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Research article

Primate neural retina upregulates IL-6 and IL-10 in response to a herpes simplex vector suggesting the presence of a pro-/anti-inflammatory axis

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ABSTRACT

Injection of herpes simplex virus vectors into the vitreous of primate eyes induces an acute, transient uveitis. The purpose of this study was to characterize innate immune responses of macaque neural retina tissue to the herpes simplex virus type 1-based gene delivery vector hrR3. PCR array analysis demonstrated the induction of the pro-inflammatory cytokine IL-6, as well as the anti-inflammatory cytokine IL-10, following hrR3 exposure. Secretion of IL-6 was detected by ELISA and cone photoreceptors and Muller cells were the predominant IL-6 positive cell types. RNA *in situ* hybridization confirmed that IL-6 was expressed in photoreceptor and Muller cells. The IL-10 positive cells in the inner nuclear layer were identified as amacrine cells by immunofluorescence staining with calretinin antibody. hrR3 challenge resulted in activation of NFκB (p65) in Muller glial cells, but not in cone photoreceptors, suggesting a novel regulatory mechanism for IL-6 expression in cone cells. hrR3 replication was not required for IL-6 induction or NFκB (p65) activation. These data suggest a pro-inflammatory (IL-6)/anti-inflammatory (IL-10) axis exists in neural retina and the severity of acute posterior uveitis may be determined by this interaction. Further studies are needed to identify the trigger for IL-6 and IL-10 induction and the mechanism of IL-6 induction in cone cells.

1. Introduction

Retinal degenerative diseases such as retinitis pigmentosa and macular degeneration are significant causes of blindness in the United States affecting an estimated two million people each year. To date, there are few therapies for these diseases, but a number of approaches are under investigation, including biologics such as ranibizumab, retinal transplantation, stem cells, and gene therapy (Al-Saikhan, 2013; Brandt, 2013; Lipinski et al., 2012; Rosenfeld et al., 2006). Gene therapy offers the potential advantage of a permanent therapy.

Several different viruses have been utilized as ocular gene delivery vectors including herpes simplex virus (HSV) (Liu et al., 1999), adenovirus (AdV) (Sweigard et al., 2010), adeno-associated virus (AAV) (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008), and lentiviruses (Balaggan and Ali, 2012). Many factors must be considered when designing a vector for ocular gene delivery including selection of viral vector, delivery route, cellular target, and choice of promoter (Brandt, 2013). The large size of the HSV genome, compared to the genome of adeno or lentiviruses, makes it possible to insert larger genes, or multiple genes, when used as a gene delivery vector (Spencer et al., 2001).

Our work in rodents showed that gene delivery with HSV vectors did not induce inflammation of the eye (Brandt et al., 1997; Spencer et al., 2000). In contrast, we found that HSV, AdV, and lentiviral vectors induced a transient inflammatory response in

Abbreviations: HSV, herpes simplex virus; AdV, adenovirus; AAV, adeno-associated virus; NHP, non-human primate; IL, interleukin; TLR, Toll-Like Receptors; pfu, plaque forming units; PR, photoreceptors; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; ILM, internal limiting membrane; FITC-PNA, FITC conjugated *Arachis hypogea* lectin; NFκB, nuclear factor kappa-beta; gp130, signal-transducing β-subunit glycoprotein 130; IL-6R, IL-6 α-receptor; IF, immunofluorescence; ON, overnight; RT, room temperature.

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non-human primate (NHP) eyes ((Liu et al., 1999); unpublished data). The pro-inflammatory cytokine IL-6 has been detected in inflammatory eye diseases and HSV-1 ocular infections, suggesting a role in virus-induced inflammation (Biswas et al., 2006; De Vos et al., 1992; Feghali and Wright, 1997). Transient inflammation (Ikeda et al., 2009), or the induction of Th2 cytokines including IL-10 (Bennett, 2003), following subretinal injection of lentiviral and AAV vectors has also been reported. Studies using a murine cytomegalovirus retinitis model and experimental autoimmune uveitis suggest that retinal cells may suppress intraocular inflammation via stimulation of suppressor cytokines such as IL-10, IL-27, and SOCS (Blalock et al., 2013; Lee et al., 2011). The findings that both IL-6 and IL-10 have been associated with various forms of inflammation in the neural retina raises the possibility that a pro-/anti-inflammatory axis exists and the severity of uveitis could depend on which of the axes is predominant.

In this study, we found increased expression of both IL-6 and IL-10 in macaque neural retina tissue exposed to the HSV-1 gene delivery vector hrR3. We chose to study neural retina *ex vivo* because it is the tissue contacted directly following intra-vitreal injection of vectors and we wished to study early innate response of the retina in the absence of infiltrating cells. Cone and Muller cells were the predominant IL-6 positive cell type in the neural retina, while IL-10 staining was detected in amacrine cells. hrR3 activated NFκB (p65) in Muller cells, but not in cone photoreceptors, even though they expressed IL-6. The induction of IL-6 and IL-10 did not require viral replication. These results suggest that combined effects of the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 may determine the extent of ocular inflammation following viral gene delivery in the primate eye.

