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Triamcinolone attenuates macrophage/microglia accumulation associated with NMDA-induced RGC death and facilitates survival of Müller stem cell grafts

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ABSTRACT

Retinal ganglion cell (RGC) death in glaucoma models is associated with accumulation of activated microglia, a sign of neural degeneration which has been shown to constitute a barrier for transplant cell survival and migration. This study investigated the use of triamcinolone (TA) to control macrophage/microglia accumulation in a model of RGC depletion to create a permissive environment for stem cell grafting. Intravitreal NMDA alone or in combination with TA was used to induce rapid onset of RGC death in 3–4 week old Lister hooded (LH) rat eyes prior to Müller stem cell transplantation into the vitreoretinal space. The effect of NMDA on RGC death and microglial accumulation was assessed immunohistochemically, whilst electroretinography (ERG) was used to assess RGC function. Post transplantation, survival of grafted cells and their association with microglia were also examined by immunohistochemical methods. Intravitreal injection of NMDA alone resulted in severe macrophage/microglia accumulation associated with extensive RGC death 4–7 days post-treatment. Combination of NMDA with TA significantly reduced microglial numbers in the RGC when compared to NMDA only treated eyes while still depleting the retina of RGC. At the same time, NMDA/TA treatment also caused functional RGC loss as demonstrated by reduction of the scotopic threshold response. Upon transplantation with Müller stem cells, NMDA/TA treatment caused significant reduction in the number of transplant associated macrophage/microglia compared to eyes treated with NMDA alone. On this basis it is proposed that intravitreal injection of TA may be useful as an effective anti-inflammatory agent to control macrophage/microglia accumulation induced by RGC death, thereby creating a retinal environment permissive to cell transplantation studies.

1. Introduction

Current experimental models of glaucoma include transient ischemia reperfusion injury (Adachi et al., 1996; Selles-Navarro et al., 1996), episcleral vein sclerosis using hypertonic saline injections (Morrison et al., 1997) and diode laser treatment causing episcleral vein damage and trabecular meshwork scarring (Levkovitch-Verbin et al., 2002) amongst others. These models are designed to cause increase in intra-ocular pressure (IOP) which often results in retinal ganglion cell (RGC) depletion. However the chronic intra-ocular hypertension and RGC death caused by these models also induces considerable microglial activation within the retina (Naskar et al., 2002). Our work has previously shown that activated microglia constitutes a barrier to transplant cell migration and integration (Singhal et al., 2008; Bull et al., 2008), making these models unsuitable for studies investigating the biological activity of stem cells or their derived progenies. N-methyl D-aspartate (NMDA) is a chemical agonist of the NMDA receptors of the glutamate family. These receptors are highly calcium permeable and their excessive stimulation results in excitotoxic damage to specific neurons (Sattler and Tymianski, 2001). RGCs, which undergo apoptotic cell death following intravitreal NMDA injections in rat eyes (Lam et al., 1999), are known to possess these receptors in abundance and are particularly vulnerable to NMDA mediated excitotoxic damage (Sucher et al., 1997).

Triamcinolone (TA) is an FDA approved synthetic corticosteroid that is widely used in various anti-inflammatory applications, particularly in the eye, where it has been used intra-vitreally to resolve diabetic macular oedema (Cunningham et al., 2008; Jonas et al., 2004a,b) and to reduce post-operative inflammation after retinal surgery (Mankowska et al., 2008). This study investigated whether a combination of intravitreal NMDA and TA could generate a rat model of RGC depletion that was free of microglial activation and which potentially may be used for stem cell transplantation studies.

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2. Methods

2.1. Preparation of NMDA and triamcinolone acetonide (TA) for intravitreal injection

Based on preliminary work and doses previously used by others (Lam et al., 1999), 2 μl of 40 mM NMDA (M3262, Sigma–Aldrich, UK) diluted in serum free DMEM was injected intra-vitreally in each eye. In order to administer NMDA and TA together, both chemicals were prepared separately but mixed together (1 μl of each) immediately before the intravitreal injections. To keep the total volume of the injection constant, NMDA was prepared at a concentration of 80 mM and 1 μl of this solution was mixed with 1 μl of TA at a concentration of 80 mg/ml prepared as described below.

For the injections, TA (Kenalog) 40 mg/ml was centrifuged to allow the particles to settle at the bottom of the tube, enabling removal of the excipient. These particles were then washed with sterile saline for injection before being resuspended in the same solution to achieve a concentration of 80 mg/ml. One μl of this solution yielded a final concentration of 1.33 mg/ml in the rat eye (presumptive rat vitreal volume of 55–60 μl), which was equivalent to the concentration of 1 mg/ml usually used in humans (Gao et al., 2004).

