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ARTICLE

The Role of Ultraviolet-B in Corneal Healing Following Excimer Laser in situ Keratomileusis

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Corneal photoablation with the 193 nm argon fluorid excimer laser during photorefractive keratectomy (PRK) in high diopter range is frequently associated with subepithelial haze and consequent refractive regression due to avascular corneal wound healing. The wound healing response can be augmented by Ultraviolet-B (UV-B) exposure originating from sun or solarium. Clinically Laser in situ Keratomileusis (LASIK) even in high diopter range is associated with less subepithelial haze and regression than PRK. In an animal model, the morphologic changes of the rabbit cornea were evaluated following LASIK and secondary UV-B exposure. Light microsopic changes were found to be insignificant. Transmission electron microscopy (TEM) normal epithelium, epithelial adhesion structures and normal anterior stroma showed in the LASIK treated UV-B irradiated rabbit eyes. Around the peripheral LASIK cut, migrating keratocytes with pseudopodia were observed. Under the flap (160 µm depth) the overall stromal collagen structure was normal, some activated keratocytes and mild extracellular matrix formation within and around keratocytes were noted. Within activated keratocytes TEM showed prominent rough endoplasmic reticulum, Golgi apparatus, mitochondria and extracellular vacuoles, which showed resolution with time. These changes were much milder than in PRK treated-UV-B irradiated eyes. Secondary UV-B caused no long-term disturbance in corneal transparency in LASIK and UV-B treated rabbit eyes. (Pathology Oncology Research Vol 8, No 1, 41–46, 2002)

Keywords: Laser in situ Keratomileusis (LASIK), Ultraviolet-B, corneal transparency, subepithelial haze, activated keratocytes, extracellular matrix

Introduction

Deep corneal photoablation with the 193 nm ArF excimer laser using traditional techniques (mechanical deepithelialization) of photorefractive keratectomy (PRK) is frequently associated with clinically significant subepithelial haze and secondary refractive regression.[1,2,3,4,5,6,7,8] For higher corrections, refractive surgeons have shifted to other techniques, such as Laser In Situ Keratomileusis (LASIK) suggested by Pallikaris in 1994.[9] LASIK causes less postoperative haze and better refractive stability.

In a previous publication the authors reported the role of secondary ultraviolet-B (UV-B) during corneal healing following traditional excimer laser PRK (10). In that study, substantial metabolic activation with abundant extracellular matrix production and secondary increase in corneal thickness with abnormal proteoglycan deposition were demonstrated.[10] This explains the role of secondary UV-B exposure in subepithelial haze and refractive regression, observed clinically by others as well.[1,2,4,5,6,7,8] Since the introduction of LASIK, less haze and refractive regression have beenreported in the literature,[11,12,13,14,15,16] and it can be speculated that environmental UV exposure is less harmful after LASIK technique, than after traditional PRK. The present study reports on the morphologic changes after observed secondary UV-B irradiation on LASIK-treated corneas.

Materials and Methods

The animals used in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research. A total of 32 pigmented chinchilla rabbits

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(range, 2.5-3.0 kg) were used in the study. The animals received a pretreatment intramuscular sedation by ketamine (25 mg/kg), and were anaesthesized by intravenous ketamine (25 mg/kg) combined with xylazine (2 mg/kg). Propacaine hydrochloride was instilled into the right eye and the eyelids were held open by a speculum. A 130 µm thick central corneal flap was made using the Chiron System-ALK-E automated keratome (Claremont, California). The flap of the night eyes was gently lifted and in 16 eyes each a –5.0 Dpt (45 µm), and in the other 16 eyes a –10.0 Dpt (90 µm) photoablation was performed in the stromal bed by the Aesculap-Meditec MEL 60 (Heroldsberg, Germany) 193 nm argon-fluorid (ArF) excimer laser. The operative energy and repetition rate were set by the manufacturers at 250 mJ/cm^2 and 20 Hz, respectively. Afterwards, the corneal flap was replaced and the animals received topical antibiotics (tobramycin) three times a day until complete reepithelization. The left eye remained untreated.

