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SYNCHROTRON X-RAY DIFFRACTION AND HISTOCHEMICAL STUDIES OF NORMAL AND MYOPIC CHICK EYES

Keywords: Myopia, sclera, cornea, proteoglycans, collagen, X-ray diffraction

ABSTRACT. Synchrotron X-ray diffraction patterns were obtained from the cornea and posterior sclera of control and myopic chicks. No significant difference was found in the interfibrillar or in the intermolecular spacing of the collagen fibrils from the corneas of control and myopic chicks. The intermolecular spacing of myopic sclera was shown to be significantly \((p < 0.01)\) higher than in controls.

Sclera and cornea from normal and myopic chicks were stained for proteoglycans using the 'critical electrolyte' method of Scott and Orford (1981). In the sclera, two morphologically distinct types of proteoglycans could be distinguished: one small and usually elongated \((\approx 20 \text{ nm})\), the other larger and irregularly shaped. The small proteoglycans were seen binding preferentially to the 'd' and 'e' bands of the collagen fibrils. Small proteoglycans were also present within the fibrils, these were usually \(\approx 10 \text{ nm}\) in diameter although sizes up to \(30 \text{ nm}\) were observed. Longitudinal sections of fibrils revealed that these intrafibrillar proteoglycans were chiefly orientated parallel to the axis of the collagen fibrils, and preferentially located along the gap region of the fibrils.

No difference was observed in the binding sites of the proteoglycans between normal and myopic sclera. The larger proteoglycans were also seen aggregated into clumps, which were usually associated with spaces between collagen bundles. The differences between control and myopic sclera appear to be quantitative rather than qualitative suggesting that the scleral expansion in myopia is an enhanced form of normal scleral growth.

Introduction

This paper details an investigation into the structure and distribution of the collagen and proteoglycans distributed within the cornea and sclera of normal and myopic chick eyes. Within the cornea and sclera of the eye, the collagen fibres are responsible for resisting the tensile forces, while the hydrophilic proteoglycans provide a positive swelling pressure to keep the tissue inflated. Proteoglycans have also been reported to control collagen fibril diameter (Scott, 1984), to be involved in wound healing in cornea (Rawe et al., 1991), and have recently been implicated in the process of scleral elongation in the development of myopia (Rada et al., 1991).

Myopia in humans is usually associated with increased length of the vitreous chamber (Gernet, 1981; Hōsaka, 1988). In chicks, an increase in vitreous chamber length leading to myopia can be induced by visual form deprivation (Wallman et al., 1978). In chicks there is also often an associated increase in corneal curvature (Gottlieb et al., 1987).

A number of theories have been proposed to account for the increase in vitreous chamber length. One suggestion is that increased intraocular pressure could cause the sclera to stretch passively thus increasing the vitreous chamber size. However, Perkins (1981) failed to find any consistent increase in
intraocular pressure in humans with myopia. Abnormalities in the appearance of the sclera of humans with high myopia (Curtin et al., 1979) has lead to suggestions that the sclera of myopic individuals is somehow weakened leading to stretching of the sclera under normal intraocular pressure. Findings by Raviola and Wiesel (1985), who showed that sclera thinning was associated with myopia in monkeys, and by Curtin and Teng (1957) who reported scleral thinning in humans with high myopia, suggests that some form of stretching may take place. However treatment of the sclera in tree shrews with lathrytic agents did not result in sclera elongation in chicks (McBrien et al., 1991). In chicks the development of partial field myopia is not associated with overall thinning of the sclera. However, in chicks the sclera is divided up into an inner cartilaginous layer and an outer fibrous layer; and Gottlieb et al., (1991) report that in myopic eyes the cartilaginous layer becomes thicker while the fibrous layer becomes thinner, which again suggests the possibility of stretching of the fibrous sclera.

Christensen and Wallman (1991) have shown that in myopic chicks there is an increase in sclera dry weight as well as increases in DNA syntheses and soluble protein content, together with increased hydration in myopic chick scleras. While recent work (Rada et al., 1991) has shown that, compared to control, the myopic chick sclera contains increased levels of the small proteoglycan decorin, and greatly increased levels of the proteoglycan aggrecan. The workers propose that the increased levels of aggrecan are involved in extension of the myopic sclera leading to ocular enlargement, although the exact mechanism by which this occurs is still unclear.