2. Material and methods

2.1. Virus

High titer stocks of wild type HSV-1 strain KOS, and HSV-1 hrR3, were prepared in Vero cells (Grau et al., 1989), and purified on sucrose gradients as previously described (Visalli and Brandt, 1993). The hrR3 vector contains an insertion of the *Escherichia coli* β-galactosidase gene into the large subunit of HSV-1 KOS ribonucleotide reductase (UL39, ICP6) (Cai and Brandt, 2008; Goldstein and Weller, 1988). Precautions were taken during virus preparation and all experiments to minimize endotoxin levels. High titer viral stocks were tested for endotoxin levels with the ToxinSensor Chromagenic LAL Endotoxin Assay Kit (Genscript, L00350, Piscataway, NJ). Vector preparations contributed ≤0.5 endotoxin units (EU)/ml in all experiments. Complete media (DMEM/F-12 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) contained similar levels of endotoxins (≤0.5 EU/ml).

2.2. Macaque retina tissue

Eyes from euthanized rhesus macaques (*Macaca mulatta),* or cynomolgus macaques (*Macaca fascicularis*), were obtained as they became available from the UW Primate Center or Covance (Madison, WI). No monkeys were deliberately sacrificed for these studies. Macaque eyes were kept on ice and dissected immediately after sacrifice. Posterior eye cups were incubated with PBS/1 mM EDTA for 30 min at 37 °C to loosen neural retina tissue and separate the retina from RPE cells. Neural retina tissue was rinsed in PBS and utilized in *ex-vivo* experiments as indicated.

2.3. RNA isolation

Cynomolgus macaque neural retina tissue was incubated overnight in complete media or complete media containing 1.8×10^8 plaque forming units (pfu) of hrR3 at 37 °C in 5% CO_2. Neural retina tissue from 3 different cynomolgus macaques for each condition was used to provide biological triplicates. Tissues were rinsed in PBS prior to homogenization in TRIzol reagent (Ambion/Life Technologies, Grand Island, NY, #15596-026). RNA isolation was performed following the TRIzol Reagent protocol. DNase digestion (Qiagen, Valencia, CA, RNase-Free DNase Set, #79254) was completed prior to RNA cleanup on RNeasy spin columns (Qiagen, RNeasy Mini Kit, #74104). RNA was eluted in RNase-free H_2O and quantitated on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, #ND-1000).

2.4. PCR

500 ng of purified neural retina RNA per sample was converted into cDNA (Qiagen, RT^2 First Strand Kit, 330401). Each cDNA synthesis reaction was then run on a Rhesus Macaque Innate and Adaptive Immune Response RT^2 Profiler PCR Array (Qiagen, #PAQQ-052ZA) using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, #330520) and an ABI 7300 cycler. Results from biological triplicates were grouped and compared using the Qiagen RT^2 Profiler PCR Array Data Analysis v3.5. β-actin, β-2-microglobulin, and glyceraldehyde 3-phosphate dehydrogenase were utilized as housekeeping genes for data normalization. RT^2 qPCR primer assays were performed for each cDNA with IL-6 (Qiagen, #PPQ09482B) or IL-10 (Qiagen, #PPQ01623B) and β-actin primers (Qiagen, #PPQ08986B) following the standard protocol. Primer assay data were analyzed by the $\Delta\Delta C_T$ method and results expressed as fold-change in gene expression.

2.5. Viral replication

Cynomolgus macaque neural retina tissue from three animals was incubated with 6.8×10^7 pfu of hrR3 or KOS in complete media for 1 h at 37 °C in 5% CO_2. A sample of the supernatant was removed and labeled as "input virus." Neural retina tissue was rinsed twice with PBS to remove unbound virus, complete media was added, and tissue was incubated for 24 h at 37 °C. Culture media was removed and labeled as "24 h virus." Samples were titered on Vero cells (Grau et al., 1989), and mean titers plotted as pfu/ml.

2.6. ELISA

Neural retina tissue, obtained from two cynomolgus and one rhesus macaque, was cultured for 24 h at 37 °C in complete media or complete media containing 6.6×10^7 pfu hrR3. Supernatants were collected and analyzed in duplicate with a Monkey IL-6 Instant ELISA Kit (eBioscience, San Diego, CA, #BMS641INST). Duplicate OD values were averaged, and pg/ml IL-6 determined from the standard curve. A two sample *t*-test was performed on mean pg/ml values assuming equal variance ($n = 3$, $p = 0.0002$). Cleared supernatants from media or hrR3 treated macaque neural retina tissue were assayed in duplicate for IL-10 with a Monkey IL-10 Instant ELISA kit (eBioscience, #BMS642INST).

2.7. Immunofluorescence

Macaque neural retina tissue was cultured for 24 h at 37 °C in complete media or complete media containing 6.6×10^7 pfu of hrR3. Tissue pieces were washed in PBS, fixed in 4% paraformaldehyde/PBS, paraffin embedded, and sectioned.