Twenty-one days after LASIK, 8 rabbits from both group were exposed to 100 mJ/cm^2 UV-B light (280-315 nm) in an UV-chamber for 7 minutes as described previously.[10] The peaks of UV-irradiation were at 306 and 313 nm. The energy density was measured in front of the rabbit eyes. The right eye was held open by a speculum, and 0.9% sterile saline was instilled onto the eye every 30 seconds to prevent drying of the cornea. The remaining 8-8 animals did not receive UV-B irradiation.

Anterior stromal haze was assessed biomicroscopically, using the scale of Hanna et al[17] every 2 weeks for 2 months.

Four animals were sacrificed with an overdose of intravenous sodium phenobarbital 4 weeks, and the remaining 4 animals 8 weeks following UV-B exposure. The same applied for the control group. The eyes were fixed in 4% paraformaldehyde plus 1% glutaraldehyde, the dissected, and half of each eye processed through graded alcohols and embedded in paraffin wax. Sections 4 µm thick were stained with haematoxylin-eosin. Portions of corneal tissue from the remaining half of the eyes were post-fixed in 4% osmium tetroxyde, dehydrated and embedded in Epon resin. Semi-thin sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate before evaluation with a Philips transmission electron microscope (TEM).

Results

Clinical findings with slit lamp biomicroscopy

Flap preparation and the laser surgery were uneventful. Following LASIK-only, no subepithelial haze could be observed with the slit lamp in the sub-flap area. Around the flap cut a thin circular line of haze, which characteristically appeared around the 2nd and 3rd postoperative week could be observed in most of the eyes. After secondary UV-B irradiation, mild photokeratitis was observed. One month after UV-B exposure, no haze was detected in the -5.0 Dpt (45 µm) group. A minimal haze, with an average grade of 0.5 (range 0 to 1.5),[4] was observed in the –10.0 D (90 µm) group in the sub-flap area. In the –10.0 D LASIK-UV-B treated group, clinically detectable haze showed resolution with time. At the end of the 4th week, the sub-flap haze disappeared. Around the cut-line, circular haze was similar as described above in the LASIK-only group, and persisted up to the follow-up time.

Flap cut region, light microscopy

Light microscopic evaluation after LASIK-only showed normal epithelial and anterior stromal morphology in the corneal centre. A so-called epithelial plug and a curvilinear scar corresponding to the keratome incision line were observed in the anterior stroma. Outside the section line, no epithelial downgrowth was found. In the –5.0 D (45 µm) and –10.0 D (90 µm) groups following LASIK and UV-B, similar epithelial hyperplasia and curvilinear scar were observed in the cut line as in the LASIK-only groups.

Flap-cut region, transmission electron microscopy (TEM)

Using transmission electron microscopy, in the LASIK-only treatedrabbit eyes, around the flap edge epithelial basement membrane break, i.e.: along the blade cut, keratocytes with pseudopodia formation could be noted (*Figure 1*). Within and around these keratocytes some intra- and extracellular vacuoles were observed, but the majority of cells showed no vacuolization. No changes were detected in the surrounding stromal collagen architecture (*Figure 2*). In the -5.0 D (45 µm) LASIK group 4 weeks following UV-B irradiation, similar changes as described in LASIK-only eyes (epithelial basement membrane rupture, pseudopodia formation, fibroblast migration and extracellular vacuoles within and around the keratocytes)

