Light-Scattering and Ultrastructure of Healed Penetrating Corneal Wounds

Russell L. McCally,¹,² David E. Freund,¹ Andrew Zorn,³ Jennifer Bonney-Ray,¹ Rhonda Grebe,² Zenaida de la Cruz,² and W. Richard Green²

PURPOSE. To investigate quantitatively for the first time the relationship between light-scattering and ultrastructure of semitransparent scars resulting from penetrating wounds in rabbit cornea.

METHODS. Penetrating wounds, 2 mm in diameter, were made in the central cornea and allowed to heal for 3.6 to 4.5 years at which time the rabbits were killed. The scar and corneal thickness outside the scar were measured using ultrasonic pachymetry. Corneas were excised immediately and their transmissivity was measured from 400 to 700 nm. The tissue was then prepared for transmission electron microscopy. Transmission electron micrographs (TEMs) were analyzed to determine fibril positions and radii. Scattering was calculated using the direct summation of fields (DSF) method.

RESULTS. Scar thickness averaged 0.26 ± 0.04 mm, and the scars were flat. Thickness outside the scars averaged 0.40 ± 0.04 mm. Three scars were moderately transparent, five were less transparent, and one was much less transparent. The wavelength dependence of the measured total scattering cross-section was indicative of the presence of voids (lakes) in the collagen fibril distribution, and lakes were evident in the TEMs. The images showed enlarged fibrils and some showed bimodal distributions of fibril diameters. Calculated scattering was characteristic of that expected from regions containing lakes—a finding consistent with the scattering measurements.

CONCLUSIONS. Despite the long healing time, these scars remained highly scattering. A combination of lakes, disordered fibril distributions, and a significant population of enlarged fibrils can explain the scattering. A possible cellular contribution cannot be ruled out. (Invest Ophthalmol Vis Sci. 2007;48:157–165) DOI:10.1167/iovs.06-0935

Charles Cintron adapted a model originally developed by Heydenreich¹ to investigate healing of penetrating corneal wounds.²⁻⁴ This highly successful model consisted of removing a centrally located, 2-mm diameter, full-thickness button. Such wounds heal by first forming a fibrin clot, after which cells invade and ultimately produce an avascular network of collagen fibrils.²⁻⁵ The model had advantages of simplicity, reproducibility of healing with minimal complications, and the production of scar tissue suitable for analysis.⁶ Of note, it has been reported that the initially opaque scars ultimately become “transparent.”²⁻⁵ This and other biochemical observations have led to the initial suggestion that rabbit cornea exhibited a capability for true regeneration of stromal tissue.³ However, more extensive investigations showed that the regeneration was incomplete at the biochemical and ultrastructural level,⁷ and the statement that the wounds became transparent was qualified to their becoming less opaque and sometimes transparent.⁵

Two primary physical factors lead to the transparency of normal corneas—a third factor—the thinness of the normal cornea—is less applicable to the scars that are the subject of the present investigation.⁶ First, collagen fibrils in the stroma are weak scatterers because their radius is much smaller than the wavelength of visible light, and their refractive index is close to that of their surroundings. Second, destructive interference among the fields scattered by different fibrils reduces the scattering from that which would occur if the fibrils scattered independently of one another. A quantity called the total scattering cross-section incorporates both of these effects.⁷⁻⁹ It can be determined (within a multiplicative constant) from measurements of transmissivity.⁵,¹⁰,¹¹ The total scattering cross-section is proportional to a quantity known as the interference factor, which shows how much the scattering is reduced. It depends on the spatial distribution of the collagen fibrils about one another and, to a lesser extent, on the spatial distribution of fibril scattering strengths.⁹ The interference factor is central to most transparency theories.⁷,¹²⁻¹⁶ It can be calculated from the fibrillar structures shown in transmission electron micrographs (TEMs) and it provides a quantitative measure of the degree (or lack thereof) of fibrillar ordering.⁹ Alterations in the spatial distribution of fibrils that reduce destructive interference, or changes that cause the fibrils to scatter more efficiently, reduce transparency and, if severe, lead to corneal opacity. In normal cornea, keratocytes do not contribute significantly to light-scattering, except under the special condition of specular-scattering, which occurs in reflective confocal microscope images.⁶,¹¹,¹⁷,¹⁸ Because at present there is no quantitative theory to describe scattering from corneal cells,¹⁸ it is not possible to separate their possible contribution to reduced transmissivity in corneal wounds from that of the disrupted fibrillar matrix.

