ORIA

The Ophthalmic Research Institute of Australia

Research Milestones

Advancing eye research

Highlights of ORIA research

During the past ten years the ORIA has provided $4,637,233 to support 123 annual projects in institutions and departments throughout Australia.

The ORIA has provided funds to research all major eye diseases including macular degeneration, glaucoma, low vision, lens and cataract, and diabetic retinopathy.

The ORIA has also supported the formation of the Australian and New Zealand Ophthalmic Surveillance Unit which focuses on rarer eye diseases.

Highlights of ORIA research

In 2002, the $250,000 distributed in grants by the ORIA provided the basis for more than $2.5 million in federal funding. In 2003, another $250,000 provided by the ORIA resulted in $3.5 million in additional funding.

The ORIA receives no government funding.

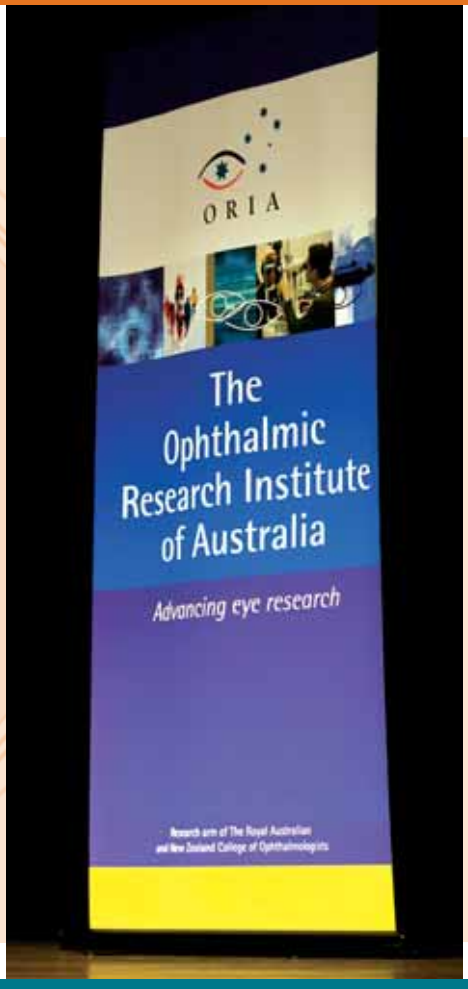
In an endeavour to encourage new investigators, the ORIA has provided a percentage of its available annual funds to support this type of research. The ORIA has provided new investigator funding support for the previous four years. It is an innovative funding approach.

The ORIA continues to be in the forefront of eye research funding in Australia by collaborating with meetings and seminars, academic support such as the Annual Endowment to the First Chair of Ophthalmology in Melbourne and raising awareness into eye disease.

Eye disease in Australia

- Each year, 10,000 Australians go blind.
- Despite the common misconception, vision loss is not restricted to the ageing and can affect people at any point in their lives.
- As vision is lost, the incidence of depression increases and reliance on family, friends and community services doubles.
- Vision loss leads to lower rates of employment, greater social isolation and emotional distress.
- The impact of low vision and blindness is greater than the disability burden for breast cancer, prostate cancer, melanoma and HIV AIDS combined.
- Considering these factors, it is little wonder that blindness ranks alongside cancer as the health condition Australians fear most.[1]

[1] *Source* – The Eye Foundation, www.eyefoundation.org.au



ORIA – advancing research into eye disease

The ORIA was come a long way since it was founded in 1953 by a group of ophthalmologists who were concerned with the need to advance eye research in Australia. The Institute's first major project was to assist in the founding of the first Chair of Ophthalmology in Australia, which was established in Melbourne, and has since played a major role in eye research. The ORIA is now the research arm of the Royal Australian and New Zealand College of Ophthalmologists whilst maintaining its own independent corporate structure.

Grants awarded each year have enabled many ophthalmologists, and those scientists in related fields, to enrich Australian medicine and surgery through their contribution to clinical practice, medical education and medical research.

Grants are made to research workers in all fields of eye disease throughout Australia. These applications are considered by the ORIA's Research Advisory Committee which decides on the merit of the project and the individuals with original ideas so that institutions and workers are supported. In 2010 the Research Advisory Committee consists of 14 ophthalmologists and scientists from all states in Australia as well as New Zealand. The Committee represents experts in all fields of ophthalmology and vision science. To further enhance the independence of the annual process and to ensure the very highest of advice, each application is forwarded to at least two outside referees for their opinions.

We can't guarantee that the research we fund will find a cure for some of the more challenging eye diseases many Australians suffer from. Nonetheless, real breakthroughs in eye disease treatment since the ORIA's inception have been realized through the benefit of research. You will see from the following update reports of projects funded during 2009 that investigators are progressing well with their research, continuing to benefit all Australians who have debilitating eye disease.

Mark Daniell – Chairman

Progress reports on just some of the research funded by the ORIA within Australian Institutions in 2009.

The ORIA and its supporters funded twelve one-year projects and four two-year projects.

Prof Stuart Graham, Dr Mark Butlin and Prof Albert Avolio from the Australian School of Advanced Medicine at Macquarie University, Sydney were awarded a grant for their project ...

Central blood pressure and arterial waveform analysis in glaucoma and association with vascular factors

The pathogenic mechanisms involved in glaucoma still remain controversial. Elevated intraocular pressure (IOP) is clearly associated but up to a third of glaucoma patients never manifest elevated IOP (normal tension glaucoma (NTG)), and not all patients respond to pressure reduction, with some continuing to progress despite normalised IOP. Vascular mechanisms have been proposed as an additional mechanism to IOP.

While an association between systemic blood pressure (BP) and glaucoma has been established in many incident studies (eg BMES), progressive glaucomatous disease has frequently been linked to systemic hypotension or more precisely to reduced ocular perfusion pressure, supporting the role of ocular blood flow in the pathogenesis[1]. Several recent major long term studies have supported a link between glaucoma and reduced ocular perfusion pressure[2,3]. In studies of systemic hypertension it is now accepted that arterial stiffness, as measured by pulse wave velocity (PWV) and augmentation index (AI), is associated with increased risk of cardiovascular events and mortality. The Rotterdam study (2006) showed that even in apparently healthy people, arterial stiffness remains an independent predictor of heart disease and stroke. The arterial stiffness, or decreased elasticity of vessels, increases the aortic central and pulse pressures, with a greater pressure wave reflection.

The central aortic pressure can be accurately estimated using radial

tonometry and applying a transfer function[4]. Central aortic pressure is often lower than brachial BP, which may have implications in disease such as glaucoma where reduced perfusion pressure has been implicated. From the derived aortic waveform an augmentation index (AI) can also be calculated to measure arterial stiffness.

In this study we used the SphygmoCor pulse wave analyser (SphygmoCor, Sydney Aust) to measure brachial BP and radial pulse waveforms, and to then derive a central BP and AI. The waveform can be further studied by determining ratios of systolic to diastolic time and area under the pulsation curve, as indirect markers of perfusion in diastole (eg Form Factor and Subendocardial Viability Ratio – SEVR). We collected individual data on the type of glaucoma (POAG vs NTG), history or presence of disc haemorrhages, peripapillary atrophy, presence of spontaneous retinal venous pulsations (SVP), migraine, smoking history, hyperlipidemia, medications (including statins) and whether the glaucoma has been stable or progressing.

We examined 126 glaucoma patients (90 POAG and 36 NTG) who were on treatment and who had been followed for at least three years and had several reliable visual fields such that glaucoma progression could be determined on the basis of the Humphrey GPA program. We also tested 50 age and sex matched controls. Data was analysed using multiple linear stepwise regression using blood pressure variables with and without ocular variables in the model.

