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Experimental Eye Research 78 (2004) 553–560
EXPERIMENTAL EYE RESEARCH
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Keratocyte reflectivity and corneal haze

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Received 4 July 2003; accepted 8 July 2003

Abstract

Corneal transparency is a remarkable characteristic that is essential for vision. Biophysical models of corneal transparency have entirely focused on the stromal extracellular matrix and disruption of the regular array of collagen fibres as the main reason for corneal haziness. Therefore, disorder of corneal transparency has traditionally been explained by a combination of three main factors: (1) abnormal water content (i.e. swelling or edema); (2) abnormal collagen fibre diameter, spacing, and orientation (i.e. scar tissue or fibrosis); and (3) abnormal accumulation of macromolecules (proteins, glycosaminoglycans, lipids, etc.) as in many corneal dystrophies. Here, clinical and experimental data are provided to support the concept that corneal keratocytes, which are normally invisible and transmit light, may show intense light scattering in injured corneas. Thus, the existence of a fourth group of corneal transparency disorders is proposed that predominantly are associated with abnormal cellular-based reflections from multiple layers of stromal keratocytes. In this group of patients, the light scattering structures (keratocyte nuclei, cell-body, and cell-processes) cannot be discriminated using standard slit-lamp biomicroscopy but requires a confocal microscopic examination. Despite their importance, almost nothing is known about the physical basis for the invisibility and haziness of the keratocytes. A more comprehensive model to understand corneal transparency is needed and should include the interaction of visible light with the physical structure of the keratocyte and its subcellular constituents.
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Keywords: corneal stroma; keratocytes; corneal transparency; haze development; light scattering

1. Introduction

Transparency is defined as the property of transmitting light without appreciable scattering so that structures lying beyond are seen clearly. How transparency is attained in an inhomogeneous structure as the cornea has puzzled many researchers over the years. Although the cornea is not perfectly clear, it scatters only a few percent of the incident light across the visible spectrum, and yet allows for light transmission in the forward direction. Attempts to understand corneal transparency have focused on the ultrastructure and scattering properties of the stroma that constitutes about 90% of the total corneal volume. The stroma has a thickness of about 500 μm in humans with bundles (lamellae) of highly ordered collagenous fibres and ground substance (water, salts, proteoglycans, glycoproteins, etc.) being the main structural components of the extracellular matrix. All traditional theories to explain corneal transparency, including the lattice-theory of Maurice (1957) and the spatial-criterion by Goldman and Benedek (1967) and others (Goldman et al., 1968; Hart and Farrell, 1969; Benedek, 1971; Farrell et al., 1973), have focused on light propagation in the stromal extracellular matrix. But the human cornea also contains as many as 2·0–3·5 million keratocytes (Møller-Pedersen et al., 1994; Møller-Pedersen, 1997) that occupy as much as 9–17% of the total stromal volume (Hahnel et al., 2000). Each keratocyte is flat (~1 μm) with numerous cell-processes up to 50 μm long that extend in many directions, giving the cell a stellate appearance (Fig. 1(A)) (Hogan et al., 1971; Kuwabara, 1975; Jester et al., 1994; Müller et al., 1995; Somodi and Guthoff, 1995; Hahnel et al., 2000). The larger cell-processes branch into a complex network of delicate, interconnected cytoplasmatic protrusions that covers a significant area of the lamellae (Fig. 1(B) and (C)). It is estimated that each human corneal keratocyte covers an area of about 1000 μm^2 in the frontal plane (Hahnel et al., 2000). Thus, the density, volume, and size estimates of the keratocytes suggest that light travelling through the human

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DOI:10.1016/S0014-4835(03)00208-2