In this investigation we have used two techniques. Synchrotron X-ray diffraction has enabled us to obtain the mean centre-to-centre spacing between fibrils in the stoma and the mean centre-to-centre spacing between collagen molecules within the fibrils, from cornea and sclera at normal physiological hydration, and without any processing of the tissue. In addition, the 'critical electrolyte' technique of staining proteoglycans (Scott and Orford, 1981) was employed to look at the distribution of proteoglycans in chick cornea and sclera. The aim of this project was two-fold. Firstly, to examine the collagen/proteoglycan structure and arrangement in the previously uninvestigated normal chick cornea and sclera. The second part of the work involved looking for abnormalities in the collagen and proteoglycan components of myopic cornea and sclera, in order to gain some insight into the mechanism of scleral expansion in myopia.

Materials and Methods

Normal and visual deprivation myopia corneas

Myopia was induced in one eye of the chicks using the form deprivation technique described by Wallman et al., (1978), the other eye on the chick was left untreated and acted as a control. The chicks were killed at 6 weeks and the eyes stored wrapped in clingfilm and frozen at −40°C until required. Previous studies have shown that the freezing process has no effect on the interfibrillar or intermolecular spacings or on the distribution of proteoglycans in the stroma (Quantock et al., 1990). All of the scleral samples investigated came from the posterior region of the sclera, adjacent to the optic nerve.

X-ray diffraction

X-ray diffraction patterns were recorded at the Science and Engineering Research Council synchrotron source at Daresbury UK. Since the cornea is a weakly diffracting system it was necessary to use the very intense X-radiation produced by a synchrotron source in order to obtain a usable pattern. The derivation of the X-ray patterns in cornea has been described by Meek et al., (1991).

The X-ray patterns were recorded on Caeverken AB film (Caeverken, Strängnäss, Sweden). The exposed X-ray films were scanned using an Ultrascan XL laser microdensitometer (LKB instruments Inc., Gaithersburg, MD, USA) and background scatter was subtracted from the trace before measurement.

X-ray patterns arising from the packing of the fibrils were obtained on a low-angle camera, this camera was able to record reflections derived from spacings between ~30 nm to ~100 nm, and was calibrated from the 67 nm axial periodicity in rat tail tendon. The beam dimensions at the detector were 4 × 0.5 mm, the wavelength was 0.154 nm,
and the specimen to film distance was either 2.5 m or 4 m. Measurement of the first-order diffraction ring from the packing of the collagen fibrils within the corneal stroma allowed the Bragg spacing to be obtained. The Bragg spacing was increased by a factor of 1.12 to give the centre-to-centre spacing of the collagen fibrils (the interfibrillar spacing); this factor takes into account the liquid-like packing of the collagen fibrils in the corneal stroma (Worthington and Inouye, 1985).

X-ray patterns deriving from the lateral packing of the molecules within the collagen fibrils were obtained on a high-angle camera, this camera was able to record reflections derived from spacings from \( \sim 0.3 \text{ nm} \) to \( \sim 2 \text{ nm} \), and was calibrated from 0.305 nm spacing in calcite. The beam had a diameter of 1 mm at the detector, and a wavelength of 0.1488 nm. The specimen to film distance was 11 or 12 cm. Measurement of the first-order diffraction ring from the packing of the collagen molecules allowed the intermolecular Bragg spacing to be obtained. The Bragg spacing was increased by a factor of 1.11 to give the mean centre-to-centre spacing of the collagen molecules (intermolecular spacing). The factor of 1.11 assumes that the collagen molecules are packed in a 'pseudo-hexagonal' lattice which is the most probable lateral arrangement of the molecules in the fibrils (Maroudas et al., 1991).

The X-ray data were obtained 'blind' from samples identified only by numbers; the final results were then subjected to paired t-tests to assess the significance of any differences in spacings between control and myopic specimens.