In this article, for the first time, we relate experimental measurements of light scattering from healed penetrating wounds to the ultrastructure depicted in TEM of the wounds via calculations of the total scattering cross section and the interference factor.

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MATERIALS AND METHODS

Animals

In conducting the experiments, we adhered to the ARVO Statement for the Use of Animals in Research. Two-millimeter-diameter penetrating wounds in the central corneas of five New Zealand White rabbits were produced at different times in Charles Cintron’s laboratory at the Schepens Eye Research Institute, as described previously. The rabbits were ultimately delivered to the Wilmer Eye Institute where they were housed until the time of the light-scattering experiments (3.5–4.5 years after wounding).

Before being killed for light-scattering measurements, rabbits were anesthetized with an intramuscular injection of xylazine and ketamine hydrochloride in the proportions: 60% of 20 mg/ml xylazine to 40% of 100 mg/ml ketamine by volume. They were killed by an overdose of pentobarbital/phenothion sodium (Beuthanasia; Schering-Plough Animal Health, Omaha, NE) administered in an ear vein and the eyes enucleated. The scar thickness and the thickness of the adjacent unwounded cornea were measured with an ultrasonic pachymeter, and the scars were photographed. The corneas were then excised with a scleral ring and mounted as described previously. They were bathed in physiologic saline (BSS; Alcon, Fort Worth, TX) during the entire excision and mounting procedure. After the corneas were mounted, the hydrostatic pressure across the specimen was raised to 18 mm Hg, and the mounted cornea was inserted in the scattering apparatus where it also was bathed in physiologic saline. Transmission at 500 nm was monitored for 10 minutes before measurements were taken, to ensure the stability of the preparation.

Transmission Measurements

Transmission measurements were made in an apparatus similar to that described in Farrell et al. except for several improvements (primarily in the electronics). The improvements have not altered the optics that ensure that the incident and transmitted light beams have very low angular divergences and that the collection optics minimize the collection of forward scattered light.

The transmissivity, \( F_\lambda \), at a wavelength \( \lambda \) is given by

\[
F_\lambda = \frac{I_\lambda}{I_\infty} = \exp(-\rho_\sigma_\lambda t),
\]

where \( I_\infty \) is the irradiance of the light transmitted through the cornea, \( I_\lambda \) is the irradiance of the light incident on the cornea, \( t \) is the thickness of the cornea, \( \rho \) is the number density of fibrils in a typical lamella, and \( \sigma_\lambda \) is the total scattering cross-section. To account for cornea-to-cornea variations in scar thicknesses, we average the quantity \(-\ln F_\lambda \lambda \)/t = \( \rho \sigma_\lambda \). In the Results section, it is shown that the values of \( \rho \sigma_\lambda \) vary from scar to scar, but that they can be broken into three groups to facilitate discussion.

Electron Microscopy

Immediately after the scattering measurements, the mounted corneas were removed from the apparatus, and while still under pressure, phosphate-buffered 1% glutaraldehyde 4% formaldehyde fixative was dripped on the anterior surface for 7 minutes. At this point, the cornea was sufficiently rigid to retain its shape when it was removed from the mount. Fixation was continued overnight followed by postfixation in 1% phosphate-buffered osmium tetroxide for 2 hours. Corneas were then dehydrated in a graded series of ethanol, stained with uranyl acetate in 100% ethanol, passed through two changes of propylene oxide and infiltrated in a 1:1 propylene oxide and resin mixture (modified Luft’s medium [based on LX 112; Ladd Research Industries, Burlington, VT, and dodecenylsuccinic anhydrid DDSA]) overnight. The infiltration process was continued by immersing the samples in a 100% resin mixture in a vacuum for 2 hours. Samples were then polymerized overnight at 37°C and then at 58°C to 66°C for 2 days. Ultrathin sections were doubly stained with tannic acid-uranyl acetate and lead citrate solutions. Tannic acid is an improved stain for elastin and collagen fibers. Samples were examined and photographed with a transmission electron microscope (JEM 100B; JEOL, Tokyo, Japan).

