LONG-TERM CORNEAL KERATOCTYE DEFICITS AFTER PHOTOREFRACTIVE KERATECTOMY AND LASER IN SITU KERATOMILEUSIS

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ABSTRACT

Purpose: To measure changes in keratocyte density up to 5 years after photorefractive keratectomy (PRK) and laser in situ keratomileusis (LASIK).

Methods: This was a prospective, nonrandomized clinical trial. Eighteen eyes of 12 patients received PRK to correct a mean refractive error of –3.73 ± 1.30 D, and 17 eyes of 11 patients received LASIK to correct a mean refractive error of –6.56 ± 2.44 D. Corneas were examined by using confocal microscopy before and 6 months, 1 year, 2 years, 3 years, and 5 years after the procedures. Keratocyte densities were determined in five stromal layers in PRK patients and in six stromal layers in LASIK patients. Differences between preoperative and postoperative cell densities were compared by using Bonferroni-adjusted paired *t* tests.

Results: After PRK, keratocyte density in the anterior stroma was decreased by 39%, 42%, 45%, and 47% at 6 months, 2 years, 3 years, and 5 years, respectively ($P < .001$). At 5 years, keratocyte density was decreased by 20% to 24% in the posterior stroma ($P < .05$). After LASIK, keratocyte density in the stromal flap was decreased by 22% at 6 months ($P < .02$) and 37% at 5 years ($P < .005$). Keratocyte density in the anterior retroablation zone was decreased 18% ($P < .005$) at 1 year and 43% ($P < .005$) at 5 years. At 5 years, keratocyte density was decreased by 19% to 22% ($P < .05$) in the posterior stroma.

Conclusions: Keratocyte density is decreased in the anterior stroma after PRK and in the stromal flap and the retroablation zone after LASIK for up to 5 years. Posterior stromal keratocyte deficits are first noted at 5 years.

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INTRODUCTION

Development of the excimer laser by Trokel and coworkers[1] has resulted in a marked increase in refractive surgery in the past decade. Photorefractive keratectomy (PRK) sculpts the corneal surface by using an excimer laser to remove a layer of the anterior stroma. In laser in situ keratomileusis (LASIK), an anterior corneal flap is cut and an excimer laser removes a layer of the middle stroma. The epithelium and the anterior stroma are preserved during LASIK, and this is thought to modify the corneal wound-healing response when compared with PRK.[2]

The corneal stroma is populated by keratocytes, whose nuclei are visible in confocal microscopy. Human corneal keratocytes remodel structural proteins to maintain homeostasis, mediate wound repair, migrate in response to injury, and die, through apoptosis, in response to wounding.[3-6] Studies of human corneas after PRK and LASIK by histologic methods and confocal microscopy demonstrate a period of active wound healing augmented by activated keratocytes (≤6 months after surgery) and followed by a long period of corneal remodeling associated with quiescent keratocytes.[7-10] Keratocyte density estimated by confocal microscopy and light microscopy decreases in the first 3 years after PRK and LASIK.[9-14] It is not known whether this gradual loss of keratocytes continues beyond 3 years after laser refractive surgery or whether keratocyte density recovers. We also do not know the consequences of keratocyte loss to the stroma, or whether there is a minimum number of keratocytes needed to maintain the health of the cornea.

The clinical confocal microscope provides a means of repeated noninvasive examination of corneal keratocyte nuclei. The keratocyte density estimated by using confocal microscopy is consistent with density estimated by using light microscopy, DNA distribution, or vital dyes.[9,10,15-17] In this prospective comparative trial, we extended our previous observations[11-14] of keratocyte density after PRK and LASIK to 5 years after each procedure.

METHODS

PATIENTS

We studied 18 eyes of 12 patients (three men, nine women) who received PRK and 17 eyes of 11 patients (one man, 10 women) who received LASIK at the Mayo Clinic, Rochester, Minnesota, between July and October 1998. PRK patients were 40 ± 7 years old (range, 22 to 53 years) and had a mean preoperative refractive error of –3.73 ± 1.30 D (range, –1.25 to –5.75 D). LASIK patients were 32 ± 9 years old (range, 22 to 50 years) and had a mean preoperative refractive error of –6.56 ± 2.44 D (range, –2.00 to –11.00 D). All patients had a complete ophthalmologic examination before surgery to ensure that the cornea and anterior segment were normal.

Patients who had had previous ocular surgery, who had glaucoma or diabetes, or who were taking topical ocular medications were excluded. After surgery, none of the patients had a second operation or wore contact lenses beyond 5 days. One LASIK patient failed to return for the 5-year examination. Each patient gave informed consent to participate after the nature and possible consequences of the study had been explained. The study was approved by the Institutional Review Board of Mayo Clinic and followed the Declaration of Helsinki recommendations for research involving human subjects.

PRK PROCEDURE

A 6.3-mm-diameter circular area of epithelium was removed by using the laser-scrape technique. The corneal surface was ablated by using a VISX Star 2 excimer laser (VISX, Santa Ana, California) to a mean planned depth of 46 ± 18 µm (±SD; range, 13 to 90 µm). Postoperatively, patients wore a bandage soft contact lens (SofLens 66, Bausch & Lomb Inc, Rochester, New York) until the cornea epithelialized (2 to 5 days). Topical medications were ofloxacin 0.3% (Ocuflox, Allergan Inc, Irvine, California), four times daily until epithelialization was complete, and fluorometholone 0.1% (FML, Allergan Inc), four times daily with a taper over 8 to 12 weeks.

LASIK PROCEDURE

A Hansatome microkeratome (Chiron Vision Corp, Claremont, California) was used to create a flap with a superior hinge and a planned thickness of 180 µm. The actual mean flap thickness was 160 ± 28 µm.[13] The midstroma was ablated by using a VISX Star 2 excimer laser to a mean planned depth of 63 ± 26 µm. Postoperatively, topical medications were ofloxacin 0.3% four times daily for 5 days and fluorometholone 0.1% four times daily with a taper over 3 weeks.

