Expression of collagen genes in postnatal rabbit sclera

Thu K. Moe,[1] Ph D, Ji Ziliang,[1] MSc, Amutha Barathi,[1] BVSc, Roger W. Beuerman,[1,2,3] Ph D

[1] *Singapore Eye Research Institute, Singapore.*
[2] *Department of Ophtholmology, National University of Singapore, Singapore.*
[3] *Louisiana State University Eye Center, Louisiana State University Health Sciences Center, New Orleans, LA, USA.*

Correspondence and reprint requests:
Professor Roger W. Beuerman, Singapore Eye Research Institute, MD9, # 01-15, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260.

Abstract

Aims: To examine the differential expression of collagens in the rabbit sclera at several stages of early postnatal development from 1 day to 4 weeks.
Materials and methods: Total RNA was extracted from the sclera of 2 groups of rabbits at each time point and real-time polymerase chain reaction analysis was employed. The amount of message for each collagen gene was compared with the corresponding level for 1 day mRNA. Histological examination was performed for sclera at 1 day and 4 weeks.
Results: Real time polymerase chain reaction allows the quantification of message level. The mRNA level for collagen α1 (I), α2 (I), and α1 (III) were highest 1 day after birth and declined gradually during the observation period. Collagen α2 (XI) demonstrated a relatively similar trend of expression. Histological analysis showed that scleral fibroblast density was greatest at 1 day.
Conclusion: Collagens play a crucial role in active tissue construction during the postnatal developmental period and changes in the level of transcription of these genes could affect the overall structure of the sclera.

Key words: *Collagen, Gene expression, Histology, Polymerase chain reaction*

Introduction

Collagen is an important component of connective tissue, and determines the biomechanical properties of the tissue. In sclera, it accounts for 50% to 70% of the dry weight, and is responsible for the strength and resilience of the tissue. Collagen type I is the major component of sclera and the remainder is largely collagen type III.[1,2] Fibroblasts are the only known source of structural protein in the sclera. Changes in the content or organization of scleral collagen may be important in several common disorders of the eye.

Myopia is a relatively common refractive error and is characteristically associated with enlargement of the eye, especially in the axial length.[3] Influences on the scleral fibroblast are thought to play a role in the regulation of the eye's axial length during the development of myopia as well as during normal postnatal ocular development. Studies with myopic animal models have shown that enlargement of the eye is associated with thinning of the posterior sclera, resulting from reduced deposition of collagen and proteoglycan.[4] Siegwart and Norton have recently shown that α1 (I) collagen mRNA level was 35% lower in 5-week-old myopic tree shrews after 11 days of monocular form deprivation.[5]

Although there are several reports on biochemical and structural changes in collagens and proteoglycans in the sclera of myopic animal models and in high myopia in humans,[6,7] there is little research into regulation of collagen genes in the sclera, in particular in normal postnatal development. The present study demonstrates the differential expression of collagen α1 (I), α2 (I), α1 (III), and α2 (XI) in the rabbit sclera from 1 day to 4 weeks of postnatal development.

We have previously reported that commonly known housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β actin, and hypoxanthine phosphoribosyltransferase (HPRT) were differentially expressed during this observation period, whilst the 18S rRNA gene showed

constant expression.[8] Hence, we used 18S rRNA as the internal control gene in this study.

Materials and methods

Total RNA extraction and cDNA synthesis

New Zealand White rabbits of different ages were obtained from the Laboratory Animal Center of the National University of Singapore. The animals were used in accordance with the *Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Research*. Two randomly chosen rabbits from each of 2 litters of 1-day-old and 1-, 2-, and 4-week-old animals were sacrificed. The posterior sclera was dissected from each eye and isolated from the adjacent and adherent tissues. The optic nerve and nerve head were not included. The sclera of each group of each litter was immediately frozen in liquid nitrogen. Two sclera were pooled together and used for RNA isolation.