Image Analysis and Scattering Calculations

Micrographs (magnification 64,800×) were scanned at 300 dpi on a scanner (PowerLook 1000; UMAX, Milton Keynes, UK). This resolution ensured that the smallest fibrils would be at least 8 pixels in diameter. Image analysis was performed on a computer (Macintosh, Apple Computer, Cupertino, CA) using an augmented version of the public domain NIH Image program (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The general outline of the image-processing algorithm that was used to obtain the fibril positions and their radii has been described previously.

Light-scattering was calculated from fibril positions and radii by using the direct summation-of-fields (DSF) method. This method enables the estimation the ensemble average of the total scattering cross section and the interference factor by using a single TEM, and it can be applied to assemblies of fibrils that have arbitrary spatial distributions and arbitrary distributions of diameters, as those encountered in scarred corneas. The calculations herein account for the individual fibril diameters as described by Freund et al.

Estimates for the refractive indices of the fibrils and extracellular matrix were obtained using the Gladstone-Dale law of mixtures and the refractive increment law, as described previously. Calculations of refractive indices require assumptions of collagen concentration, how it is distributed in the fibrils, and the concentration of components in the extracellular matrix. These quantities are unknown for these scars; thus, for the purposes of this study, we assumed that they are the same as for normal cornea. We also assumed that the fibrils contain a fixed percentage of collagen; thus, all fibrils have the same index of refraction—indeed, independent of their diameters.

RESULTS

Despite the long healing time, these wounds remained highly scattering. Figure 1 shows examples from the three scattering groups that were identified as described later. It is apparent that scattering in the scars is not uniform. The narrow slit photograms also show that the scarred region is flat and thinner than the adjacent unwounded cornea. The average thicknesses of the scars and adjacent cornea were respectively 0.26 ± 0.04 and 0.40 ± 0.04 mm. Others have obtained similar results for scar thickness in healed penetrating wounds.

Table 1 lists values of \( \rho \sigma_\lambda \) obtained from transmission measurements on the nine scarred corneas that were investigated. (The cornea from the left eye of rabbit C4 was damaged and could not be used.) These results show that \( \rho \sigma_\lambda \) from the identically prepared scars are in three distinct groups as delineated in the table. Group 1 had the lowest scattering. The average \( \rho \sigma_\lambda \) in group 2 is 1.74 times greater than the group 1 average at 400 nm and 1.56 times greater at 700 nm. The Wilcoxon-Mann-Whitney test showed that the differences in average values are significant at all wavelengths (\( P < 0.04 \)). The single cornea in group 3 had the highest values of \( \rho \sigma_\lambda \)—ranging from 1.5 times greater than the group 2 average at 400 nm to 2.38 times greater at 700 nm.

Figure 2 shows the average fraction of light transmitted, \( \langle F_\lambda \rangle \), as a function of wavelength for the three groups. \( \langle F_\lambda \rangle \) was determined from the average of \( \rho \sigma_\lambda \) in Table 1, assuming that the scars had the average thickness noted earlier (i.e., \( t = 0.26 \) mm). The effect on transmissivity resulting from the differences in scattering in the three groups is obvious.