Overall we found no significant difference between systemic or derived central aortic BP parameters between glaucomas and normals. However there were several findings that emerged from the regression analysis. Brachial pulse pressure was lower in the combined glaucoma group compared to controls ($p<0.003$), and also in the POAG group when analysed separately, but not the NTG group. In the arterial waveform analysis POAG was associated with a higher SEVR ($p<0.01$) and a lower brachial Form Factor than NTG ($p<0.02$). Disease progression was associated with disc haemorrhages as expected ($p<0.02$) but also negatively associated with spontaneous venous pulsations ($p<0.002$). The latter is in keeping with reports by Morgan et al that SVPs are reduced in glaucoma. When the ocular parameters (haems, SVP, peripapillary atrophy) were not included in the regression model, the main predictor of disease progression was a decreased SEVR ($p<0.02$). Smoking history also showed a weaker association with glaucoma ($p<0.03$) but not progression.

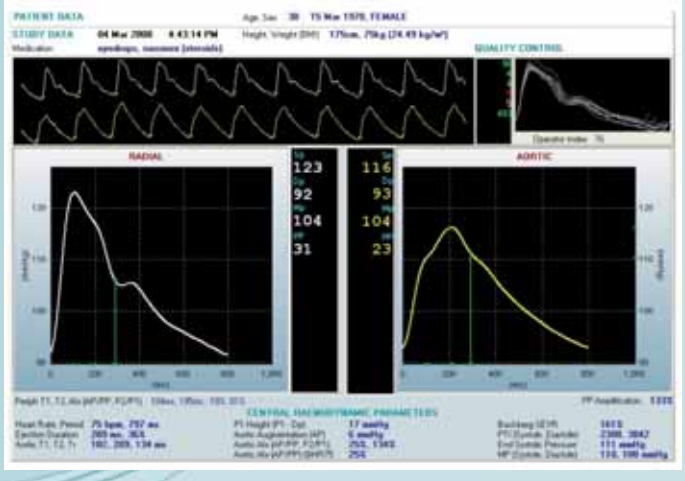


Fig 1. Example of glaucoma patient with reduced pulse pressure and high diastolic BP.

Since glaucoma pathogenesis is still poorly understood, and there is increasing evidence that vascular factors somehow play a role, this study sought to further examine aspects of blood pressure and pulsatility that might lead to an understanding of mechanism. There does seem to be an association with altered vascular waveforms and reduced pulse pressure, but the study did not show as strong a link with arterial stiffness or central blood pressure parameters as we had hoped. The findings however did support the notion that patients with glaucoma should seek to modify and control factors that can impact on vessel walls such as smoking, hyperlipidaemia and systemic hypertension, as these changes may have a negative effect. The finding that loss of SVPs is a risk factor for progression is novel. The results have recently been prepared for publication.

References

1. Tielsch, JM, Katz, J, Sommer, A, Quigley, HA, Javitt, JC. *Arch Ophthalmol* 1995, 113, 216-221.
2. Leske, MC, Heijl, A, Hyman, L, Bengtsson, B, Dong, L, Yang, Z. *Ophthalmology* 2007, 114, 1965-1972.
3. Leske, MC, Wu, SY, Hennis, A, Honkanen, R, Nemesure, B. Group., BS. *Ophthalmology* 2008, 115, 85-93.
4. Chen, CH, Nevo, E, Fetics, B, Pak, PH, Yin, FCP, Maughan, L, Kass, DA. *Circulation* 1997, 95, 1827-1836.

Dr Weiyong Shen from the Save Sight Institute, Sydney Eye Hospital, Sydney received an ORIA Young Investigator Grant for his project ...

Glial dysfunction in diabetic retinopathy

In the proposed study, we hypothesized that Muller glial disruption contributes significantly to blood retinal barrier breakdown in retinal vascular diseases including diabetic retinopathy. Experiments have been performed *in vitro* to test the cell specificity of a selective glial toxin, DL-alpha-aminoadipic acid (DL-alpha-AAA), using a number of ocular cells and then we investigated changes in the retinal vasculature after subretinal injection of DL-alpha-AAA in normal and diabetic rats. One conference presentation and one paper have been resulted from this work. In addition, results from this project have led us to extend the study from rodents to non-human primates. This was executed in collaboration with Prof Zhizhong Ma in Peking University Eye Center during 2009–2010. Partial results of this study have been used as preliminary data for an NHMRC project grant application commencing in 2011.

Experiment 1: To test the cell specificity of DL-α-AAA in vitro

First, we investigated the cell specificity of DL-α-AAA on normal human astrocytes (NHAs), rMC-1 Müller cells, bovine retinal vascular endothelial cells (BRVECs), human retinal pericytes and RPE cells in vitro. Cell death was observed in NHAs and rMC-1 Müller cells after 16 hr-treatment. With NHAs, 3.5% of cells were killed after treatment with 10mM of DL-α-AAA ($P<0.05$ vs 0mM) but there was no significant increase in the numbers of dead cells after treatment with 0.1, 1 or

5mM of DL-α-AAA. With rMC-1 Müller cells, a significant increase in cell death was observed with a concentration as low as 1mM of DL-α-AAA. Treatment of rMC-1 Müller cells with 1, 5 and 10mM of DL-α-AAA resulted in 7.6%, 95.1% and 94.6% respectively of cells dying. By contrast, there was no evidence of damage to BRVECs, retinal pericytes and RPE cells after 16 hours of continuous exposure to 10mM of DL-α-AAA based on observations of cell morphology and cell viability staining. In addition, changes in cellular metabolic activity were quantitatively measured using the Alamar blue assay. With NHAs, a significant inhibition of cellular metabolic activity was observed with 10mM of DL-α-AAA but not with 0.1, 1 and 5mM concentrations. The cellular metabolic activity of rMC-1 cells showed a concentration-dependent reduction after treatment with 1, 5 or 10mM of DL-α-AAA. There was no reduction in cellular metabolic activities in BRVECs, pericytes and RPE cells when they were exposed to 10mM of DL-α-AAA for the same period. Results from this experiment indicate that DL-α-AAA is particularly toxic to glial cells including Müller cells and astrocytes among the types of ocular cells tested.

Results of this study have been used as preliminary data for an NH&MRC Grant

Experiment 2: To study Müller glial changes after subretinal injection of DL-α-AAA in normal rats

To investigate changes in the retinal glia after subretinal injection of DL-α-AAA, double label immunohistochemistry (IHC) was performed using antibodies against glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP). In normal and PBS injected retinas, the GS antibody labelled Müller cells along their entire cellular extent, particularly on the somata within the inner nuclear layer and their endfeet at the inner and outer limiting membranes. GFAP staining was confined to filamentous structures encircling blood vessels in the innermost retina. At four days after injection of DL-α-AAA, there was a decrease in GS immunoreactivity in the somata and outer endfeet of Müller cells, accompanied by increased GFAP immunoreactivity in the retina. At 14 and 60 days after injection of DL-α-AAA, GS immuno-reactivity seemed to have recovered to a certain extent or was even upregulated in some Müller cells but strong GFAP immunoreactivity persisted from 4 to 60 days in the injected area. Results from this experiment suggest that subretinal injection of DL-α-AAA induced long-term disruption of Müller cells in the rat retina.