Total RNA was isolated using TRIzol Reagent (Life Technologies, Louisville, USA) in accordance with the manufacture's instructions. Total RNA was quantified by spectrophotometer (Genesys 5, Thermo Spectronic, Rochester, USA) and the integrity of the samples was analyzed by 260/280 nm absorbance ratios and by 1.2% agarose gel electrophoresis. Total RNA was treated with DNase and reverse transcription was carried out with 1 μg of total RNA from each sample by SUPERSCRIPT II (Life Technologies, Louisville, USA) using 0.5 μg of random primer in accordance with the manufacture's instructions.

Real-time comparative polymerase chain reaction

Real-time comparative polymerase chain reaction (PCR; SYBR Green, Foster City, USA) was performed in a 96-well microtiter plate format on a ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, USA) equipped with a Sequence Detection System (SDS) software version 1.6.3. PCR was performed using SYBR Green PCR master mix following the manufacturer's protocol with slight modification. The primers for collagen α1 (I), α2 (I) and α1 (III) were obtained from previously published primer sequences.[9,10] Collagen α2 (XI) primer was constructed using Primer Express software version 1.0 (PE Applied Biosystems, Foster City, USA) using human, mouse, and rat sequence homologous region (**Table 1**). Quantum RNA Classic II 18S Internal Standard (Ambion, Austin, USA) was used as an endogenous control. The 18S primer-competimer ratio of 2:8 was used in all experiments. To standardize and evaluate scleral gene expression, aliquots of the same cDNA preparation were used as templates in all PCR reactions. PCR reactions and dissociation curve analysis were performed following the protocols as previously described.[8]

Table 1. Primer sequences and sizes of polymerase chain reaction products.

Gene	Primers	Sizes
Collagen α1 (I)	5'-GATGTGCCACTCTGACTGG-3' (Fw) 5'-ACATCGATGATGGGCAGGC-3' (Rev)	536 bp
Collagen α2 (I)	5'-GCATTGCATACATGGATGAGG-3' (Fw) 5'-GCACAATGCTCTGATCAATCC-3' (Rev)	455bp
Collagen α1 (III)	5'-TTATAAACCAACCTCTTCCT-3' (Fw) 5'-TATTATAGCACCATTGAGAC-3' (Rev)	255 bp
Collagen α2 (XI)	5'-TGGCAAGGCTGGACGAAG-3' (Fw) 5'-CCTGAGCACCTGTATCACC-3' (Rev)	154 bp

Data analysis by comparative C_T ($\Delta\Delta C_T$) method

The C_T values were used and represent the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value (optimal DRn) was first detected. The calculation for comparative C_T ($\Delta\Delta C_T$) method was previously described.[11]

Measurement of axial length and histology processing

Six animals of each of 2 litters at 1 day and 1, 2, 4, and 6 weeks old were sacrificed and axial lengths of the eyes were measured with a digital caliper (accuracy and reliability, ±0.03 mm). The posterior sclera from 1-day-old and 4-week-old rabbit eyes were fixed with 10% formal saline for 24 hours and embedded in paraffin. From paraffin blocks, 5 μm sections were cut and the sections were stained by the hematoxylin and eosin (H&E) method.

Results

Specific amplification

Dissociation curve analysis demonstrated that each of the primer pairs described in **Table 1** amplified a single product with a distinct Tm (melting temperature) as shown for collagen α1 (I) in **Figure 1** at 87.3°C. The Tm of collagen α2 (I), α1 (III), and α2 (XI) at 81.4°C, 75.1°C, and 85.7°C, respectively, were obtained from a similar analysis. Once the predicted length of each product had been confirmed