Figure 3 shows that the quantity \( \lambda^2 \rho \sigma_\lambda \) has a linear dependence on \( \lambda \) (i.e., it can be expressed in the form \( A + BA \), which
is a straight line of slope \( B \). This implies that the total scattering cross-section has the functional form

\[
\sigma(\lambda) = \frac{A}{\lambda^2} + \frac{B}{\lambda^3}
\]

where \( A \) and \( B \) are constants. The first term in equation 2 has the same wavelength dependence as the scattering cross-section of an isolated fibril and is characteristic of fibrils having either short-ranged order or homogeneous disorder in their positions.\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\)\(^18\)\(^19\)\(^20\)\(^21\)\(^22\)\(^23\)\(^24\) The existence of the second term in equation 2 has been associated with the presence of regions in the stromal lamellae that are devoid of fibrils.\(^10\) Such regions have often been called “lakes.”\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\) Their effect is to increase scattering.\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^25\)\(^26\) Moreover, the fact that the slopes (i.e., the values of \( B \)) become progressively greater from group 1 to group 3 suggests that void regions become more prevalent in the corneas having greater scattering.

TEMs from corneas in the three groups were analyzed to characterize the fibril distributions and to determine fibril positions and diameters. As discussed later, these data (from suitable TEMs; note that the DSF method, which is used to calculate scattering, requires that there be a rectangular region containing ~1000 or more parallel fibrils [in cross section], to obtain adequate statistics) were used to calculate the scattering that would be expected from the depicted structures. Figure 4 shows four of the TEMs that were analyzed. Fibril positions and diameters were obtained from the entire depicted regions; the rectangles outline the regions that were subsequently used in the DSF light-scattering calculations. Figure 5 shows the distributions of fibril diameters from the TEM in Figure 4. Figure 4a from the midstromal region of a cornea in Group 1 shows several small lakes, which confirms the prediction of their existence based on the scattering measurements. Of note, the distribution of fibril diameters obtained from this micrograph (cf., Fig. 5a) is bimodal (i.e., it has two distinct peaks). To our knowledge bimodal distributions of fibril sizes have not been previously observed in either scarred or normal cornea. Figures 4b and 4c, respectively, from the anterior and posterior regions of corneas in group 2, show several lakes, and 4b shows a population of very large fibrils. The diameter distributions from these micrographs are unimodal (cf., Figs. 5b and 5c). Figure
4d is from the anterior stroma of the cornea in group 3. This region contains large lakes, a broad distribution of fibril diameters (cf., Fig. 5d), and disorganized fibrils.

Table 2 summarizes the fibril statistics from all the TEMs that were analyzed. It shows that several had bimodal diameter distributions. The average position of the distribution peaks for the micrographs in group 1 having bimodal distributions are at 325 Å and 400 Å, whereas the average position of the peaks for those in group 2 are at 330 Å and 470 Å.

It is important to note the TEMs chosen for fibril analysis and from which scattering could be calculated have more fibrillar order than was typical throughout the scars. Many regions in the scars had a much less order arrangement of fibrils. Such distributions are not amenable to DSF scattering calculations, but are consistent with the high level of scattering found in the measurements. Although not presented herein, lower-magnification micrographs from all three groups show regions of disorganized lamellar structures, deposits of granular material, and some partially degenerated keratocytes, some of which contain vacuoles filled with granular material. In general, all these characteristics are more prevalent in the corneas in groups 2 and 3 and are consistent with the higher scattering observed in these groups.

The DSF method9,21 was used to calculate the total scattering cross-section for rectangular regions in the micrographs listed in Table 2 which contained ~1000 or more fibrils. The calculated total scattering cross sections and the fibril number densities obtained from the TEM were used in equation 1 to determine the fraction of light that would be transmitted through corneas assumed to have the structures depicted in the rectangular regions that were analyzed. The calculations also assumed a corneal thickness of 0.26 mm (i.e., the average thickness of the scars). The average values of the product of the fibril number density and the computed total scattering cross-section, \( \langle \rho r(\lambda) \rangle \), were determined for the micrographs in each of the three groups and were used to compute \( \langle F_\lambda \rangle \) for each of the groups. These results are shown in Figure 6. The Wilcoxon-Mann-Whitney test showed that the differences in \( \langle \rho r(\lambda) \rangle \) for the micrographs in groups 1 and 2 are significant at all wavelengths \( (P < 0.035) \). Because only two micrographs from the cornea in group 3 were analyzed, no statistical test could be performed; however \( \langle \rho r(\lambda) \rangle \) for the group-3 cornea is five or more times the group-2 average at all wavelengths.