Experiment 3: To study retinal vascular changes after sub-retinal injection of DL-α-AAA in normal and diabetic rats

To monitor changes in the retinal vasculature after disruption of Müller cells by DL-α-AAA, fundus fluorescein angiography (FFA) was performed periodically after subretinal injection of PBS or DL-α-AAA in rats. Subretinal injection created a bleb in the superior quadrant of the retina that could be easily identified with fundus photography (Fig. 1A). One day after the injection, there was no obvious leak in the area injected with DL-α-AAA except at the site of needle penetration (Fig. 1B, star). Retinal vascular telangiectasis and leak was observed as early as four days post injection and persisted for at least 8 weeks

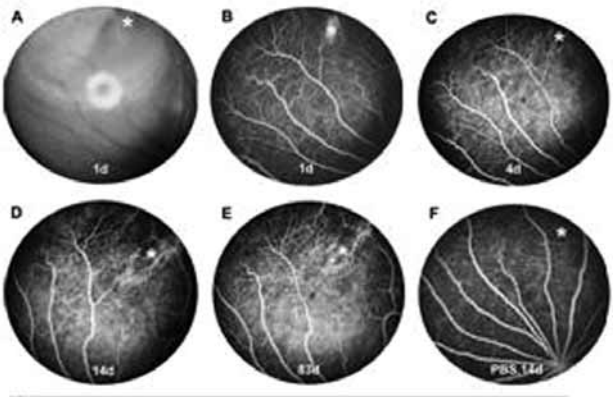


Fig.1

after a single injection (Fig. 1C-E). Subretinal injection of PBS did not induce obvious retinal vascular leak and RPE disturbance at any time (Fig. 1F). Changes in the blood retinal barrier after subretinal injection of DL-α-AAA or PBS were quantitatively studied by measurement of retinal vascular permeability to fluorescence labelled dextran FD-4 (Fig. 1G). The levels of retinal vascular leakage were shown as a ratio to the averaged values of not-treated retinas. Retinal vascular permeability increased slightly in PBS-injected group but there was no statistical difference compared with the not-treated group. Subretinal injection with DL-α-AAA significantly increased the vascular permeability at 4 and 14 days post injection, respectively (Fig. 1G, *P<0.01 and †P<0.05, n=5~10 in each group).

To correlation of retinal vascular changes with glial disruption after DL-α-AAA injection, retinal wholemounts were double-stained for isolectin B4 and vimentin to correlate vascular abnormalities with glial disruption after subretinal injection of DL-α-AAA (Fig. 2). In the normal retina, IB4 staining revealed smooth and well-defined retinal vessels, accompanied by even and regular vimentin immunoreactivity (Fig. 2A-D). Subretinal injection of PBS produced slight disturbance of the retinal vasculature, accompanied by increased vimentin immuno-reactivity in a limited area around the site of needle penetration (Fig. 2E-H). However, subretinal administration of DL-α-AAA dramatically activated retinal glial cells and disturbed the retinal vasculature, as evidenced by enhanced immunoreactivity of vimentin that was accompanied by prominent tortuosity of retinal capillaries across the injected area, and changes in the retinal vasculature which correlated well with zones of disruption of glial cells in DL-α-AAA injected eyes (Fig. 2I-L).

To test whether Müller glial disruption in diabetic retinas

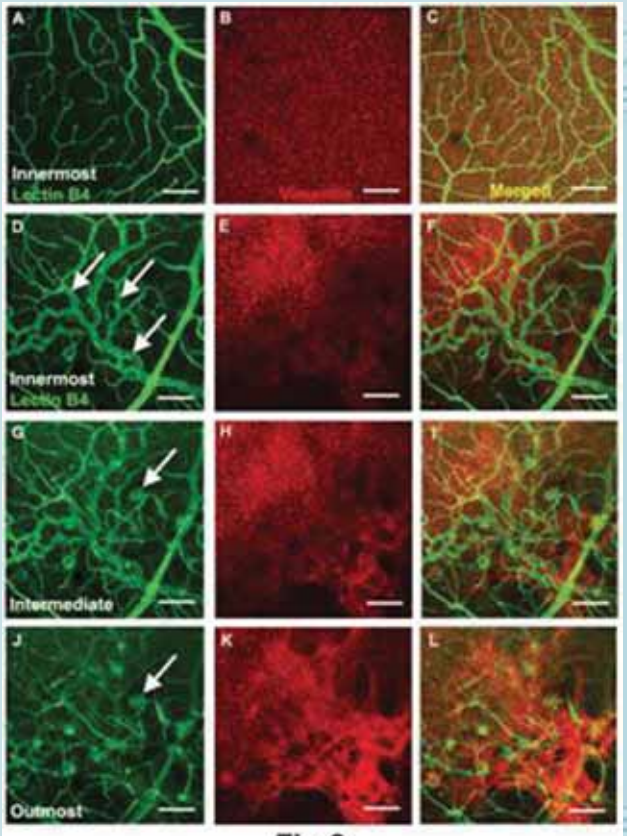


Fig.2

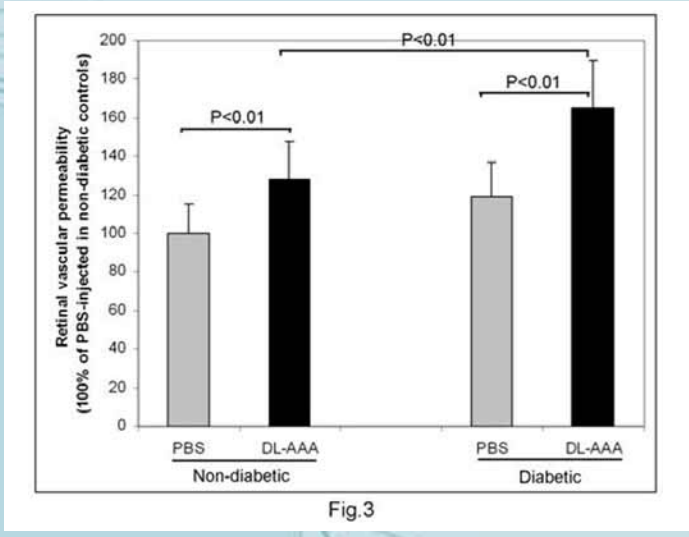


Fig.3

exacerbates blood-retinal barrier breakdown, rats were rendered diabetic by intraperitoneal injection of streptozotocin (STZ, 65mg/kg) or citrate buffer and animals with blood glucose levels>13.8mmol/L were considered as diabetic. Rats received subretinal injection of DL-α-AAA (50 g) or PBS six weeks after diabetes and the BRB was quantitatively measured at two weeks after injection. Our results demonstrated that further disruption of the retinal glia by DL-α-AAA in diabetic rats exacerbated BRB breakdown (Fig. 3, n=8 in each group).

In summary, the results obtained from this study are consistent with a model in which retinal glial disturbance directly induces

abnormalities of the retinal vasculature. In pathological situations like diabetes, further disruption of Müller cells by DL-α-AAA exacerbates breakdown of the BRB. Our findings may be of considerable clinical relevance to understand the molecular and cellular mechanisms of DR in the human retina.

Publications

1. Shen WY, Li S, Chung SH, Gillies MC. Retinal vascular changes after glial disruption in rats. *J Neurosci Res* 2010;88:1485-1499.
2. Shen WY, Zhang J, Chung SH, Hu YT, Ma ZZ and Gillies MC. Retinal changes after subretinal injection of DL-α-aminoadipic acid in non-human primates. ARVO poster 2009.
3. Shen WY, Zhang J, Chung SH, Hu YT, Ma ZZ and Gillies MC. Submacular DL-alpha-aminoadipic acid eradicates primate photoreceptors but does not affect luteal pigment or the retinal vasculature. *Invest Ophthalmol Vis Sci* submitted.

