Structure of Corneal Scar Tissue: An X-Ray Diffraction Study

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ABSTRACT Full-thickness corneal wounds (2 mm diameter) were produced in rabbits at the Schepens Eye Research Institute, Boston. These wounds were allowed to heal for periods ranging from 3 weeks to 21 months. The scar tissue was examined using low- and wide-angle x-ray diffraction from which average values were calculated for 1) the center-to-center collagen fibril spacing, 2) the fibril diameter, 3) the collagen axial periodicity *D*, and 4) the intermolecular spacing within the collagen fibrils. Selected samples were processed for transmission electron microscopy. The results showed that the average spacing between collagen fibrils within the healing tissue remained slightly elevated after 21 months and there was a small increase in the fibril diameter. The collagen *D*-periodicity was unchanged. There was a significant drop in the intermolecular spacing in the scar tissues up to 6 weeks, but thereafter the spacing returned to normal. The first-order equatorial reflection in the low-angle pattern was visible after 3 weeks and became sharper and more intense with time, suggesting that, as healing progressed, the number of nearest neighbor fibrils increased and the distribution of nearest neighbor spacings reduced. This corresponded to the fibrils becoming more ordered although, even after 21 months, normal packing was not achieved. Ultrastructural changes in collagen fibril density measured from electron micrographs were consistent with the increased order of fibril packing measured by x-ray diffraction. The results suggest that collagen molecules have a normal axial and lateral arrangement within the fibrils of scar tissue. The gradual reduction in the spread of interfibrillar spacings may be related to the progressive decrease in the light scattered from the tissue as the wound heals.

INTRODUCTION

The cornea forms part of the tough outer protective coating of the eye, and is also the primary refractive component. Transparency is generally believed to depend upon a restriction in the cross-sectional diameter of the collagen fibrils and on regularity in the packing of the collagen fibrils, which produces destructive interference of scattered incident light (Maurice, 1957; Hedbys, 1961; Benedek, 1971). The factors that restrict the growth of collagen fibrils are uncertain, although proteoglycans (Scott, 1984), hydroxylysine-linked disaccharides (Harding et al., 1980), and type V collagen (Linsemayer et al., 1993) have all been implicated. On the other hand, collagen fibril packing depends on the ground substance between the fibrils, which is composed primarily of proteoglycans and glycoproteins such as type VI and XII collagens. Proteoglycans play an important role controlling corneal hydration and are thought to be involved in the maintenance of fibril separation (Borcherding et al., 1975; Scott, 1985). An alteration in the structure or the relative amounts of the collagen or of the proteoglycans can result in a degradation of the optical properties of the cornea. The lack of transparency in human corneal scars has been ascribed to the increased thickness and diminished number of collagen fibrils in the tissues (Schwartz and Keyserlingk, 1969). Rabbit corneal scars, however, do not exhibit the large variation in collagen fibril diameter (Cintron et al., 1978).

Penetrating wounds in rabbit cornea heal after a prolonged period to form transparent tissue (Cintron and Kublin, 1977). However, transmission electron microscopy and biochemical analysis of collagen in the healing wounds showed that the dimensions of the fibrils and the pattern of collagen intermolecular cross-linking did not return to normal (Cintron et al., 1978). We have now used this rabbit model to measure a range of structural parameters using synchrotron x-ray diffraction. X-ray diffraction has the advantage over electron microscopy that the tissue hydration can be maintained during the x-ray exposure so that processing artifacts can be eliminated. Furthermore, x-rays, like light, pass through the entire corneal thickness, and the results thus represent averages throughout the corneal tissue, unlike electron microscopy, which is of necessity highly selective.

The low-angle x-ray pattern from the cornea has two components. The equatorial pattern consists of an intense first-order reflection from the packing of the constituent collagen fibrils followed by a series of weaker subsidiary maxima. (Goodfellow et al., 1978). The meridional pattern arises from the axial stagger between adjacent molecules, which produces the so-called *D*-periodicity observed in the electron microscope (Meek et al., 1981). The wide-angle pattern contains the usual collagen molecular reflections, including one corresponding to a Bragg spacing of about 1.5 nm that arises from the molecular packing within the fibrils (Meek et al., 1991). In this paper, we monitor changes in collagen molecular and fibrillar spacing and fibril diameters as a function of the age of the scar tissue to gain insight into the possible causes of light scattering from this tissue.