Figure 7 displays the wavelength dependence of the average total scattering cross section for the three groups. In agreement with the experimental results in Figure 3, the calculated values of \( \langle (\lambda/550)^2 \rho r(\lambda) \rangle \) for each group also have a linear dependence on wavelength and the slopes become progressively greater from group 1 to group 2 to group 3. This result is consistent with TEMs from the three groups.

**DISCUSSION**

Cintron et al.2,5 reported that scars from 2-mm penetrating corneal wounds became less opaque and in some cases appeared

**Table 1. Experimental Scattering Levels**

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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</thead>
<tbody>
<tr>
<td>C4R*</td>
<td>C8L</td>
<td>C11R</td>
</tr>
<tr>
<td>λ (nm)</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>400</td>
<td>2.841</td>
<td>3.188</td>
</tr>
<tr>
<td>600</td>
<td>1.054</td>
<td>1.656</td>
</tr>
<tr>
<td>650</td>
<td>0.941</td>
<td>1.512</td>
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Data are \( \rho r \), expressed in inverse micrometers.
* Column headings are cornea scar number with the wound age in years.
transparent after healing 1.5 to 2 years. This article presents the first quantitative measurements of light-scattering and their relationship to ultrastructure of such scars. Transmissivity in normal rabbit cornea ranges from 0.88 at 400 nm to 0.97 at 700 nm.10 The scars from the lowest scattering group had transmissivities ranging from 0.39 at 400 nm to 0.69 at 700 nm (cf., Fig. 2), values comparable to those of rabbit corneas swollen to twice the normal thickness.10 Therefore, although these scars were less opaque than in their early stage of healing (photographs not shown), even those in the lowest-scattering group did not approach what could reasonably be described as being transparent. It is important to note, however, that measurements of transmission average over the diameter of the incident beam (\(1/11011\) mm) and through the entire depth of the cornea. Slit-lamp views of the scars (cf., Fig. 1) show that the scars were not homogeneous. They had some regions that scattered much less than others. It also is noteworthy that scars from opposite eyes of the same rabbit were in different scattering groups for three of the four pairs of corneas (cf., Table 2). Moreover, some scars in Group 2 had healed for the same time as the scars in Group 1. This suggests that the healing process and partial recovery of transparency is probably influenced primarily by slight (unintended) variations in the initial wounding.

The total scattering cross-section of normal transparent rabbit cornea varies as \((1/\lambda^3)\).6,10,11,15,25,26 This dependence, which is the same as that of an isolated fibril, is indicative of short-ranged ordering in the fibril positions.9,15,16,25 However, if fibril positions were to become disordered so that the fibrils scattered independently of one another, scattering would increase because there would be less destructive interference, but the scattering cross section would still vary as \((1/\lambda^3)\).6,14,15,25 The scattering data in Figure 3 are inconsistent with this mechanism for explaining the high level of scattering from the scars; because, if this were the case, the product \((\lambda/550)^3 \langle \rho_r(\lambda) \rangle\) would have been a constant, independent of wavelength. As discussed previously, the results in Figure 3 show that the measured total scattering cross-sections for the scars have the functional form \(A/\lambda^3 + B/\lambda^2\). This form is indicative that the scars contain intralamellar fibril-free regions (vis., “lakes”) surrounded by regions of disordered fibrils.6,10,25,27 Other investigators have reported that lakes persist in scar tissue up to 9 months, but they stated that they did not appear to be of sufficient size and number to affect transparency.29 In our study, the lakes persisted up to 4.5 years (Fig. 4) and the scattering data in Figure 3 suggest that, in fact, lakes are a significant factor producing the high level of scattering in the scars.

