Keratocyte Density in the Human Cornea After Photorefractive Keratectomy

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Objective: To perform a quantitative analysis of keratocyte density in human corneas after photorefractive keratectomy (PRK).

Methods: In a prospective comparative trial, 24 eyes of 14 patients received PRK to correct refractive errors of between –1.25 diopters (D) and –5.75 D. Corneas were examined by using confocal microscopy before and 1 day, 5 days, and 1, 3, 6, 12, 24, and 36 months after PRK. Keratocyte nuclei were counted in 5 stromal layers in 3 to 6 scans per eye per visit. Keratocyte density in each layer post-PRK was compared with the density in the corresponding layer of the pre-PRK full stroma (included stroma that would later be photoablated) and the pre-PRK future unablated stroma (thickness adjusted by omitting the future ablation depth) (Bonferroni-adjusted paired t test).

Results: Keratocyte density in the anterior 10% of the post-PRK stroma decreased by 25% ($P=0.002$), 41% ($P<0.001$), 40% ($P<0.001$), 43% ($P<0.001$), and 45% ($P<0.001$) at 3, 6, 12, 24, and 36 months compared with the anterior 10% of the pre-PRK full stroma and was reduced by 15% at 36 months ($P=0.02$) compared with the anterior 10% of the pre-PRK future unablated stroma.

Conclusion: After PRK, keratocyte density in the anterior stroma is not restored to the high-density levels found in the preoperative stroma.

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PHOTOREFRACTIVE keratectomy (PRK) uses an excimer laser to precisely photoablate graded amounts of anterior corneal stroma to induce a change in corneal refraction. Although clinical results are good, variable predictability of refractive and visual outcomes, regression of the initial refractive effect, and subepithelial haze remain clinical concerns.1-2 These adverse effects have, in part, been attributed to qualitative or quantitative variations in corneal keratocytes and their subsequent effect on corneal wound healing.3-5 An early loss of keratocytes after PRK followed by keratocyte repopulation has been demonstrated in rabbit histologic studies.6,6 Primates8,12 and human histologic studies suggest an early increase in anterior stromal keratocytes that may persist for as long as 6 months postoperatively. However, histologic studies are invasive and cannot be used to study keratocyte density in vivo. An objective quantification of changes in keratocyte density after PRK may enhance our understanding of corneal wound healing.

The clinical confocal microscope provides a means of repeated noninvasive examination of the cornea at the cellular level.16 Confocal microscopy has been used to quantify keratocyte density in normal human and rabbit corneas in vivo, and these measurements have been validated by comparing cell density measured by confocal microscopy with density estimated from histologic sections of the same tissue.17,18 After PRK, corneal keratocyte density has previously been subjectively3-5,19,20 and quantitatively21 measured using confocal images. In the current study, sequential changes in keratocyte density and variation of density with depth in the central human cornea were measured for 3 years after PRK by using confocal microscopy.

METHODS

SUBJECTS

Twenty-four eyes of 14 subjects (3 men and 11 women) aged 22 to 53 years (mean ± SD age, 40 ± 7 years) were enrolled prospectively in a nonrandomized fashion from July through October 1998. All participants were patients of the Mayo Clinic, Rochester, Minn. The mean ± SD preoperative spherocylindrical refractive error was −3.73 ± 1.30 D (range, −1.25 to −5.75 D). All

From the Departments of Ophthalmology (Drs Erie, Patel, McLaren, and Bourne) and Biostatistics (Mr Hodge), Mayo Clinic, Rochester, Minn. The authors have no relevant financial interest in this article.
subjects were white. None of the subjects had a history of anterior segment disease, ocular trauma or surgery, diabetes mellitus, or the use of ocular medications. Systemic medications were permitted, unless they were known to affect the cornea or the anterior segment. Contact lens wear was discontinued within 2 weeks (soft lenses) or 3 weeks (rigid gas-permeable lenses) of enrollment in the study. All eyes had normal anterior segments, intraocular pressures (≤22 mm Hg), and fundi. Our institutional review board approved this study, and all subjects gave informed consent after the nature and possible consequences of the study were explained to them.