Transmission electron microscopy
Cornea and sclera were prepared for electron microscopy using the 'critical electrolyte' method of staining proteoglycans described by Scott and Orford (1981). This technique allows the specific staining of the anionic glycosaminoglycan part of the proteoglycans by the cationic, electron opaque dye, cuprolinic blue. Small (0.5 mm\(^2\)) pieces of tissue were stained overnight in 0.05% cuprolinic blue and 0.1 M MgCl\(_2\) in 25 mM sodium acetate buffer pH 5.7. The samples were then rinsed three times (for 15 min each time) in the buffer solution containing glutaraldehyde and MgCl\(_2\). After this, the samples were stained 'enblock' in three changes of 0.5% aqueous sodium tungstate. This was followed by a conventional ethanol dehydration and embedding procedure. Sections were cut from the cured blocks using an Ultracut E ultramicrotome, and were stained with aqueous uranyl acetate before being examined with a Philips 301 TEM.

Results

X-ray diffraction
Table 1 gives the details on the radius of corneal curvature (mm) and the refractive error (diopters) for the normal and experimental chick eyes.

The low and high-angle X-ray results from cornea and sclera from control and normal chicks are summarized in Table 2. There are no low-angle results for sclera because the distribution of fibril diameters and fibril spacings in this tissue are too disordered to produce an X-ray pattern.

Low-angle results. The results from low-angle X-ray diffraction gave a mean interfibrillar spacing of 54.6 \( \pm \) 5.9 nm for control corneas, and a spacing of 58.3 \( \pm \) 5.3 nm for myopic corneas. No significant difference (p > 0.2) was found between the two types of cornea.

High-angle results. The results from high-angle X-ray diffraction give a mean intermolecular spacing of 1.10 \( \pm \) 0.05 nm for control corneas, and a spacing of 1.14 \( \pm \) 0.06 nm for myopic corneas. No significant difference (p > 0.2) was found in intermolecular

<table>
<thead>
<tr>
<th>Radius of corneal curvature (mm)</th>
<th>Refractive error (diopters)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>Myopic</td>
</tr>
<tr>
<td>Normal</td>
<td>Myopic</td>
</tr>
<tr>
<td>4.09</td>
<td>4.01</td>
</tr>
<tr>
<td>4.00</td>
<td>4.05</td>
</tr>
<tr>
<td>3.85</td>
<td>3.25</td>
</tr>
<tr>
<td>4.04</td>
<td>3.96</td>
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<tr>
<td>3.96</td>
<td>3.73</td>
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Table 2. *Interfibrillar and intermolecular spacings from cornea and posterior sclera of normal and myopic eyes of 6-week-old chicks. The spacings are given in nm.*

<table>
<thead>
<tr>
<th>Spacing</th>
<th>Normal</th>
<th>Myopic</th>
<th>Normal</th>
<th>Myopic</th>
<th>Normal</th>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>cornea</td>
<td>60.0</td>
<td>60.0</td>
<td>1.13</td>
<td>1.13</td>
<td>1.15</td>
<td>1.19</td>
</tr>
<tr>
<td>cornea</td>
<td>52.5</td>
<td>61.4</td>
<td>1.09</td>
<td>1.17</td>
<td>1.17</td>
<td>1.20</td>
</tr>
<tr>
<td>Intermolecular</td>
<td>47.2</td>
<td>50.3</td>
<td>1.02</td>
<td>1.05</td>
<td>1.12</td>
<td>1.20</td>
</tr>
<tr>
<td>sclera</td>
<td>61.4</td>
<td>56.2</td>
<td>1.13</td>
<td>1.11</td>
<td>1.14</td>
<td>1.19</td>
</tr>
<tr>
<td>Intermolecular</td>
<td>52.1</td>
<td>63.8</td>
<td>1.10</td>
<td>1.22</td>
<td>1.21</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Spacing between the two types of cornea. High-angle X-ray patterns were also obtained from the intermolecular spacing of collagen molecules within the collagen fibrils in the scleral stroma. A mean intermolecular spacing of 1.16 ± 0.03 nm was obtained for normal sclera, and a spacing of 1.20 ± 0.02 nm for myopic sclera. Myopic sclera was found to have a significantly (p < 0.01) higher intermolecular spacing than the control sclera.

**Transmission Electron Microscopy**

**Cornea**

The corneas of both control (Fig. 1a) and myopic (Fig. 1b) chicks appeared very similar to each other. The collagen fibrils in both types of cornea were regular in appearance and approximately 25 nm in diameter. The cuprolinic blue-stained proteoglycans appeared to be of similar size and numbers in both tissues, morphologically there appeared to be only one type. It proved impossible to localize where along the collagen fibril the proteoglycans were binding, as the collagen fibrils were of too small a diameter to allow the collagen banding pattern to be clearly resolved.