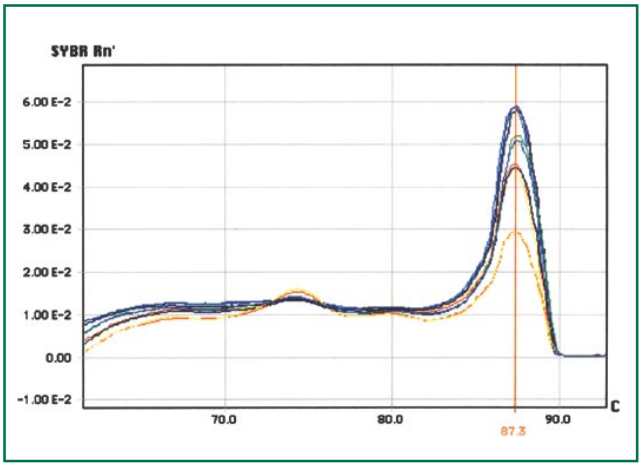

Figure 1. Dissociation curve analysis of collagen α1 (I) rRNA. The dissociation curve analysis was performed at the end of the polymerase chain reaction and the melting temperature of the specific polymerase chain reaction product is seen as a single peak in a first derivative plot. The diagram shows the dissociation curve of collagen α1 (I) rRNA from the triplicates of each cDNA sample (1 day and 1, 2, and 4 weeks) and the melting temperature of collagen a1 (I) rRNA is 84.4°C.

