Short Communication

Organotypic Retinal Explant Cultures as *In Vitro* Alternative for Diabetic Retinopathy Studies

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Summary

Diabetic retinopathy (DR) is a major cause of vision loss and one of the most common and debilitating complications of diabetes. Research to prevent DR is hindered by a lack of experimental model systems that faithfully reproduce the disease pathology, in particular for type 2 diabetes, which requires prolonged disease progression in animals to develop some hallmarks of DR. Here, we introduce an alternative *in vitro* model system for DR, based on serum-free, organotypic rodent retinal explant cultures, which allow physiological and pharmacological manipulation of the retina for up to two weeks under tightly controlled conditions. Retinal explant cultures have the advantage of isolating direct neuronal consequences of diabetic conditions from indirect systemic effects mediated via the retinal vasculature or the immune system. Exposed to conditions emulating type 1 or type 2 diabetes, retinal explants displayed elevated cell death rates among inner retinal neurons as well as photoreceptors, with a particularly strong loss of cone photoreceptors. Our results support a direct impact of diabetic conditions on retinal neurons and may help explain color vision defects observed in DR patients. This serum-free *in vitro* DR model avoids the animal suffering of established DR models and reduces the overall number of animals needed for such research. It should prove useful to study the mechanisms of neuronal cell death caused by DR and to screen for potential future DR treatments.

Keywords: retina, diabetes, animal models, photoreceptors, cell death

1 Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes and a leading cause of vision impairment worldwide. Although it is generally considered a microvascular disorder, studies in humans and animal models have found evidence for retinal neurodegeneration occurring before the onset of vascular alterations (Barber et al., 2011; Vujosevic and Midena, 2013). Thus, a central question in DR research is whether neuroretinal pathology is a consequence of vascular defects or whether diabetic conditions directly cause retinal neurodegeneration. Currently, DR research is hindered by a lack of disease models that faithfully reproduce the retinal

phenotype of diabetes, in particular type 2 diabetes (Lai and Lo, 2013). One of the most commonly used type 1 diabetes models, the injection of streptozotocin in rodents, usually produces a subtle retinal phenotype, with thinning of the ganglion cell layer (Robinson et al., 2012; Martin et al., 2004). Genetic DR models include the Ins2Akita mouse, a type 1 diabetes model carrying a mutation in the insulin-2 gene. Among type 2 diabetes animal models, mice with mutations in the leptin gene or receptor (ob/ob, db/db) may be useful to study DR (Lai and Lo, 2013; Robinson et al., 2012; Ly et al., 2014). However, a general problem with type 2 diabetes models is the long time-frame and high interindividual variability in their pathophysiology. Overall, most type 1 and 2 diabetes

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Tab. 1: Experimental conditions, medium composition, type of diabetes emulated, and group abbreviations used in this study

Experimental condition	R16 medium composition	Diabetes	Abbreviation
Control	19 mM glucose 2 µg/ml insulin	=	Control
Osmotic control	19 mM glucose 2 µg/ml insulin 15 mM mannitol (4 days)	=	Mannitol
No insulin	19 mM glucose 0 µg/ml insulin (4 days)	Type 1	No INS
High glucose, no insulin	34 mM glucose (4 days) 0 µg/ml insulin (4 days)	Type 1	HG, No INS
High glucose	34 mM glucose (4 days) 2 µg/ml insulin	Type 2	HG
2-deoxyglucose	19 mM glucose 2 µg/ml insulin 20 mM 2-deoxyglucose (24 h)	Type 2	2-DG

models exhibit only minor changes in the neuroretina in spite of prolonged disease progression and the associated cost of animal suffering.