**Sclera**

The cartilaginous region of the chick sclera contains large amounts of proteoglycans but is devoid of fibrillar collagen. Chondrocytes were frequently observed in this matrix. The numbers of chondrocytes present appeared to be the same in normal and in myopic sclera. Figure 2a and b show chondrocytes from the cartilaginous region of the normal and myopic sclera respectively. It can be seen from these figures that the chondrocytes are surrounded by aggregations of large proteoglycans. The empty spaces surrounding the chondrocytes may be an artifact caused by the low osmolarity and pH of the 'critical electrolyte' staining solution.

The fibrous region of the sclera contains bundles of collagen fibrils, the collagen fibrils themselves are of various diameters, and are closely associated with proteoglycans. Figure 3a and b shows the fibrous region of chick sclera for normal and myopic chicks. Both Figures show that in some regions there are empty spaces between the collagen bundles, these spaces were frequently observed to be associated with aggregations of large proteoglycans.

Within the fibrous part of the sclera the fibrils are frequently in contact with each
other, some adjacent collagen fibres were observed running in opposite directions (with regard to the collagen banding pattern), while others had their banding patterns running in the same direction. Two sizes of proteoglycan were observed in the stroma, small (~20 nm long) proteoglycans which were commonly observed bound to collagen fibrils. Larger proteoglycans (>40 nm), fairly irregular in shape were also present in quantity, and were sometimes observed attached to the collagen fibrils. These large proteoglycans were frequently seen aggregated in clumps. Figure 4a and b show proteoglycans attached to specific regions of the collagen fibril. The nomenclature used in this paper to describe the banding of the D-period of the collagen fibrils is that used by Hodge and Schmitt (1960). In both myopic and control specimens proteoglycans were occasionally observed bound to most points along the D-period of the collagen fibril, however the most commonly observed binding site was the 'd' and 'e' bands of the collagen fibrils.

As well as both large and small proteoglycans which bind to the exterior of the collagen fibril there are also small proteoglycans present within the collagen fibrils of both normal and myopic sclera. These small proteoglycans are usually orientated parallel to the axis of the collagen fibril. Figure 5a and b show small proteoglycans present within the collagen fibrils from control and myopic sclera, the proteoglycans appear to be preferentially located along the gap region of the D-period of the collagen fibril; (the gap region is located between the c2 and e bands along the collagen fibril).

Figure 6 shows transverse sections through the fibrous sclera of normal (Fig. 6a) and myopic chicks (Fig. 6b). Both Figures clearly show the presence of intrafibrillar proteoglycans. A few of the intrafibrillar proteoglycans observed were quite large (up to 30 nm in diameter) although the majority were approximately 10 nm in diameter. The intrafibrillar proteoglycans tended to be more common towards the outer surface of the sclera, and were occasionally associated with what appeared to be 'holes' in the collagen fibrils.

Discussion

The results of the X-ray study show that there are no significant differences in interfibrillar spacing or in intermolecular spacing between normal and myopic chick corneas. However, myopic sclera was shown to have a significantly higher intermolecular spacing than normal. A possible explanation for the increased intermolecular spacing in sclera is that the myopic tissue is more hydrated. It has been shown that increased hydration in sclera results in increased intermolecular spacing (Fullwood, 1992), and recent work by Christensen and Wallman (1991), has shown that the sclera of myopic chicks is more hydrated than normal sclera. Since most of the swelling pressure in sclera is the

Fig. 3. Shows the fibrous sclera of normal (A) and myopic (B) chicks. In both cases numerous large proteoglycans are visible associated with the collagen bundles. Aggregations of these proteoglycans (arrows) are also sometimes evident in spaces between the collagen bundles. Note also the chondrocyte (C) in (B) ×12,000.

Fig. 4. Shows the fibrous sclera of normal (A) and myopic (B) chicks. It is evident that in both cases the small extracellular proteoglycans (arrow heads) are attached preferentially to the 'd' and 'e' bands of the collagen fibrils. A; ×205,000. B; ×142,000.

Fig. 5. Shows the fibrous sclera of control (A) and myopic (B) chicks. Both micrographs show longitudinal sections through large diameter collagen fibrils, and in both cases small intrafibrillar proteoglycans (arrow heads) are evident within the fibrils and are preferentially located along the gap regions of the fibrils. ×142,000.