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MATERIALS AND METHODS

The production of scars was carried out at the Schepens Eye Research Institute, Boston, MA. At all times, animals were treated in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Adult rabbits weighing approximately 2.5 kg were anesthetized with an intravenous injection of sodium pentobarbital and topical application of proparacaine to each eye before corneas were wounded. A 2 mm diameter, full-thickness wound was made in one eye of each rabbit (Cintron et al., 1973); the other cornea was used as a control. Rabbits were allowed to heal for 3 and 6 weeks and 2, 7, 14, and 21 months and were then sacrificed with an overdose of sodium pentobarbital. Corneas were excised, wrapped tightly in cling film, and stored on ice while being transported from Boston to the synchrotron in the UK. The tissue hydration was monitored by weighing the corneas at frequent intervals during the experiments.

X-ray diffraction

Diffraction patterns were recorded at the Engineering and Physical Sciences Research Council synchrotron source at Daresbury, UK. The tissues were contained in a moist atmosphere in airtight cells to prevent dehydration. Low-angle patterns ($0.01\ nm^{-1} < R < 0.15\ nm^{-1}$) were recorded using a long camera (2.4–4 m), 90% of which was under vacuum to minimize air scattering. The effective beam dimensions were 0.5×2 mm and the x-ray wavelength was 0.154 nm. The first-order reflection in the equatorial pattern required an exposure of 30–120 s and the higher order required about 30 min. No visible specimen damage was apparent even after long exposures. Occasionally, more than one pattern was recorded from a specimen by repositioning the specimen within the beam between exposures.

Wide-angle patterns ($0.25\ nm^{-1} < R < 3.3\ nm^{-1}$) were recorded on a short camera (11–12 cm) filled with helium to minimize air scatter. The beam had a circular cross-section of diameter 0.5 mm and a wavelength of 0.1488 nm. The exposure time was 4–5 min.

The x-ray patterns were recorded on Caeverken Reflex 25 film (Caeverken, Strangness, Sweden), and the exposed films were scanned using an Ultrascan XL laser microdensitometer (LKB Instruments Inc., Gaithersburg, MD).

The average center-to-center fibril spacing was calculated from the first equatorial reflection in the low-angle x-ray pattern (Goodfellow et al., 1978, Sayers et al., 1982) The Bragg spacing was multiplied by a factor of 1.12 on the assumption that collagen fibrils are packed with liquidlike order (Worthington and Inouye, 1985). The shape of the profile of this reflection was used to obtain an estimate of the order in the fibril packing. This was done by normalizing for exposure time, subtracting the background (using a least squares polynomial fit), smoothing and converting the data to real space. The ratio of the maximum height of the peak to the width at half height (R_1) was calculated and expressed as absorbance units per nm. This ratio was independent of the specimen thickness and of the exposure of the x-ray films.

The subsidiary maxima in the low-angle pattern are thought to arise principally from the scattering by the individual cylindrical collagen fibrils with essentially no sampling by the interference function due to the fibril packing (Worthington and Inouye, 1985). Therefore, the first of these would be expected to occur at

$$R = 5.14/2\pi r_0 \quad (1)$$

where R is the reciprocal space coordinate of the reflection and r_0 is the fibril radius, and the numerical factor derives from a Bessel function (Vainshtein, 1966). The position of the maximum in the diffraction pattern, therefore, may be used to find R and hence estimate r_0 (Worthington and Inouye, 1985). The validity of using only one reflection for this estimate has been assessed previously (Meek and Leonard, 1993). The ratio of the height to the width at half height of the first subsidiary maximum (R_2) was computed to give an indication of how the diameter variation changed during scar formation.

The reflection near 1.5 nm in the wide-angle pattern from corneal stroma has been shown to originate from the packing of the individual collagen molecules within the fibrils (Meek et al., 1991). The Bragg spacing was multiplied by 1.11 (Maroudas et al., 1991) to generate the intermolecular spacing, on the assumption that the molecules are packed in a quasi-hexagonal array.