PHOTOREFRACTIVE KERATECTOMY PROCEDURE AND POSTOPERATIVE REGIMEN

Photorefractive keratectomy was performed using the VISX STAR laser (VISX Inc, Santa Clara, Calif) with a wavelength of 193 nm, a fixed pulse rate of 6 to 8 Hz, and a radiant exposure of 160 mJ/cm². The epithelium was removed by using the laser-scrape technique (43 µm epithelial ablation followed by manual scrape of the remaining epithelial cells with a blunt spatula). The mean ± SD planned ablation depth was 46 ± 18 µm (range, 13-90 µm). Emmetropia was attempted in all cases. Immediately after ablation, the cornea was cooled for 30 seconds by irrigation with cold balanced salt solution.

After the PRK procedure, patients wore a bandage soft contact lens (SoFlens 66; Bausch & Lomb Inc, Rochester, NY) until the cornea epithelialized (2-3 days). Topical medications consisted of 0.3% preservative-free ketorolac tromethamine (Acular PF; Allergan Inc, Irvine, Calif) for 4 doses over 2 days, 0.3% ofloxacin (Ocuflax; Allergan Inc) 4 times daily until epithelialization was complete, and 0.1% fluorometholone (FML; Allergan Inc) 4 times daily with a taper over 3 months.

A bandage soft contact lens was already in place on all eyes at the 1-day examination. To protect against possible corneal abrasion, a bandage soft contact lens was placed for approximately 15 minutes on all eyes at the 5-day examination and on 4 eyes at the 1-month examination. A bandage contact lens was not used thereafter.

CONFOCAL MICROSCOPY IN VIVO

Corneas were examined by using a tandem scanning confocal microscope (Tandem Scanning, Reston, Va) before and at 1 day, 3 days, and 1, 3, 6, 12, 24, and 36 months after PRK. The method of examination was described in an earlier article. Briefly, 0.3% proparacaine hydrochloride (Bausch & Lomb Pharmaceuticals, Inc, Tampa, Fla) was instilled into the eye to be examined. One drop of 2.5% hydroxypropyl methylcellulose (CIBA Vision Ophthalmics, Atlanta, Ga) optical coupling medium was placed on the tip of the objective lens. The objective was aligned to the visual axis of the eye and manually advanced until the medium contacted the central cornea. The position of the objective was adjusted to provide an en face view of the central cornea to confirm correct alignment. The patient self-fixed on a bright target with the contralateral eye to minimize eye movements.

A full-thickness scan, consisting of a series of confocal images, was recorded as the focal plane was advanced at approximately 78 µm/s from anterior to the epithelium to the endothelium. Digital images were stored on a computer workstation (Indy; Silicon Graphics Inc, Mountain View, Calif). Each image represented a coronal section (x-axis × y-axis) of 475 µm × 350 µm and a depth of field (z-axis thickness) of 9 µm. Each image was separated from the adjacent image by an average of 2.6 µm. Images were acquired by either setting the camera in a fixed-gain mode, with a constant gain, voltage, and black level, or in automatic-gain mode, with these parameters automatically adjusted by the camera throughout image acquisition. The cornea was scanned through its full thickness 4 to 8 times per visit.

THICKNESS MEASUREMENTS

An intensity profile of back-scattered light from selected confocal images was obtained as described previously. Images were acquired with the camera operating in its fixed-gain mode. Intensity was estimated from the mean grayscale value in a 300 × 300 pixel area in the center of each image. Peaks in intensity corresponded to the superficial epithelium, the sub-basal nerve plexus, the most anterior keratocytes, and the endothelium. The video image corresponding to each intensity peak was displayed, and the first focused video image of each corneal region was identified and used in the determination of thickness. In profiles generated from corneas after PRK, a peak corresponding to anterior stromal haze was often present, as confirmed by the presence of increased reflectivity of keratocytes in the corresponding video image.