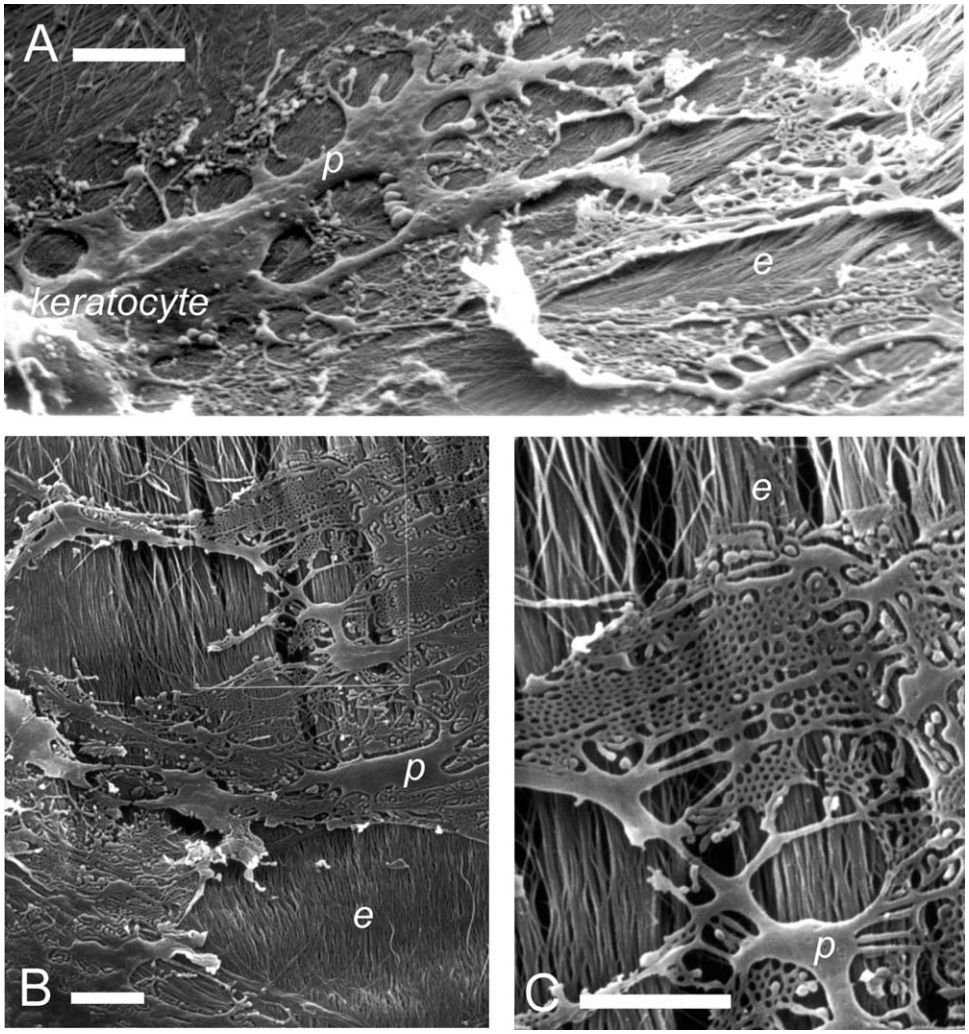

Fig. 1. Scanning electron micrographs demonstrating the extension and complexity of the human corneal keratocyte. Note that (A) only shows a portion of the cell. Also note the complex network of delicate, interconnected cytoplasmatic protrusions (*p*) that covers a large area of the collagen fibres (*e*). (C) is a magnification of the region marked with a rectangle in (B). Bars indicate 5 μm. The photos were kindly provided by Linda J. Müller who prepared the tissue as described in Müller et al. (1995).

cornea has to pass through up to 100 layers of keratocytes with light-scattering properties. However, the keratocytes are discounted in the current models for corneal transparency as stated by Maurice (1957): "In between the lamellae lie a few flattened cells…; these represent a very small fraction of the volume and they may be ignored…". There appears to be no theoretical or experimental data to support this assumption, and the keratocytes were mainly ignored to reduce the complexity of the transparency models.

Indeed the keratocytes have the potential to scatter light. Using slit-lamp biomicroscopy at non-specular angles, the oval keratocyte nuclei are visible in the normal human cornea as numerous, low reflecting points that scatter light in all directions (Fig. 2(A) and (B)). By changing the angle of incident light toward the specular angle, the keratocyte nuclei show up dramatically and act like little mirrors as elegantly demonstrated by Maurice using his scanning slit specular microscope (Maurice, 1974; Gallagher and Maurice, 1977). By contrast, the stellate cytoplasm of the keratocytes remains invisible (at all angles of incident light) and does not cause much scatter under normal conditions. These observations using slit-lamp biomicroscopy are even more apparent when the normal stroma is examined using in vivo confocal microscopy that essentially is analogous to a magnified, non-specular slit-lamp examination of an optical slice of the tissue. Using in vivo confocal microscopy, the cytoplasm of the normal keratocytes appears to transmit light completely, whereas the low reflecting keratocyte nuclei appear to be the principal structures backscattering light in the normal stroma (Fig. 2(C)). It therefore seems reasonable that a more comprehensive model to understand corneal transparency also considers the interaction of light with the physical structure of the keratocyte including the nucleus, cytoskeleton, and smaller organelles. Such a model should contain a detailed description of changes in the index of refraction and scattering properties when light passes from the extracellular matrix and through the plasmalemma,