CONFOCAL MICROSCOPY

Corneas were examined by using a tandem scanning confocal microscope (Tandem Scanning Corporation, Reston, Virginia) before and at 6 months and 1 year, 2 years, 3 years, and 5 years after PRK and LASIK. The method of examination has been previously described.[17] Briefly, 2.5% hydroxypropyl methylcellulose (Goniosol, CIBA Vision Ophthalmics, Atlanta, Georgia) optical coupling medium was placed on the tip of the objective lens, and the lens was advanced until the solution contacted the cornea. The objective lens was aligned with the center of the cornea by centering the light and dark rings of the epithelial image. After alignment, the focal plane was scanned through the cornea at approximately 72 µm per second from anterior to posterior. Digital images were captured with the video camera in an automatic-gain mode (gain was set by the camera to optimize image brightness) and were stored on a computer workstation (Indy, Silicon Graphics Inc, Mountain View, California) at 30 frames per second. We also recorded images with the camera gain fixed manually to estimate the brightness of scattered light from the cornea. Brightness of these images was adjusted for brightness of a fluorescent glass model cornea. Each image represented a coronal section of 475 µm × 350 µm (horizontal × vertical), was separated from the adjacent image by an average of 2.4 µm, and had a depth of field of 11.9 µm.[18] Four to eight scans were recorded at each visit. All scans were within the central 4 mm of the cornea, although not in the identical region each time. Typically, keratocyte density was assessed from one scan from each eye with the least transverse movement and no anteroposterior movement.

Changes in confocal image contrast[18] and cell brightness that could affect estimates of cell density were evaluated over the 5-year study period. Cell brightness was calculated as the mean intensity of individual cells in scans recorded with the gain of the video camera fixed. Image contrast was calculated as the difference between the cell brightness and brightness of the background region around the cell divided by the brightness of the background region. Contrast was determined from the same scans used to assess cell density, with the video gain automatically controlled by the camera.

STROMAL LAYERS

In PRK, five layers were considered (Figure 1) as described previously: (1) 0% to 10% (anterior), (2) 11% to 33%, (3) 34% to 66%, (4) 67% to 90%, and (5) 91% to 100% (posterior) depth.[12,14] In the pre-PRK and post-PRK cornea, the boundaries of the stromal layers were determined relative to the depth of 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, but without visible endothelial cells. In the pre-PRK cornea, the thickness of Bowman's layer was the distance between the first focused image of subbasal nerves and the most anterior keratocytes.

In LASIK, six layers of stroma were considered (Figure 2) as described previously: (1) anterior half of the stromal flap, (2) posterior half of the stromal flap, (3) anterior half of the 100-µm-thick retroablation zone, (4) posterior half of the retroablation zone, (5) posterior 66% to 90% of the pre-LASIK stroma, and (6) posterior 91% to 100% of the pre-LASIK stroma.[11,13] Small bright objects in the anterior stroma of all post-LASIK scans were used to identify the flap interface in postoperative corneas.[9-13] The thickness of the stromal flap (distance from the most anterior keratocytes to the flap interface) and the stromal bed (distance from the flap interface to the endothelium), determined at the 1-month post-LASIK scan, was used to delimit the corresponding anterior and posterior stromal layers in the pre-LASIK cornea. This left a gap between the flap and the bed in the preoperative cornea, a gap that represented the tissue destined for ablation. Cell density in this gap was not assessed. This allowed us to compare the same tissue layers in the pre- and post-LASIK stroma (Figure 2). After LASIK, the posterior 66% to 90% and 91% to 100% of stroma were determined from the pre-LASIK stromal thickness (distance between the most anterior keratocytes and the endothelium).
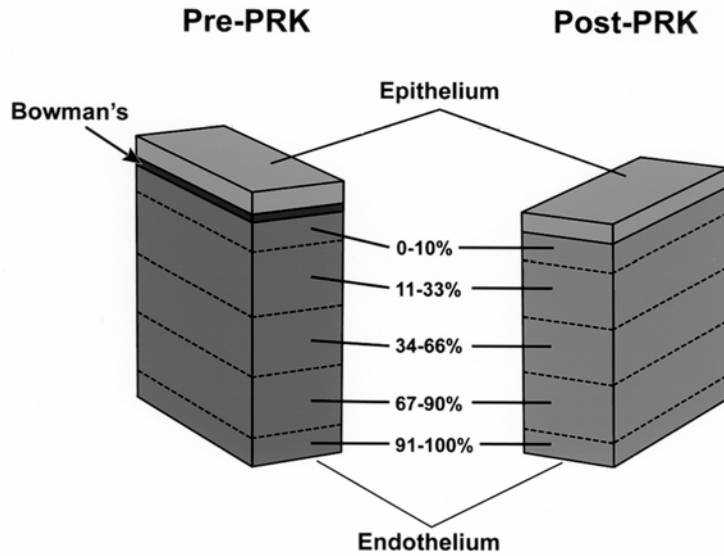
FIGURE 1

Schematic representation of the five stromal layers studied in pre-PRK and post-PRK cornea. Stromal layers in the pre-PRK cornea are compared with corresponding layers in the post-PRK cornea.
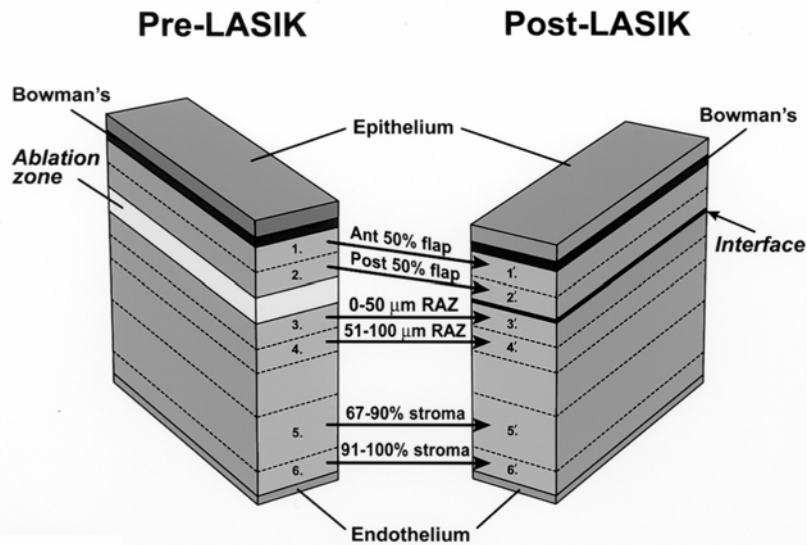

FIGURE 2

Schematic representation of the six stromal layers studied in pre-LASIK and post-LASIK cornea. The thickness of stroma equivalent to the ablation depth (ie, ablation zone) in the pre-LASIK cornea was omitted from analysis to allow comparison of the same tissue layers preoperatively and postoperatively. RAZ = retroablation zone.