The wavelength dependence of the calculated scattering is consistent with the wavelength dependence determined from the measurements (cf., Figs. 5, 7). Both the calculations and measurements show that the quantity \((\lambda/550)^3 \langle \rho_r(\lambda) \rangle\) depends linearly on \(\lambda\), and that the magnitude and slopes of the linear fits increase from group 1 to group 2 to group 3. The
calculated values of $\sigma_0(\lambda)$ are much lower, however, than the measured ones. Consequently, the calculated transmissivities are much greater than the measured values (Figs. 2, 6). This is because the measurements probe the entire depth of tissue, including all the highly disorganized regions noted previously. Thus, the lack of quantitative agreement would be expected because, as noted previously, the DSF method can only be applied to regions that contain a sufficient number ($\geq 1000$ or more) of parallel fibrils. The DSF method implicitly assumes that the fibrils shown in cross section, which are used in the calculations, are arranged parallel to one another over a reasonable distance (at least several wavelengths). Light waves scattered from parallel fibrils can interfere, whereas waves scattered from nonparallel or tangled fibrils cannot. As noted in the Introduction, destructive interference among the waves scattered from the parallel fibrils in the stromal lamellae is a major factor underlying corneal transparency. Nonparallel or tangled fibrils scatter independently of one another and greatly increase the overall scattering. We are unaware of any theory for calculating scattering from tangled fibrils.

**FIGURE 5.** (a–d) Fibril diameter distributions from the micrographs in Figures 4a–d. The distribution from cornea C8LM15983 in (a) is bimodal, with distinct peaks at 320 Å and 390 Å. Bimodal diameter distributions have not been reported previously for such scars.

<table>
<thead>
<tr>
<th>Table 2. Fibril Statistics</th>
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<tbody>
<tr>
<td>Cornea/EM</td>
</tr>
<tr>
<td>C4RM/15993*</td>
</tr>
<tr>
<td>C4RP/15995*</td>
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<tr>
<td>C8LM/15984*</td>
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<tr>
<td>C8LM/15989*</td>
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<tr>
<td>C8RM/15987*</td>
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<td>C8RM/15988*</td>
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* TEMs used for DSF calculations.
The total scattering cross-section $\sigma_t(\lambda)$ can be written in the form

$$\sigma_t(\lambda) = \langle \sigma_n(\lambda) \rangle \sigma_s(\lambda).$$  (3)

where $\langle \sigma_n(\lambda) \rangle$ is the total scattering cross-section of an average isolated collagen fibril and $\sigma_s(\lambda)$ is the interference factor that measures the degree of order in the spatial arrangement of fibril axes and fibril sizes.\(^9,21\) The magnitude of $\sigma_s(\lambda)$ provides a quantitative measure of the degree of fibrillar ordering.\(^6,9,30\)

As used herein, it also accounts for variability in scattering strengths that result from differences in fibril sizes.\(^9\) The interference factor can have values between zero (corresponding to crystalline order) and one (corresponding to random order).\(^30\)

In normal rabbit cornea $\sigma_s(\lambda)$ is $\sim 0.11$ in the anterior stroma and $\sim 0.085$ in the posterior stroma, and it is essentially independent of wavelength in both regions.\(^9\) These values are indicative of a substantial degree of fibrillar ordering in the normal transparent cornea. In contrast, the average values of $\sigma_n(550)$ obtained from the DSF calculations are, respectively, $0.18 \pm 0.13$, $0.38 \pm 0.22$, and $0.80 \pm 0.33$ for groups 1, 2, and 3. Thus, all groups have less fibrillar order than normal cornea. The progressive increases in the average values of $\sigma_n(550)$ from groups 1 to 3 indicate that disorder in the spatial distribution of fibrils and the resultant decrease in destructive interference in the scattered fields, also is an important factor in the increased scattering.

The mean fibril diameters in Table 2 are in close agreement with the larger of the reported values for normal rabbit cornea\(^9,31,32\) (however, the 476 Å diameter for C25RA15675 is an exception, in that it is larger). The mean diameters are also in reasonable agreement with earlier measurements obtained from TEMs of 1.5-year-old scars\(^2\) and with x-ray diffraction measurements of 21-month-old scars.\(^29\) In general, however, the diameter distributions in the scars are much broader than those in normal rabbit cornea, as evidenced both by their standard deviations and by their ranges. The standard deviations in Table 2 range from 27 Å (which was for a cornea in group 1) to 115 Å, whereas those for normal cornea range from 20 Å to 28 Å.\(^9\) Rawe et al. reported an SD of 70 Å based on their x-ray diffraction measurements of a 21-month-old scar.\(^29\) The range of diameters obtained in this study is greater than Cintron et al.\(^2\) found in 1.5 year old scars (100–500 Å), perhaps because we analyzed more micrographs and larger regions. Table 2 shows that most of the TEMs we analyzed contained populations of fibrils with diameters greater than 500 Å.