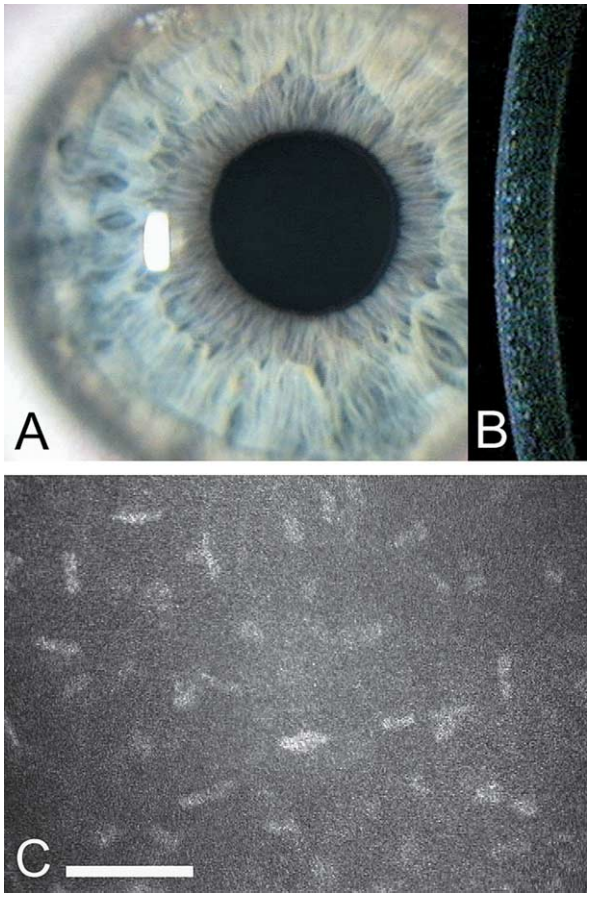

Fig. 2. Slit-lamp biomicroscopy (A, B) of a normal human cornea. Note the multiple small spots that scatter light in the optical cross-section of the stroma (B). In vivo confocal microscopy (C) of the mid-stroma in a normal human cornea showing that the keratocyte nuclei are the only structures scattering light, whereas the cytoplasm appears invisible. Bar indicates 100 μm.

travels through the cytoplasm through subcellular structures and out on the back of the keratocyte into the extracellular matrix again. There is already evidence to suggest that marked differences in the index of retraction between the keratocyte and the surrounding extracellular matrix lead to corneal haze development (Møller-Pedersen et al., 1998a,b, 2000; Jester et al., 1999).

Here three clinical cases are presented that all showed significant stromal opacity by slit-lamp biomicroscopy and highly reflective keratocytes by in vivo confocal microscopy. The cases include one patient who received a penetrating keratoplasty, one patient with sterile keratitis, and one patient who underwent a photorefractive keratectomy (PRK). In all three cases, the increased cellular backscatter from multiple layers of stromal keratocytes appeared to be the principal reason for the cornea to lose its transparency. Thus, it seems that each layer of keratocytes may act as a semi-transparent mirror that can alter its reflectivity based on various environmental stimuli, leading to changes in the total corneal light scattering. To further illustrate this point, manual epithelial debridement was performed in three rabbit corneas. The scrape injury killed all keratocytes in the subepithelial region and left a continuous approximately 100 μm acellular zone in the anterior stroma. This region devoid of keratocytes showed considerably less backscattering of light than the normal stroma, demonstrating that keratocytes are a principal source of light scattering in the normal transparent cornea. Moreover, the anterior stroma developed significant haze as the acellular zone became repopulated with highly reflective wound healing keratocytes.

2. Methods

2.1. Patients and examinations

Three patients with corneal opacities were included in the study because the identity of their light scattering structures could not be revealed by slit-lamp biomicroscopy. The patients gave informed consent and received a full ophthalmological examination, photo-slit-lamp biomicroscopy, and in vivo confocal microscopy (Tandem Scanning Corporation; Reston, VA, USA) (Ivarsen et al., 2002). *Case 1*: Three months earlier, a 74-year-old woman with Fuchs' dystrophy had received an 8 mm penetrating corneal graft in her left eye. The postoperative treatment included systemic corticosteroids (30 mg prednisolone for 1 week; tapered over 3 months), and topical chloramphenicol and prednisolone (ointment four times a day). The surgery and immediate postoperative period were uneventful with no symptoms or signs of graft rejection. *Case 2*: A 35-year-old woman was referred with a two-month history of stromal opacities of unknown origin in the left cornea. During the observation period, the eye had appeared non-inflamed and all microbiological tests (for bacteria, virus, and chlamydia) were negative. The tentative diagnosis remained a sterile keratitis. *Case 3*: One month earlier, a 27-year-old man had received a 6 mm diameter, −7·5 dioptre PRK in this right eye using a MEL 70 G-Scan excimer laser (Asclepion, Jena, Germany). He had no previous medical history except for nine years of soft contact lens wear. The postoperative treatment included topical chloramphenicol (three times a day for 1 week) and prednisolone (three times a day).