KERATOCYTE DENSITY

Two images with no motion artifact were selected from each stromal layer for assessment of cell density for a total of 10 images in PRK and 12 images in LASIK assessed per eye per visit. In the most anterior stromal layer, one of the two images was always the most anterior image containing keratoctyes. The selected confocal images before PRK and LASIK and at all visits after PRK and

LASIK were randomly presented to an observer who was masked to the patient, time after surgery, and stromal layer. Keratocyte nuclei (cells) were identified as bright objects in a predefined area (0.109 mm^2) of each selected image to calculate keratocyte density (cells/mm^3) by using a custom computer program (Figure 3).[17] The mean cell density in each layer after PRK and LASIK was compared with the mean cell density in the corresponding layer before PRK and LASIK (Figures 1 and 2). The full-thickness density was estimated by dividing the number of cells in a full-thickness stromal column with a cross-sectional area of 1 mm^2 by the stromal thickness. The number of cells in the column was estimated from the mean density in each analyzed frame weighted by the distance between frames.[17] Cell density estimates are approximately 30% higher in the current study than in our previous studies[11-14] because image depth of field was recalculated to be 11.9 µm in the current study rather than 16 µm, which was used in previous studies.[18]
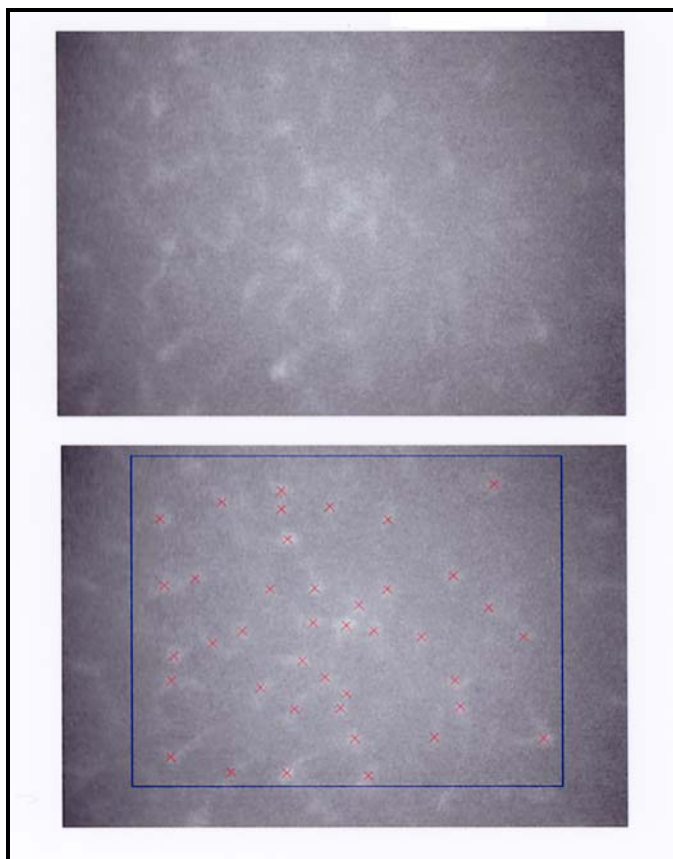

FIGURE 3

Method to determine keratocyte density. Keratocyte nuclei (top) were manually counted to determine keratocyte density (cells/mm^3) in two images from each stromal layer, as described by Patel and associates.[17] By convention, nuclei overlapping the edges of the bounding box were counted on only two sides (left and lower side).

We examined intraobserver variability of cell density assessment by repeating estimates of cell density of a subset of subjects after a 2-year interval (between 3 and 5 years after surgery). Seven subjects were randomly selected from the PRK group, and nine were selected from the LASIK group. After their 5-year examinations, cell densities in their 3-year examinations were reassessed in the same frames as they were originally assessed.

STATISTICAL ANALYSIS

Keratocyte densities at all postoperative visits were compared with keratocyte densities before surgery by using a paired *t* test. Significances of differences were Bonferroni-adjusted for five comparisons. For each cornea, the slope of a line fitted to the logarithm of density versus time for the five measurements between 6 months and 5 years was calculated for each stromal layer. The mean slope for each layer was compared to zero by Student *t* tests. Tests were checked for the effects of potential correlations between measurements from both eyes of the same subject by using generalized estimating equation models.[19] The results of these models were

similar to results of the standard tests, and only the results of the standard tests are presented. The significance of the differences between cell density assessed from the same frames at 3 and 5 years was determined by using a paired *t* test. All statistics were calculated by using SAS software (SAS Institute Inc, Cary, North Carolina). A *P* value of <.05 was considered statistically significant.

RESULTS

Keratocyte densities before and after PRK and LASIK are shown in Tables 1 and 2. After PRK, keratocyte density was decreased by 39% in the anterior 10% of the stroma at 6 months ($26,050 \pm 3,395$ cells/mm^3) compared with density in the anterior 10% before PRK ($43,313 \pm 7,925$ cells/mm^3, $P < .001$, Table 1). Between 6 months and 5 years, keratocyte density in the anterior stroma continued to decrease at a rate of 3.4% per year ($P = .02$, Table 3). At 5 years after PRK, keratocyte density in this layer was reduced by 47% ($23,007 \pm 6,140$ cells/mm^3; $P < .001$, Table 1). Keratocyte density in the middle and posterior stroma did not change at each visit for up to 3 years (Table 1, Figure 4). At 5 years after PRK, a significant decrease in keratocyte density was first measured in the posterior two thirds of the stroma ($P < .005, .03,$ and $.04$; Table 1; Figure 4). Between 6 months and 5 years, keratocyte density in the full-thickness stroma decreased at an average annual rate of 3.2% (Table 3), more than seven times the normal annual rate of 0.45%.[17]

By 6 months after LASIK, keratocyte density was decreased by 23% ($P = .02$), 19% ($P < .005$), and 15% ($P = .004$) in the anterior stromal flap, posterior stromal flap, and anterior retroablation zone, respectively, when compared with the density in the same layers before LASIK (Table 2). Between 6 months and 5 years, keratocyte density continued to decrease in these layers, and by 5 years, densities were reduced by 32%, 42%, and 42% of pre-LASIK densities, respectively ($P < .001$, Table 2, Figure 5). Keratocyte density was decreased from pre-LASIK densities in the posterior retroablation zone ($P = .02$), the posterior 66% to 90% layer ($P < .001$), and the posterior 91% to 100% layer ($P = .04$) at 5 years, but not earlier (Table 2, Figure 5). Between 6 months and 5 years, keratocyte density in the full-thickness stroma decreased at an average annual rate of 4.2% (Table 4), 10 times the annual rate in normal corneas of 0.45%.[17] The highest rates of decrease (7.2% and 8.4%) were adjacent to the flap interface (Table 4).