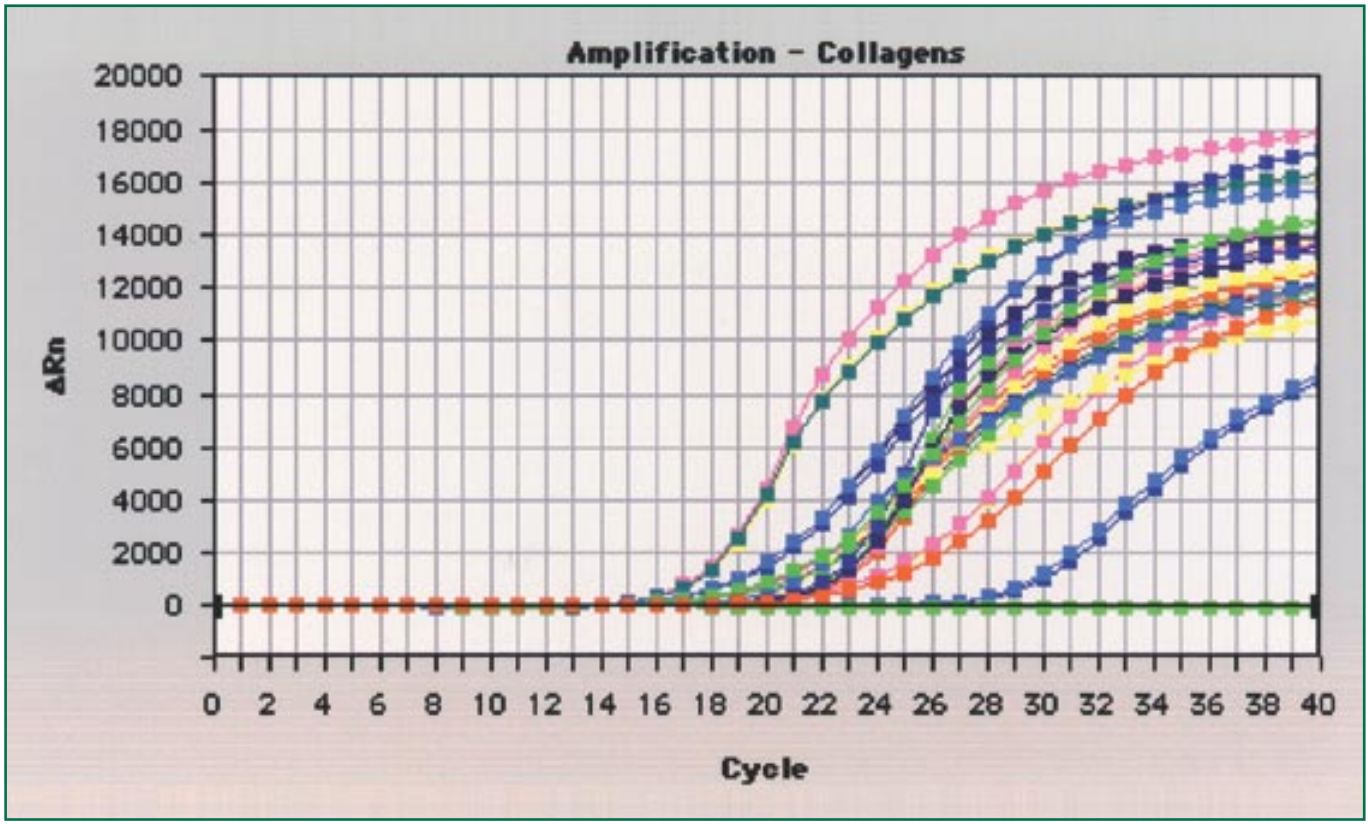

Figure 2. Amplification plot of collagen α1 (I) and 18S rRNA internal standards. The triplicates of each cDNA (1 day, 1, 2, and 4 weeks) of both samples were amplified in the same microtiter plate. The C_T value of each amplification reaction was exported and analyzed.

by agarose gel electrophoresis, the Tm was used to identify specific products in subsequent analyses.

Comparative analysis by real time polymerase chain reaction

Figure 2 shows the PCR amplification pattern for collagen α1 (I) and the 18S rRNA internal standards. Each collagen gene was amplified in triplicate with internal standards in the same microtiter plates. The average C_T value for collagen α1 (I) was used to determine the number of copies of collagen genes, ΔC_T and $\Delta\Delta C_T$, and the relative number of copies for each gene of interest compared to the 1 day mRNA of that gene (**Table 2**). The average C_T values for collagen α2 (I), α1 (III) and α2 (XI) and the corresponding internal standards were used to calculate the relative number of copies for each gene compared with the corresponding 1-day mRNA (data not shown). **Figure 3** shows the differential expression of collagen genes during the observation period. Collagen α1 (I), α2 (I) and α1 (III) demonstrate the same expression patterns. The relative amount of messages for these collagens was the highest at 1 day after birth. Collagen α1 (I), α2 (I) and α1 (III) mRNA levels declined after 1 day and reached the levels of 0.013-, 0.01-, and 0.06-fold lower than that of 1-day mRNA, respectively. At 2 weeks, the levels were relatively similar to the 1-week level: 0.024-, 0.01-, and 0.13-fold lower than that of the 1-week mRNA level, respectively. These were followed by lower expression levels at 4 weeks: 0.007-, 0.002-, and 0.008-fold that of the 1-day mRNA level, respectively (**Figures 3a, b,** and **c**). Whereas collagen α2 (XI) showed high expression at 1 day, followed by a lower level of expression at 1 week (0.26-fold that of 1 day). At 2 weeks, the mRNA level increased up to 1.24-fold that of the 1 day mRNA level, followed by lower levels at 4 weeks (0.17-fold that of the 1 day mRNA level) [**Figure 3d**].

Axial length of the eye and histological analysis

The axial lengths of the rabbit eyes averaged 6.94 mm and 7.09 mm for the left and right eyes, respectively, 1 day after birth. This gradually increased to 8.97 mm and 9.03 mm, respectively, at 1 week, and reached 10.33 mm and 10.51 mm, respectively, at 2 weeks. Continued growth

Table 2. Comparative quantification of collagen α1 (I), using comparative C_T method.

Time	Collagen α1 (I) Average C_T	18S rRNA Average C_T	ΔC_T Collagen α2 (I)-18S rRNA	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T\ 1\text{-day}}$	Collagen α1 (I) (Relation to 1-day with range)
1 day	13.60 ± 0.05	17.25 ± 0.24	-3.65 ± 0.25	0.00 ± 0.25	1 (0.84-1.18)
1 week	18.59 ± 0.11	15.99 ± 0.03	2.59 ± 0.12	6.25 ± 0.12	0.013 (0.012-0.014)
2 weeks	17.29 ± 0.09	15.55 ± 0.20	1.73 ± 0.22	5.38 ± 0.22	0.024 (0.02-0.027)
4 weeks	20.40 ± 0.18	16.88 ± 0.08	3.52 ± 0.20	7.17 ± 0.20	0.007 (0.006-0.008)