Dr Krisztina Valter-Kocsi, Dr James Wong, Prof Jan Provis and Dr Michele Madigan from the Visual Science Group, Research School of Biological Sciences, ANU, Canberra were awarded a grant for their project ...

Complement proteins and photoreceptor death in light-induced retinal degeneration

Activation and compromised regulation of the complement system are important in the development and progression of age-related macular degeneration (AMD), however it remains unclear as to what initiates these immune processes in the retina. This study aims to investigate if photoreceptor death itself could be the triggering event that activates immune responses, which if not controlled adequately, eventually lead to the development of AMD. We used a light-induced retinal degeneration rat model to assess the relationship between photoreceptor cell death and complement system activation.

To date we have further characterised changes in the rat retina during and following exposure to strong light. The area with the most severe damage ('hot spot') is always found to be at the centre of vision in the rat retina – this may be considered the functional equivalent of the human macula. In this region, the outer barrier structure that protects the retina from the outside environment breaks down following exposure to damaging bright light. Disruption of the barrier allows invasion of immune cells, such as choroidal microglia into the retina. These immune cells are responsible for clean up of damaged cells, but may themselves cause further tissue damage. We observed histological changes in the 'hot spot' following exposure to light that are very similar to those seen in human 'dry' (atrophic) AMD. The

Prof Jan Provis

results of this study are published and in press[1].

We also investigated how microglia are activated and gather specifically around the 'hot spot' in the retina. Monocyte chemo-attractant protein (MCP-1 or Ccl2) was seen in the retina as early as 12 hours after light exposure began which correlated closely with the beginning of large-scale photoreceptor loss. Ccl2 expression continued to increase and by the end of the light exposure period, a significant number of Müller cells, the resident macroglial cells of the retina, showed positive labelling for Ccl2 in the 'hot spot'. Accumulation of microglial cells in the 'hot spot' closely followed the time course of Ccl2 up-regulation. Activated microglial cells were also found to express Ccl2 which can then further attract other immune cells to this location. A manuscript describing the results of this study is in preparation[2].

More recently we found that invading microglia also expressed complement system proteins. The complement system is a key group of proteins that are responsible for the recognition of potentially toxic agents and the initiation of clean-up of damaged or dead cells within the tissue. Activation of the 'up-stream' members of the complement system can then initiate a cascade of events, which, if not controlled adequately, can lead to further cell loss. We examined the temporal relationship between the activation of genes of the complement system and photoreceptor cell death, and found that complement gene activation closely follows the onset of photoreceptor cell death. When the presence, location and regulation of complement proteins in the retina was studied, we found complement proteins (C1q and C3), in normal healthy retina, located in retinal vessels only. After retinal light damage, expression of complement proteins became more prominent not only in the vessels but also in the retinal tissue itself, mostly in the 'hot spot'.

From these studies, we now have a better understanding of the sequence of events in the development of retinal degeneration, which could be similar to processes occurring in different types of disease, with the common element of the loss of photo-receptors. It seems that exposure to bright light causes photoreceptor damage and loss in the central area of vision, that initiates the up-regulation of a chemo-attractant molecule (Ccl2) in the Müller cells, and signals to microglia cells in the surrounding tissue. This leads to the activation and recruitment of glial cells from the surrounding vessels into the damaged area. Once the barrier protecting the retina is damaged, it allows the large-scale invasion of glial cells, from the choroidal vasculature, into the retina. These cells, apart from cleaning-up the debris in the tissue, also produce complement proteins as well as a chemo-attractant, to recruit even more glial cells. This might be responsible for the close temporal relationship of the up-regulation of

... a few short treatments with 670nm light can prevent severe photoreceptor loss

complements to photoreceptor cell death.

Recently we found that 670nm near-infrared light is beneficial to the retina. Our data showed that a few short treatments with 670nm light can prevent severe photoreceptor loss and can ameliorate the loss of retinal function in the light-damage rat model. We conducted gene microarray experiments to assess the effects of bright light on the expression of retinal genes and the effects of 670nm light treatment in the healthy control and light-exposed retina. Exposure to bright light induced a 1000-fold increase in Ccl2 gene regulation, and a 2.5-fold increase in C3 gene expression. Treatment with 670nm light, prior to light damage, reduced the expression of both genes by around 50%. The gene microarray project is submitted and under review[3]; the study of the effects of 670nm light treatment on retinal integrity and inflammatory status is being prepared for publication[4].

References

1. Rutar, M, Provis, J, Valter, K. Brief exposure to damaging light causes focal recruitment of macrophages and long-term destabilization of photoreceptors in the albino rat retina. Curr Eye Res 2010.
2. Rutar, M, Natoli, R, Valter, K, Provis, J. Expression of the chemokine Ccl2 by Muller cells following light-induced retinal degeneration: implications for microglial recruitment.
3. Natoli, R, Zhu, Y, Valter, K, Bisti, S, Eells, J, Stone, J. Gene and non-coding RNA regulation underlying photoreceptor protection: microarray study of dietary anti-oxidant saffron and photobiomodulation in rat retina.
4. Albarracin, R, Valter, K. Photobiomodulation protects the retina from light-induced photoreceptor degeneration.

Presentations

Valter, K. The dark and bright side of light. Invited seminar at the University of Queensland, Brisbane, January 2010.

Valter, K. Damaging light and healing light – the benefits of photobiomodulation. Invited seminar at Nencki Institute of Experimental Biology, Warsaw, Poland, September 2009.

Valter, K. Promising approaches to the prevention and treatment of retinal degeneration. Invited seminar at Singapore Eye Research Institute, July 2009.

Valter, K. Light – a double-edged sword: light damage and photobiomodulation. Invited seminar at Center for Complex Systems and Brain Sciences (Prof Janet Blanks, dir), Florida Atlantic University, Boca Raton, Florida, USA, April 2009.

Valter, K, Albarracin, R. Retinal protection by photobiomodulation. NVRI Symposium on Visual Processing 2009, Melbourne.

Valter, K, Rutar, M, Provis, J. Complement activation in retinal degenerations. XIVth International Symposium on Retinal Degeneration, 2010, Canada.

Rutar, M, Natoli, R, Valter, K, Provis, J. Mechanisms of neuroinflammatory activation in the neural retina following light-induced retinal degeneration. ARVO, Fort Lauderdale, Florida, US.

Provis, J, Rutar, M, Natoli, R, Valter, K. Role of the complement system in acute and chronic models of retinal degeneration. ANS, 2010, Sydney.

Rutar, M, Natoli, R, Valter, K, Provis, J. Chemokine-mediated guidance of the neuroinflammatory response by Müller cells following light-induced retinal degeneration. ANS, 2010, Sydney.

A/Prof Anthony Kwan, Prof Traian Chirila and A/Prof Damien Harkin from the Queensland Eye Institute in Brisbane were also awarded funding for ...

Development of an artificial silk membrane for retinal pigment epithelial cell growth

Background

This project addresses the growing interest in techniques for treating retinal disease and especially that related to dysfunction of the retinal pigment epithelium (RPE) in our ageing population. The ultimate goal of the research is to develop improved techniques for growing new RPE cells in the laboratory with the view to subsequent transplantation. One of the key challenges associated with this work is the development of a substrate on which to grow the RPE cells while in the laboratory and which can subsequently be used as a carrier during transplantation back into the body. In preliminary studies we had demonstrated that a commercially available cell line derived from human RPE cells (ARPE-19) can be grown on transparent membranes produced from fibroin – a fibrous protein isolated from silk which by virtue of being present in silk sutures has had a long history of clinical use and thus is likely to be well tolerated when implanted into the eye. Our initial aims were to further optimise attachment of RPE cells to fibroin membranes and to evaluate the effects of this material on RPE morphology. Funding received from ORIA however enabled us to leverage additional funds from the Prevent Blindness Foundation (Queensland) which in turn enabled us to expand the number of experimental aims to include: modifications to the fibroin membrane structure to improve permeability and hence improved ability to support movement of nutrients and waste products following transplantation, establishment of techniques for growing RPE from donor human tissue, and evaluation of donor RPE cell responses to cultivation on fibroin membrane. We are pleased to report that significant progress has been made in addressing these experimental aims as summarised below.

