Aim: To characterise temporal changes in corneal wound repair at the LASIK flap margin.

Methods: 18 rabbits received monocular LASIK and were evaluated during 6 months using slit lamp and in vivo confocal microscopy. In three corneas, the exposed stroma was stained with DTAFl. At various time points, corneas were processed for histology and stained for nuclei, f-actin, ED-A fibronectin, α-smooth muscle actin, TGF-β1, TGF-β2, TGF-β receptor II, and CTGF.

Results: At day 1, leucocytes migrated from the conjunctival vessels into the cornea. Near the limbus, the leucocytes were organised in long chains stretching towards the flap edge. From day 4, elongated fibroblasts migrated from the periphery to align in a circumferential band (approximately 250 μm wide) next to the flap edge. The lateral extension of this stromal band was delimited by the incisional gap in the epithelial basement membrane. TGF-β1, TGF-β2, TGF-β receptor II, and CTGF were expressed in the band from day 2. Myofibroblasts were identified at week 3 and over time a 50 μm thick layer of fibrotic matrix was deposited. Concurrently, the peripheral circumferential band became narrower (width decreasing to 33% (SD 7%) at 4 months; n = 5) and showed an increased organisation with a gradual decline in reflectivity. At all time points, keratocytes within and below the flap remained quiescent and only minimal fibrosis developed at the interface.

Conclusions: Fibrotic wound repair following LASIK is restricted to a narrow band peripheral to the corneal flap edge. The lateral extension of the fibrosis is sharply delimited by the incisional gap in the epithelial basement membrane. The fibrotic wound healing at the LASIK flap margin is associated with myofibroblast transformation and wound contraction and involves a TGF-β signalling pathway.

Laser in situ keratomileusis (LASIK) is a modern refractive surgical procedure that involves temporary displacement of a hinged corneal tissue flap during laser treatment of the underlying stromal bed. LASIK is associated with a high risk of complications related to the corneal flap including epithelial ingrowth, flap melting, and traumatic flap dislocation. These complications may be related to the clinically observed lack of corneal wound healing that allows the corneal flap to be separated from its stromal bed for an indeterminate time after surgery. Thus, the LASIK interface is readily accessible for re-treatment more than 1 year after the initial procedure, and corneal flap dehiscence has been observed several years after uneventful surgery. Using slit lamp biomicroscopy, a white reflecting band is typically observed in the corneal flap periphery after LASIK (Fig 1). Over time, this circumferential band undergoes characteristic changes in width, texture, and reflectivity. The band is generally believed to represent corneal wound healing; however, its exact nature, localisation, and significance have not been thoroughly investigated. Using a rabbit eye model, the present study characterises temporal changes in corneal wound repair at the LASIK flap margin. Various components of the wound healing response are studied, including initial inflammation, TGF-β growth factor expression, keratocyte phenotypic transformations, deposition of extracellular matrix (fibrosis), and wound contraction.

MATERIALS AND METHODS

Study design

Eighteen New Zealand White rabbits (weight 4.0–5.5 kg) with normal corneas received monocular LASIK and were evaluated during 6 months using slit lamp and in vivo confocal microscopy. In three corneas, the exposed stroma was vitally stained during surgery with 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF; Sigma, Denmark); a green fluorescent probe that binds covalently to collagen, allowing detection of unstained, newly deposited matrix. Before surgery and at all examinations, animals were anaesthetised topically with oxybuprocaine 0.8%, and systemically with midazolam 2.0 mg/kg and Hypnorm 0.3 ml/kg (fentanyl 0.315 mg/ml, and fluanisone 10 mg/ml; Janssen Pharmaceuticals, Beerse, Belgium). Preoperatively, two drops of ciprofloxacin 3 mg/ml and chloramphenicol 0.5% were applied. Postoperative treatment (three times per day) included topical ciprofloxacin for 2 days and topical chloramphenicol and systemic buprenorphine (0.05 mg/kg) for 5 days. At selected time points, animals were killed by injecting sodium pentobarbital 150 mg/kg, and the corneas were processed for histology. The study was approved by the Danish Animal Experiments Inspectorate, and all animals were housed and treated according to the ARVO statement for the use of animals in ophthalmic and visual research.