In vitro studies using cell culture may provide valuable insights into the cellular processes triggered by diabetic conditions, for instance into the direct cytotoxic effects of high glucose conditions (Matteucci et al., 2015). Yet, cell cultures cannot reproduce the complex interplay of different neuronal cell types that is characteristic of the retina. Retinal explants conserve the histotypic context of the retina, yet undergo rapid vasoregression and may therefore be useful to study the direct effects of hyperglycemia and hypoinsulinemia, independent of vascular- or immune-related effects. This may allow identification of the initial molecular mechanisms of DR. Serum-free, organotypic retinal explant cultures have previously been used in studies on hereditary retinal diseases (Caffe et al., 2001; Sahaboglu et al., 2014) and for drug screening purposes (Paquet-Durand et al., 2010). Serum free and chemically defined culture conditions allow accurate and reproducible control of experimental conditions, which makes these systems excellent tools for quantitative metabolic as well as pharmacological studies. Here, we show that *in vitro* simulated diabetic conditions induce neuronal cell death in both the inner and outer nuclear layers (INL, ONL) of the retina with cone photoreceptors most severely affected. Taken together, our findings provide evidence that neuronal cell death in DR can occur independent of vascular defects or immune system

activation, and demonstrate the utility of retinal explant cultures as a novel tool to study DR disease mechanisms and to test potential treatments. Compared to *in vivo* experimentation, retinal explant cultures offer the additional advantage that the suffering of live animals is eliminated and that the number of animals needed is greatly reduced, in agreement with the 3R concept of Russell and Burch.

2 Animals, materials and methods

Animals

Wild-type C3H mice devoid of the *rd1* mutation (Sanyal and Bal, 1973) were housed under standard white cyclic illumination, had free access to food and water, and were used irrespective of gender. All personnel entrusted with animal care and handling had received appropriate training, guidance, and supervision, as specified in the German law on animal protection (TierSchG). The procedures were reviewed and approved by the Tübingen University review board (§4 TierSchG v. 22. 5. 2014; 26. 11. 2015), and performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and visual research. Post-natal (P) day 5 animals were killed by decapitation; both eyes were used for explant preparation. The total number of animals used in this study was 35, yielding 70 retinal explant cultures.

Organotypic retinal explant cultures

Retinal explants obtained from P5 wild-type mice were cultured as previously described (Caffé et al., 2001; Sahaboglu et al., 2014). Briefly, the retina was placed with the pigment epithelium facing down on cell culture inserts (Millicell, Merck Millipore) with R16 complete medium (CM) containing 19 mM glucose + supplements (Caffé et al., 2001), which was replaced every two days. The cultures were incubated at 37°C in 5% CO₂. From P5 to P7 cultures were kept in CM, afterwards treatments were applied until P11 for all conditions except for 20 mM 2-deoxy-glucose (2-DG), which was applied for 24 h from P10 to P11. Treatments were: 15 mM D-mannitol as osmotic control (Mannitol), removing insulin from the CM (No INS), adding 15 mM glucose to CM and removing insulin (HG, No INS), and adding 15 mM glucose to CM (HG) (Tab. 1).

Histology, immunostaining and TUNEL assay

Explants were fixed at P11 for 45 min at room temperature (RT) with 4% PFA, cryoprotected in graded sucrose solutions, embedded in OCT (Leica-Microsystems), and flash-frozen on liquid nitrogen. 12 µm cryosections were collected on glass slides and stored at -20°C. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche Diagnostics) was used for the *in situ* detection of dying cells. For immunohistochemistry, cryosections were dried at 37°C and incubated in blocking solution (10% normal goat serum, 1% BSA and 0.3% Triton in PBS). Primary antibodies (cleaved caspase-3 1:200, Cell Signaling 9064; PKCα 1:100, Novus Biologicals NB200586; glycogen phosphorylase 1:1000, kindly provided by Prof. Pfeiffer-Guglielmi; cone arrestin 1:1000, Merck Millipore AB15282) were applied overnight at 4°C. Subsequently, sections were incubated with corresponding secondary antibodies, conjugated to Alexa Fluor 488 or 566 (Molecular Probes), for 1 h at RT in the dark. Finally, sections were counterstained with DAPI and mounted with Vectashield (Vector Labs).