2.2. Animals and procedures

Three normal New Zealand White rabbits (weight 4·5–5·0 kg) received a monocular, central 6 mm diameter manual epithelial debridement using a hockey knife. Surgery and examinations were preceded by anaesthesia as previously reported (Ivarsen et al., 2002). Postoperative treatment (three times a day for 5 days) included systemical buprenorphine (0·05 mg kg^{-1}) and topical ciprofloxacin and chloramphenicol. The study was approved by the Danish Animal Experiments Inspectorate, and all rabbits were

housed and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. The animals were evaluated preoperatively, at day four, and weekly for 2 months using photo-slit-lamp biomicroscopy and in vivo confocal microscopy.

3. Results

3.1. Patients with stromal haze

Case 1 (*penetrating keratoplasty*): By three-month post-surgery, the central region of the corneal graft showed fine reticular haze (Fig. 3(A)). In the optical cross-section, the haze was confined to the anterior half of the stroma (Fig. 3(B)). Corneal thickness measured 0·56 mm by optical pachymetry, and there was no sign of ocular inflammation. In vivo confocal microscopy revealed highly reflective keratocytes in the anterior stroma (Fig. 3(C)), whereas the keratocytes in the optically clear posterior stroma remained quiescent with a morphology similar to that in the normal cornea (Fig. 2(C)). It should be noted that the image in Fig. 3(C) represents a single confocal frame with an optical z-axis thickness of approximately 9 μm (Petroll et al., 1993). However, real-time advancement of the focal plane even more clearly suggested that the reflective structures in Fig. 3(C) represent the cytoplasmatic protrusions of an interconnected meshwork of keratocytes. This interpretation of the in vivo histopathological findings is supported by the close correlation to the scanning electron micrograph shown in Fig. 1(A) and to previous ex vivo observations of the human corneal keratocyte morphology (Hogan et al., 1971; Müller et al., 1995; Somodi and Guthoff, 1995; Jester et al., 1999; Hahnel et al., 2000).

Case 2 (sterile keratitis): Slit-lamp biomicroscopy of the central cornea showed diffuse haze in the anterior stroma with multiple nodules of more enhanced reflectivity (Fig. 4(A) and (B)). The central cornea appeared slightly edematous with a thickness of 0·61 mm by optical

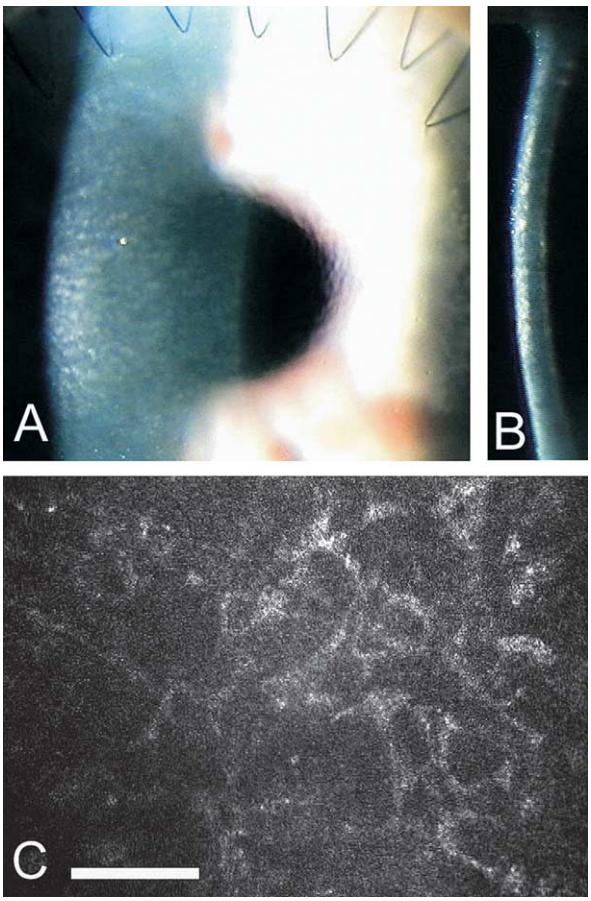

Fig. 3. Slit-lamp biomicroscopy (A, B) of a penetrating corneal graft with anterior stromal haze by three-month post-surgery. In vivo confocal microscopy (C) showing keratocytes with visible cytoplasm in the anterior part of the stroma, whereas there appears to be no increased light scattering from the extracellular matrix. Bar indicates 100 μm.