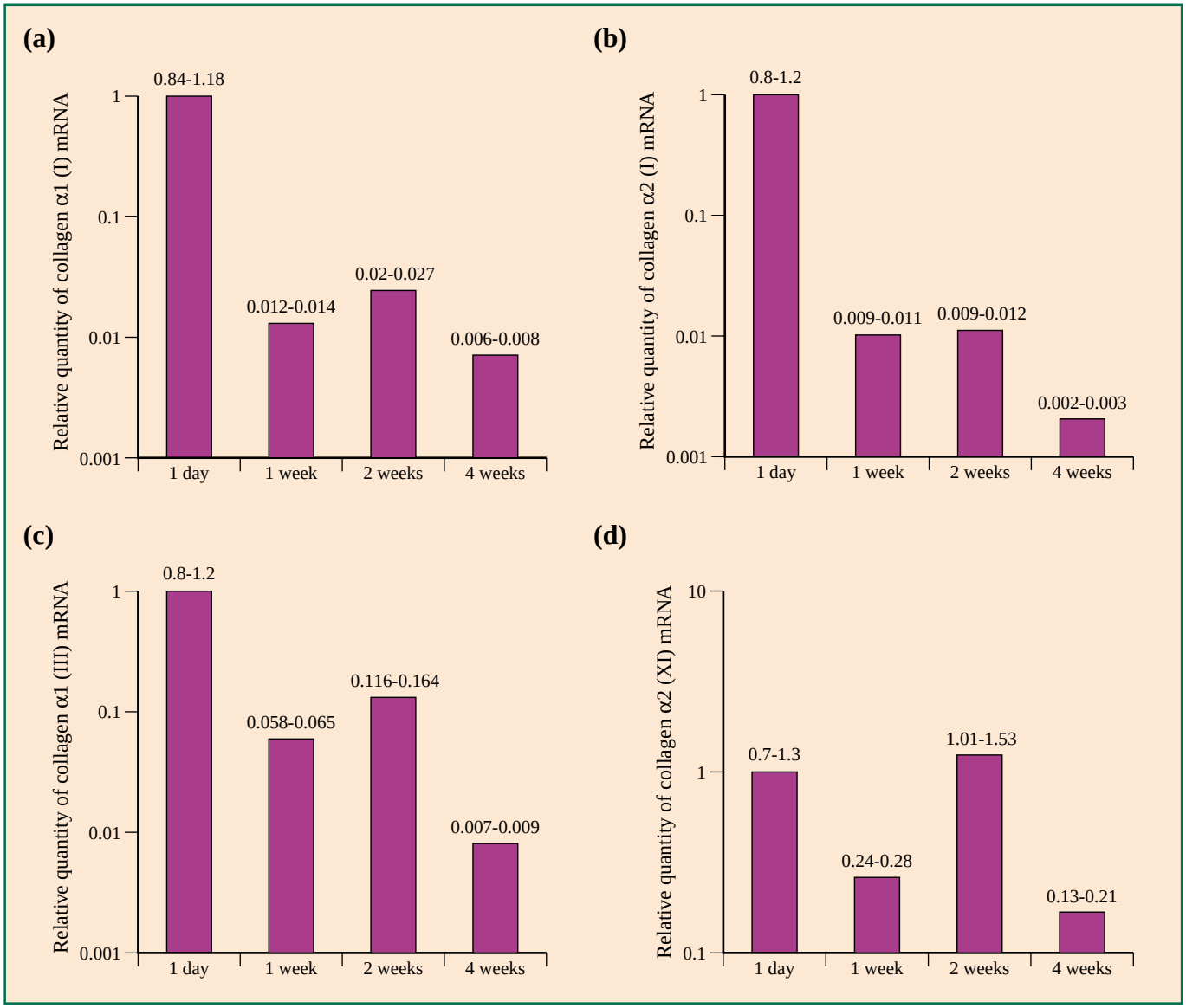

Figure 3. Bar graphs show the relative gene expression of (a) collagen α1 (I), (b) α2 (I), (c) α1 (III), and (d) α2 (XI) to the corresponding 1-day mRNA level (range) after normalization with 18S rRNA internal standard. The mRNA levels of collagen α1 (I), α2 (I), and α1 (III) at 1 day are highest and decreased during 4 weeks of observation. The collagen α2 (XI) also shows the lower mRNA level at 4 weeks after birth.

produced values of 13.32 mm and 13.39 mm, respectively, at 4 weeks and 14.99 mm and 15.05 mm, respectively, at 6 weeks after birth **(Figure 4)**. **Figures 5a** and **b** show the microscopic structure of the posterior sclera at 1 day and 4 weeks after birth. At 1 day, there were many fibroblast cells embedded in the collagen fibers (**Figure 5a**). However, the 4-week-old posterior sclera sample shows abundant collagen fibers that are easily seen as wavy, unbranched fibers — in between them are fewer fibroblast cells (**Figure 5b**).

Discussion

The sclera, together with the cornea, forms the outer tunic of the eye. The sclera provides a protective coat for the eye and possesses the tensile strength to withstand the considerable expansive force generated by the intraocular pressure. Collagen type I has been reported to be the major component of the sclera and the rest was thought to be collagen type III.