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Expression of Toll-like receptors in human retinal and choroidal vascular endothelial cells

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ABSTRACT

Toll-like receptors (TLRs) are a family of proteins that initiate the innate immune response in reaction to invading microbes. Studies confirm the expression of TLRs in a variety of ocular tissues and cells, and it has also been suggested that selected TLRs may be associated with geographic atrophy and neovascularisation in age-related macular degeneration, diabetic retinopathy and other vascular and inflammatory diseases of the ocular posterior segment. However, TLR expression and localisation in the retinal and choroidal vasculature has not been defined. A better understanding of differential TLR expression in the choroid and retina, particularly in endothelial cells would improve our knowledge of vascular and inflammatory diseases in the posterior segment of the eye. In this study the gene (mRNA) expression of TLRs 1–10 was investigated using RT-PCR and comparative qPCR and the protein expression and localisation of selected TLRs (3, 4, 6 and 9) were examined using western blotting, flow cytometry and immunofluorescent staining. PCR showed gene expression of TLR1-6 and 9 in human choroidal endothelial cells (hCEC) and TLR2-6, 9 and 10 in human retinal endothelial cells (hREC). Western blotting detected TLR3, 4 and 9 proteins in both hCEC and hREC with higher levels in hCEC, whilst TLR6 protein was not detectable in either endothelial cell type. Flow cytometry detected all four TLRs (3, 4, 6 and 9) on the cell surface and intracellularly, TLR6 expression was detectable but low. The expression and localisation of TLR3, 4 and 9 were confirmed by immunofluorescent staining in endothelial cells and whole tissue sections and their functionality tested by expression of IL-6 (ELISA) in response to stimulation with specific TLR ligands. This study has, for the first time, identified the differential expression and localisation of TLRs in intraocular endothelial cells. This profiling will help inform our understanding of different retinal and choroidal vascular diseases, as well as the development of future treatments for intraocular vascular diseases.

1. Introduction

Toll-like receptors (TLRs) are a family of proteins that initiate the innate immune response. They act as a critical link between innate and adaptive immunity, triggering an immediate response against invading microbes through recognition of pathogen associated molecular patterns (PAMPs). In addition, TLRs recognize endogenous damage-associated molecular patterns (DAMPs), which include heat shock proteins, uric acid crystals, high-mobility group box 1, hyaluronan, heparin sulphate, mRNA, surfactant protein A and various products of the extracellular matrix such as fibronectin and fibrinogen (Sato et al., 2009). TLRs are primarily expressed in immune cells, such as macrophages, dendritic cells, B cells and natural killer cells (Takeda et al., 2003). In addition, they are also differentially expressed in a variety of other cell types and tissues, including intestinal (Cario and Podolsky, 2000), nasal (Lin et al., 2007), endometrial epithelium (Jorgenson et al., 2005; Schaefer et al., 2004), fibroblasts (Ospelt and Gay, 2010), cancer cells

Abbreviations: AMD, age-related macular degeneration; EC, endothelial cell; CNV, choroidal neovascularisation; RPE, retinal pigment epithelium; hCEC, human choroidal endothelial cells; hREC, human retinal endothelial cells.

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(Szajnik et al., 2009; Szczepanski et al., 2009) and glia and neurons in the central nervous system (Larsen et al., 2007).

A series of studies confirm the expression of TLRs in a variety of ocular tissues and cells (Bonini et al., 2005; Chang et al., 2006; Iqbal et al., 2005; Jin et al., 2007; Kumar et al., 2004; Zhang and Schluesener, 2006) including TLR3 and 4 in the retinal pigment epithelium (RPE) (Elner et al., 2005; Kumar et al., 2004) and TLR2, 3, 4 and 9 the conjunctiva (Bonini et al., 2005; Chang et al., 2006). Healthy human corneal cells also contain measurable levels of TLR1-10, with TLR1, 2, 3, 4, 6 and 9 with the highest rate of expression (Jin et al., 2007). TLRs have been associated with a number of ocular vascular and inflammatory diseases. TLR3 variations are associated with geographic atrophy (GA) and choroidal neovascularisation (CNV) (Ambati, 2011; Yang et al., 2008). Klettner et al. (2013) reported the induction of cell death in RPE cells by TLR3 activation as a possible mechanism of GA. The authors also describe an increased TLR3 induced secretion of VEGF at higher concentrations in RPE cells (Klettner et al., 2013). Increased peripheral blood mononuclear TLR2 and 3 have also been reported in patients with neovascular age-related macular degeneration (AMD) (Zhu et al., 2013). It has also been suggested that TLR3 activation in retinal vascular endothelial cells (EC) leads to the production of sE-Selectin, sICAM-1 and interferon beta in retinal vasculitis patients, and that pre-treatment with anti-TLR3 antibodies may inhibit the production of these molecules (Lee et al., 2007). Different inflammatory diseases of the posterior ocular segment show predilection for particular vascular beds, for example, punctate inner choroidopathy (PIC) and multiple evanescent white dot syndrome (MEWDS) appear to preferentially affect the inner choroid (Machida et al., 2008). Other diseases, for example, systemic lupus erythematosus (SLE) may affect both retinal and choroidal vasculature to different extents (Nag and Wadhwa, 2006; Trivedi and Greidinger, 2009).