Progress

1. Optimisation of RPE cultivation techniques

While the ARPE-19 cell line is highly regarded as a suitable model of normal RPE function, care must be taken to grow these cells in the correct way in order that they will develop a morphology resembling that displayed by RPE cells in vivo. The principal considerations required to achieve this result are cell density, time in culture and use of low serum concentrations in the culture medium. Over the last 12 months, careful use of these parameters has enabled us to produce cultures of RPE cells from the ARPE-19 cell line which now more closely resemble the normal epithelial structure observed in vivo (refer to Fig 3a). Ultimately, our research will need to be repeated using cultures of RPE cells freshly isolated from donor human tissue since this source is likely to best represent the behaviour of RPE cells in patients. It must be said that growing human RPE cells from adult donor tissue is a challenging task owing to a number of logistical and technical issues. Nevertheless, over the last 9 months we have methodically explored a number of published methods and have now achieved a fair degree of success using a technique developed by the Engelmann's group in Dresden (Engelmann and Valtink 2004; Valtink

and Engelmann 2009). The key components to success in our hands time that tissue has been stored prior to cell isolation and using a combination of enzymes during tissue digestion as outlined in the group's reports. An example of cultures produced using this approach is shown below in Figure 1.

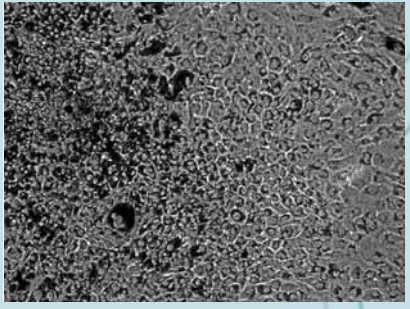
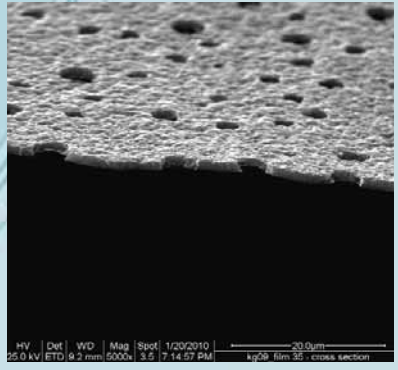


Figure 1: Culture of donor RPE cells derived from cadaveric tissue established using techniques developed by the Engelmann's group. On the left hand side of the image can be seen some smaller cells that still retain a large amount of melanin pigment. Cells towards the right hand side of the picture have begun to lose pigment as has been shown previously to occur during cultivation *in vitro*.

2. Modification of fibroin membranes to facilitate permeability

In preliminary studies, we had used fibroin membranes of between 50 to 100 microns in thickness which while supporting the attachment and growth of RPE cells in vitro was considered to be too thick to be used in vivo. Moreover, observations made during processing of the fibroin membranes for routine histological studies suggested that the membranes would not be sufficiently permeable to allow efficient movement of nutrients and waste products following transplantation. Hence a formulation of 'porous ultra-thin' fibroin membranes was developed by drying 3–5 micron thick films of fibroin solution (created using a casting table) which were mixed with a small amount of polyethylene glycol (PEO). During the drying process, the PEG accumulated as localised droplets of approximately 2–3 microns in diameter which could then be subsequently removed by further washing in water resulting in miniature pores across the width of the membrane (Figure 2). Further analysis by scanning electron microscopy combined with routine histological techniques confirms that a significant percentage of the pores traverse the full thickness of the membranes and thus should facilitate free movement of nutrients and waste products. The actual degree of permeability is now being confirmed using a variety of fluorescently labelled molecules of differing sizes that are representative of the molecules that would need to cross the membranes *in vivo*.

Figure 2: Scanning electron microscopy image of our 'porous ultra-thin' fibroin membrane which we hypothesize will facilitate better function of transplanted RPE cells through enabling more efficient movement of essential nutrients and waste products between the retina and underlying choroidal blood vessels.



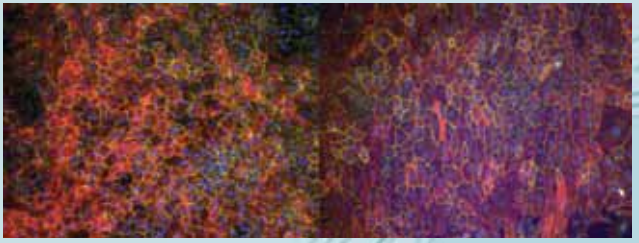


Figure 3: Comparison of long-term cultures of ARPE-19 cells grown on either conventional tissue culture plastic (left) or sheets of porous ultra-thin fibroin membrane. Following nearly two months in culture (necessary to develop epithelial phenotype) the cells were fixed and stained with three dyes to illustrate the presence of nuclei (blue), actin filaments (red) and ZO-1 (green) a protein utilised in the formation of tight junctions between adjacent RPE cells. While some areas in each culture remain poorly organised (absence of green ring around cells) the similarity between cultures is encouraging and indicates that fibroin is worthy of further exploration as a substrate for RPE cell transplantation.

3. Assessment of RPE morphology on fibroin membranes

Within the field of RPE research, it is widely accepted that RPE cells grown in the laboratory display a very different morphology to that present in the body unless careful consideration is given to the culture conditions. Moreover, any RPE culture that is designed for transplantation should be carefully evaluated to ensure that it displays what is often referred to as a 'polarised' morphology. The conditions optimised in section 1 have therefore been applied to cultures grown on our porous ultra-thin fibroin membrane. Importantly, these cultures have been grown in parallel with those on conventional tissue culture plastic. Thus far, our studies of RPE phenotype using markers for actin filaments and tight junctions indicate a high degree of similarity between the cultures (Figure 3) and further experiments in progress using additional markers (antibodies to keratin 8/18 and RPE-65) and transmission electron microscopy will soon reveal further details on the degree of culture differentiation and polarity that has been achieved. Preliminary results obtained using RPE cultures derived from cadaveric donor tissue confirm that these cells also attach and grown on fibroin membranes and studies of cell phenotype are in progress.

Publications

A Kwan, S Cheng, T Chirila. Development of tissue-engineered membranes for the culture and transplantation of retinal pigment epithelial cells. In Biomaterials and Regenerative Medicine in Ophthalmology. Chapter 15. p. 390-408. Publisher: Woodhead Publishing Ltd (2010)

D Harkin, KA George, AMA Shadforth, S Cheng, AS Kwan, TV Chirila. Development of an Ultra-Thin Fibroin Membrane for RPE Cell Transplantation. *Invest Ophthalmol Vis Sci* 2010;51: EAbstract 5248.

References

Engelmann, K and M Valtink (2004). 'RPE cell cultivation'. *Graefes Arch Clin Exp Ophthalmol* 242(1): 65-7.

Harkin, DG, KA George et al. (2010). Development of an ultra-thin fibroin membrane for RPE cell transplantation. Association for Research in Vision and Ophthalmology. Fort Lauderdale, FL, USA.

Valtink, M and K Engelmann (2009). 'Culturing of retinal pigment epithelium cells'. *Dev Ophthalmol* 43: 109-19.