[1,2] Another independent study revealed that collagen types V and VI were also detected in the aged human sclera.[12] The present study demonstrates that collagen α1 (I), α2 (I), α1 (III), and α2 (XI) are present and differentially expressed in rabbit sclera during postnatal development from 1 day to 4 weeks. The amount of message for collagen α1 (I), α2 (I), and α1 (III) appeared to be the highest at 1 day after birth and decreased to the lowest at 4 weeks after birth. A relatively similar trend of expression was also observed for collagen α2 (XI), showing the lowest expression at 4 weeks after birth.

We have previously reported that the axial growth rate of the rabbit eye appeared to be the highest (0.28 mm/day) during 1 to 7 days after birth and declined to 0.12 mm/day at 4 to 6 weeks old.[13] The present study supports these findings and suggests that collagens probably play a major

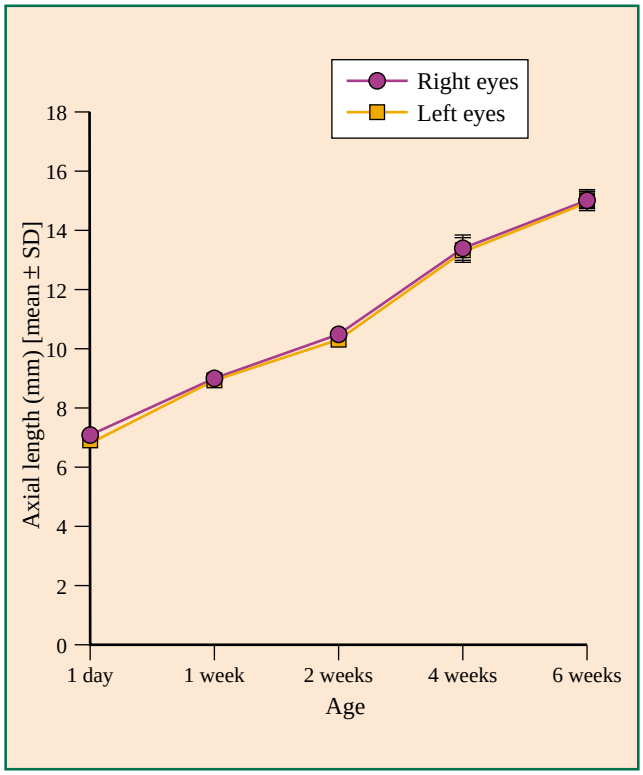

Figure 4. Mean ± SD of the axial length of the eyes at 1 day and 1, 2, 4, and 6 weeks after birth. The axial length of the eye is substantially increased during the first 2 to 6 weeks.