To assign uncertainty values to the x-ray data, two methods were used. Where the number of measurements was less than or equal to two, the uncertainty was estimated from the precision at which measurements could be made. Where three or more results were averaged, the standard deviation was calculated. Where possible, significance was assessed by Student's t-tests.

Electron microscopy

After the x-ray experiments had been performed, one scar from each age of healing was used for electron microscopy. The tissues were cut out and divided into four segments. These were then fixed in 2.5% (w/v) glutaraldehyde in 25 mM sodium acetate buffer, dehydrated through an ethanol series, and embedded in Polarbed (Agar Scientific Ltd., Stansted, Essex). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 410 transmission electron microscope (Eindhoven, The Netherlands). The number of collagen fibrils was counted in 10 1-μm^2 areas from anterior to posterior regions of the tissues, and the results were averaged. The significance of the results was assessed by Student's t-tests.

RESULTS

A series of collagen meridional reflections were present in the x-ray patterns from all the scar tissues. In every case D was 65 nm, the normal value for corneal collagen (Marchini et al., 1986).

The average interfibrillar spacings were calculated from the first-order equatorial reflections, and the results are presented in Table 1. After 21 months, the spacing was slightly elevated compared with normal ($p < 0.05$). It was clearly apparent from the x-ray patterns that the diffuseness of the diffraction rings varied according to the age of the scar. Fig. 1 shows a three-dimensional representation of a series of linear scans across the equatorial reflections (after normalization and background subtraction). Each scan represents a different time period, and the profile from a normal cornea is shown on the diagram for comparison. The gradually increasing intensity of the reflection as the tissue is remodeled is readily apparent. This may be caused by an increase in the number of nearest neighbors (i.e., in the packing density) of the fibrils but may also be affected by other factors such as the thickness of the specimen. To quantify the shape of each peak in Fig. 1, the ratio of the height to the width at half height was calculated, and these data, labeled R_1, are presented in

TABLE 1 The average interfibrillar spacing in rabbit corneal scar tissue and the ratio of peak height to peak width at half height (R_1)

Age of scar	Interfibrillar spacing	Number of diffraction patterns	Ratio, R_1 (10^{-2})
3 weeks	63.0 ± 5 nm	1	0.3
6 weeks	66.1 ± 5 nm	1	0.3
7 months	71.8 ± 0.6 nm	3	0.8
14 months	71.8 ± 2.8 nm	4	2.2
21 months	72.8 ± 2.5 nm	3	3.9
Control	66.3 ± 3.5 nm	2	6.9

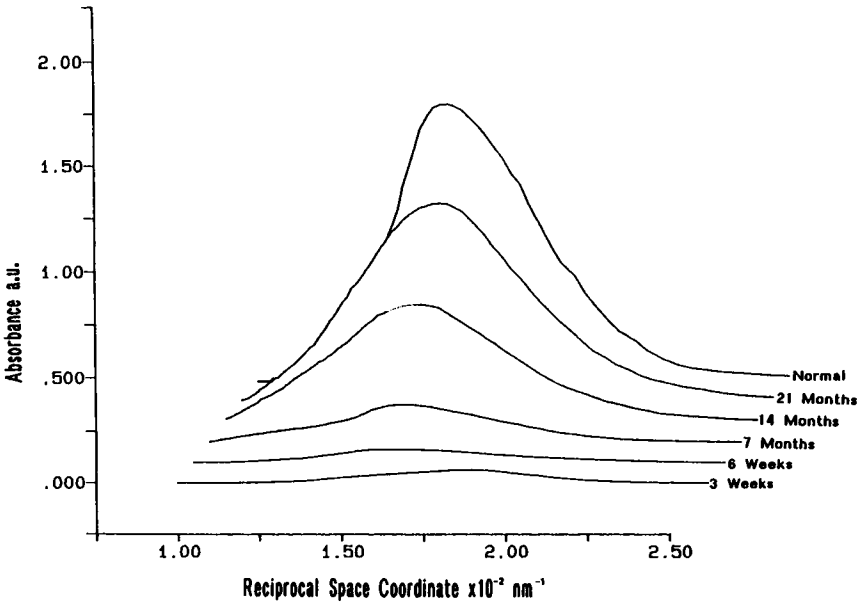

FIGURE 1 A 3D representation of a series of linear scans across the first-order low-angle equatorial reflections from scars of various ages and from normal rabbit cornea.