When keratocyte densities were randomly assessed in the same frames by the same masked investigator after 2 years, the mean difference in cell density was $640 \pm 4,274$ cells/mm^3 in the PRK patients and $128 \pm 2,185$ cells/mm^3 in the LASIK patients. These differences were not significantly different from zero (paired *t* test, $P = .70$ and $P = .87$, respectively). The average ratio of the difference between the first and second measurement to the mean of the two measurements was 0.029 for PRK and 0.005 for LASIK. The minimum detectable differences between the first and second measurements by the same observer were 4,050 cells/mm^3 for PRK and 1,730 cells/mm^3 for LASIK (paired *t* test, $\alpha = .05$, $\beta = .20$).

Figures 6 and 7 demonstrate brightness of cells and background as well as contrast in images throughout the study. Cell and background brightness varied by 10 to 15 intensity units but did not increase or decrease consistently throughout the study. Cell contrast increased after surgery, reached a maximum at 6 months, and returned to presurgery levels at 1 year. From 1 to 5 years, cell contrast did not change.
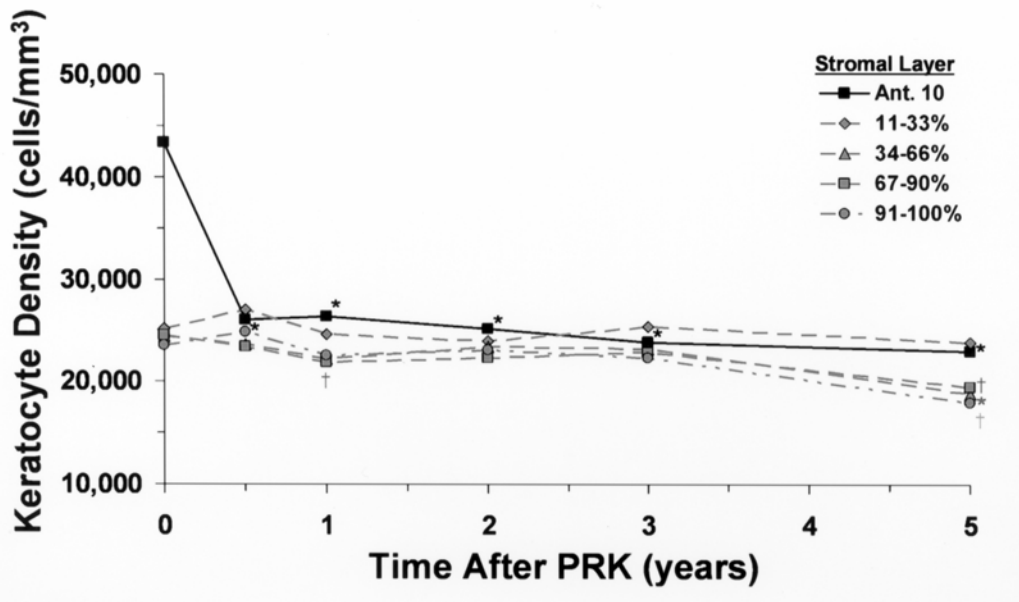

FIGURE 4

Keratocyte density before and after PRK. In the anterior 10% of the stroma, keratocyte density was diminished at all post-PRK visits relative to density before PRK. Cell density in most remaining stromal layers was not decreased until 5 years after PRK. *$P < .005$ and †$P < .05$, compared with densities before PRK.

TABLE 1. KERATOCYTE DENSITY (MEAN ± SD, CELLS/MM^3) BEFORE AND AFTER PHOTOREFRACTIVE KERATECTOMY (PRK)											
STROMAL LAYER (% DEPTH)		*TIME AFTER PRK*									
	Pre-PRK	**6 mo**	*P**	**1 yr**	*P**	**2 yr**	*P**	**3 yr**	*P**	**5 yr**	*P**
0% to 10% (anterior)	43,313 ± 7,925	26,050 ± 3,395	<.001	26,318 ± 5,258	<.001	25,142 ± 5,712	<.001	23,846 ± 5,975	<.001	23,007 ± 6,140	<.001
11% to 33%	25,121 ± 2,813	27,042 ± 4,776	NS	24,592 ± 3,511	NS	23,921 ± 6,672	NS	25,369 ± 4,390	NS	23,869 ± 4,984	NS
34% to 66%	24,388 ± 4,864	23,632 ± 5,054	NS	22,240 ± 5,459	NS	23,416 ± 4,922	NS	23,221 ± 3,816	NS	18,826 ± 5,204	<.001
67% to 90%	24,473 ± 3,367	23,416 ± 3,499	NS	21,830 ± 4,802	NS	22,337 ± 5,449	NS	22,897 ± 3,591	NS	19,532 ± 5,855	.02
91% to 100% (posterior)	23,524 ± 5,003	24,775 ± 4,234	NS	22,564 ± 4,065	NS	23,050 ± 4,362	NS	22,337 ± 3,895	NS	17,935 ± 6,667	.04
Full thickness	26,221 ± 2,897	24,755 ± 3,302	NS	23,301 ± 3,564	.001	23,776 ± 3,596	.004	23,459 ± 3,394	.009	21,017 ± 4,533	<.001

NS = not significant (*P* > .05).
*Significance of differences compared to pre-PRK by using Student's paired *t* test (Bonferroni-adjusted for five comparisons).