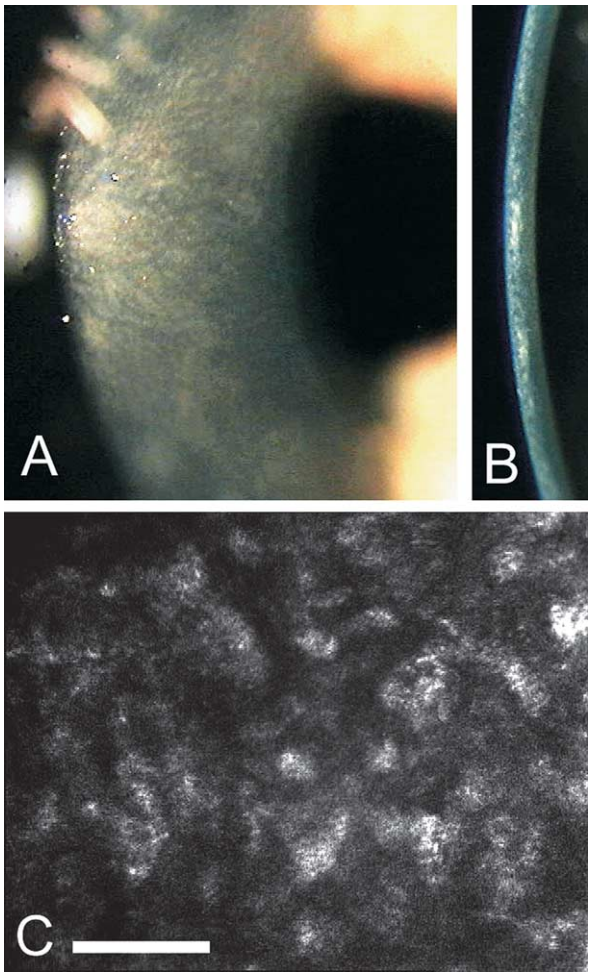

Fig. 4. Slit-lamp biomicroscopy (A, B) of a sterile keratitis with focal areas of haze in the anterior stroma. In vivo confocal microscopy of the anterior stroma (C) showing keratocyte nuclei and cytoplasm with enhanced reflectivity. Bar indicates 100 μm.

pachymetry. In vivo confocal microscopy revealed highly reflective keratocytes with prominent nuclei and visible cell-processes in the anterior stroma (Fig. 4(C)). By contrast, the posterior stroma, epithelium, and endothelium appeared normal.

Case 3 (*photorefractive keratectomy*): By one month post-PRK, this patient showed moderate corneal haze confined to the subepithelial stroma (Fig. 5(A) and (B)). The spherical equivalent refraction was −0·5 dioptre, and the central corneal thickness was 0·45 mm by optical pachymetry. In vivo confocal microscopy revealed increased cellular-based reflections from multiple layers of keratocytes in the subepithelial stroma. The cells appeared activated with highly reflective nuclei and visible cytoplasm (Fig. 5(C)), whereas the keratocytes in the posterior stroma remained normal with low-reflecting nuclei and transparent cytoplasm.

3.2. Light scattering after epithelial debridement

In all animals, the epithelial scrape injury induced characteristic changes in corneal light scattering. By day four, the anterior stroma showed a dark region of approximately 100 μm (Fig. 6(A), arrow) that had almost no light scattering as compared to underlying normal stroma. This low-scattering region appeared completely acellular with no visible keratocytes by in vivo confocal microscopy (Fig. 6(B)). By contrast, the posterior stroma contained a normal population of quiescent keratocytes. Over the next 2 weeks, the acellular zone gradually became repopulated by reflective wound healing keratocytes with a spindle-shaped morphology consistent with cell-migration (Møller-Pedersen et al., 1998a,b). By week three, the anterior stroma had a hazy appearance (Fig. 6(C), arrow) with a considerable higher reflectivity than the posterior