There are differences in the localisation of TLRs in different cell types. Traditionally, TLR3, TLR7 and 9 are considered endosomal, i.e. they are predominantly or entirely intracellular and localise to cell endosomes, whilst TLR1, 2, 4–6 are predominantly expressed on the cell surface (Akira et al., 2006; Kadowaki et al., 2001; Kumar et al., 2009; Makela et al., 2009; Matsumoto et al., 2003; Trivedi and Greidinger, 2009). It is also reported that there are significant variations in the expression of endosomal TLRs in different cell types (Trivedi and Greidinger, 2009). Cho et al. (2009a), Pegu et al. (2008), and Kleinman et al. (2012), have suggested significant cell surface localisation of TLR3 in EC, and that this surface localisation is responsible for non-specific siRNA mediated angiogenesis inhibition in mice. However, in RPE cells, TLR3 appears dispersed throughout the cell cytoplasm (Kumar et al., 2004).

In addition to TLR activation in RPE and other adjacent tissues, it is possible that direct activation in intraocular EC occurs, and could determine the site predilections and progression of eye diseases through signal transduction and activation of transcription factors. The presence and expression pattern of TLRs in human choroidal endothelial cells (hCEC) and retinal endothelial cells (hREC) remains unknown. If TLR3 localisation in hCEC is mainly endosomal, rather than cell surface localised, the non-specific siRNA activation of TLR3 would only have limited effect in controlling hCEC proliferation, for example in CNV. In addition, if the expression of TLR3 is similar in hREC and hCEC, it would be more difficult to selectively or independently modulate TLRs in these vascular beds.

In this study we used several methodologies to investigate the expression of TLRs in hCEC and hREC. A better understanding of TLR expression in hCEC and hREC will lead to an improved knowledge of the neovascular and inflammatory diseases in the posterior segment of the eye.

2. Materials and methods

This research received approval from the local research ethics committee, Nottingham Q1060301.

2.1. Isolation of human choroidal and retinal microvascular endothelial cells

Fresh posterior segments free of any known ocular disease were obtained from the Manchester Eye Bank and hCEC and hREC isolated and cultured as previously published (Stewart et al., 2011). The retina and choroid were gently teased from the sclera in turn and separately cut into 1 mm pieces irrigated with sterile phosphate buffered saline (PBS, Sigma Aldrich, Gillingham, UK), which were then incubated in 0.1% (w/v) collagenase I (Sigma Aldrich) in serum free medium (SFM, EGM2-MV, Lonza, Wolverhampton, UK) for 1 h at 37 °C. The cells were isolated with 35 μL of anti-CD31 coated Dynabeads (Life Technologies, Paisley, UK), and the endothelial cells were then put in endothelial growth medium (Lonza) and seeded onto 35 mm culture dishes coated with 5 μg/mL fibronectin (Sigma Aldrich) for 4 h. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and the medium was changed every 2 days.

Cells were passaged once when they were 90–100% confluent, seeded onto 6-well plates (5×10^4 cells per well) for the different experiments. Both sexes were represented amongst the donors, aged between 55 and 91 years (average 70).

2.2. Reverse-transcription polymerase chain reaction

Total RNA was extracted from hCEC and hREC using an RNeasy Mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. The lysate was homogenized by QIAshredder spin column (Qiagen), and centrifuged for 2 min at full speed. An equal volume of 70% ethanol was added to the homogenized lysate and transferred to an RNeasy spin column. Buffer RW1 and Buffer RPE were applied to wash the spin column membrane. Finally, total RNA was eluted with 30 μL RNase-free water. All the washing and elution steps mentioned above were followed with stepwise centrifugation. RNA was quantified by Nanodrop spectrophotometer and the quality was determined by agarose gel electrophoresis. Reverse transcription was performed in a 40 μL final volume containing 2000 ng total RNA using the QuantiTect Reverse Transcript kit (Qiagen). The samples were incubated with genomic DNA Wipeout Buffer at 42 °C for 2 min. Reverse-transcription master mix was prepared containing Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix (Qiagen). Cycle parameters were set at 42 °C for 30 min and 95 °C for 3 min. RT-PCR was performed using ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma Aldrich) using human TLR1-10 RT-primer set (InvivoGen, Toulouse, France). Amplification conditions were set at 95 °C for 2 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, and 72 °C for 5 min cDNA were analysed by ethidium-bromide-stained 2% (w/v) agarose gels to determine the presence and size of cDNA. 10 μL of 6× sample loading buffer (New England Biolabs Ltd, Herts, UK) was added to each 50 μL PCR reaction, which was mixed and loaded into separate wells in the gels. Gels were run at 100 V until the blue dye approached the end of the gels. Negative controls (non-template control and no reverse transcriptase control) were also run. A 100 base pair DNA ladder (New England Biolabs) was used to estimate the size of DNA molecules. After electrophoresis, the gels were illuminated with an ultraviolet lamp and imaged using a digital camera.