TABLE 2. KERATOCYTE DENSITY (MEAN ± SD, CELLS/MM^3) BEFORE AND AFTER LASIK

STROMAL LAYER	Pre-LASIK	6 mo[†]	P*	1 yr[†]	P*	2 yr[†]	P*	3 yr[†]	P*	5 yr[‡]	P*
Anterior flap	47,004 ± 6,896	41,063 ± 4,876	.02	37,863 ± 7,910	<.002	37,156 ± 7,071	.005	34,619 ± 5,307	<.001	31,976 ± 4,072	<.001
Posterior flap	34,277 ± 5,461	27,605 ± 4,571	<.005	24,999 ± 4,300	<.001	25,821 ± 5,141	<.001	21,892 ± 3,239	<.001	19,715 ± 4,378	<.001
Anterior RAZ	28,793 ± 4,023	24,474 ± 3,811	<.004	23,560 ± 3,981	<.003	21,389 ± 3,929	<.001	21,229 ± 4,712	<.001	16,559 ± 2,676	<.001
Posterior RAZ	27,599 ± 4,787	26,074 ± 2,907	NS	26,279 ± 2,681	NS	27,451 ± 2,973	NS	25,913 ± 3,336	NS	22,943 ± 3,286	.02
66% to 90% (posterior)	29,753 ± 4,275	27,467 ± 3,823	NS	27,559 ± 3,760	NS	27,879 ± 3,719	NS	26,668 ± 3,587	NS	23,429 ± 3,359	<.001
91% to 100% (posterior)	28,770 ± 5,457	27,444 ± 4,694	NS	27,879 ± 3,205	NS	25,091 ± 3,672	NS	27,490 ± 3,205	NS	23,454 ± 3,463	.04
Full-thickness	31,108 ± 4,984	28,337 ± 2,863	NS	27,533 ± 2,757	.004	27,491 ± 2,693	.05	26,320 ± 1,972	.005	22,982 ± 1,829	<.001

NS = not significant (*P* > .05); RAZ = retroablation zone.
*Significance of differences compared to pre-LASIK by using Student's paired *t* test (Bonferroni-adjusted for five comparisons).
[†]Seventeen eyes of 11 patients.|
Sixteen eyes of 10 patients.

TABLE 3. CHANGE IN KERATOCYTE DENSITY BETWEEN 6 MONTHS AND 5 YEARS AFTER PHOTOREFRACTIVE KERATECTOMY (PRK)

STROMAL LAYER	RATE OF CHANGE (% PER YEAR)*	P[†]
0% to 10% (anterior)	–3.4 ± 5.4	.02
11% to 33%	–1.6 ± 4.9	.15
34% to 66%	–4.4 ± 3.3	<.001
67% to 90%	–3.5 ± 4.8	.007
91% to 100% (posterior)	–7.5 ± 8.4	.002
Full-thickness	–3.2 ± 2.8	<.001

*Instantaneous annual rate of change (%) in keratocyte density (mean ± SD).
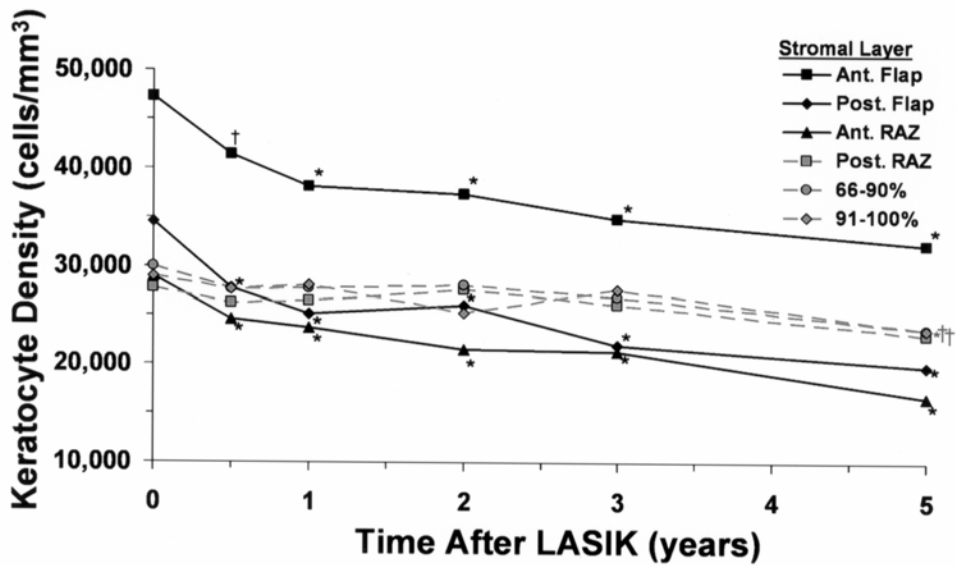†Student t test versis zero.

FIGURE 5

Keratocyte density before and after LASIK. In the anterior and posterior stromal flap and the anterior retroablation zone (RAZ), keratocyte density was decreased at all post-LASIK visits from density before LASIK. Cell densities in all remaining stromal layers were first decreased at 5 years after LASIK. *P < .005 and †P < .05, when compared with densities before LASIK.

TABLE 4. CHANGE IN KERATOCYTE DENSITY BETWEEN 6 MONTHS AND 5 YEARS AFTER LASIK

STROMAL LAYER	RATE OF CHANGE (% PER YEAR)*	P†
Anterior flap	–4.3 ± 3.2	<.001
Posterior flap	–7.2 ± 4.3	<.001
Anterior RAZ (0 to 50 µm)	–8.4 ± 3.7	<.001
Posterior RAZ (51 to 100 µm)	–2.6 ± 4.1	.02
Posterior 66% to 90%	–3.5 ± 3.4	<.001
Posterior 91% to 100%	–3.1 ± 2.2	<.001
Full-thickness	–4.2 ± 2.2	<.001