Corneal thickness was the distance between the first focused image of the superficial epithelium and the endothelium. Epithelial thickness was the distance between the first focused image of the superficial epithelium and the sub-basal nerve plexus. When sub-basal nerves were not visible in images after PRK, we determined epithelial thickness as the distance between the first focused image of the superficial epithelium and the first focused image of anterior keratocytes. The Bowman layer was the distance between the first focused image of the subbasal nerve plexus and the most anterior keratocytes. Stromal thickness was the distance between the first focused image of the most anterior keratocytes and the last focused image of the posterior keratocytes having a reflected light intensity similar to other images of the stroma but without images of endothelial cells. We corrected depth measurements for the nonlinear separation of video images by counting the number of images between the image of the objective surface and the surface of the epithelium.

An estimate of the stromal thickness destined to be ablated, or the actual photoablation depth, was obtained as the surgically induced stromal thinning (S_bis) between the pre-PRK (S_pre) and 1 month post-PRK (S_post) measured stromal thickness:

\[ S_{bis} = S_{pm} - S_{post} \]

The pre- and post-PRK stroma was subdivided by depth into 5 layers: 0% to 10% (anterior), 11% to 33%, 34% to 66% (middle), 67% to 90%, and 91% to 100% (posterior). In the post-PRK cornea, the boundaries of the stromal layers were determined relative to the most anterior keratocyte layer. The post-PRK stromal thickness was compared with 2 pre-PRK stromal thicknesses: the pre-PRK full stroma and the pre-PRK future unablated stroma (Figure 1). The pre-PRK full stroma included the anterior stroma that would later be photoablated. In this case, the boundaries of the pre-PRK stromal layers were determined relative to the most anterior keratocytes immediately posterior to the Bowman layer. In the pre-PRK future unablated stroma, the anterior stroma equal to the actual photoablation depth (as measured at 1 month after PRK) was omitted from the analysis. In this case, the boundaries of the pre-PRK stromal layers were determined relative to the most anterior keratocytes that would remain after the future PRK ablation. This allowed direct comparison of the same tissue layers in the pre- and post-PRK stroma.

KERATOCYTE DENSITY MEASUREMENT

Keratocyte density was measured from images of one confocal scan of the central cornea with the camera in its automatic-gain mode. All scans were reviewed, and the single scan with
the least anterior-posterior and lateral ocular movement was selected for analysis.

Two images with well-defined bright objects from each layer were selected for analysis. In the anterior 10% of the stroma, 1 of the 2 selected images was the most anterior countable image. The images were presented in random order to one observer (J.C.E.) who was masked to the subject and the examination visit. The same observer manually counted keratocyte nuclei (bright objects) in each of the 10 selected images per scan per examination by using an interactive computer program (Figure 2). The number of cells in a predetermined area was used to determine keratocyte density (cells/mm²). The mean cell density in each layer after PRK was compared with the mean cell density in (1) the corresponding layer of the pre-PRK full stroma and (2) the same tissue layer of the pre-PRK future unablated stroma.

DATA ANALYSIS

Groups were compared using a paired t test if data were distributed normally or the Wilcoxon signed-rank test if they were not. P values were Bonferroni-adjusted for multiple comparisons. P<.05 was considered statistically significant. The general estimating equation model was investigated to account for the potential correlation between 2 eyes of the same individual. In all cases, the conclusions were the same as the results of the standard statistical tests, so only the standard results are reported. In eyes requiring a reoperation, all data after the reoperation were excluded from analysis.

RESULTS

No complications were encountered during PRK or postoperatively. Five (21%) of 24 eyes underwent a reoperation. One eye required a reoperation at 7 months after PRK to treat an initial undercorrection, and 4 eyes required a reoperation at 13 months after PRK to treat myopic regression.