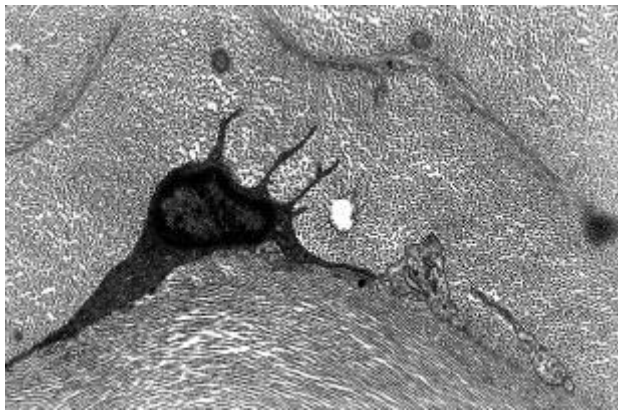

Figure 1. Fibroblast-like keratocytes shows fil- and lamellipodia (× 5000)

could be observed around the flap edge. No qualitative or quantitative differences could be detected concerning flap-edge keratocytes within the –5.0 D and –10.0 D LASIK-UV-B treated groups. No epithelial downgrowth was found in the central cornea in either of the groups.

Central cornea and sub-flap area, light microscopy

Epithelium, epithelial basement membrane and keratocyte morphology by light microscopy under and above the flap was were found to be normal 4 and 8 weeks following LASIK-only treatment. Using light microscopy, in the -5.0 D (45 µm) and -10.0 D (90 µm) LASIK and UV-B irradiated eyes, keratocytes were uniform, collagen fibers laid parallel, in regular arrangement. Under the flap, keratocyes demonstrated no light microscopically detectable changes.

Central cornea and sub-flap area, TEM

Above the flap, epithelium, epithelial adhesion structures and anterior stromal morphology assessed by TEM were normal. Under the flap, some activated keratocytes were noted with increased number of rough and smooth endoplasmic reticuli, Golgi apparatus and mitochondria in deeper stroma. Within and around the keratocytes, vacuoles were observed filled with amorphous, electron-lucent material (*Figure 3*). Some of the vacuoles had round shape, but many of them were irregular. At 4 weeks the localization of vacuoles was mainly within the keratocytes; at 8 weeks they were located mainly around the keratocytes. In the

–10.0 D (90 µm) group 4 weeks following UV-B irradiation, metabolic activation affected a larger stromal thickness (about 1/3 thicker), and the vacuoles had also larger diameter. Other deeper keratocytes showed pseudopodia and filipodia, pointing toward the plane of the keratotome incision, indicating cell movement toward the place of tissue injury. Some keratocytes exhibited fibroblast-like transformation. The structure of collagen lamellae around activated keratocytes was slightly disorganized, but these irregularities were confined to the surroundings of the keratocytes, elsewhere the parallel structure of collagen lamellae was intact (*Figure 4*). The sub-flap collagen fiber disturbance in the affected area was less than the size of the keratocyte. Further stromal collagen structure was entirely normal. There was no regenerated collagen between the stromal flap and the ablated stroma, except around the wound margin.

The main differences between the –5.0 D and –10.0 D LASIK-UV-B irradiated groups were the thickness of the affected stroma the number and diameter of the vacuoles and the number of activated keratocyes, i.e.: the differences were mainly quantitative. There were no signs of keratocyte activity outside the incision (peripheral cornea), and the cells in this area were normal. Ultrastructural changes showed resolution of extracellular matrix production over time. In the eyes receiving –5.0 D LASIK-UV-B, sub-flap metabolic activation was not

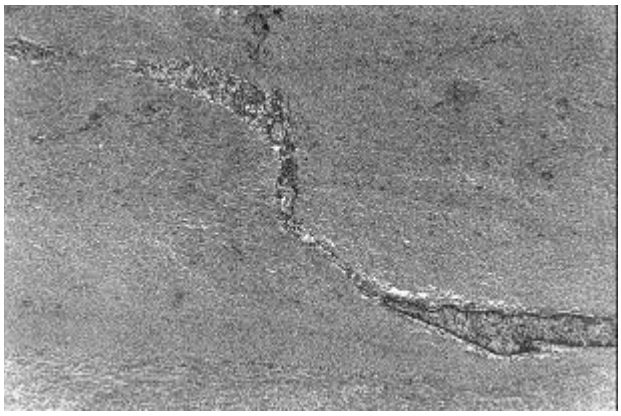

Figure 2. Elongated cellular processes suggesting migration of a keratocyte through stromal collagen lamellae (× 5000)