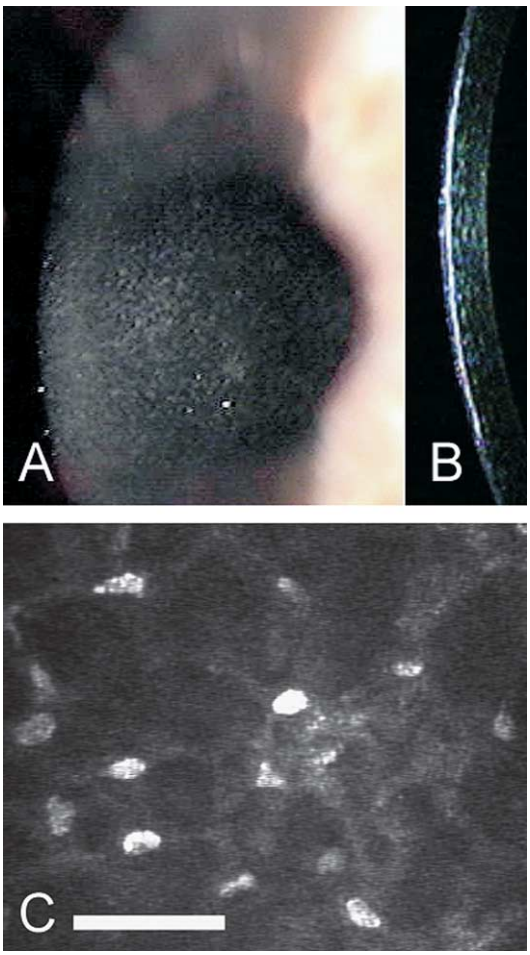

Fig. 5. Slit-lamp biomicroscopy (A, B) of a cornea with subepithelial haze by one month post-PRK. In vivo confocal microscopy (C) of the anterior stroma showing keratocytes with highly reflective nuclei and visible cell-processes. Bar indicates 100 μm.

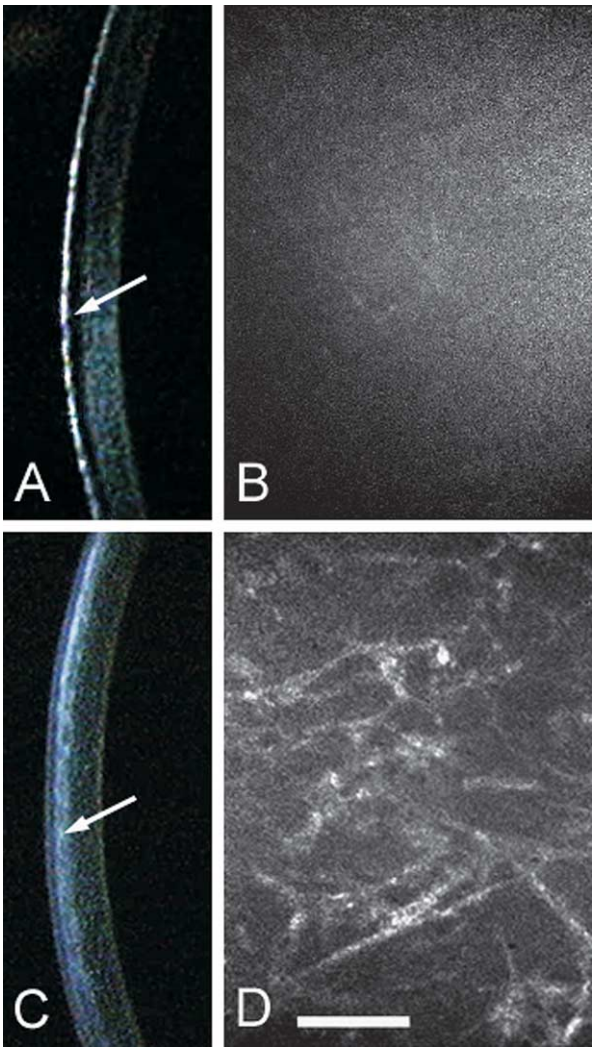

Fig. 6. Slit-lamp and in vivo confocal microscopy of the same rabbit cornea by 4 days (A, B) and 3 weeks (C, D) after manual epithelial debridement. The confocal images on the right are obtained in the regions marked with arrows on the left. By day four, the scrape injury had generated a 100 μm zone in the anterior stroma (A, arrow) with almost no light scattering as compared to the underlying normal stroma. The low-scattering region showed no keratocytes and appeared acellular (B). By 3 weeks, the anterior stroma showed an elevated reflectivity (C, arrow) as the region became repopulated by reflective wound healing keratocytes (D). Bar indicates 100 μm in (B) and (D).

stroma. In the region of haze, the wound healing keratocytes showed an increased cellular-based reflectivity (Fig. 6(D)). Over the next 5 weeks, the keratocyte morphology and the reflectivity of the anterior stroma gradually normalized.

