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Comparative effects of posterior eye cup tissues from myopic and hyperopic chick eyes on cultured scleral fibroblasts

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ABSTRACT

The role of individual ocular tissues in mediating changes to the sclera during myopia development is unclear. The aim of this study was to examine the effects of retina, RPE and choroidal tissues from myopic and hyperopic chick eyes on the DNA and glycosaminoglycan (GAG) content in cultures of chick scleral fibroblasts. Primary cultures of fibroblastic cells expressing vimentin and α-smooth muscle actin were established in serum-supplemented growth medium from 8-day-old normal chick sclera. The fibroblasts were subsequently co-cultured with posterior eye cup tissue (full thickness containing retina, RPE and choroid) obtained from untreated eyes and eyes wearing translucent diffusers (form-deprivation myopia, FDM) or −15D lenses (lens-induced myopia, LIM) for 3 days (post-hatch day 5–8) ($n = 6$ per treatment group). The effect of tissues (full thickness and individual retina, RPE, and choroid layers) from −15D (LIM) *versus* +15D (lens-induced hyperopia, LIH) treated eyes was also determined. Refraction changes in the direction predicted by the visual treatments were confirmed by retinoscopy prior to tissue collection. Glycosaminoglycan (GAG) and DNA content of the scleral fibroblast cultures were measured using GAG and PicoGreen assays. There was no significant difference in the effect of full thickness tissue from either FDM or LIM treated eyes on DNA and GAG content of scleral fibroblasts (DNA 8.9 ± 2.6 μg and 8.4 ± 1.1 μg, $p = 0.12$; GAG 11.2 ± 0.6 μg and 10.1 ± 1.0 μg, $p = 0.34$). Retina from LIM eyes did not alter fibroblast DNA or GAG content compared to retina from LIH eyes (DNA 27.2 ± 1.7 μg *versus* 23.2 ± 1.5 μg, $p = 0.21$; GAG 28.1 ± 1.7 μg *versus*. 28.7 ± 1.2 μg, $p = 0.46$). Similarly, the choroid from LIH and LIM eyes did not produce a differential effect on DNA content (DNA LIM 46.9 ± 6.4 *versus* LIH 51.5 ± 4.7 μg, $p = 0.31$). In contrast, scleral fibroblast DNA was greater in co-culture with RPE from LIM eyes than the empty basket and DNA content less for co-culture with RPE from LIH eyes (LIM: 72.4 ± 6.3 μg *versus* empty basket: 46.03 ± 1.0 μg; $p = 0.0005$ and LIH: 27.9 ± 2.3 μg *versus* empty basket: 46.03 ± 1.0 μg; $p = 0.0004$). GAG content was lower with RPE from LIM eyes (LIM: 27.7 ± 0.9 μg *versus* empty basket: 29.5 ± 0.8 μg, $p = 0.021$) and was higher with RPE from LIH eyes (LIH: 33.7 ± 1.9 μg *versus* empty basket: 29.5 ± 0.8 μg, $p = 0.010$). Choroid from LIM eyes induce a relative increase in scleral GAG content e.g. (LIM: 32.5 ± 0.7 μg *versus* empty basket: 29.5 ± 0.8 μg, $p = 0.0004$) while, choroid from LIH eyes induced a relative decrease in scleral GAG content (LIH: 18.9 ± 1.2 μg *versus* empty basket: 29.5 ± 0.8 μg, $p = 0.0034$). GAG content of cells in co-culture with choroid from LIM *versus* LIH treated eyes was significantly different (32.5 ± 0.7 μg *versus* 18.9 ± 1.2 μg respectively, $p = 0.0002$). In conclusion, these experiments provide an evidence for a directional growth signal that is present (and remains) in the *ex-vivo* RPE/choroid, but that does not remain in the *ex-vivo* retina. The identity of this factor(s) that can modify scleral cell DNA and GAG content requires further research.