RAZ = retroablation zone.
*Instantaneous annual rate of change (%) in keratocyte density (mean ± SD).
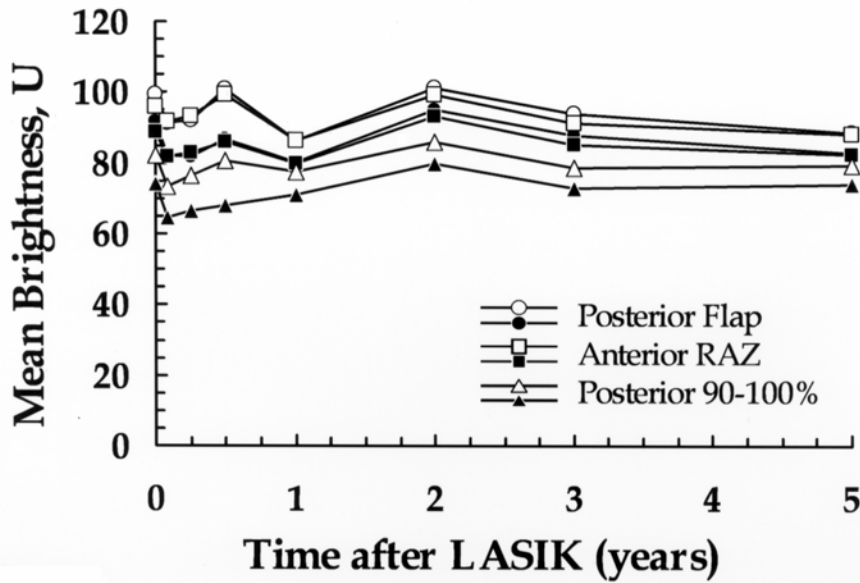†Student *t* test versus zero.

FIGURE 6

Change in cell and background brightness over time. Mean brightness of cells (open symbols) and the background area immediately surrounding the cells (solid symbols) in the layers immediately anterior and posterior to the interface, and the posterior 90% to 100% of stromal thickness after LASIK. Brightness was adjusted for variations in the illumination brightness and sensitivity of the video camera by using measurements of a fluorescent glass standard. Units of brightness are arbitrary and represent the digitized output of the video camera. Brightness remained steady between 1 and 5 years when keratocyte density decreased, suggesting that potential changes in confocal image brightness did not affect our ability to identify cells.

FIGURE 7
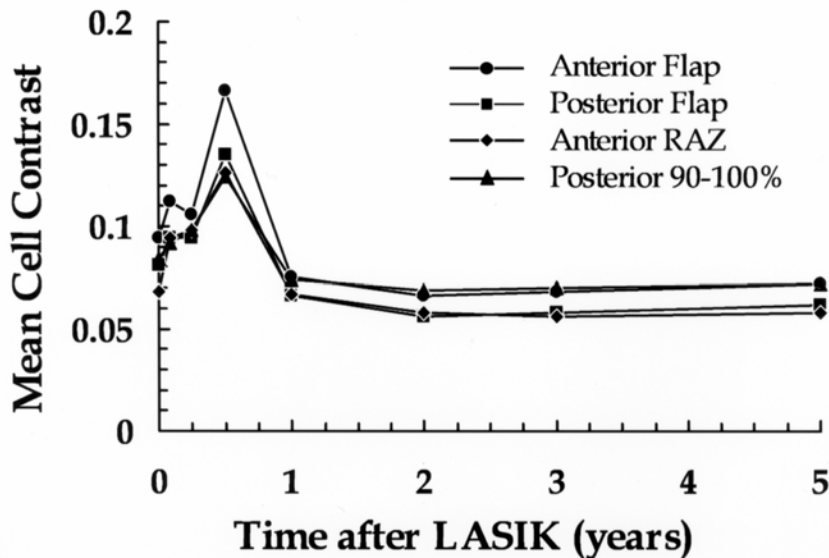
Change in contrast over time. Mean contrast between cells and background in the same frames that were used to determine cell density. After LASIK, contrast increased to a maximum at 6 months and then returned to pre-LASIK contrast by 1 year. Contrast remained steady between 1 and 5 years, suggesting that potential changes in confocal image contrast did not affect our ability to identify cells.

DISCUSSION

This prospective 5-year longitudinal clinical trial demonstrates a gradual loss of keratocytes from the anterior stroma after PRK and from the stromal flap and the stroma immediately posterior to the ablation interface after LASIK. By 5 years after both procedures, keratocyte loss was also significant in the posterior stroma.

In the normal cornea, keratocyte density is highest in the anterior 5% to 10% of the stroma—approximately 40% higher than cell density in the middle and posterior stroma.[16,17] During PRK, this keratocyte-rich anterior stroma is removed by photoablation. Although keratocytes are able to divide and migrate after wounding,[5,6,20] our study shows that keratocytes do not repopulate the post-PRK anterior stroma to the densities in this layer before PRK for at least 5 years. Rather than repopulate the anterior stroma, keratocyte density decreases an additional 12% between 6 months and 5 years (approximately 3.6% per year). This rate of keratocyte loss is approximately nine times faster than the 0.4% per year loss in the normal aging cornea.[17] Early after wounding by PRK, corneal keratocytes disappear through apoptosis mediated by cytokines released from injured epithelium.[7] Some investigators have suggested that apoptosis could also be responsible for long-term losses of anterior keratocytes through continued epithelial-stromal interactions or cell-matrix interactions.[3,13]

Keratocytes in the middle and posterior stromal layers (11% to 100% depth) appear to be less affected by PRK than the anterior keratocytes were. Cell density in these layers was unchanged through 3 years relative to density before PRK. At 5 years after PRK, however, keratocyte loss first became significant in the middle and posterior stroma. The reason for this delayed loss is unclear, but small molecular signals to undergo apoptosis could be directed to deeper stromal keratocytes via gap junctions.[21]

In earlier studies, keratocyte density decreased in the stromal flap and anterior retroablation zone as early as 1 month after LASIK.[10,11] Our current data demonstrate that this early keratocyte loss in the anterior and posterior stromal flap and in the anterior retroablation zone progresses to deficits of 32%, 42%, and 42% of pretreatment densities, respectively (*P* < .005), by 5 years after LASIK. In a recent study of 13 postmortem LASIK corneas, Dawson and coworkers[10] were unable to detect a decrease in keratocytes with increasing postoperative time, but their cross-sectional investigation lacked both central corneal control densities and the statistical power to detect longitudinal differences similar to ours. In histopathologic studies,[4,7] epithelial and stromal injuries induced by the microkeratome caused keratocyte apoptosis in the stromal layers anterior and posterior to the lamellar interface. This localized loss has been attributed to epithelial debris, including apoptosis-inducing cytokines, being tracked into the interface by the microkeratome blade.[7] Recently, implanted epithelial cells (both viable cells and degenerating cells) have been found in the LASIK interface years after surgery.[9,10] These implanted epithelial cells may contribute to the localized long-term keratocyte deficits through release of apoptotic cytokines that diffuse along the interface and into the central stroma.