Bimodal distributions of fibril diameters have not been reported for scars of this type. Fibrils having diameters near the low peak of the bimodal distributions found in this study have diameters within the range reported in normal cornea. However, the diameters of fibrils near the high-diameter peak for the group 2 corneas are greater than any that have been found in normal corneas. Moreover, the number of fibrils near the high peak in both groups 1 and 2 is greater than those near the low peak for every cornea except one. Age is unlikely to be a factor in explaining either the broad diameter distributions or the bimodal diameter distributions. Kanai and Kaufman\(^33\) found that collagen fibril diameters in aged (80-year) human corneas are essentially the same as in younger corneas; whereas Daxer et al.\(^34\) found fibril diameters of 308 ± 10 Å and 322 ± 10 Å for human corneas that were, respectively, less than and greater than 65 years of age. Both of these values are within the range of those reported for normal human cornea and both have small standard deviations.

The x-ray data suggest that the fibrils in older scars produced in an identical manner as this work have hydration levels close to normal. This led Rawe et al.\(^29\) to conclude that the larger fibrils either have a different molecular arrangement or a larger number of collagen molecules per fibril. Either of these conclusions would justify our assumption that the fibrils have a fixed percentage of collagen, and therefore that their refractive indices would be independent of their diameters. Because the single fibril scattering cross-section, $\langle \sigma_n(\lambda) \rangle$, is proportional to the fourth power of fibril diameter,\(^9,21\) the population...
of large fibrils would be expected to contribute significantly to the scattering.

The effects of the significant populations of large fibrils in both the unimodal and bimodal distributions are manifested in the isolated fibril scattering cross sections. The mean values of $\langle \sigma_T(550) \rangle$ calculated from the group-1 and -2 TEMs are respectively $(1.63 \pm 0.59) \times 10^2 \AA^2$ and $(2.59 \pm 0.53) \times 10^2 \AA^2$. The Wilcoxon-Mann-Whitney test showed that the differences in the averages are significant ($P = 0.02$). The mean value of $\langle \sigma_T(550) \rangle$ calculated from the two TEMs from the group-3 cornea is $8.6 \times 10^2 \AA^2$. Although no statistical test was performed, it is obviously much greater.

Although it is possible that keratocytes contribute to the increased scattering, their potential contribution is difficult to assess because at present there is no theory that can quantitatively predict cellular scattering in the cornea. 18 The TEMs that were examined did contain some degenerating keratocytes. It is possible that their refractive indices differ from those of normal keratocytes,35,36 but even the refractive indices of normal keratocytes are unknown.18 The way that nonspecular angular scattering from normal cornea scales with scattering angle and wavelength indicates that scattering is due to the fibrillar matrix, and keratocytes do not contribute significantly.17 In any event it is unlikely that scattering from keratocytes or other corneal cells such as myofibroblasts has the same wavelength dependence as the scattering from these scars.37 It is clear, however, that a comprehensive theory for cellular scattering is needed and that it would contribute not only to a better understanding of light-scattering from wounded cornea, but also to scattering from normal cornea.18,38

In summary, scars from 2-mm diameter penetrating wounds remained highly scattering even after 3.6 to 4.5 years of healing. Analysis of light-scattering measurements suggested that “lakes” or voids in the collagen fibril distribution were a significant factor contributing to the high level of scattering. TEMs from the scars confirmed the presence of the lakes and some also showed bimodal distributions of fibril diameters. Analysis of the TEM using the DSF method showed that, in addition to scattering from lakes, disorder in fibrillar organization and the effects of significant populations of greatly enlarged fibrils on the isolated fibril scattering cross sections are significant factors contributing to increased scattering.

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References