Another new investigator who was awarded a grant was Dr Alex Hewitt from the Centre for Eye Research, Melbourne for his project ...

Refinement of putative loci predisposing to glaucomatous optic neuropathy

Glaucoma is the most common cause of optic neuropathy worldwide and if detected early, blindness is preventable. The aims of this project were to identifying genetic variants which lead to glaucoma blindness. Individual risk profiling could prevent glaucoma blindness by pre-symptomatic screening followed by tailored therapy after disease is detected. Analogous to fire fighting, just as total fire bans and smoke alarms prevent catastrophic damage, knowledge of the genomic profile of at-risk individuals could ensure that patients receive adequate pre-symptomatic clinical screening and early intervention. Additionally, just as all fires (i.e. oil-based versus electrical versus wood) cannot be extinguished with just with any form of hydrant, it is likely that understanding the molecular mechanisms underpinning a particular patient's disease would allow for the use of individualised medicine.

Glaucoma is the most common cause of optic neuropathy worldwide and if detected early, blindness is preventable

The candidate gene approach has been the basis for hundreds of genetic investigations. A specific-hypothesis driven approach relies on understanding the biology of the disease and precise gene function. For many complex diseases, such as primary open angle glaucoma, the underlying biology is poorly understood and the number of potential candidate genes is legion. We used a genome-wide association design. Genome-wide association holds great promise, particularly in the case of common complex diseases, because no prior information about underlying molecular mechanisms is required. Unfortunately, the high cost of genome-wide arrays has been the major barrier inhibiting the widespread utilisation of such study design. Well-powered GWA investigations generally cost more than AUS$1.5 million. Equimolar pooling of DNA heralds as the most promising approach for cost reduction. In equimolar pooling, rather than individually genotyping each subject in each of the case and controls cohorts, the equimolar DNA pools of all 'cases' and all 'controls' are constructed separately and then genotyped.

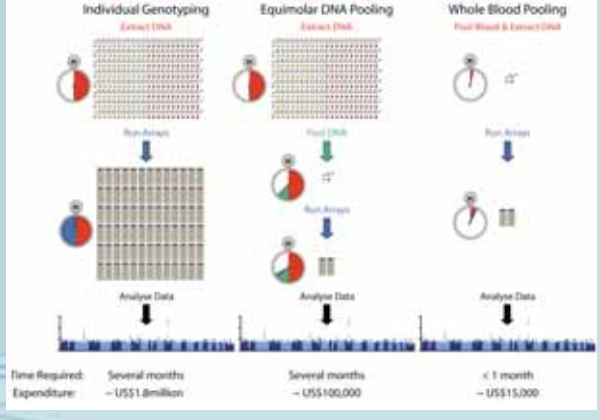


Figure 1

ORIA funding allowed us to further explore economical methods for genome-wide association. In particular we showed for the first time that rather than pooling samples at the DNA level, whole blood samples could be pooled allowing for only a small number of DNA extractions and substantial reduction in time required to undertake large-scale gene-discovery investigations. Compared with individual genotyping, pooling can cost 100-fold less.

This novel methodology was utilised to investigate the genomic architecture of OAG and identified a number of putative disease-associated regions. Three case-control cohorts were investigated. Pools were constructed using two independent OAG cohorts – of case subjects with severe glaucoma blindness and age-matched normal controls – from South Australia and Tasmania. The South Australian case cohort represented the first spoke of the Australian and New Zealand Registry of Advanced Glaucoma, whilst the Tasmanian cohort was recruited through the Glaucoma Inheritance Study in Tasmania (GIST). Additional control subjects for the less severe GIST glaucoma cohort were selected through the Blue Mountains Eye Study.

Five SNPs at Xq25 were found to be strongly associated with OAG (χ^2 range: 14.4-22.9). The mean r^2 between the SNPs at this locus was 0.921. The effect at this locus was greater in the subjects who had advanced glaucoma. Following individual genotyping, 18 autosomal SNPs were found to have a significant ($p \leq 0.001$) allelic or genotypic association with OAG. Three of these remained highly significant on joint analysis of the full cohort. Being homozygous for the high risk allele in at least two of these three loci, conferred an OR for developing OAG equal to 2.13 (95%CI: 1.57-2.88; $\chi^2=26.5$, $p=2.64 \times 10^{-7}$), when compared to people who had no high risk allele in the homozygous state. This effect increased to an OR of 3.43 (95%CI: 2.11-5.60; $\chi^2=28.9$, $p=7.62 \times 10^{-8}$) when analysis was refined to solely those cases at the severe end of the OAG disease spectrum.

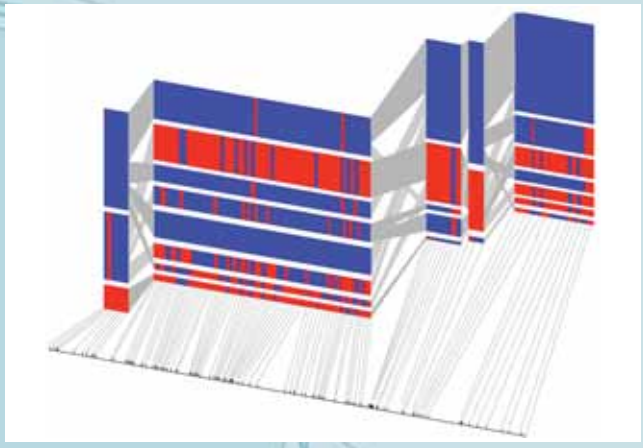


Figure 2. Haplotype Block Structure in the HapMap Centre d'Etude du Polymorphisme Humain population of the novel glaucoma locus on Xp25. Image based on the work of Dr Ben Fry, colours represent allelic variants and the z-offset emphasises the transition between blocks.

Quantile-quantile plots of test statistics obtained from the genome-wide association of exudative age-related macular degeneration and blind primary open angle case-control DNA pools.

Clearly further work is required before the association of variants at these novel loci with OAG is definitively proven. Replication in additional disease cohorts, in addition to functional molecular studies,

will remain essential facets of such work. A caveat on such investigations is that should the true causal variant(s) at any of these loci be identified, then they are likely to have a greater effect size than that observed here.

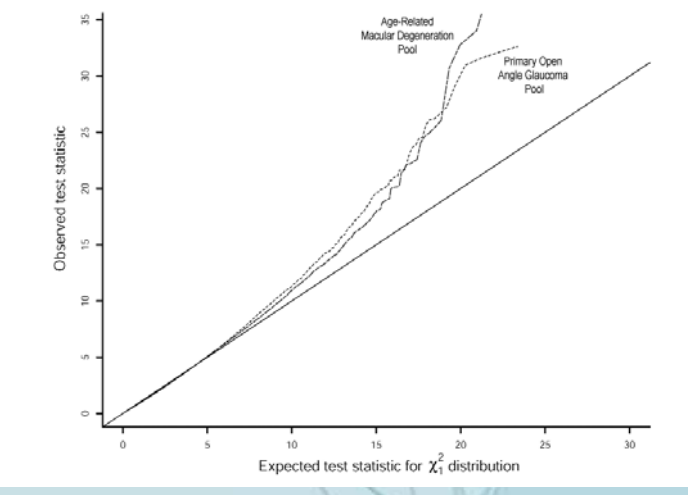


Figure 3

In summary, ORIA funding allowed the identification of novel putative genetic risk factors for glaucoma through the use of well-characterised ethnically homogeneous cohorts, coupled with the power of high density genetic markers. Our strategy of concentrating analysis on the cases with definitive end-stage glaucomatous visual field loss was chosen to maximize the probability of finding strong novel genetic associations. Additionally, our design of undertaking GWA on a subset of the full cohort is well established. Despite being labour-intensive, there is a very high chance that novel genes accounting for the major common genetic contribution to many inherited human diseases could be uncovered relatively cheaply using equimolar DNA or whole blood pooling.