Alternatively, a causal relationship between decreased keratocyte density and decreased innervation after LASIK has been hypothesized.[11-13,22] Müller and coworkers[23] documented direct innervation of keratocytes by stromal nerves. A normal stromal keratocyte population may depend on a normal density of corneal nerves. Transplanted corneas, for example, have both keratocyte[24] and nerve deficits.[22] We recently showed that subbasal nerves were still reduced 21% by 5 years after LASIK, although nerve densities were not significantly different from preoperative densities (Erie JC, et al, ARVO Meeting, 2005, E-abstract 4355). What effect, if any, a reconstituted nerve density has on future keratocyte densities is unknown. It is likely that the epithelium, stroma, and nerves all participate, to varying degrees, in the homeostasis of stromal keratocytes. Their eventual effect on keratocyte density after PRK and LASIK will require longer follow-up.

The clinical significance of a reduced keratocyte population after PRK and LASIK and its effect on the long-term health of the cornea is unknown. Wilson and coworkers[7] suggested that the high density of keratocytes in the anterior stroma provides some form of protection against infection of the corneal epithelium and minimizes posterior extension of infections. A normal keratocyte density, however, does not seem to be necessary for corneal clarity or good vision after PRK and LASIK. All of our study patients had clear corneas and a corrected visual acuity of 20/25 or better at all postoperative visits. Similarly, Rajan and coworkers[25] have shown good clinical results for up to 12 years after PRK. Recently, Dawson and coworkers[9] measured the image brightness from a 30-µm-thick optical section that included the 4- to 6-µm-thick interface scar in 13 postmortem LASIK corneas and found it not to differ from normal corneas. Although this measurement had the statistical power to detect only very large differences in backscattered light, the result is nevertheless consistent with clinical impressions that the hypocellular interface scar does not degrade vision. The possibility of a long-term effect of keratocyte insufficiency cannot be ruled out, however, given the many functions of these cells.

Estimates of keratocyte density after PRK and LASIK by confocal microscopy have potential limitations, which we investigated as possible sources of spurious results. First, manual assessment of keratocytes is subjective and could be affected by intraobserver variability over our 5-year study period. Previous evaluations of keratocyte estimates in normal corneas have shown intraobserver variability to be approximately 7.8%.[18] In the current study, keratocyte density estimated on two occasions separated by 2 years differed by 2.9% after PRK and 0.5% after LASIK, and these differences were not statistically different from zero. Second, changes in backscattered light, cell brightness, and image contrast after PRK and LASIK could limit one's ability to accurately identify keratocytes in confocal images. Cell and background brightness varied somewhat after both PRK and LASIK, although they did not vary in a way that was consistent with the changes in cell density. Contrast between cells and the immediate background, which is important for identifying bright objects as cells, increased to a peak at 6 months after LASIK, but then decreased to pre-LASIK contrast at 1 year. This change in contrast is consistent with the appearance and disappearance of highly reflective, activated wound-response keratocytes.[10,26,27] Image contrast remained steady between 1 and 5 years. Keratocyte density decreased during this same time. If contrast had affected our ability to estimate keratocyte density, contrast should have changed as apparent cell density changed.

The characteristics of cell images, scatter of light, cell brightness, and image contrast did not likely affect our ability to assess keratocyte density in this study.

All LASIK procedures were performed by using a superior hinge with a mean flap thickness of 160 µm. It is possible that a nasal hinge or a thinner corneal flap could affect keratocytes differently. Additionally, our LASIK mean midstromal ablation depth was 17 µm greater than the PRK mean surface ablation depth. The difference in the amount of stromal tissue removed in the PRK and LASIK groups may also contribute to the observed differences in keratocyte density.

In summary, we used a noninvasive method to study long-term changes in keratocyte density in human corneas after PRK and LASIK. By measuring and following changes in keratocyte density in various layers of the cornea after these procedures, one may better understand their long-term biologic and clinical consequences.

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PEER DISCUSSION

DR ROGER F. STEINERT. Only a few long-term studies of corneal laser refractive surgery outcomes are available. These studies are difficult to accomplish. Younger patient populations such as those undergoing refractive surgery tend to be mobile, reducing availability. Happy patients are not motivated to return for even routine exams, much less complex investigations. Unhappy patients may not wish to revisit the surgeon's practice. Funding for these studies is difficult to obtain. This five-year study utilizing confocal microscopy to examine keratocyte density after corneal refractive surgery is, therefore, particularly remarkable.

The principal findings are 1) an immediate decline in keratocytes at all layers of the cornea after either photorefractive keratectomy (PRK) or laser in-situ keratomileusis (LASIK), and 2) a trend toward ongoing keratocyte decline throughout 5 years of follow-up.

The previously observed acute decline in keratocyte density in the stroma underlying the ablation zone in PRK has been attributed to the release of apoptosis-inducing cytokines from the injured epithelium and stroma. But how can this effect persist for at least 5 years? Is it due to the lack of Bowman's layer in PRK patients and the presence of implanted epithelium, not clinically evident, in LASIK flap interfaces? Denervation is another possible factor, although the authors' own studies have documented full return of sub-basal corneal nerves by 5 years postoperatively. A multivariate analysis of clinical variables including the amount of optical correction, vision outcomes, age, and gender, among other factors, might help identify other correlations with keratocyte depletion.

A weakness of this investigation is the absence of a control group. Six patients in the PRK group and five patients in the LASIK group were studied unilaterally. The authors do not state whether the fellow eye remained untreated or were excluded for other reasons. If untreated, these eyes would have been ideal controls. The failure to have a contemporaneous control group leaves unanswerable concerns about possible artifact in the measurements and information about the natural history of keratocyte decline due to aging. A frequently cited figure, from another study by Dr Bourne and coworkers, is that keratocyte density declines by about 4% per decade. The decline in keratocyte density in this study certainly exceeds that figure. But, absent a control group, we do not know with any certainty the natural history of keratocyte density for this population and with the methodology employed here.