4. Discussion

Many corneal diseases are associated with development of opacities in the stroma. Previous attempts to explain haze development have generally focused on the disruption of the collagen fibre array as the principal source of the increased light scattering (Maurice, 1957; Goldman and Benedek, 1967; Goldman et al., 1968; Hart and Farrell, 1969; Benedek, 1971; Farrell et al., 1973). Based on this knowledge, slit-lamp biomicroscopy has traditionally been used to divide corneal transparency disorders into three main groups: (1) conditions associated with an abnormal water content (i.e. swelling or edema); (2) conditions associated with abnormal collagen fibre diameter, spacing, and orientation (i.e. scar tissue or fibrosis); and (3) conditions associated with abnormal accumulation of macromolecules (proteins, glycosaminoglycans, lipids, etc.) as in many corneal dystrophies. As shown here, there appears to be a fourth group of corneal transparency disorders that predominantly are associated with abnormal cellular-based reflections from multiple layers of stromal keratocytes. In this group of patients, examination using slit-lamp biomicroscopy is not sufficient to reveal the light scattering structures. Therefore, in the present study, the *z*-axis localization of slit-lamp detectable corneal haze was correlated directly to the corresponding histopathology using in vivo confocal microscopy. This approach revealed a group of patients, where the keratocyte nuclei, cell-body, and cell-processes were the main intracorneal structures showing enhanced light scattering. In the three cases presented here (penetrating keratoplasty, sterile keratitis, and PRK), the reflectivity of the anterior stromal keratocytes had been environmentally altered without any obvious changes in keratocyte numbers (Figs. 3(C), 4(C) and 5(C) compared with Fig. 2(C)). One of these corneas (case 2) also showed slight edema which may have contributed to the increased light scattering. As expected, the PRK-treated cornea (case 3) contained reflective wound healing keratocytes similar to the scrape-injured rabbit corneas (Fig. 5(C) compared with Fig. 6(D)) and confirming previous clinical and experimental studies (Møller-Pedersen et al., 1998a,b, 2000). However, in two of the patients (cases 1 and 2), it was unexpected to find reflective cells. Thus, hazy keratocytes may be an unidentified contributor to increased light scattering in many patients with corneal opacities after different types of stress and injury. In most transparency disorders, however, there seems to be one predominant factor causing the haze; for example: (1) edema in bullous keratopathy; (2) scarring after keratitis or trauma; (3) accumulated macromolecules in granular corneal dystrophy; and (4) reflective keratocytes during the first six to nine months post-PRK. But in some patients, corneal haze may be induced by a combination of two or more of the four factors (edema, scarring, accumulated macromolecules, and reflective keratocytes) that may contribute to a different extent in each individual patient. Obviously, it is important to identify the specific light scattering structures before rational strategies for their treatment and prevention can be initiated.

4.1. Optics of the extracellular matrix

In the stromal extracellular matrix, the two main components have a non-uniform index of refraction, about 1·47 for the collagen fibres as compared to 1·35 for the surrounding ground substance (proteoglycans and other macromolecules) (Maurice, 1957). Commonly, this imbalance would produce a quite milky structure, yet the cornea scatters only a few percent of the incident light across the visible spectrum. According to the lattice-theory of Maurice (1957), the extracellular matrix is transparent because of the physical arrangement of the collagen fibres that forms a lattice of uniform diameter and spacing. In a lattice structure, light scattered to the side by each individual fibres is cancelled by destructive interference from the electromagnetic waves scattered from the neighbouring fibres. Still, undiffracted light is allowed to propagate in the forward direction. A different hypothesis was later proposed by Goldman and Benedek (1967) and others (Goldman et al., 1968; Hart and Farrell, 1969; Benedek, 1971; Farrell et al., 1973) who added the spatial-criterion. According to this theory, stromal transparency does not require a strict lattice arrangement but is a consequence of the refractive index fluctuations occurring over very small distances. Thus, the stromal extracellular matrix is transparent because of the small diameter (~30 nm) and spacing (~60 nm between the centres) of the collagen fibres as compared to the wavelength of visible light (~400 to 700 nm). Significant scattering will occur when the index of refraction fluctuates over distances larger than half the shortest wavelength of visible light (~200 nm), and structures whose size matches the wavelength of light will scatter most strongly.