Publications

1. Hewitt AW, Chappell AJ, Straga T, Landers J, Mills RA, Craig JE. Sensitivity of confocal laser tomography versus optical coherence tomography in detecting advanced glaucoma. *Clin Experiment Ophthalmol*. 2009 Dec;37(9):836-41;
2. Hewitt AW, Wu J, Green CM, Lai T, Kearns LS, Craig JE, Mackey DA. Systemic disease associations of familial and sporadic glaucoma: the Glaucoma Inheritance Study in Tasmania. *Acta Ophthalmol*. 2010 Feb;88(1):70-4.
3. Craig JE*, Hewitt AW*, McMellon AE, Henders AK, Ma L, Wallace L, Sharma S, Burdon KP, Visscher PM, Montgomery GW, MacGregor S.* Rapid inexpensive genome-wide association using pooled whole blood. *Genome Res*. 2009 Nov;19(11):2075-80. *Equal contribution by authors.
4. Sherwin JC, Hewitt AW, Bennett SL, Baird PN, Craig JE, Mackey DA. Primary open angle glaucoma in subjects harbouring the predicted GLC1L haplotype reveals a normotensive phenotype. *Clin Experiment Ophthalmol*. 2009 Mar;37(2):201-7.
5. Hewitt AW, Sanfilippo P, Ring MA, Craig JE, Mackey DA. Mortality in primary open-angle glaucoma: 'two cupped discs and a funeral'. *Eye* (Lond). 2010 Jan;24(1):59-63.

Dr Shiwani Sharma, A/Prof Jamie Craig and A/Prof Nicolas Voelcker from the Department of Ophthalmology at Flinders Medical Centre in Adelaide, South Australia received project funding for ...

Determining the involvement of non-genetic factors in pseudoexfoliation syndrome

Pseudoexfoliation (PEX) syndrome is an age-related disorder characterized by accumulation of fibrillar extracellular deposits on the anterior lens capsule. Histologically, pathological extracellular deposits are seen in all tissues surrounding the anterior chamber of the eye. Prevalence of PEX syndrome widely varies in different populations but increases with age. Prevalence of the disease is higher in females than males in some populations. PEX syndrome is the major risk factor for glaucoma, the second leading cause of blindness worldwide. Associated cataract is the most common cause of surgical intervention required by PEX patients. These patients have a higher rate of complications from cataract surgery. Retinal vein occlusion associated with PEX can lead to profound visual loss. Besides ocular pathology PEX syndrome increases the risk of heart disease and stroke. Both genetic and environmental factors are thought to contribute to the disease. Molecular studies to date indicate that oxidative stress and over-production and reduced turnover of extracellular matrix are involved in the disease pathology. However the disease pathogenesis is poorly understood.

Genetic studies have shown that genetic variants of the *LOXL1* (*lysyl oxidase-like 1)* gene are associated with the risk of PEX syndrome in several populations. The product of the *LOXL1* gene is involved in the formation of elastic fibres in tissues that provide resilience to tissues. Thus genetic association of *LOXL1* with PEX syndrome is consistent with the involvement of extracellular matrix proteins in the pathophysiology of the disease. However this genetic association does not explain the variation in disease prevalence in different populations. Therefore additional genetic and/or non-genetic factors are thought to be involved in PEX syndrome.

Epidemiological studies from Australia and India suggest that PEX syndrome is more common in people who experience increased exposure to ultraviolet (UV) radiation from sunlight. UV-A radiation is the major component of sunlight reaching the earth's atmosphere. While the cornea blocks the most of UV-A radiation, some reaches the lens and can induce stress response in the lens epithelial and fibre cells. As the lens epithelium is involved in the production of pathological PEX deposits seen on the anterior lens capsule in PEX syndrome patients, this project aimed to determine, in the lens epithelial cells, the effect of UV-A on expression of those genes implicated in PEX pathophysiology. The study has made considerable progress and the work is ongoing.

Studies suggest that PEX syndrome is more common in people who experience increased exposure to ultraviolet radiation from sunlight.

Progress to date

For UV-A exposure of cultured lens epithelial cells under aseptic conditions, a UV-A transilluminator was manufactured by the Biomedical Engineering, Flinders Medical Centre. The method for culturing primary lens epithelial cells from lens capsule specimens

obtained from patients undergoing cataract surgery was developed. Although growth of lens epithelial cells from the explants in culture was observed from almost every explant (n=12), the cells did not survive sub-culturing, necessary for obtaining sufficient cell numbers for experiments. Hence, primary lens epithelial cultures were found unsuitable for the study. We are instead using an established cell line derived for the human lens epithelium, SRA 01/04, for this study. The SRA 01/04 cells were exposed to various doses of UV-A, and cell viability assayed at multiple time points after treatment. Untreated cells were used as control. A single dose of 6.3 J/cm^2 UV-A resulted in 80% viable cells after 6, 16 and 24 hours post-treatment. Contrarily, repeating treatment with the same dose of UV-A on consecutive days significantly reduced cell viability. Therefore a single treatment with 6.3 J/cm^2 of UV-A is being used as the optimum exposure for SRA 01/04 cells. The SRA 01/04 cells treated with this optimum UV-A exposure and untreated cells have been collected 6 hours after the treatment for RNA extraction. RNA from three replications of treated and untreated SRA 01/04 cells will be used for gene expression analysis. For gene expression analysis, microarray analysis will be performed on the Illumina platform using the Human WG-6 v3.0 Expression BeadChip array at the Australia Genome Research Facility. This expression array includes more than 27,000 reference sequence RNA, more than 7,000 annotated mRNA and more than 12,000 Unigene contigs. Instead of determining the effect of UV-A exposure on expression of only those genes thus far implicated in PEX, its effect on the entire human transcriptome will be determined to gain a complete understanding of its influence on the genes expressed in the lens epithelial cells. The genes differentially expressed between treated and untreated cells will be validated by quantitative real-time RT-PCR (reverse transcription-polymerase chain reaction). Furthermore, suitable housekeeping genes for use in quantitative real-time RT-PCR in the validation phase of the study have been selected. Six housekeeping genes, namely, *ACTB* (Beta-actin), *GAPDH, HPRT1* (hypoxanthine guanine phosphori-bosyl-transferase 1), *TBP* (TATA binding protein), *UBC* (ubiquitin C) and *HBMS* (hydroxy-methyl-bilane synthase) were amplified from cDNA from SRA 01/04 cells initially by end-point PCR to test the specificity of primer pairs and optimise PCR conditions for amplification. Subsequently, real-time RT-PCR was performed on cDNA from SRA 01/04 cells using the gene-specific primers for each housekeeping gene and SYBR green Mastermix. Specificity of the PCR products was confirmed by melt-curve analysis and agarose gel electrophoresis. Real-time PCR has been optimised for two of the six housekeeping genes, namely, *ACTB* and *GAPDH*. Quantitative real-time PCR will be performed to determine the stability of expression of these housekeepers in UV-A treated and untreated SRA 01/04 cells. These housekeeping genes will be used in the validation phase of the study.

This study is expected to reveal the genes whose expression is influenced by UV-A exposure in lens epithelial cells. It is also expected to reveal the effect of UV-A exposure on expression of those genes already implicated in PEX syndrome.

A/Prof Ian Trounce and Prof Jonathan Crowston from the Centre for Eye Research, Melbourne, were awarded project funding for ...