KERATOCYTE DENSITY

Central keratocyte density in the pre-PRK full stroma, the pre-PRK future unablated stroma, and the post-PRK stroma is shown in Table 1 and Figure 3.

Pre-PRK Full Stroma vs Post-PRK Stroma

In the pre-PRK full stroma, the full-thickness stroma was included in the analysis (Figure 1). Keratocyte density in the anterior 10% of the post-PRK stroma never returned to the high levels of the anterior 10% of the pre-PRK full stroma; density decreased by 30% (P<.001), 25% (P=.002), 41% (P<.001), 40% (P<.001), 43% (P<.001), and 45% (P<.001) at 1, 3, 6, 12, 24, and 36 months after PRK (Figure 4).

Pre-PRK Future Unablated Stroma vs Post-PRK Stroma

In the pre-PRK future unablated stroma, the anterior stroma equal to the depth of ablation, as measured at 1 month after PRK, was omitted from the analysis (Figure 1). At 5 days after PRK, keratocyte density in the most anterior keratocyte layer (17462±3710 cells/mm²) was significantly less than the cell density in the next deeper analyzed image at 5% to 10% stromal depth (23299±3352 cells/mm²; P=.01). No difference in cell density between

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Table 1

<table>
<thead>
<tr>
<th>Layer Description</th>
<th>Pre-PRK Full Stroma</th>
<th>Post-PRK Stroma</th>
<th>Pre-PRK Future Unablated Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%-10%</td>
<td>67%-90%</td>
<td>34%-66%</td>
<td></td>
</tr>
<tr>
<td>11%-33%</td>
<td>34%-66%</td>
<td>11%-33%</td>
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<td>34%-66%</td>
<td>34%-66%</td>
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<td>67%-90%</td>
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<tr>
<td>91%-100%</td>
<td>91%-100%</td>
<td>91%-100%</td>
<td></td>
</tr>
</tbody>
</table>

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Figure 1. The 5 layers of the post–photorefractive keratectomy (PRK) stroma (B) were compared with the corresponding 5 layers of the pre-PRK full stroma (A; stromal thickness included the anterior stroma that would later be photoablated) and the same 5 layers of the pre-PRK future unablated stroma (C; direct comparison of the same pre- and post-PRK tissue layers was possible by omitting the thickness of the anterior stroma equivalent to the future depth of ablation).

Figure 2. Keratocyte density was determined by manually counting keratocyte nuclei (X’s) in selected confocal images using an interactive computer program. Objects overlapping the edges of the bounding box were counted on only 2 sides of the box (left and lower sides).
Comparison of keratocyte density in the pre–photorefractive keratectomy (PRK) full stroma with density in the post-PRK stroma at 1, 2, and 3 years after PRK. The pre-PRK full stroma includes the anterior stroma that would later be photoablated. *P values are for post-PRK vs pre-PRK keratocyte density. NS indicates not significant.

Figure 5

**Keratocyte Morphology**

Before PRK, keratocyte nuclei appeared as bright oval or bean-shaped objects. Cellular processes were not evident. The preoperative anterior keratocytes were smaller, more numerous, and more tightly packed than the posterior keratocytes.

At 1 day after PRK, keratocyte nuclei were the same in size, shape, and reflectivity when compared with quiescent keratocytes in the same tissue layer of the preop-

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Table 1. Central Keratocyte Density Before and After Photorefractive Keratectomy (PRK)*