4.2. Keratocyte optics

The keratocyte is a complex, inhomogeneous structure with various organelles and subcellular constituents surrounded by a plasmalemma (Hogan et al., 1971; Kuwabara, 1975; Müller et al., 1995). For the keratocyte to be transparent, light must pass through it without being scattered. As the photons travel through the cell, they will interact with the cellular components that may act as significant scatterers depending on their size, shape, and refractive index. Ultrastructural studies have revealed

the size and shape of most organelles, but there are currently no details about the refractive index distribution inside the keratocyte. Since most of the organelles will have different refractive indices, the structural inhomogeneities across of the cytoplasma will result in a spatially varying index of refraction. Similar to the extracellular matrix, only intracellular objects with a diameter larger then 200 nm will be major scatterers provided there is a refractive index mismatch. The largest structure in the cytoplasm is the nucleus with a diameter of up to 30 μm. Accordingly, the keratocyte nuclei are by far the most important scatterers in the normal stroma as detected using both slit-lamp and in vivo confocal microscopy (Figs. 2 and 6(A), (B)). Among the other organelles, the mitochondria, the Golgi apparatus, and the rough endoplasmic reticulum have a length of up to 1000 nm (Müller et al., 1995). Also lipid inclusion bodies and aggregates of glycogen granules have diameters in the range of 500–1000 nm (Kuwabara, 1975). Another potential scattering source is the border between the keratocyte and the extracellular matrix, where there may be big fluctuations in the index of refraction. This is particularly important because the dimensions of the delicate cell processes (Fig. 1(C)) are comparable to the wavelength of light. It seems mandatory for maintaining keratocyte invisibility that the overall refractive index of the cytosol matches the refractive index of the extracellular matrix. It is therefore likely that the keratocyte contain some sort of mechanism to smooth out the fluctuations in index of refraction within the cytoplasm and match the overall refractive index with the extracellular matrix. It was recently proposed that intracellular crystallin proteins may represent such a mechanism (Jester et al., 1999).

Crystallins are water-soluble globular proteins that are present in high amounts in the keratocyte cytoplasm. Two of the most abundant, aldehyde dehydrogenase class 1 and transketolase, account for about 28% of the total water-soluble protein in the cytoplasm of normal rabbit keratocytes (Jester et al., 1999). The functions of the crystallin proteins are currently unknown but they may absorb ultraviolet radiation and protect the keratocyte cytoplasm from oxidative stress. Due to their high concentrations, they may also play a structural role similar to the crystallin proteins inside the lens, where they provide a transparent matrix for the passage of visible light. Due to their dipolar electrical properties, it can be speculated that the crystallin proteins may induce some kind of ordering in the form of a moving lattice inside the keratocyte cytoplasm. Such a dynamic grid of uniform protein particles could essentially be functioning similar to the lattice of collagen fibres in the extracellular matrix as described by Maurice (1957). The concentration and compaction of crystallins would then determine the intracellular index of refraction and thereby the light-scattering properties. In accordance with this hypothesis, a marked reduction (>50%) in crystallin protein levels was found in highly reflective wound healing keratocytes isolated from freeze-injured rabbit corneas (Jester et al., 1999). Additionally, PRK-treated rat corneas showed decreased crystallin mRNA levels for up to three-months post-surgery (Schultz, 2001). Furthermore, development of corneal transparency in neonatal mice and rabbits correlated closely to the appearance of the quiescent and transparent keratocyte phenotype that has a high expression of crystallin proteins (Jester et al., 2003; Song et al., 2003). On the other hand, aldehyde dehydrogenase class 3 deficient knockout mice showed normal transparent corneas (Nees et al., 2002). Thus, many questions need to be answered before the crystallin hypothesis can be accepted. Of specific interest will be identification of the spatial distribution and organization of the crystallins inside the keratocyte cytoplasm, and how they interact with the cytoskeleton, plasmalemma, and various organelles. Whatever the underlying mechanism may turn out to be, the keratocytes have a potential for dynamic alteration of their light scattering properties and can vary their cellular transparency analogous to a semi-transparent mirror. Indeed, the keratocyte cytoplasm holds an important key for further understanding of the biological and biophysical aspects of corneal transparency and clouding. Further insight into this issue may lead to new and effective strategies for pharmacologic manipulation of corneal clarity in the many corneal diseases associated with development of stromal haze.

Acknowledgements

The author thanks Linda Müller for providing the electron micrographs and for sharing her extensive knowledge on the ultrastructure of the corneal keratocytes and extracellular matrix. James V. Jester, David Maurice, and Russel L. McCally are acknowledged for the many inspiring discussions over the years on corneal transparency and biophysics. Anders Ivarsen helped with the confocal examinations and surgery on the rabbits, whereas the patients received surgery by Jesper Hjortdal.

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