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Research article

N-methyl-N-nitrosourea-induced neuronal cell death in a large animal model of retinal degeneration *in vitro*

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ABSTRACT

N-methyl-*N*-nitrosourea (MNU) has been reported to induce photoreceptor-specific degeneration with minimal inner retinal impact in small animals *in vivo*. Pending its use within a retinal transplantation paradigm, we here explore the effects of MNU on outer and inner retinal neurons and glia in an *in vitro* large animal model of retinal degeneration. The previously described degenerative culture explant model of adult porcine retina was used and compared with explants receiving 10 or 100 μg/ml MNU (MNU10 and MNU100) supplementation. All explants were kept for 5 days *in vitro*, and examined for morphology as well as for glial and neuronal immunohistochemical markers. Rhodopsin-labeled photoreceptors were present in all explants. The number of cone photoreceptors (transducin), rod bipolar cells (PKC) and horizontal cells (calbindin) was significantly lower in MNU treated explants ($p < 0.001$). Gliosis was attenuated in MNU10 treated explants, with expression of vimentin, glial fibrillary protein (GFAP), glutamine synthetase (GS), and bFGF comparable to *in vivo* controls. In corresponding MNU100 counterparts, the expression of Müller cell proteins was almost extinguished. We here show that MNU causes degeneration of outer and inner retinal neurons and glia in the adult porcine retina *in vitro*. MNU10 explants display attenuation of gliosis, despite decreased neuronal survival compared with untreated controls. Our results have impact on the use of MNU as a large animal photoreceptor degeneration model, on tissue engineering related to retinal transplantation, and on our understanding of gliosis related neuronal degenerative cell death.

1. Introduction

Retinal transplantation as a potential cure for patients suffering from retinal degenerative disease has been explored in the laboratory for almost three decades using a multitude of animal models (Turner and Blair, 1986; Ghosh and Ehinger, 2000; Aramant and Seiler, 2004; Tucker et al., 2014). In spite of intensive efforts, successful experimental retinal transplantation has yet to be demonstrated and thus the prospect of lab bench-to-clinical treatment translation remains elusive.

We and others have shown that non-disrupted retinal tissue grafts, i. e full-thickness retina, in small and large animal models of photoreceptor degeneration show excellent long-term survival with proper photoreceptor organization (Ghosh et al., 2004; Seiler and Aramant, 1998). However, to restore visual function in a diseased eye, grafted photoreceptors also need to form synaptic connections with host inner retinal cells, a phenomenon which is hampered by remaining host photoreceptor and reactive glial cells even in severely degenerated retinas (Ghosh et al., 2007; Wanner et al., 2008). Interestingly, neuronal integration has been demonstrated if the outer retina of the host is disrupted mechanically or by ischemia, indicating that selective removal of photoreceptors prior to transplantation may be required for successful fusion of graft-host neurons (Ghosh et al., 1999; Zhang et al., 2003).

The alkylating carcinogen N-methyl-N-nitrosourea (MNU) has been reported to induce photoreceptor-specific apoptotic degeneration rapidly with minimal inner retinal remodeling in small animals (Wan et al., 2006, 2008; Chen et al., 2014; Rösch et al., 2014; Yoshizawa et al., 2009). Mice receiving MNU systemically show rapid retinal degeneration, but also severe systemic toxic effects, whereas intravitreal application of MNU results in photoreceptor degeneration with minimal effects on general health (Rösch et al., 2014; McCormick et al., 1998).

The photoreceptor-selective preponderance of MNU toxicity is theoretically attractive from a tissue engineering perspective for

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retinal transplantation. An ideal scenario is that the treatment selectively removes all diseased photoreceptors in the host prior to actual transplantation, after which, formation of neuronal connections between grafted photoreceptors and the remaining inner retina of the host is facilitated. However, general and retina-specific toxicity data from large animals is thus far limited. As a first step towards exploring selective photoreceptor removal in a large animal model of retinal degeneration, we for the present paper have chosen to expose adult porcine retinal cultures to MNU. The adult porcine retina *in vitro* has been well characterized previously, and offers excellent opportunity to isolate and explore biochemical and biomechanical factors affecting retinal cell health (Taylor et al., 2013a, 2013b; Kobuch et al., 2008; Kaempf et al., 2008). In addition, the *in vitro* setup allows for excellent dosing control when exploring effects of drugs, neurotrophic factors and other compounds. Adult porcine retinal explants display an accelerated degree of photoreceptor degeneration compared with genetically engineered *in vivo* models, but retain a substantial amount of cells after 5 days *in vitro* (Taylor et al., 2013a; Petters et al., 1997). Thus, the adult retinal explant model may be regarded as a rapid *in vitro* equivalent of retinal degeneration relevant for future transplantation experiments in which a diseased host retina is pretreated to remove all outer nuclear cells hampering graft-host integration. We here report the effects of MNU treatment on adult porcine retinal grafts with focus on outer and inner retinal neuronal survival as well as effects on Müller cell structure and function.