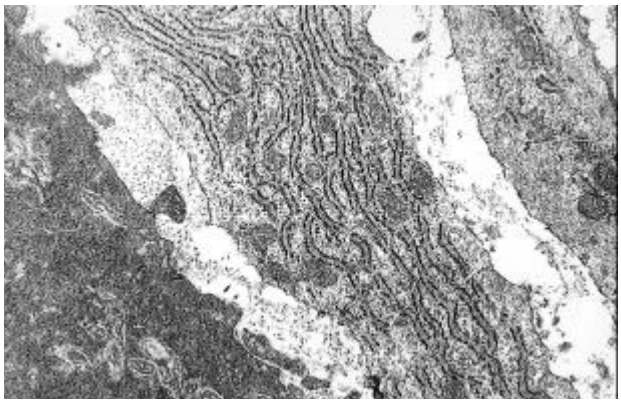

Figure 3. Abundant rough endoplasmic reticulum and excessive extracellular matrix production in a rabbit cornea 4 weeks after –10.0 Dpt LASIK and UV-B exposure. (× 13000)

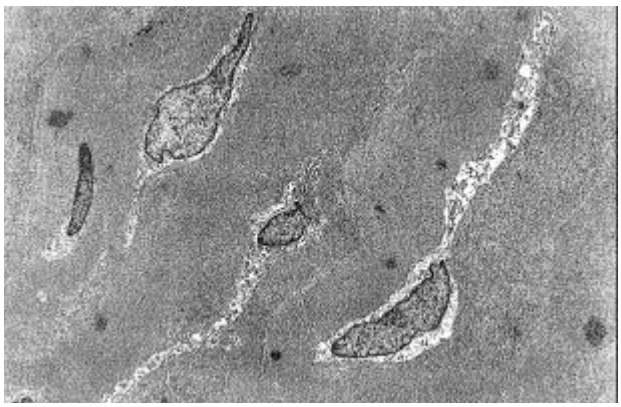

Figure 4. Deep stromal keratocytes 4 weeks after LASIK only. Note the slightly edematous cytoplasm and non-membrane bound vacuoles in the cell processes. (× 4000)

detectable 8 weeks following UV-B exposure, no pseudopodia or fibroblast-like transformation were found. In eyes treated with –10.0 D LASIK-UV-B, sub-flap keratocyte morphology was normal, activated keratocytes were confined to the flap edge. Vacuoles were localized around the keratocytes and not intracellularly.

Discussion

LASIK was developed and clinically accepted because it preserves epithelium, epithelial nerve plexi around the flap hinge, epithelial junctional structures, Bowman's membrane and a thin anterior stromal layer. It was presumed that by preserving these anatomical structures, less haze and regression are to be expected,[9,10] although regression after LASIK has also been reported.[18] Clinically, most of the expectations are met. However, LASIK is surgically more demanding, and the learning curve for the surgeon is longer. Consequently, the focus of debate is regarding the range of myopia best suited for LASIK, versus traditional PRK.[11,14,19,20,21]

In this study we examined the role of secondary UV-B on corneal wound healing processes after LASIK surgery. The same UV-setting and haze assessment criteria were used as previously described[10,17], in order to be able to compare wound healing in PRK and LASIK after secondary UV-VB exposure. The purpose was to answer the following question: does UV-B cause morphologically detectable metabolic activation of keratocytes after LASIK comparable to that described in PRK? The harmful effects of UV-B on PRK treated patients were demonstrated in a previous study.[10] Furthermore, the analysis of Corbett et al,[21] who studied the environmental and other risk factors for regression of PRK, found that regression was higher in eyes that underwent higher dioptric or smaller diameter treatment, or were exposed to solar radiation as well as sun beds. Females taking oral contraceptives or patients with ocular surface disorders were also at risk, as were those who showed regression after the treatment of the first eye.[21] Among the parameters mentioned above, sun exposure and sun beds are avoidable environmental risk factors, especially in countries with continental climate. For haze development and regression during the time span of avascular corneal wound healing following PRK, sun beds may harbor high risk, especially among young females.[10] No evidence was found in the literature concerning post-LASIK haze development following UV-B exposure during the same time span.