Table 1. R_1 was very low in 3- and 6-week-old scars, indicating a wide variation of nearest neighbor interfibrillar spacings. As the age of the scar increased, R_1 increased, but after 21 months it had not returned to normal. The increase in R_1 indicates a measurably higher degree of interfibrillar order with increasing age of the scar, although the average interfibrillar spacing remained slightly above normal. Visual examination of the scar tissue confirmed previous observations (Cintron and Kublin, 1977; Cintron et al., 1978; Hassell et al., 1983). After 3 or 6 weeks the scar tissue was very opaque, whereas after 21 months it was only slightly opaque.

The average fibril diameter was calculated for normal and scar tissue at physiological hydrations from the first subsidiary maximum in the low-angle pattern (Table 2). For the control tissue, the mean diameter was 41.7 ± 0.4 nm, whereas in scar tissue it was generally slightly higher ($p < 0.05$). The change in the spread of diameters with increasing age of the scar was quantified by calculating the ratio of peak height to peak width at half height, R_2. The results indicated that after 14 months of wound healing, within the experimental resolution, the range of fibril diameters found in scar tissue had returned to normal (Table 2).

Wide-angle diffraction was used to calculate the intermolecular spacing of the collagen in the full-thickness scar tissue. At each age examined, the scar tissue had a lower intermolecular spacing than the controls (Table 3). The reduction was significant at 3 weeks ($p < 0.03$) and 6 weeks ($p < 0.02$) but was not significant thereafter. It was interesting to note that all the wide-angle patterns contained reflections not seen in the controls; the most prominent of these occurred at ~0.45 and 0.90 nm. The intensities of these extra reflections reduced with increasing age of scar.

The intermolecular spacing of the collagen molecules in a fibril depends on the hydration of the fibrils: when the cornea dries, the intermolecular spacing reduces (Fullwood et al., 1992). A control rabbit cornea and a 3-week and a 2-month corneal scar were dried in a hot air drying cabinet until the weight became constant. The wide-angle x-ray patterns were then produced and the intermolecular spacings were calculated (Table 4). On drying, the intermolecular

TABLE 2 The average fibril diameters of collagen in rabbit corneal scar tissue, and the ratio of peak height to peak width at half height (R_2) of the first subsidiary equatorial reflection

Age of scar	Average diameter	Number of diffraction patterns	Ratio, R_2 (10^{-2})
6 weeks	>39.2 nm	2	
7 months	43.5 ± 0.9 nm	2	0.11
14 months	42.9 ± 1.0 nm	4	0.16
21 months	43.4 ± 0.7 nm	2	0.17
Control	41.7 ± 0.4 nm	2	0.16

TABLE 3 The intermolecular spacing in rabbit corneal scar tissue (hydrated)

Age of scar	Average intermolecular spacing	Number of diffraction patterns
3 weeks	1.67 ± 0.06 nm	2
6 weeks	1.62 ± 0.06 nm	2
7 months	1.72 ± 0.06 nm	2
14 months	1.75 ± 0.04 nm	4
21 months	1.75 ± 0.06 nm	2

TABLE 4 The intermolecular spacing in rabbit corneal scar tissue (hydrated and dry)

Age of scar	Intermolecular spacing (wet)	Intermolecular spacing (dry)	Number of diffraction patterns
3 weeks	1.67 ± 0.07 nm	1.60 ± 0.03 nm	1
2 months	1.69 ± 0.06 nm	1.47 ± 0.03 nm	1
Control	1.78 ± 0.06 nm	1.42 ± 0.02 nm	1

Only one x-ray pattern was obtained from each specimen.