The measurement of keratocyte density is fraught with technical challenges. The study provides no data on corneal thickness. A change in thickness would lead to a decline in keratocyte density if the amount of extracellular matrix increases relative to cell numbers. The image provided has a relatively low contrast, and the reported measured contrast is also low, which could lead to increased error in counting cells. In addition, keratocytes are not the only cell population in the stroma, but confocal microscopy may not adequately distinguish keratocytes from Schwann cells and bone marrow derived cells, for example. Finally, the inability of confocal microscopy to detect a keratocyte does not necessarily mean that a keratocyte is really absent.

The authors are to be congratulated for observations that are notable and, if confirmed and extended, raise concerns that excimer laser ablation may ultimately lead to a decline in keratocytes below the level necessary to maintain extracellular matrix turnover. One can speculate that this loss might lead to corneal ectasia. I am unaware of any natural disease caused by progressive loss of keratocytes, although it is not possible that the excimer laser has created a new iatrogenic disease. These findings require validation in other controlled studies and using other techniques, but such studies will be challenging to formulate and accomplish.

ACKNOWLEDGEMENTS

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DR CHRISTOPHER J. RAPUANO. Were the manual counts performed in a masked fashion? Were the counts at six months performed at the same time, and by the same people, as the counts at five years? In other words, were the six-month counts done four years ago by different people than the current counts?

DR STEVEN E. WILSON. I encourage you to use the same technology to look at patients who are having mitomycin prophylactic treatment for prevention of haze; probably 90 percent of refractive surgeons are using mitomycin without any long-term data as to the effect. It is clear the reason mitomycin works so well is that it eliminates 100 percent of all corneal cells in about 20 percent of the anterior cornea. Similar to your concerns, I think they are even magnified in those patients because data after six months in the animal model shows that none of those cells have returned. What happens in the future since we have limited experience with these types of patients? In 10 to 20 years, are we going to see anterior corneal necrosis or other problems? Your type of study could give us more data about that in humans.

DR GEORGE O. WARING III. How did you see the interface? That is, what landmarks were easy to see? It is certainly hard to see with a slit lamp. What would you speculate is the reason that the cells do not divide and repopulate? Are they like endothelial cells,

which we don't think they are? The keratocytes are certainly capable of reproducing under stress and making hypercellular scars. What is going on here in terms of recovery?

The database maintained by Edelhauser, Grossniklaus, and Dawson at Emory now has about 150 eye bank specimens of human corneas of patients that have had LASIK. The main finding in the central area is a hypocellular scar. That scar does not re-populate itself with keratocytes and the findings are similar to what you've reported. At the edge of the scar, which is also the edge of the flap, there is a hypercellular scar with myofibroblasts and fibroblasts. This appears as a regular normal scar tissue, presumably from epithelial stromal interaction. This means you can lift the LASIK flap indefinitely after LASIK. My longest personal LASIK flap lift is 12 years, and it was done very easily. We have performed biomechanical studies now at Emory up to eight years post-operatively and find that the strength of the lamellar wound is about 2 percent of the normal cornea. So that flap does not heal normally in the center and perhaps this is related to the absence or the decrease of keratocytes in that area, or the decrease in epithelial stromal interaction.

DR JAY C. ERIE. Regarding Dr Rapuano's question, the selected confocal images before and after PRK and LASIK were randomly presented to a single observer who was masked to the patient, time after surgery, and stromal layer. All images were counted at the same time – after the 5-year exam.

We thank Dr Wilson for his comments regarding mitomycin and its effect on keratocytes.

Dr Waring, the interface is easily seen at all times after LASIK when using the confocal microscope. This is due to highly reflectile particles in the interface that are seen with confocal microscopy but not by biomicroscopy. The work by Dawson and coworkers [1] has shown that the reflectile particles are vacuoles within interface keratocytes. Additionally, epithelial cells and plastic particles are also introduced by the microkeratome. We do not have an explanation for the observed keratocyte deficits after these surgical procedures. Possibilities include chronic denervation, chronic apoptosis, and increased susceptibility to ultraviolet light damage.

Regarding Dr Steinert's discussion: first, we recognize that we did not have concurrent controls and, as such, cannot rule out the possibility that changes in cell density could have resulted from systematic changes in our microscope and subjective estimation of density. .Artifacts with the confocal microscope were a concern to us, especially when we began seeing declining cells in the posterior stroma at five years. Could this be due to changes in image contrast, meaning, are there cells there that we just cannot see and that is why we are not counting them? We investigated image contrast and looked at differences between cell brightness and background brightness over that five-year period. We found that cell contrast did increase and decrease in the first year, consistent with the migration of activated keratocytes into the area and then out again. But, at one year through five years, there was no change in image contrast. During this time we did see a decrease in cells. So, we do not think that subtle changes in the microscope or image contrast changes accounted for our observations.

We did use both eyes for some patients. To account for this, we used the generally estimating equation model, which corrects for potential bias between two eyes of the same observer. Eyes that required a surgical enhancement for an initial undercorrection were excluded from the study. This is the reason some patients only had one eye included in the study.

We studied thickness changes in the same group over the same time period and found that stromal thickness increased 5.5 µm (0.3% / year) in the LASIK group and 2.2 µm (0.1% / year) in the PRK group. Therefore we do not think thickness changes account for our observed keratocyte changes. We also recognize that the bright white objects we are counting may not necessarily be only keratocyte nuclei. They could be other corneal cells. But others have shown, as we have, that keratocyte density estimates using confocal microscopy are similar to keratocyte density estimates using light microscopy. [2,3] We do recognize that some keratocytes are bone marrow-derived and confocal microscopy cannot distinguish between the two different types of keratocytes. What are the clinical implications, if any? For comparison, Dr William Bourne has demonstrated that 10 or 20 years after corneal transplant surgery, both keratocytes and nerves in the graft are reduced 40 to 50 percent, yet the corneas remain clinically clear.

Regarding normal age-related keratocytes cell loss, Dr Steinert referred to our study where we demonstrated, by using confocal microscopy, a 0.4 percent loss per year. As far as we are aware, this is the only published estimate of age-related keratocyte loss.

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