role in providing material for the rapid structural demands during this growth period. Indeed, at 4 weeks the overall axial length of the eye is approximately 60% that of an adult. Furthermore, derangement and disorganization of the collagen structure in the sclera could lead to the situation of thinning of the sclera as found in myopia. Beighton reported that defective organization of collagen–fibril bundles observed in Ehlers-Danlos syndrome was associated with a severe form of myopia.[14]

Norton and Rada reported that hydroxyproline levels were significantly reduced at the posterior sclera of tree shrews after 21 days of form deprivation.[4] Siegwart and Norton recently demonstrated that collagen α1 (I) mRNA level was 35% lower in the deprived eyes compared to that of control eyes.[5] The present study also agreed with the previous findings and suggested that postnatal development of the eye and form deprivation of the eye would undergo the same process of active tissue remodeling that affects the growth of eye. However, further studies need to be done to understand the types of collagens involved in this process and the changes in the levels of transcripts.

At 4 weeks after birth, the increase in thickness of the sclera with several layers of collagen bundles, and fewer fibroblast cells compared to 1-day-old sclera were observed by light microscopic examination in this study. These findings were supported by the high level of collagen gene transcripts found in the first week after birth. The changes in the protein levels of collagens during the postnatal developmental period are currently being investigated.

Acknowledgements

This work was supported by the following grants, National Medical Research Council Grant, NMRC MH95:03, and National Institutes of Health, NEI EY12416.