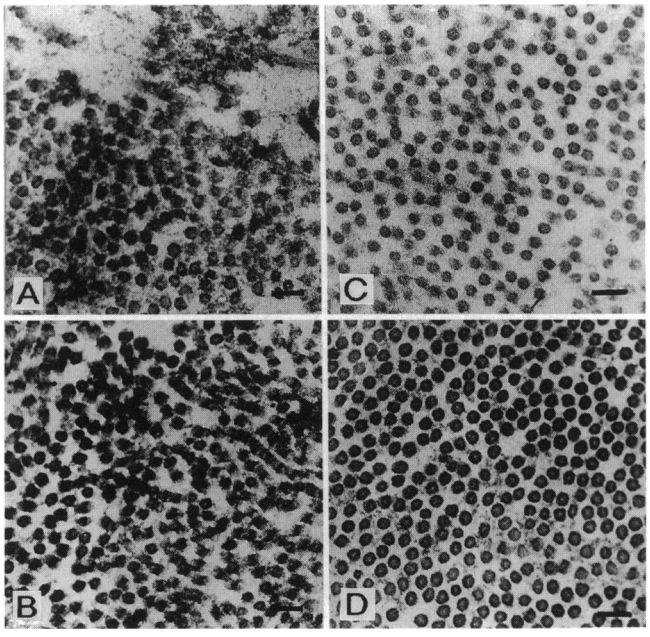

FIGURE 2 Cross-sections of collagen fibrils in rabbit scar tissues (A, B, C) and normal rabbit (D). The ages of the scars were: (A) 3 weeks, (B) 7 months, (C) 14 months. (Bar = 100 nm).

spacing of the normal cornea reduced by 20%, whereas the intermolecular spacing of the scar tissues reduced by only 4% (3 weeks) and 13% (2 months).

After the x-ray diffraction experiments were complete, several of the scars were embedded and sectioned for electron microscopy. One scar from each age of healing was used, except for 14 months where two were used (a and b; see Table 5). From images of cross-sections such as those in Fig. 2, the number of collagen fibrils were counted in $10 \times 1\ \mu m^2$ regions, and the results were averaged (Table 5). The results showed that the density of collagen fibrils significantly increased ($p < 0.001$) in scar tissue from 3 weeks to 21 months, but failed to reach that of the control cornea.

DISCUSSION

Transparency of the cornea is thought to be due to the narrow uniform diameter of the collagen fibrils and the ordered packing of the fibrils. Hart and Farrell (1969) and Benedek (1971) have shown theoretically that short-range order in the corneal stroma should be sufficient to ensure transparency. The results from the work on the packing arrangement of the cornea by Sayers et al. (1982) confirm the presence of order extending to no more than ~120 nm. The loss of corneal transparency in corneal scar tissue may be due to a number of factors: 1) a loss of short-range order of the collagen fibrils, 2) a change in the number density of the fibrils, 3) an increase in diameter of some of the collagen fibrils (scattering goes as the fourth power of the fibril diameter (Benedek, 1992)), 4) a change in the refractive index of the ground substance or of the collagen fibrils, and 5) the presence of "lakes" (areas devoid of collagen fibrils; such areas would be expected to scatter light if they are greater than half the wavelength of light. (Benedek, 1971)).

TABLE 5 The number of collagen fibrils per μm^2 measured from transmission electron micrographs of rabbit corneal scar tissue

Age of scar	Fibril number (per μm^2)*
3 weeks	161.3 ± 46.4
6 weeks	168.0 ± 31.6
7 months	211.9 ± 40.3
14(a) months	217.2 ± 38.0
14(b) months	316.0 ± 77.5
21 months	230.3 ± 49.9
Control	326.2 ± 69.7

* ± refers to the standard deviation

Diffraction from the collagen fibrils shows that their arrangement was highly disordered in the early stages and gradually became more ordered as the age of the scar increased (Table 1). However, a normal fibril arrangement was not achieved after 21 months healing. The increase in the interfibrillar spacing suggests a reduction in the packing density compared with normal cornea. The reduced intensity of the first-order equatorial reflection is probably due to an increase in the variation of nearest neighbor distances possessed by each fibril. Both these results are consistent with the electron microscope observations that showed a reduced but gradually increasing fibril number density that, after 21 months, failed to return to normal.