<table>
<thead>
<tr>
<th>Keratocyte Density, Mean ± SD, cells/mm³</th>
<th>Full Thickness</th>
<th>Regional Densities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%-10% (Anterior)</td>
<td>11%-33%</td>
</tr>
<tr>
<td>Pre-PRK Full stroma (n = 24)</td>
<td>19 608 ± 2236</td>
<td>32 380 ± 5848</td>
</tr>
<tr>
<td>Pre-PRK Future unablated stroma (n = 24)</td>
<td>18 110 ± 1947</td>
<td>20 930 ± 2819</td>
</tr>
<tr>
<td>Post-PRK 1 d (n = 24)</td>
<td>18 835 ± 3207</td>
<td>22 589 ± 4908§</td>
</tr>
<tr>
<td>Post-PRK 5 d (n = 24)</td>
<td>18 718 ± 2653</td>
<td>22 456 ± 4367§</td>
</tr>
<tr>
<td>Post-PRK 1 mo (n = 24)</td>
<td>19 861 ± 2447‡</td>
<td>22 813 ± 4001§</td>
</tr>
<tr>
<td>Post-PRK 6 mo (n = 24)</td>
<td>18 612 ± 2406§</td>
<td>24 434 ± 3636§</td>
</tr>
<tr>
<td>Post-PRK 12 mo (n = 23)†</td>
<td>17 304 ± 2572§</td>
<td>19 560 ± 3795§</td>
</tr>
<tr>
<td>Post-PRK 24 mo (n = 19)†</td>
<td>17 686 ± 2609§</td>
<td>18 613 ± 4112§</td>
</tr>
<tr>
<td>Post-PRK 36 mo (n = 19)†</td>
<td>17 372 ± 2443§</td>
<td>17 720 ± 4308§</td>
</tr>
</tbody>
</table>

*Paired comparisons were performed with a t test. *P values were Bonferroni-adjusted for 8 comparisons (adjusted *P = unadjusted *P × 8). Only *P values for significant differences are reported.

†Data from eyes requiring a reoperation were excluded.

‡P < .01 vs pre-PRK full stroma keratocyte density.

§P < .05 vs pre-PRK future unablated stroma keratocyte density.

¶P < .001 vs pre-PRK future unablated stroma keratocyte density.

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At 5 days after PRK, keratocytes in the anterior stroma showed bright nuclei and visible cell processes in some eyes. These highly visible cells have been interpreted to represent activated keratocytes or repair fibrocytes. The number of eyes with activated keratocytes peaked at 3 months after PRK. By 12 months, keratocyte morphology was similar to that before PRK (Figure 6, Table 2). When present, activated keratocytes appeared in the most anterior keratocyte layer and extended posteriorly to depths ranging from 10 to 57 µm (Table 2).

Keratocytes are distributed nonuniformly throughout the anterior-posterior stroma of the human cornea and density is highest in the most anterior stroma. The first part of the current study compared the keratocyte density in post-PRK stroma with cell density in pre-PRK full stroma (preoperative stromal thickness not adjusted for ablation depth; Figure 1). The data showed that the dense keratocyte population found in the preoperative anterior stroma was partially or completely removed during PRK photoablation, and this high keratocyte density was not reconstituted in the post-PRK anterior stroma. Specifically, keratocyte density in the anterior 10% of the post-PRK stroma decreased by 41%, 40%, 43%, and 45% at 6, 12, 24, and 36 months after PRK when compared with the anterior 10% of the pre-PRK full stroma. Consequently, the distribution of keratocytes after PRK also changed; keratocytes were now distributed uniformly, rather than nonuniformly, throughout the anterior-posterior stroma.