Surgery

One week before LASIK, the nictating membrane was removed in all eyes. During LASIK surgery, the eye was gently proptosed and a hinged corneal flap was cut using a microkeratome (Supratome; Schwind, Kleinostheim, Germany). The flap was lifted and the stromal bed received a 6 mm diameter, −8.0 dioptres correction using a MEL 70 G-Scan excimer laser (Asclepion, Jena, Germany). After careful repositioning of the flap, a bandage soft contact lens (7.4 mm radius of curvature; Dk/t = 27; Igel Rx 67 Spheric UV; Ultravision International, Beds, UK) was inserted for 2 days to prevent flap dislocation. In three corneas, DTAFl staining was performed at surgery by irrigating the flap and stromal bed with a sterilised solution (pH 7.4) of 0.5% DTAFl.
projections of through-focusing z-series of the flap edge were
Using in vivo confocal microscopy, three dimensional surface
image analysis (two measurements at each time point).
peripheral circumferential band was measured using digital
biomicroscopy. Subsequently, the relative width of the
photographed at week 1, 2, 8, and 16 using slit lamp
five animals, the same region of the flap margin was
on two consecutive days during the first week. In a group of
rabbits was evaluated. However, to avoid alteration of the
surgery, the flap margin and adjacent regions were examined
4, and 6 months. At each time point, a minimum of two
Wound healing at the LASIK flap edge 1273
All rabbits were evaluated preoperatively using slit lamp and
in vivo confocal microscopy (Fig 2A). To illustrate temporal changes in morphology, the band is
shown at higher magnification (rectangle) at 1 month (B), 6 months (C), and 12 months (D). Note the characteristic changes in width, texture, and reflectivity.
dissolved in 0.2 M sodium bicarbonate. After 1 minute, the
stained surfaces were rinsed with sterile saline and the flap
was repositioned.

**Slit lamp and in vivo confocal microscopy**

All rabbits were evaluated preoperatively using slit lamp and
in vivo confocal microscopy as previously reported. After
surgery, the flap margin and adjacent regions were examined
daily for the first week, then at 1, 2, 3, and 4 weeks, and at 2,
4, and 6 months. At each time point, a minimum of two
rabbits was evaluated. However, to avoid alteration of the
wound healing response, the same animal was not examined
on two consecutive days during the first week. In a group of
five animals, the same region of the flap margin was
photographed at week 1, 2, 8, and 16 using slit lamp
biomicroscopy. Subsequently, the relative width of the
peripheral circumferential band was measured using digital
image analysis (two measurements at each time point).
Using in vivo confocal microscopy, three dimensional surface
projections of through-focusing z-series of the flap edge were
generated, and representative two dimensional and three
dimensional images were contrast adjusted.

**Histology**

Corneal tissue was obtained for histology at day 2 and 4, and
at week 1, 2, and 3 post-LASIK (10 animals in total with two
rabbits at each time point). Five corneas were processed for
histology at 6 months as were the three DTAF stained
corneas. The tissue was fixed in situ by anterior chamber
perfusion (4% formaldehyde for 3 minutes), excised, dis-
sected, embedded in Tissue-Tek (Sakura, Tokyo, Japan), snap
frozen in liquid nitrogen, and stored at 280°C. Serial cryostat
cross sections (thickness approximately 5 µm) were cut, air
dried, and fixed in acetone for 10 minutes. To detect f-actin,
sections were stained with Alexa Fluor 568 conjugated
phalloidin (1.1 µmol/l for 3 hours; Molecular Probes,
Leiden, Netherlands). Fibronectin and α-smooth muscle actin (α-SMA) were detected by incubating sections over-
night with mouse anti-human ED-A fibronectin (clone DH1;
1:200; Biostrend, Cologne, Germany), respectively mouse anti-
human α-SMA (clone 1A4; 1:400; Sigma, Denmark). To
detect selected growth factors and receptors, sections were
incubated overnight with one of the following primary
antibodies: goat anti-human transforming growth factor β1
(TGF-β1; 1:100; Santa Cruz Biotechnology, CA, USA); mouse
anti-human transforming growth factor β2 (TGF-β2; clone
8607.211; 1:75; R&D Systems, Minneapolis, MN, USA); goat
anti-human transforming growth factor β receptor II (TGF-
βRII; 1:100; Santa Cruz Biotechnology, CA, USA); and goat
anti-human connective tissue growth factor (CTGF; 1:12500;
a generous gift from Dr Gary Grotendorst). Primary
antibodies were visualised with one of the following Alexa
Fluor 568 conjugated antibodies (Molecular Probes, Leiden,
Netherlands): goat anti-mouse IgG (1:100 for 30 minutes)
and donkey anti-goat IgG (1:100 for 30 minutes). Co-
localisation of cell nuclei was performed using Hoechst
33342 (2 µg/ml; Molecular Probes, Leiden, Netherlands).
Control experiments included evaluation of tissue from
unoperated animals, use of unspecific primary antibodies,
omission of primary or secondary antibodies, and
peadsorption of primary antibodies with corresponding growth factors
to ensure specificity). Sections were evaluated using a Zeiss
Axiovert 135 inverted microscope, equipped with a 20×
ojective (NA = 0.75) and a zoom adaptor (range 0.4–2.0 ×).
Selected images were overlaid and contrast adjusted.

