; Winkler et al., 2014) that mainly focuses on the identification of epithelial defects. Given that both cell types are damaged in DM, our model includes a 3D co-culture model of human corneal stromal cells and neurons which allows us for the first time to investigate the interactions between the two cell types within the human tissue.

In this study we developed a novel 3D *in vitro* model that mimics the *in vivo* stromal-nerve interactions during healthy and DM conditions. We co-cultured healthy human fibroblast cells, T1DM cells, and T2DM cells on along with the SH-SY5Y cell line that was further differentiated into human neuronal cells on our 3D system. The SH-SY5Y cell line was used for this study, as it is one of the most established, well characterized lines used to study neuronal growth. This model is the first of its kind and may provide new insights for a better understanding of the corneal complications associated with DM. Furthermore, this model could assist in the development of novel therapeutic agents for the treatment of diabetic keratopathy and diabetic neuropathy complications.

2. Materials and methods

2.1. Ethics and inclusion criteria

All procedures adhered to the tenets of the Declaration of Helsinki. Healthy human corneal samples were obtained from the National Disease Research Interchange (NDRI) and the diabetic corneal samples were obtained from Oklahoma Lions Eye Bank. The Oklahoma University Health Sciences Centre Institutional Review Board (IRB) was notified and appropriate permission was obtained prior to experimental initiation. Inclusion criteria for the diabetic donors included clinical diagnosis of T1DM or T2DM and absence of other unrelated diseases or ocular pathology. The healthy/control group included corneal samples from donors with no history of ocular trauma or systemic diseases. For each group in this study, cells were isolated from at least 4 donor corneal tissue samples ($n \geq 4$). Diabetic related complications like acute cerebral infarction, cerebrovascular accident, complications from end stage renal disease, or respiratory failure were the causes of death for the diabetic groups whereas the cause of death for healthy controls varied from accidental to non-diabetic related diseases like head trauma, acute segment elevation myocardial infarction, subarachnoid hemorrhage and cardiac arrest. In this study the average age for each group was as follows: Healthy: 57y/o, T1DM: 55y/o, and T2DM: 59y/o with no significant age differences noted between the groups. The duration of DM was from 3 to 30 years, with a mean of 15.5 years.

2.2. Primary cell isolation and culture

HCFs, T1DMs and T2DMs cells were isolated as per our previously optimized protocol. Initially the corneal epithelium and endothelium are removed from the stroma by scraping with a razor blade. Further, the stromal tissues are cut into small pieces of size ~ 2×2 mm and placed into T25 culture flaks. This is followed by making the explants adhere to the bottom of the flask at 37 °C for about 30 min where Eagle's Minimum Essential Medium (EMEM: ATCC: Manassas, VA) containing 10% fetal bovine serum (FBS: Atlantic Biological's; Lawrenceville, CA) and 1% Antibiotic (Gibco Antibiotic-Antimycotic, Life technologies) is added to the flasks carefully without disturbing the explants. Upon 100% confluence the cells are passaged into T75 culture flasks and further cultured before being processed for further analysis (Karamichos et al., 2012; Priyadarsini et al., 2014).

2.3. 3D construct assembly, maintenance, and co-cultures

Our 3D constructs for HCFs, T1DM, and T2DM cells were assembled as per our previously optimized protocol (Karamichos et al., 2009; Karamichos et al., 2010; Karamichos et al., 2011a,b; Karamichos et al., 2012). 1×10^6 cells/well were seeded on polycarbonate membrane inserts with 0.4-μm pores (Corning Costar; Corning Incorporated, Corning, NY, USA) and cultured in EMEM containing 10% FBS and 1% antibiotic (Fig. 1). Further, the cells were treated with 0.5 mM 2-O-α-D-glucopyranosyl-L-ascorbic acid (American Custom Chemicals Corporation, San Diego, CA, USA) to stimulate ECM secretion and assembly. The cultures were maintained for 3 weeks and fresh media was supplemented every other day during the entire study period.

After 3 weeks, 8×10^3 cells/well of SH-SY5Y human neuroblastoma cells were seeded on top of our assembled 3D constructs and differentiation was initiated (Fig. 1). SH-SY5Ys were treated with 10 μM Retinoic Acid (Sigma Aldrich, USA) which was added to EMEM containing 1% FBS and cultured for 5 days. Cells were then switched to a treatment of serum free media of EMEM containing 2 nM of Brain-derived neurotrophic factor (BDNF) (Sigma Aldrich) and cultured for 2 additional days before they were processed for qRT-PCR and Immunofluorescence.

2.4. Real-time PCR

All samples were evaluated for mRNA expression by qRT-PCR as previously described (Karamichos et al., 2011a,b; Karamichos et al., 2014a,b; Priyadarsini et al., 2014). Briefly, Ambion RNA mini extraction kit (Ambion TRIzol Plus RNA Purification Kit: Life technologies, Carlsbad, CA) was used to extract total RNA. This was followed by synthesis of cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. We used TaqMan gene expression of both (Applied Biosystems, Foster City) GAPDH (Hs99999905_m1) and 18S (Hs99999901_s1) as our housekeeping genes in the HCFs, T1DM, and T2DM. Furthermore, no changes were noted in either one between the three cell types in our housekeeping genes. ACTA2 (Hs00426835_m1), COL1A1 (Hs00164004_m1), COL3A1 (Hs00943809_m1), COL5A1 (Hs00609133_m1), IGF1 (Hs01547654_m1) and IGF1R (Hs00609566_m1), NRF1(Hs00192316_m1), PGC1A (Hs01016719_m1) and TFAM (Hs00273372_s1) were investigated.

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