The x-ray diffraction showed minimal changes in the average fibril diameters in scar tissue from those in the controls (Table 2). However, the results suggest that in early scar tissue, the range of fibril diameters is greater than that present in normal tissue, but after extended healing it returns to normal (within the experimental resolution). The present observations confirm previous electron microscopic studies showing that the average collagen fibril diameter does not change markedly from normal (Cintron et al., 1978). Furthermore, although we have shown that the range in the fibril diameters is increased in scar tissue up to 14 months relative to normal adult cornea, previous ultrastructural studies have demonstrated that this range remains greater than normal for 1.5 years (Cintron et al., 1978). This difference might be due to biological variability or to the limited number of specimens we were able to examine.

The x-ray data indicate that collagen molecules have a normal axial arrangement within the fibrils of scar tissue (D = 65 nm) and a near normal lateral spacing (Table 3) suggesting that the hydration of the collagen fibrils is close to its physiological level. Therefore the increased collagen fibril diameters are due either to a different molecular arrangement or to a larger number of collagen molecules per fibril, and the latter would indicate that the collagen fibrils have a normal refractive index. When a 3-week scar tissue was dried, the reduction of the intermolecular spacing was less compared with dry normal tissue. This reduction in the intermolecular spacing was closer to normal in dry 2-month scar tissue. It appears, therefore, that in early scar tissue,

factors controlling the collagen molecular arrangement are altered. In scar tissue the composition of the fibrils is known to change. It has been shown that there is an increase in type V collagen relative to type I collagen in healing corneal tissue (Cintron et al., 1981, 1988). Chick corneal collagen fibrils are composed of type I and V collagen (Linsenmayer et al., 1993). It has been suggested by these authors that the fibril diameter is partially determined by the proportion of type I and V collagen in the heterotypic fibrils. This may also alter the response of the lateral spacing of the collagen molecules to drying. Other factors may also contribute, such as the number and nature of the intermolecular bonds (Cannon and Cintron, 1975) and the level of glycosylation (Cintron, 1974).

The additional wide-angle reflections (corresponding to Bragg spacings of 0.45 and 0.90 nm) may be related to similar reflections previously reported from macular dystrophy corneas (Quantock et al., 1992). Macular dystrophy is a corneal condition caused by a storage disorder of proteoglycans. The corneal stroma displays certain ultrastructural similarities to that of scar tissues, including the presence of "lacunae" and large chondroitin/dermatan sulphate filaments when visualized with the cationic dye Cuprolinic blue. It is thought that these high-angle reflections may arise from the sugar repeat distances in these large proteoglycan molecules (Quantock et al., 1992).

The visual observations clearly showed that the scar tissue under study was opaque, but with increasing age the transparency of the tissue improved. However, even at 21 months the transparency of the tissue was still reduced compared with normal. Electron microscopy has shown that in early stages of scar tissue development, (up to a few months), there are considerable areas free of collagen fibrils that would scatter light and cause the tissue to be opaque. In the 7- to 21-month scar tissue the light scattering may result from the presence of lakes in the tissue, an increase in the fibril diameter or the loss of short-range order. The results suggest that the fibril diameter, though increased, would not increase light scattering sufficiently to effect transparency to the extent noticed. Electron microscopy has shown that lakes persist in scar tissue up to 9 months but these do not appear to be of sufficient size and number to affect transparency greatly. The x-ray reflection that changed most with increasing age was the first-order equatorial reflection in the low-angle pattern. From our interpretation of the changes in this reflection it seems that, as the scar tissue increases in age, it is the level of fibril order that increases, and with it the packing density and the transparency of the tissue. Fibril order is dependent on the interaction of molecular components between the fibrils. Recent analyses of corneal stromal "ground substance" have shown that, in addition to proteoglycans, type VI and XII collagens are present (Takahashi et al., 1993; Oh et al., 1993, Cintron et al., 1994). These interfibrillar collagens associate with proteoglycans and fibrillar collagens, respectively, and may play an important role in stabilizing the collagen fibril structure. Future studies will examine the interactions of these interfibrillar macromolecules in healing corneas.

Data correlating the return of normal proteoglycan synthesis with decreases in opacity have been previously published (Hassell et al., 1983). These studies suggested that collagen interfibril order is important in corneal transparency. The present study tests this hypothesis by showing that collagen interfibril order does change with time consistent with the decrease in tissue opacity. Furthermore, the present study shows that neither fibril diameter nor intermolecular spacing of the fibrillar collagens are related to corneal opacity during wound healing as previously believed.

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