What a depleted anterior stromal keratocyte population after PRK means for the long-term health of the human cornea is unknown. There may be no need to replace keratocytes to reach the high density found in the preoperative anterior stroma. Additionally, reduced keratocyte density in the anterior stroma has been measured in patients after laser-assisted in situ keratomileusis (LASIK) and in patients with keratoconus who wear contact lenses. Apoptotic keratocyte loss immediately after PRK or epithelial scrape injury resulting in an acellular region of anterior stroma has been well documented in rabbit studies. Similarly, Balestrazzi et al measured a 5- to 8-µm region of acellular anterior stroma at 3 months after PRK in a human histologic study. Recently, Ambrosio et al confirmed that human keratocytes undergo apoptosis in response to epithelial debridement in a manner similar to rabbits. The tandem scanning confocal microscope is not capable of detecting a completely acel-
The current study provides new long-term data demonstrating that keratocyte density in the anterior stroma early after PRK when landmarks such as the epithelium and subbasal plexus have not yet re-formed. Therefore, the current study cannot address the presence or absence of a zone of acellular anterior stroma immediately after PRK. However, the current study did demonstrate a significant reduction in keratocyte density in the most anterior confocal image compared with the next deeper analyzed confocal image (5%-10% stromal depth) at 5 days after PRK. This difference in keratocyte density would not be expected in the normal cornea and was not observed at other examinations after PRK, including 1 day after PRK. This measured decrease in keratocyte density in the most anterior confocal image at 5 days after PRK may represent the apoptotic cell loss demonstrated by Ambrosio et al. Apoptotic keratocyte loss immediately after PRK triggers a subsequent proliferation of keratocytes. In the highly reactive rabbit, the volume void of keratocytes is repopulated within a few days postoperatively. It is hypothesized that this increase in keratocyte number occurs by cell migration or mitosis. In the second part of the current study, keratocyte density in the post-PRK stroma was compared with keratocyte density in the pre-PRK future unablated stroma. In the pre-PRK future unablated stroma, the thickness of the anterior stroma destined to be photoablated was omitted from the analysis. This allowed a direct comparison of keratocyte density in the same tissue layers of the pre- and post-PRK stroma. Investigators have previously provided evidence for a functional, 3-dimensional keratocyte network that implies lateral and anterior-posterior communication. The presence of an anterior-posterior communication network between keratocytes may, in part, explain the apparent influence of surface excimer ablation on keratocytes deeper in the stroma.

The current study provides new long-term data demonstrating that keratocyte density in the anterior 10% of the post-PRK stroma continues to decrease by approximately 5% per year between 1 and 3 years postoperatively when compared with the same tissue layer preoperatively (pre-PRK future unablated stroma). The decline in anterior keratocyte density was statistically significant at 36 months after PRK. By contrast, keratocyte density in the middle and posterior stroma remained unchanged between 1 and 3 years after PRK. Fini hypothesized that this decline in keratocyte density after PRK represents apoptotic cell loss. Alternatively, Veal suggested that denervation may play a role in the diminished density of keratocytes. Mitoaka et al noted a similarity between reinnervation of the cornea and recovery of keratocyte density after LASIK. After PRK, however, Erich et al found no association between corneal reinnervation and keratocyte density.

The current study provides no confocal microscopy morphologic evidence of activated keratocytes or repair fibrocytes at 1 day after PRK. Activated keratocytes were first identified at 5 days after PRK in some eyes and, similar to previous human studies, were identified up to 6 months after PRK. By contrast, activated keratocytes are present for less than 1 month after LASIK when viewed by confocal microscopy. Confocal microscopy estimates of keratocyte density early after PRK have limitations. First, stromal edema at 1 and 5 days after PRK could falsely decrease keratocyte density. However, Muller et al showed that the stromal interweave architecture of the anterior stroma is resistant to swelling, which is confined mainly to the posterior stroma. Therefore, any differences in cell density between the most anterior confocal image and the next deeper image (5%-10% stromal depth) because of differential swelling should be minimal. Stromal swelling, however, would decrease the measured cell density in the middle and posterior layers and should be considered when interpreting cell densities early after PRK. Also, inflammatory cells have been identified in histologic studies during the first week after PRK in the rabbit and primate cornea. Some of the cell nuclei that were counted during the first week after PRK may have been clusters of inflammatory cells, and this would artificially increase the measured keratocyte density.

In summary, we used a noninvasive method to quantify long-term changes in keratocyte density in the human cornea after PRK. The clinical importance of the measured reduction in anterior stromal keratocyte density after PRK is unknown and may be appreciated only after longer follow-up.

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REFERENCES