Abbreviations: FDM, form-deprivation myopia; LIH, lens-induced hyperopia; LIM, lens-induced myopia; GAG, glycosaminoglycan; DNA, deoxyribonucleic acid; RPE, retinal pigment epithelium; Tx, treatment; DMEM, Dulbecco's modified Eagle's medium; DMMB, dimethylmethylene blue.

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1. Introduction

Myopia (or nearsightedness), is the most common refractive disorder of the eye, affecting about 30% of the population worldwide (reviewed in Pan et al., 2012) and up to 90% of children in South East Asian countries such as Hong Kong, Singapore, Taiwan and China (Lam et al., 2012; Wong et al., 2000; Lin et al., 2004; Wang et al., 2008). The prevalence of myopia in the United States has increased by nearly 70% over the last 30 years (from 25% to 42%, Vitale et al., 2009). Myopia is one of the leading causes of blindness around the world (McCarty and Taylor, 2000). Since high myopia (>6D) is often associated with blinding complications (Congdon et al., 2003), this has driven intense research efforts aimed at understanding the mechanisms involved in myopia development and how eye growth is regulated (Curtin, 1985; Gilmartin, 2004).

Both depriving animals of high quality form vision by covering the eye with a diffuser (form-deprivation myopia) and induced defocus (using negative lenses, lens-induced myopia, LIM or positive lenses, lens-induced hyperopia, LIH) are known to alter eye growth (Schaeffel et al., 1988; Smith et al., 2010). In animal models, the increase in the vitreous chamber depth (McBrien and Norton, 1992; Mutti et al., 1999) and alteration in scleral growth (Christensen and Wallman, 1991; Norton and Rada, 1995; McBrien et al., 2001; McBrien and Gentle, 2003) directly correlate to the amount of induced myopia. The eye growth changes are thought to be mediated through intrinsic mechanisms, since they still occur when the optic nerve has been severed (Troilo et al., 1987) and when the activity of ganglion cells is blocked with tetrodotoxin (McBrien et al., 1995). In addition, only the deprived section of the eye becomes myopic, with the nondeprived part of retina remaining nearly emmetropic (chick: Wallman et al., 1987; Schaeffel and Diedrich, 2009; tree shrew: Norton and Siegwart, 1995; monkey: Smith et al., 2009). These localised retinal signals must be transmitted to the sclera if eye size is to change and due to its location between the retina and sclera the RPE is thought to have an important role in this (Rymer and Wildsoet, 2005).

The biochemical and biomechanical properties of the sclera ultimately determine the shape and size of the eye in animal models (Rada et al., 2006; McBrien et al., 2009). Growth of the two layers of the avian sclera (cartilage and fibrous) are modulated reciprocally by visual conditions (Marzani and Wallman, 1997) i.e. DNA and GAG content showed opposite changes in cartilaginous and fibrous sclera under visually deprived conditions. In addition, the amount of DNA and soluble protein was significantly greater in the scleras of deprived eyes than in those of nondeprived eyes (Christensen and Wallman, 1991). Mammals, however, only have a fibrous sclera (reviewed in Avetisov et al., 1984; Rada et al., 2000b; McBrien et al., 2001). In this case, the extracellular matrix remodelling of the posterior fibrous sclera has been shown to result in either loss or replacement of scleral proteoglycans which leads to a myopia-associated reduction in scleral GAG synthesis (in tree shrew: McBrien et al., 2000; Norton and Rada, 1995; marmosets: Rada et al., 2000a and humans: Rada et al., 2000b). It is thus important to consider further the fibrous sclera.