**RESULTS**

**Slit lamp biomicroscopy**

Throughout the study, no dislocation of the LASIK flaps was
observed. However, immediately after surgery a narrow
circumferential gap was identified along the flap edge
(Fig 2A). Over time, characteristic changes in the morphology
and reflectivity of this region were detected. During the first
week, a well defined circular band (approximately ¼ mm
wide) appeared that in the following weeks became increas-
ingly reflective and developed a fibrillar texture (Fig 2B).
By 2 months, a gradual condensation had occurred and the band
seemed more organised (Fig 2C). At 4 and 6 months, the flap
dge reflectivity had decreased considerably, leaving only a
low reflective region (Fig 2D). Over time, the circular band
gradually became narrower (Fig 2E), measuring 100% at
1 week, 89% (SD 10%) (2 weeks), 53% (13%) (8 weeks), and
33% (7%) (16 weeks) (n = 5; sample means different at all
time points; analysis of variance; p<0.05). The temporal
changes in width, texture, and reflectivity at the LASIK flap
edge appeared to parallel those observed in humans
(compare Fig 2 with Fig 1), suggesting that the rabbit may
provide an acceptable model for LASIK surgery.

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In vivo confocal microscopy
Conjunctival and corneal inflammation
In the conjunctival vessels, numerous leucocytes were observed 24 hours after LASIK. Careful examination revealed that these cells appeared to slow down, adhere to the vessel wall, and migrate into the surrounding tissue (Fig 3A, arrowheads). Near limbus, multiple inflammatory cells were found in the anterior 40 μm stroma (Fig 3B). A noteworthy observation was the presence of long chains of inflammatory cells stretching from the periphery towards the microkeratome entry (Fig 3C); suggesting directional migration of leucocytes. The leucocytes were exclusively located peripherally to the flap edge and were not observed centrally, within, or below the flap. The inflammatory response had almost disappeared by day 2.

Flap edge morphology
From day four, spindle-shaped cells (Fig 4A, arrows) in the anterior stroma began to align in a circumferential band next to the flap edge. These elongated cells first appeared in the periphery, suggesting cellular transformation and migration of the adjacent peripheral keratocytes. By contrast, more centrally located cells within and below the flap remained quiescent (curved arrows). At 2 weeks post-LASIK, the peripheral circumferential band (measuring approximately 250 μm in width and 25 μm in depth) showed further organisation and a marked increase in reflectivity, corresponding to the biomicroscopic findings (compare Fig 4B with Fig 2B). This increase in light scattering appeared to be caused by closely packed spindle-shaped cells (Fig 4B, arrows) and deposition of extracellular material. In contrast, the adjacent cells (curved arrows) on both sides of the peripheral circumferential band appeared quiescent. Over time, the band became narrower and more organised, and the reflectivity gradually declined. Thus, at 6 months, quiescent keratocytes (Fig 4C, curved arrows) were observed in a moderately reflective extracellular matrix.

Basement membrane
At day 1 post-LASIK, the epithelial defect at the incision had healed. However, below the intact epithelium, an outer (Fig 5A, arrows) and an inner break (Fig 5B, arrows) in the basement membrane was identified; corresponding to the microkeratome entry. These sharply defined interruptions in the basement membrane were separated by a gap that delimited the lateral extension of the underlying stromal wound repair (Fig 5C, D). This noteworthy observation was further supported by a 3D reconstruction of the flap edge region (Fig 6) that clearly demonstrates the spatial relation between the basement membrane and the wound repair within the peripheral circumferential band.

Histology
At the flap margin, no major acellular zones were detected in the stroma at any time point. From week 1 post-LASIK, elongated cells with a prominent f-actin expression (Fig 7A, curved arrows) were noted between the incisional breaks in the basement membrane (arrowheads). These migratory cells stretched from the underlying and peripheral stroma, similar to the in vivo observations (compare Fig 7A to Fig 4A). Corresponding to the f-actin staining, expression of ED-A fibronectin was detected. By 3 weeks, f-actin (Fig 7B) and fibronectin (Fig 7C) were expressed in the subepithelial region, and elongated cells were no longer present in the underlying stroma, suggesting that cell migration from the periphery had ended. Concurrently, expression of α-SMA was noted, consistent with myofibroblast transformation. Serial sectioning indicated that the α-SMA expression (Fig 7D) was restricted to a minor part of the f-actin and fibronectin positive regions (compare Fig 7D with Fig 7B, C). At 6 months, expression of ED-A fibronectin or α-SMA was no longer detected, and f-actin showed only weak staining similar to the normal unwounded cornea. Still, the wound repair zone was easily identified by a prominent epithelial...
hyperplasia (up to 10 layers compared to the approximately five layers in the unwounded cornea; Fig 7). Despite this hyperplasia, epithelial ingrowth was not seen at any time point. At 6 months, an unstained, subepithelial region (approximately 50 \( \mu \)m thick) was detected in DTAFlabelled corneas (Fig 7E), demonstrating stromal fibrosis peripheral to the flap edge. By contrast, very little unstained tissue was found at the LASIK interface (arrowhead). Correspondingly, the central cornea showed a normal expression of f-actin and no staining for \( \alpha \)-SMA at any time point.