The corneal wound healing cascade is a complex process, involving epithelial-stromal and stromal-epithelial immune interactions. During wound healing, after PRK, cytokine released by migrating epithelial cells may adversely affect the course of corneal wound healing during the early postoperative period.[22] Interleukin-1 (IL-1) appears to be a master modulator of many events involved during this complex cascade. Keratocyte apoptosis is the earliest stromal event which is mediated by IL-1 released from the injured epithelium.[33] Other processes such as epithelial mitosis, migration, inflammatory cell infiltration, keratocyte proliferation, myofibroblast generation, collagen and extracellular material synthesis contribute to the wound healing cascade and are also likely to be modulated by cytokines derived from corneal cells, the lacrimal gland and possibly immune cells.[33] Within the anterior stromal keratocytes, newly synthesized collagen and hyaluronan deposition may appear,[23,24] giving rise to unwanted corneal changes, such as corneal thickening, subepithelial haze and regression. Environmental or man-made UV-B seems to augment the wound healing process of the cornea following PRK,[10] originally stimulated by the traumatic and secondary fluorescence effect of excimer laser PRK. If UV-B exposure is not repeated, metabolic changes slowly decrease and morphology returns to normal. If metabolic activation is very high, more vacuoles filled with extracellular material and new collagen are produced, leading to haze and increase of corneal refractive power, i.e.: causing refractive regression.

Apoptosis is a controlled death of cells with minimal collateral damage to surrounding cells or tissue. Helena et al[25] and Wilson[26] hypothesize that keratocyte apoptosis might be an initiating factor in wound healing response after refractive surgical procedures. They found a quantitative and qualitative difference in keratocyte apoptosis between LASIK and epithelial scrape-PRK, in favor of LASIK.[25] Epithelial injury was noted to be an important factor modulating keratocyte apoptosis, keratocytes that died along the lamellar cut of the LASIK blade were replenished in 2 to 4 days by proliferation and migration.[26] These cells are activated keratocytes which might show myofibroblastic transformation, producing new collagen, hyaluronic acid and growth factors.[26] Podskochy et al found that apoptosis is the triggering mechanism of corneal cell death after UV-B exposure.[27] The longer the wavelength of UV-B exposure, the more extensive the damage to the corneal stroma.[28] Control of the initiating response of keratocyte apoptosis might provide the key for controlling unwanted healing response following LASIK/PRK and UV-B exposure. Podskochy and Fagerholm found that the production of hyaluronan is a generalized response of cornea to injury, observable following UV-B exposure as well.[27] Corneas exposed to 310 nm of UV-B were found to show disappearance of keratocytes 3 days after exposure after 7 days following UV-B exposure, almost the whole damaged area except one fourth of the anterior stroma was repopulated by new keratocytes, staining positive for hyaluronan. The corneal structure was normal, and hyaluronan (HA) disappeared 14 days after UV-B irradiation.[27] Weber found that endogenous hyaluronan is also a part of corneal wound healing following PRK, which causes local shifts in water content with concomi-

tant shifts in corneal transparency.[24] The presence of abnormal HA reduces corneal transparency by disrupting normal spacing between collagen fibrils, creating focal changes in refractive index.[24] The newly synthesized HA is biochemically distinct from normally present stromal proteoglycans.[29] Clinically, UV-B was found to enhance keratocyte morphological response to surgical trauma following PRK. Authors in a previous experiment also showed the presence of abnormal proteoglycans in the subepithelial stroma following PRK and UV-B exposure.[10] In this experiment the thickness of the affected stroma correlated with the attempted depth of photoablation.[10] Fagerholm analyzed 17 patients, who underwent repeat-PRK. Five of the specimens stained positive for hyaluronic acid while 4 showed clinically significant corneal haze and myopic regression. Fagerholm, therefore suggested the role of HA in excessive corneal wound healing.[23] Based on the work of previous authors, it is known that the poor results of PRK are due to excessive production of HA and newly synthesized collagen fibers[10,23,27,29] in certain patients, especially those with higher correction need (deeper ablation profile) of those exposed to the sun.