2. Material and methods

2.1. Tissue culturing

All proceedings and animal treatment were in accordance with the guidelines and requirements of the EU Directive 2010/63/EU for animal experiments, the government committee on animal experimentation at Lund University, and with the ARVO statement on the use of animals in ophthalmic and vision research. Adult pigs aged 4–6 months were euthanized by an overdose of sodium pentobarbital (Apoteket, Umeå, Sweden), and eyes were harvested immediately. The neuroretinas were removed using a previously described method (Taylor et al., 2013a, 2013b). To summarize, immediately after enucleation the eyes were placed in CO_2 −independent medium (Invitrogen, Paisley, UK). The anterior segment was removed by cutting 360° at the pars plana. The neuroretinas were gently removed from the pigment epithelium using microforceps, cutting at the optic nerve head. The central portion of each neuroretina was divided into 6 pieces, measuring approximately 7 × 8 mm. In total, 8 eyes from 4 animals were used, yielding 36 specimens for culture and 2 eyes serving as normal adult *in vivo* controls. The explants were placed with the photoreceptors facing the membrane onto Millicell- PCF 0.4 μm culture plate inserts (Millipore, Billerica, MA, USA). Specimens were cultured in either baseline medium (controls; CT; n = 12) DMEM/F12 (Invitrogen) supplemented with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA), or with MNU supplementation of either 10 μg/ml or 100 μg/ml (MNU10 and MNU100, respectively; n = 12 and n = 12). The cultures were maintained in an incubator at 37 °C at 95% humidity and 5% CO_2. Explants originating from the same animal and eye were divided among the various groups to ensure no bias. The medium was exchanged every second day. Explants were fixed at 5 DIV for immunohistochemical analysis.

2.2. Histology

Histological examinations were performed as previously described (Taylor et al., 2013a, 2013b), and will only be briefly summarized here. After 5 DIV, the explants were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h at room temperature. The normal adult *in vivo* controls were fixed immediately after harvest using the same paraformaldehyde concentration for 8 h at room temperature. The explants were then infiltrated with 0.1 M Sörensens medium with increasing concentrations of sucrose, up to 25%, for cryoprotection. After this, the explants were embedded in egg albumin/gelatine medium for cryosectioning at −20 °C, with a section thickness of 12 μm. Every 10th slide was stained with hematoxylin and eosin for morphological analysis. For

Table 1
Table of primary and secondary antibodies used for immunohistochemical analysis.

Antigen	Antibody name	Target cell/structure/protein	Species	Dilution	Source	Reference
Rhodopsin	Rho4D2	Rod photoreceptor	Mouse monoclonal	1:100	Kind gift of Prof. RS Molday, Vancouver, Canada	(Taylor et al., 2013a)
PKC	Phospho-PKC (pan)	Rod bipolar cells	Rabbit polyclonal	1:200	Cell Signaling, Beverly, MA, USA	(Taylor et al., 2013a)
bFGF (basic-fibroblast growth factor)	Mouse anti-bFGF	Müller cells, astrocytes	Mouse monoclonal	1:200	Sigma Aldrich, St Louis, MO, USA	(Li et al., 1998)
NeuN (Neuronal Nuclei)	Anti- Neuronal Nuclei	Ganglion cells	Mouse monoclonal	1:100	Millipore, Billerica, MA, USA	(Johansson et al., 2010)
Cone-transducin	Anti-G protein Gγc subunit	Cone photoreceptor	Rabbit polyclonal	1:1000	Cytosignal, Ca, USA	(Engelsberg et al., 2005)
GFAP	Anti-Glial Fibrillary Acidic Protein	Activated Müller cells, astrocytes	Mouse monoclonal	1:200	Chemicon International, CA, USA	(Taylor et al., 2013a)
Calbindin	Anti-calbindin-D-28-K	Horizontal cells	Mouse monoclonal	1:200	Sigma Aldrich, St Louis, MO, USA	(Taylor et al., 2013a)
Glutamine Synthetase	Anti-glutamine synthetase	Müller cells	Rabbit polyclonal	1:2000	Abcam, Cambridge, UK	(Taylor et al., 2014)
Vimentin	Mouse anti-vimentin	Müller cells	Mouse monoclonal	1:500	Chemicon International, CA, USA	(Johansson et al., 2010)
secondary Antibody	Antibody name	Target	Species	Dilution	Source	
FITC	Anti-mouse IgG FITC conjugate	Anti-mouse	Goat	1:200	Sigma Aldrich, St Louis, MO, USA	(Taylor et al., 2014)
Rhodamine red	Rhodamine Red TM -X-conjugated	Anti-rabbit	Donkey	1:200	Jackson ImmunoResearch, PA, USA	(Taylor et al., 2014)