No expression of TGF-\( \beta \)1, TGF-\( \beta \)2, TGF-\( \beta \)RII, or CTGF was detected in the unwounded stroma of the normal rabbit cornea. Two days after LASIK, TGF-\( \beta \)1 (Fig 8A) and TGF-\( \beta \)2 (Fig 8B) were expressed anteriorly in the stroma between the basement membrane breaks. Concurrently, TGF-\( \beta \)RII and CTGF were detected in the same region. At 2 and 3 weeks, the stromal expression of the three growth factors and TGF-\( \beta \)RII had narrowed and was restricted to a thin subepithelial layer within the wound repair zone (Fig 8C–E). Six months post-LASIK, the expression of all growth factors and TGF-\( \beta \)RII was negative, similar to that of the unoperated cornea. Throughout the study, no expression of TGF-\( \beta \)1, TGF-\( \beta \)2, TGF-\( \beta \)RII, or CTGF was detected at the LASIK interface or in the stroma within or below the flap. In the epithelium and endothelium of the preoperative cornea TGF-\( \beta \)1, TGF-\( \beta \)2, TGF-\( \beta \)RII, and CTGF were weakly expressed. After LASIK, no major changes in the epithelial and endothelial expression of the three growth factors and the receptor could be identified.

DISCUSSION

The present study demonstrates that post-LASIK fibrotic wound repair in the rabbit cornea is restricted to a
circumferential band in the anterior stroma between the incisional breaks in the epithelial basement membrane. The development of fibrosis at the flap edge is preceded by a characteristic sequence of events, beginning with an initial influx of inflammatory cells. Thus, at 24 hours post-LASIK rolling, adhesion, and extravasation of leucocytes were observed in the conjunctival vessels; corresponding to the recent observations in humans. Near limbus, these inflammatory cells were organised in long chains, indicating directional migration towards the microkeratome incision. A similar organisation of leucocytes following corneal wounding has previously been recognised by Wolter and hypothesised to represent migration in preformed spaces. The directional migration and accumulation of leucocytes next to the LASIK flap edge suggest that proinflammatory cytokines and chemokines are present in this region. Evidence of such signalling molecules has been found in the tear fluid, epithelium, and in keratocytes. By contrast, the lack of leucocytes at the LASIK interface may indicate that an isolated stromal injury induces less of a chemotactic signal than when the epithelium and its basement membrane are involved. Accordingly, previous studies have showed lack of inflammation following manual epithelial debridement compared to an intense inflammation after basement membrane disruption (caused by a transpithelial photoablation including a 14 μm stromal keratectomy). In the intact cornea, the epithelial basement membrane has been reported to bind cytokines, suggesting that it may act as a barrier for signaling molecules from the epithelium or tear fluid. Thus, when the basement membrane integrity is compromised at the LASIK flap edge, signalling molecules with mitogenic and chemotactic effect on keratocytes and inflammatory cells may enter the stroma. The present finding of directional cell migration and localised activation of keratocytes (between the breaks in the basement membrane) supports this hypothesis.

In the normal cornea, TGF-β1, TGF-β2, TGF-β receptor II, and CTGF were expressed in the surface epithelium, whereas no signal was detected in the unwounded stroma. Following LASIK, activation of a TGF-β signalling pathway was
detected in the corneal stroma next to the flap edge. The expression included TGF-β receptor II (which is mandatory for TGF-β signal transduction) in the keratocytes, and TGF-β1, TGF-β2, and CTGF between the basement membrane breaks from day 2. Overall, these findings suggest that TGF-β receptor II is upregulated in the keratocytes concurrent with the release of TGF-β into the corneal stroma. The localised expression of TGF-β signalling molecules at the LASIK flap margin persisted for at least 3 weeks and was not detected by 6 months. The activation of TGF-β may account for the observed myofibroblast transformation at week 3, in accordance with previous studies.11 26 27 Moreover, the observed expression of ED-A fibronectin has been reported as essential for α-SMA expression.28 Myofibroblasts are known to cause wound contraction following, for example, full thickness incisional wounds and radial keratotomy, leading to changes.
in corneal curvature.28–31 Thus, the present finding of myofibroblasts at the LASIK flap edge may indicate an active wound contraction in the region. The slit lamp detectable changes in the width of the peripheral circumferential band (Fig 2E) support this interpretation. Speculatively, such changes in the width of the peripheral circumferential band wound contraction in the region. The slit lamp detectable myofibroblasts at the LASIK flap edge may indicate an active

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Characterisation of corneal fibrotic wound repair at the LASIK flap margin

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