Opa-1 processing and mitochondrial defects in glaucoma

Background and aims

Abnormalities in the energy generators of cells (mitochondria) are increasingly thought to play an important role in degenerative diseases of the optic nerve. This project aimed to look for specific abnormalities in the mitochondria of glaucoma patients and age-matched controls. Determining what makes the optic nerve vulnerable in glaucoma will provide a focused approach to developing new treatments.

Autosomal Dominant Optic Atrophy (ADOA) and open angle glaucoma are optic neuropathies that share certain similarities in their clinical phenotype and are both characterized by the specific loss of retinal ganglion cells. Mutations causing haploinsufficiency of Opa-1, a mitochondrial dynamin GTPase, are the most frequent cause of ADOA. Mitochondrial DNA mutations and reduced ATP production have recently also been reported in peripheral blood cells of open angle glaucoma patients.

The specific aims of this study were to:

1. determine whether the Opa-1 isoform profile is altered in peripheral blood lymphocytes of glaucoma patients, either spontaneously or in response to a mild oxidative phosphorylation (OXPHOS) insult
2. determine OXPHOS enzyme-linked ATP production in glaucoma patient mitochondria.

An ORIA presentation at its AGM 2009

Results

The first achievement of this grant was seeding the establishment of a lymphoblast cell line bank from glaucoma patients. To date we have archived over 30 cell lines, aiming to expand this collection to over 100 patients in the next year. For the present studies up to 15 cell lines were used in different experiments, together with 20 control lymphoblast lines.

Aim 1

Investigation of the Opa-1 protein isoform profile: Figure 1a shows an example of a western blot of lymphoblast cell proteins probed with an antibody to Opa-1, with the 5 known length isoforms clearly resolved in this high resolution gel. Figure 1b shows the quantification of Opa-1 protein levels for all isoforms, where no difference was seen compared to controls. Further analysis of each isoform, and analyses of Opa-1 profiles following growth of cells in galactose (which forces aerobic ATP supply via mitochondrial OXPHOS) also showed no differences between glaucoma patients and controls (not shown). In conclusion, while the experiments were completed at the expected standard, no differences in Opa-1 processing could be identified to distinguish glaucoma patients from controls.

Aim 2

We next investigated OXPHOS protein levels and function in glaucoma and control cells by western blotting. Overall levels of complexes I through V were not significantly different, but when grown in galactose medium there was significant upregulation of all complexes with a trend to higher levels again in the glaucoma patients (Figure 2). This new finding indicates that OXPHOS regulation can respond to changes in fuel availability, but also suggests that the glaucoma patient cells needed to upregulate this pathway more than control cells, suggesting a mild OXHOS impairment.

We further investigated OXPHOS function in the cell lines, assaying the maximal rate of OXPHOS-linked ATP produc-tion. In Figure 3 the data is shown for substrates that are oxidized via complex I, compared with sub-strates oxidized via complex II. The results shown are combined from four indepen-dent replicates of the glaucoma (n=9) and control (n=10) groups. The complex I-linked ATP produc-tion was significantly impaired in the glaucoma group, being 35% lower than controls, while the complex II-linked ATP production rate trended lower but was not significantly lower. This is an exciting

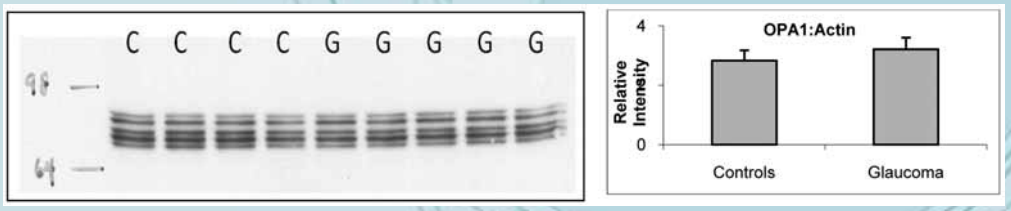


Figure 1a (left) shows the Opa-1 protein clearly resolved into 5 length isoforms. 'C'=control lymphoblast, 'G'=glaucoma patient lymphoblast. Figure 1b (right) shows the quantification of the western blot signals from a total of 9 glaucoma patients and 8 controls. There was no significant difference in total Opa-1 levels or levels of individual length isoforms.

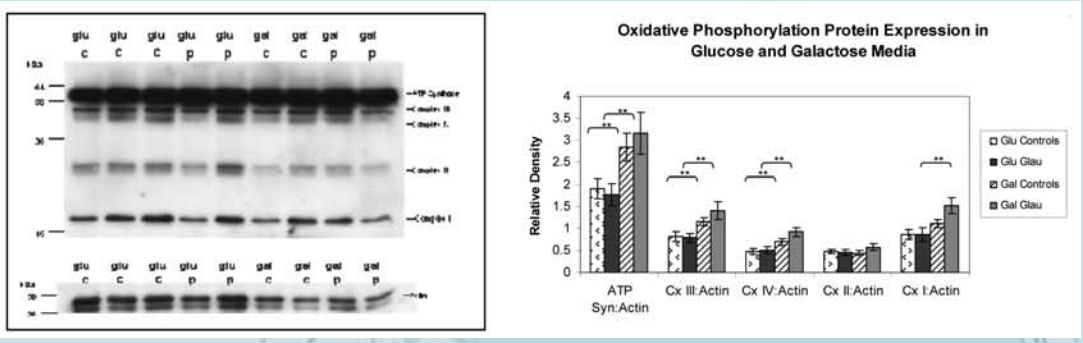


Figure 2. The panel at left shows a western blot example of lymphoblast proteins after growth in either glucose or galactose media. C=controls and P=glaucoma patients. The immunoreactive OXPHOS proteins are indicated. At right is the quantification of the blots from 9 glaucoma patients and 10 controls for each group.

finding. It suggests a mild impairment of complex I, the same enzyme complex which is impaired in the mitochondrial DNA-linked optic neuropathy Leber's Hereditary Optic Neuropathy.

In summary, in this ORIA-funded pilot study, we have established strong evidence for a mild but significant defect of complex I-linked ATP production in mitochondria of glaucoma lymphoblast cells. We are currently preparing a manuscript detailing these results. This exciting finding will form the basis of further grant applications, including an NH&MRC application. With further work, we may succeed in defining a sub-group of glaucoma patients that may benefit from new therapies aimed at improving mitochondrial function.

This exciting finding will form the basis for further grant applications, including an NH&MRC application.

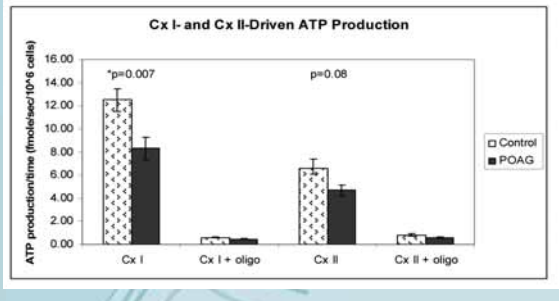


Figure 3. OXPHOS linked maximal ATP production rate in glaucoma (POAG) and control lymphoblasts. The '+oligo' rates indicate the presence of oligomycin, an inhibitor of complex V (the H+ATPase) that demonstrates the specificity of the assay. Significantly lower complex I-linked rates in the glaucoma group suggest a mild impairment of this OXPHOS complex.

Our aim is to increase available funding for research into eye disease

The ORIA's Research Advisory Committee (below), and its Chair, Prof Peter McCluskey (right)

ORIA Board and Research Advisory Committee member A/Prof Robert Casson

Acknowledgements

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