Fig. 6. Shows cross sections through the collagen fibrils of control (A) and myopic (B) chick sclera, from the same region as Figure 5. In both cases, cross sections of small intrafibrillar proteoglycans (small arrows) can be seen in the interior of the fibrils. A less common, large intrafibrillar proteoglycan, is also evident in A (large arrow). ×151,000.
result of the proteoglycans within the sclera, then it is probable that the higher hydration is caused by the increased levels of proteoglycans reported to be present in myopic chick sclera by Rada et al., (1991). Proteoglycans have charged groups on their glycosaminoglycans sidechains which attract a cloud of permiant ions producing a high osmotic potential.

Our findings have shown two different sizes of proteoglycans in both normal and myopic sclera. It seems very likely that the smaller proteoglycan is decorin ($M_r = 43,000$ Da), and the larger one probably aggregcan ($M_r > 1 \times 10^6$ Da), both of which have been isolated by Rada et al., (1991) from chick sclera. The smaller proteoglycans were observed binding preferentially to the 'd' and 'e' bands of the collagen fibrils in both normal and myopic sclera, this agrees with the observation of Quantock and Meek (1988) working on human sclera, and Young (1985) who reported binding of proteoglycans to the 'd' region in rabbit sclera. We report here for the first time the presence of small intrafibrillar proteoglycans in chick sclera, preferentially located along the gap region of the collagen fibrils. Sclera has previously been reported as containing 'almost no' intrafibrillar proteoglycans (Scott, 1991) with no indication of any preferential location within the collagen fibrils. However, Scott (1990) reports that the intrafibrillar proteoglycans present in rat-tail tendon are located along the gap region of the fibrils, this is in agreement with our findings in chick sclera. Scott suggests that in rat-tail tendon the intrafibrillar proteoglycans are attached to the surface of collagen protofibrils, and that a possible function of the proteoglycans is to inhibit the assembly of the proteofibrils into larger fibrils. If the intrafibrillar proteoglycans in chick sclera also have this role, then this suggests a possible mechanism for scleral expansion, since an equivalent mass of collagen distributed in smaller diameter fibrils has less tensile strength than the same mass of collagen distributed in fewer, large diameter, fibrils (Parry, 1988). Although we have referred to these intrafibrillar structures as 'small proteoglycans', it is possible that they are glycosaminoglycan chains directly associated with the collagen molecules within the fibrils.

The large proteoglycans observed in the sclera are frequently clumped together, and these clumps of proteoglycans are often associated with gaps between the collagen bundles. This arrangement bears a close resemblance to micrographs of the corneal stroma during wound healing when very large proteoglycans are present in spaces between the damaged lamellae (Cintron et al., 1990; Rawe et al., 1991). In wound healing the large proteoglycans are precursors of the synthesis of collagen fibrils by the keratocytes in the damaged area of the corneal stroma. It seems possible that the aggregations of large proteoglycans in chick sclera may also facilitate the increased synthesis of collagen by the chondrocytes, producing more rapid growth of the fibrous sclera in myopic chicks.

An alternate explanation for the higher intermolecular spacings in myopic sclera, is that they could be due to the collagen fibrils in the myopic chicks undergoing a more rapid turnover than in the control chicks, this might lead to the myopic collagen fibers having fewer cross-links than normal resulting in increased intermolecular spacings.

Although myopic chick sclera has been shown to have larger quantities of proteoglycans (Rada et al., 1991), this investigation has shown no difference in the preferential binding sites of the small inter- or intrafibrillar proteoglycans, or in the location and appearance of the large proteoglycans in the sclera of normal and myopic chicks. Although, the X-ray data suggests that myopic sclera may contain more intrafibrillar proteoglycans than normal. Thus, although there are differences in proteoglycan content between normal and myopic sclera these are quantitative rather than qualitative, which itself suggests that the scleral elongation which occurs in myopic chicks is simply a more enhanced form of normal scleral growth.

Acknowledgements

We would like to acknowledge the assistance of Wim Bras and Pierre Rizkallah at the Daresbury synchrotron.

The project was supported by the TFC Frost Trust, the National Keratoconus Foundation/Discovery Fund for Eye Research and the Iris Fund. We also thank the MRC, the Nuffield Foundation, and the Wellcome Trust.
References