Microscopy, quantification and statistics

Stained sections were analyzed using a Zeiss Z1 Apotome microscope and Axiovision software. The percentages of TUNEL-positive cells in INL/ONL were quantified by manually counting labeled cells in representative areas of three cryosections per retinal explant. The total number of cells was determined by dividing the INL/ONL areas through their respective average cell size. Cone photoreceptor density was quantified as number of cones per 10 µm of retinal circumference. Representative images for each experimental condition are shown in Figure S1 (<http://dx.doi.org/10.14573/altex.1603111s>). Data were analyzed using Microsoft Excel and GraphPad Prism software. Values are given as mean ± SEM. The data were compared using non-parametric Kruskal-Wallis and subsequent Dunn's multiple comparison tests. Significance levels as indicated by asterisks were: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

3 Results

Diabetic conditions cause neuronal cell death in retinal explants

Four treatment paradigms were applied to emulate diabetic retinopathy. Retinal explants were exposed to either normal or elevated glucose levels without insulin to simulate conditions similar to insulin-deficient type 1 diabetes. A third group of explants was subjected to elevated glucose levels in the presence of insulin, emulating conditions of type 2 diabetes. Fourth, explants cultured with normal glucose and insulin levels were treated for 24 h with 2-DG to block glycolysis and imitate alterations of glucose metabolism observed in certain forms of type 2 diabetes (Tab. 1).

The effects of the different treatment paradigms were characterized by quantifying the fraction of dying cells in the INL and ONL (Fig. 1A, B). Both in the untreated control and in the osmotic control with 15 mM mannitol, only low numbers of TUNEL-positive, dying cells were counted. However, a significant increase in TUNEL-positive cells was observed in the INL after treatment with high glucose, irrespective of the presence of insulin. On the other hand, omission of insulin from the culture medium also caused an increase in INL cell death. In the ONL, lack of insulin or elevated glucose levels increased cell death rates but, remarkably, elevated glucose without insulin had no obvious deleterious effect. The strongest increase in ONL cell death was observed after treatment with the glycolysis inhibitor 2-DG (Fig. 1B).

The quantification of cell rows in the INL did not reveal any significant treatment-induced changes, indicating that increased cell death had not yet translated into an overall decrease of inner nuclear layer thickness (Fig. 1C). In the ONL, only the 2-DG treatment caused a significant reduction of nuclear rows (Fig. 1D).

Activation of caspase-3 in diabetic conditions

To assess to which extent apoptosis may have contributed to retinal cell death in our experimental setup, we double-labeled retinal sections with an antibody against cleaved, activated caspase-3 and with the TUNEL assay (Fig. 1E, F). Subjected to high glucose, with or without insulin, a subpopulation of cells in both the INL and ONL displayed both cell death markers. On the other hand, the removal of insulin from the medium or inhibition of glycolysis with 2-DG did not increase caspase-3 activation in INL and ONL. Notably, the overall numbers of cells displaying caspase-3 activity were far lower than the number of TUNEL-positive cells, supporting alternative cell death mechanisms affecting the retina under emulated diabetic conditions.

Cone photoreceptors are highly vulnerable to simulated diabetes

The retina harbors a large variety of cell types in its different layers (Masland, 2012). To test whether any of these cell types were selectively affected by diabetic conditions, markers specific for rod bipolar cells (PKCα) (Haverkamp and

Wassle, 2000) and cone photoreceptors (glycogen phosphorylase) (Brennenstuhl et al., 2015) were applied to the retina. Glycogen phosphorylase labeling revealed a dramatic loss of cones under diabetic conditions, particularly in treatment paradigms that involved high glucose (Fig. 1G). Staining with the alternative cone marker arrestin confirmed cone loss under diabetic conditions (data not shown). In contrast, PKC α immunoreactivity revealed no significant change in the number of rod bipolar cells (Fig. 1H). Taken together, these results indicate a differential vulnerability of retinal cell types to diabetic conditions.

4 Discussion

For a better understanding of DR pathogenesis and to foster the development of new treatment options, reproducible and accessible disease models are urgently needed. Here, we present a multipurpose *in vitro* DR system using organotypic retinal explants cultured in a defined, serum-free medium to overcome the challenges and animal suffering associated with common DR models. We show that emulated diabetic conditions directly lead to neuronal cell death, independent of vascular alterations or immune system activation. Cone photoreceptors appear to be particularly vulnerable to diabetic conditions (Cho et al., 2000). This may correspond to the finding that color vision defects of non-vascular etiology are among the earliest signs of DR in humans (Gella et al., 2015; Wolff et al., 2015).