Signals acting on the sclera are thought to have their origin in the retina, especially in the retinal amacrine cells (Taylor and Smith, 2012) and in the retinal pigment epithelium (RPE) (Rymer and Wildsoet, 2005; Seko et al., 1994). Choroidal thickening, a mechanism (Troilo et al., 2000) which allows for rapid compensation to defocus (i.e. thickening and thinning of the choroid respectively following induced myopia and hyperopia), moves the retina forward and backward to bring the photoreceptors closer to the plane of focus (Wallman et al., 1995; Nickla and Wallman, 2010). These defocus induced changes also occur in the human eye (Read et al., 2010). The RPE is a monolayer of epithelial cells between the neural retina and choroid. It regulates the chemical composition and volume of the subretinal space (SRS) and choroid, the extracellular environments on either side of this cellular sheet (Gallemore et al., 1997). Na^+/K^+ ATPase is expressed on the apical RPE cell membrane where it is critical for net active sodium flux across the RPE from choroid to retina (Rizzolo, 1990). Chick eyes recovering from form-deprivation showed increase in Na^+ and Cl^- ion content of the outer retina (photoreceptors and RPE) and inner choroid (Liang et al., 2004; Seko, 2000). Accumulation of K^+ ions in the subretinal space in form-deprived chicks might provide a "grow" signal to the RPE (Crewther, 2000). Recent findings of Cl^- ion transport/channels gene and protein expression in chick RPE has furthered the likelihood of fluid transport across RPE being a potential mechanism involved in compensation to defocus (Zhang et al., 2011).

Evidence suggests that RPE cells synthesise and secrete many kinds of cytokines as well as express their receptors (Tanihara et al., 1997). Among these cytokines, insulin like growth factor-1 (IGF-1) was increased and IGF-1 receptors up-regulated in RPE, choroid and fibrous sclera during negative lens treatment (Penha et al., 2011). Also transforming growth factor (TGF)-β2 has been shown to be related to the development of myopia through its influence on the proliferation of scleral fibroblast cells and the production of collagen (Jobling et al., 2004). Although, the RPE has not been a major focus of myopia research, a substantial role for the RPE in modulating scleral growth has been suggested by the findings that the RPE plays a key role in driving the force behind early development of scleral cartilage in chicks (Thompson et al., 2010). Also, the RPE has been shown to stimulate proliferation of cultured scleral chondrocytes *in vitro* (Seko et al., 1994) and the destruction of retina/RPE complex by gentamycin inhibited their proliferation (Wang et al., 1998). Previous co-culture studies involving chick eyes have primarily focused on changes to the cartilaginous layer of the sclera whereas it is the fibrous layer that is structurally similar to the fibrous sclera of myopic human eyes (Avetisov et al., 1984; Rada et al., 2000b; McBrien et al., 2001). Therefore, the mechanism of retinal signal transmission and the role of posterior eye cup tissue, including RPE, in mediating changes in scleral fibroblast growth during myopia development remain unclear.

The purpose of the present study was therefore to further examine the effect of posterior eye cup tissue obtained from experimentally induced myopic and hyperopic chick eyes on DNA and GAG content of scleral fibroblasts. The hypothesis was that posterior eye cup tissues (retina, RPE and choroid) from myopic and hyperopic eyes would induce opposite effects on scleral fibroblast DNA and GAG content *in vitro*. We sought to determine which specific tissue layer contained an eye growth signal and whether the effects of individual tissue layers or combinations thereof would induce greatest effects. We compared the effects of tissue obtained from the eyes of chicks where either form-deprivation myopia (FDM) or lens-induced myopia (LIM) treatments had been used since there is much debate about whether FDM and LIM are the same or different processes (Fujikado et al., 1997). One obvious difference between these treatments is that eye growth is "open-loop" in the case of FDM, but "close-loop" in the case of LIM since visual feedback is present (Schaeffel et al., 1988). We also compared the effects of tissue obtained from LIM and LIH treated eyes.

2. Materials and methods

This study comprised two linked experiments.

i) *FDM (form-deprivation myopia) vs. LIM (lens-induced myopia).* Five-day-old chicks were fitted with either a white translucent diffuser (FDM; $n = 4$) or −15D lens (LIM; $n = 4$) over