2.2. Intravitreal injections

Adult (3–4 week old) Lister hooded (LH) rats were maintained according to the Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act (1986) and the ARVO guidelines for the use of animals. Rats were anaesthetized with an intraperitoneal injection of Ketamine HCl – 7.5 mg/100 g (Ketaset, Fort Dodge Animal Health, Southampton, UK) and Medetomidine HCl – 5 mg/100 g (Domitor, Pfizer, Sandwich, UK). Pupils were dilated using 1% tropicamide and 2.5% Phenylephrine (Chauvin Pharmaceuticals, UK). To perform the intravitreal cell injections the conjunctiva was dissected dorsolaterally down to the sclera under visualisation through an operating microscope. A small amount of viscotears gel along with a coverslip was placed over the cornea and the microscope adjusted so as to focus on the retinal blood vessels which were directly visible through the dilated pupil. Under direct visualisation through the pupil, a 30 gauge needle attached to a Hamilton syringe was inserted into the intravitreal space and the NMDA, or NMDA combined with TA, injected. LH rats were injected with NMDA/TA in one eye with the contra-lateral eye used as an untreated control.

2.3. Müller stem cell transplantation

The Müller stem cell line MIO-M1 (Lawrence et al., 2007) was used for transplantation studies to assess the effect of TA on transplant cell survival in eyes depleted of RGC by NMDA. Cells were transfected with an immunodeficiency virus type 1 (HIV-1) based lentiviral vector expressing low toxicity hrGFP from a spleen focus forming virus (SFFV) promoter as previously described (Singhal et al., 2008). Animals were immuno-suppressed with oral cyclosporine A (210 mg/l of drinking water) from day 2 before transplantation until termination of the experiment. Three days prior to transplantation, lentivirus-GFP transfected cells were plated onto a 75 cm^2 flask and allowed to reach about 70% confluence. On the day of the transplant, cells were trypsinised, counted and resuspended in serum free medium to a concentration of 2×10^4 cells/μl. Two μl intravitreal injections were given in a single eye, with the contra-lateral eyes used as untreated controls, 7 days after treatment with NMDA alone or NMDA/TA. 3–4 weeks post transplantation eyes were examined for transplant associated microglial activity using immunostaining for CD68 to identify the microglia and GFP to identify the transplanted cells.

2.4. Tissue analysis

After the different procedures, animals were terminally anaesthetized with sodium pentobarbitone. Upper segment intracardiac perfusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer was performed using a flow pump to fix the tissues. The eyes were surgically removed and transferred to 4% PFA for immersion fixation for a further 2 h. Tissues were then cryoprotected with 30% sucrose and embedded in OCT. Cryosections (16–20 μm thick) were prepared on charged glass (VWR, UK) slides for immunohistochemical staining and confocal imaging.

For whole mount immunostaining, post PFA perfusion, the eye was dissected to remove cornea and lens. Following 4 radial full thickness cuts through the walls of the remaining optic cup, the retina was separated from the underlying RPE and choroid and released with a cut around the optic nerve head. The florette shaped retina was then washed in PBS and subjected to immunostaining as described below.

2.5. Immunohistochemistry

Tissue sections/whole retinae were incubated for 1 h at room temperature with a blocking reagent consisting of PBS with 0.3% Triton and 5% Donkey Serum (Jackson Immuno Research Laboratories, Inc. 017-000-121). Primary antibodies used included antibodies to the RGC marker HuD (sc-2536, Santa Cruz biotechnology, 1 in 500), the macrophage/microglia marker ED1 (CD68) (MCA341R, Serotec, 1 in 1000), and the neurofilament protein (RT97, from the Developmental Studies Hybridoma Bank (DSHB), Iowa, USA, 1 in 2). Antibodies were diluted appropriately in blocking reagent and the tissue sections were incubated for 2 h at room temperature or overnight at 4 °C in a humidified chamber (with agitation when staining whole mounts). On completion of incubation with the primary antibody, the slides/retinae were washed in Phosphate Buffered Saline (PBS) before incubation with secondary antibodies (Alexa Fluor, Molecular Probes, 1 in 500) also diluted in blocking reagent, at room temperature for 1 h in the dark. Following three further PBS washes, nuclei were counter stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, Sigma, D9542), diluted 1 in 5000 with PBS. Retinal whole mounts were placed onto a glass slide with GCL facing up and allowed to air dry before mounting with vectashield mounting medium (Vector laboratories, UK) and a coverslip.

Apoptosis detection using the terminal dUTP nick-end labeling (TUNEL) assay was performed using the ApopTag Fluorescein *In Situ* Apoptosis detection kit (S7110, Chemicon (Millipore), MA, USA). Briefly the tissue sections were further fixed with ethanol acetic acid mix (2:1) for 5 min at −20 °C followed by terminal deoxynucleotide transferase (TdT) enzyme treatment as per kit instructions. The sections were then incubated with anti digoxigenin antibody (Abcam, ab420, 1 in 1000, mouse) followed by incubation with Alexa Fluor secondary antibody. DAPI was used to counter stain the nuclei as previously described.

Images were acquired either using a Zeiss LSM 510 meta or a Leica TCS P2 confocal microscope. For confocal microscopy, a 40× oil immersion objective was used unless otherwise stated. All image analysis was performed using the Zeiss or the Leica confocal software for image acquisition and analysis.

Counts of HUD positive or ED1 positive cells were performed within a 0.4 mm^2 area of the dorsal mid peripheral retina (site of injection) in control and treated eyes in a masked fashion and depicted as total number of cells positive for the antibody per