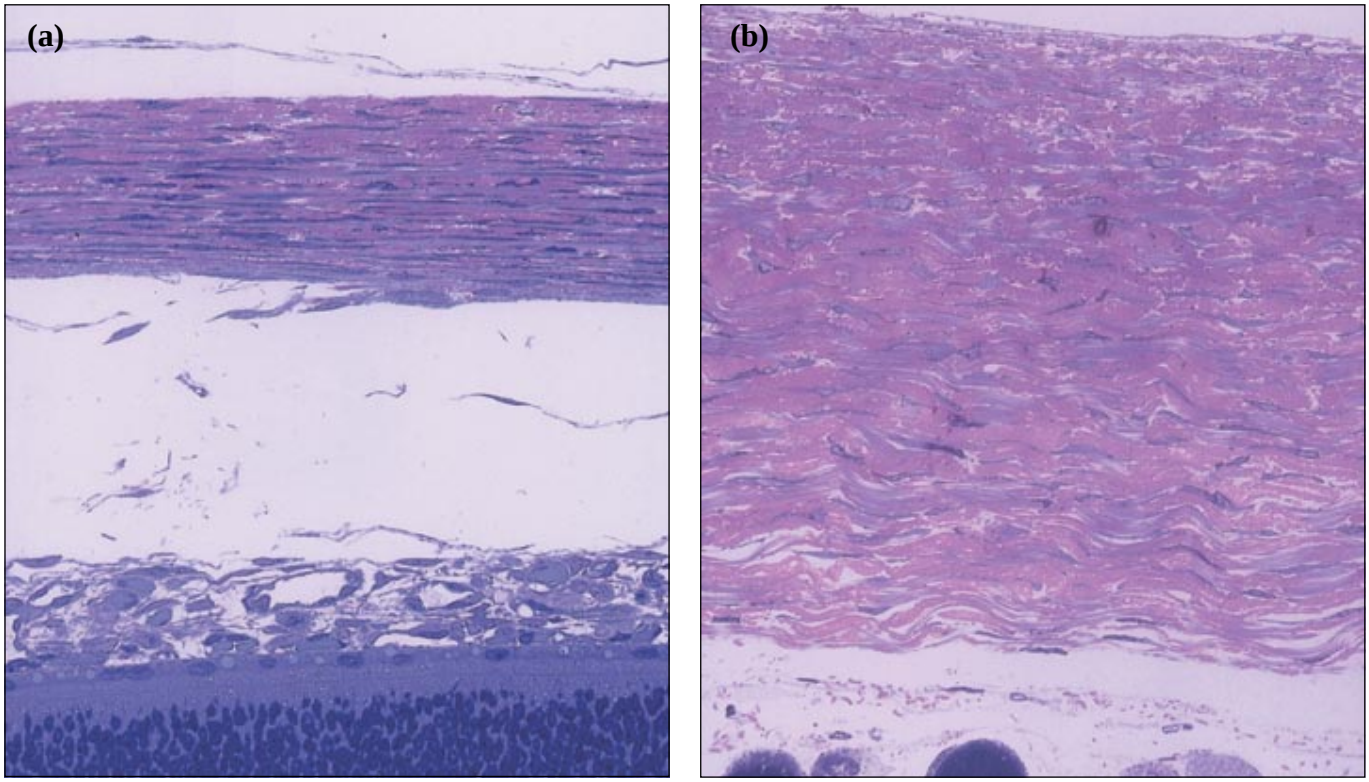

Figure 5. Histological examination shows (a) several fibroblast cells and a few collagen fibers at 1 day and (b) the 4-week sample shows abundant wavy collagen fibers and few fibroblast cells (magnification × 40).

References

1. *Schmut O. The identification of type III collagen in calf and bovine cornea and sclera. Exp Eye Res 1977;25:505-509.*
2. *Bailey AJ. Structure, function and ageing of the collagens of the eye. Eye 1987;1:175-183.*
3. *Curtin BJ, Karlin DB. Axial length measurements and fundus changes of the myopic eye. Am J Ophthalmol 1971; 1:42-53.*
4. *Norton TT, Rada JA. Reduced extracellular matrix in mammalian sclera with induced myopia. Vision Res 1995; 35:1271-1281.*
5. *Siegwart JT, Norton TT. Steady state mRNA levels in tree shrews with form deprivation myopia and during recovery. Invest Ophthalmol Vis Sci 2002;43:2067-2075.*
6. *McBrien NA, Moghaddam HO, Reeder AP, Moules S. Structural and biochemical changes in the sclera of experimentally myopic eyes. Biochem Soc Trans 1991;19: 861-865.*
7. *Avetisov ES, Savitskaya NF, Vinetskaya MI, Iomdina EN. A study of biochemical and iomechanical qualities of normal and myopic eye sclera in humans of different age groups. Metab Pediatr Syst Ophthalmol 1983;7:183-8.*
8. *Moe KT, Ziliang J, Barathi A, Beuerman RW. Differential expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β actin and hypoxanthine phosphoribosyltransferase (HPRT) in postnatal rabbit sclera. Current Eye Reasearch 2001;23:44-50.*
9. *Lee SK, Goyal M, de Miguel M, et al. Renal biopsy collagen I mRNA predicts scarring in rabbit anti-GBM disease: comparison with conventional measures. Kidney Int 1997;52: 1000-1015.*
10. *Hart DA, Boykiw R, Sciore P, Reno C. Complex alterations in gene expression occur in the knee ligaments of the skeletally mature multiparous rabbit during pregnancy. Biochim Biophys Acta 1998;1397:331-341.*
11. *Brink N, Szamel M, Young AR, Wittern KP, Bergemann J. Comparative quantification of IL-1β, IL-10, IL-10r, TNFα and IL-7 mRNA levels in UV-irradiated human skin in vivo. Inflamm Res 2000;49:290-296.*
12. *Marshall GE, Konstas AG, Lee WR. Collagens in the aged human macular sclera. Curr Eye Res 1993;12:143-153.*
13. *Thu MK, Beuerman RW, Barathi A. Gene expression and scleral growth in the rabbit [abstract]. Invest Ophthalmol Vis Sci 2000;41 (Suppl):872.*
14. *Beighton P. Serious ophthalmological complications in the Ehlers-Danlos syndrome. Br J Ophthalmol 1970;54:263-268.*