Remarkably, four days of high glucose treatment were sufficient to increase retinal cell death rates. While only photoreceptors in the ONL were found to be sensitive to perturbation of intracellular glucose homeostasis via 2-DG treatment, removal of insulin from the culture medium caused a significant increase in cell death in both INL and ONL. This supports the notion that the continuous presence of insulin in the retina plays a key role in cell survival, especially in the inner retina. Deprivation of insulin as well as high glucose caused a dramatic loss of cones. Cones are known to express insulin receptors (Rajala et al., 2013) and recent findings in secondary cone degeneration suggest a strong insulin dependency (Punzo et al., 2009; Ait-Ali et al., 2015). Interestingly, cones express glycogen phosphorylase (Brennenstuhl et al., 2015), suggesting they may use glycogen as an alternative energy source.

Studies in both animals and humans have found a thinning of all retinal layers and an increase in TUNEL-positive cells in DR, indicating widespread neurodegeneration under diabetic conditions (Barber et al., 2011; Martin et al., 2004; van Dijk et al., 2009). Yet, the mechanisms of diabetic neurodegeneration, particularly in the retina, are incompletely understood (Barber et al., 2011; Feenstra et al., 2013). Among the biochemical factors implicated in DR pathophysiology are oxidative stress, the production of advanced glycation end products (AGEs), glutamate excitotoxicity, and neuro-inflammation, eventually leading to cell death by apoptosis

Fig. 1: Diabetic conditions cause extensive neuronal cell death in the retina

Each bar color represents different experimental situations emulating diabetes-like conditions or controls, as defined in Table 1. (A, B) Bar graphs displaying the percentages of TUNEL-positive cells in both the inner nuclear layer (INL) and the outer nuclear layer (ONL). (C, D) Quantification of rows of nuclei for the INL and ONL, respectively. (E, F) Quantification of cells positive for both cleaved, activated caspase-3 and TUNEL as apoptosis markers in the INL and ONL. (G) Quantification of cone density by counting glycogen phosphorylase-positive cells per area. (H) Quantification of rod bipolar cell density by counting the fraction of PKC α -positive cells. Each dot in the bar graphs represents an individual retinal explant.

(Stitt, 2010; Zhou et al., 2011). The activity of caspase-3 is considered a hallmark of classical apoptosis. We observed a significant increase of caspase-3 activation in both INL and ONL after treatment with high glucose, with and without insulin. The percentage of cleaved caspase-3 positive cells was about 6-fold lower than the total number of TUNEL-positive cells, indicating that cell death via apoptosis was activated only in a minor fraction of dying cells. A variety of other, non-apoptotic mechanisms have been proposed to contribute to retinal cell death (Arango-Gonzalez et al., 2014), including autophagy and parthanatos (Punzo et al., 2009; Feenstra et al., 2013; Arango-Gonzalez et al., 2014). Future studies should reveal which cell death pathways different from apoptosis are triggered in diabetic conditions and which of the many cell types in the inner retina are mainly affected by these.

Taken together, this study introduces the first *in vitro* model for DR that reproduces neuronal cell death in the INL and ONL under controlled, serum free conditions. Our results support direct deleterious effects of diabetic conditions on retinal cells, particularly on cone photoreceptors. The model prompts high versatility, allowing adaptation to emulate a variety of different conditions, including features characteristic of type 1 or type 2 diabetes. Limitations of the utility of the explant model arise from the restricted duration it can be maintained in culture – about four weeks (Caffé et al., 2001), which excludes the possibility of chronic long term treatments. Another caveat is posed by the immaturity of the isolated retinas at postnatal day 5. Although they continue to develop *in vitro*, there will be some physiological differences compared to the *in vivo* situation, which might somewhat alter their response to diabetes-like conditions. However, P5 as starting point for explant culture is a necessary compromise between viability and maturity of the retinae *in vitro* (Caffé et al., 2001). Furthermore, the absence of functional vasculature and an immune system, although desirable for the investigation of strictly neuronal effects in DR, also excludes the possibility to study the role of these systems in DR. Finally, the necessity to sacrifice mice for explant preparation falls short of the general goal to fully replace the use of animals in diabetes research. Nonetheless, our model will undoubtedly prove useful for studies into the etiopathology of DR and for DR-related drug screening, and, where applicable, can significantly reduce the number of animals needed and their suffering.

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Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

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