During the LASIK-UV-B experiment we also found signs of metabolic activation within deeper stromal keratocytes. Electron-lucent vacuoles localized mainly intracellularly were noted at week 4, but mainly extracellularly at week 8 following UV-B exposure. Based on previous data[10,23,24,27,28,29] it can be hypothesized that the content of these vacuole is HA. During LASIK treatment the anterior stroma was preserved, apart from the blade cut line. Light microscopic and TEM morphology findings pertaining to the cut line were similar in LASIK-UV-B exposed eyes to the findings observed by others in LASIK-only treated eyes.[30,31] In neither of the groups was the overall structure of collagen fibers distorted under the flap, except around the affected keratocytes. Disorganisation of collagen structure did not exceed the size of the keratocyte. In our study the metabolic activation of keratocytes was milder and the thickness of affected stroma thinner, than previously noted during the PRK-UV-B experiment with the same UV-B settings.[10]

Our experimental results show that secondary UV-B exacerbates and prolongs ultrastructural changes not only in eyes treated with traditional PRK but in post-LASIK rabbit eyes, as well. However, clinical and morphological changes were less pronounced in LASIK-UV-B irradiatied eyes than in PRK-UV-B treated counterparts. The most important difference between PRK and LASIK results following UV-B exposure is that after PRK possible anterior stromal remodelling may occur in severe form, especially in the subepithelial region of the stroma. In such case the parallel structure of the anterior stromal collagen structure is disrupted, showing wavy forms and highly activated keratocytes producing excessive extracellular material, which is able to distort the regular collagen structure. This interferes with central corneal clearity. In the LASIK-UV-B eyes the structure of the anterior stroma was completely preserved and we found a milder keratocyte metabolic activation only under the keratome incision, in the deeper stroma (i.e. subepithelial keratocyes were normal). The collagen structure was completely continuous, regular fibers were detectable above and under the entire keratome incision, with practically no stromal remodelling. Because the integrity of superficial corneal layers is maintained during LASIK, the keratocyte repair process is of a lesser degree than after PRK, therefore stromal regularity can be better preserved even after secondary UV-B exposure. The smaller degree of stromal wound healing after LASIK might prevent haze development, which is one of the most important success-limiting factors in photorefractive surgery. On the other hand, epithelial downgrowth with decrease of best spectacle-corrected visual acuity is an important limiting factor of LASIK.[32] This complication was not met with during this study.

Based on our results, it can be concluded that LASIK and UV-B exposure causes less activation in deep keratocytes than does traditional PRK surgery. The metabolic activation of stromal keratocytes is localized to the subflap area and around the flap margin. In PRK the degree of keratocyte activation and anterior stromal remodelling are the most important limiting factors of the procedure, while in LASIK the corneal thickness, regularity of the keratome incision, excimer photoablation depth and possible epithelial downgrowth are more important. UV-B is able to enhance keratocyte metabolic response following LASIK, but this response is mild and confined to the immediate environment of keratocytes, leaving the parallel stromal collagen structure normal, and activated keratocytes may regain normal morphology within weeks. Stromal transparency therefore, is better maintained in post-LASIK eyes, than in post-PRK eyes following UV-B exposure. Ultrastructural results are in accordance with the clinical findings, i.e.: less haze is found in eyes treated with the LASIK method even with deep photoablation.

More studies are needed in order to establish the safe treatment range for LASIK and PRK with the smallest complication incidence. At present it seems, that at lower photoablation depth, PRK is relatively safe, while at greater photoablation depth LASIK offers quicker visual rehabilitation, less subepithelial haze and regression, and a somewhat better protection against theharmful effects of UV-B. Individual treatment planning is necessitated, however, for a each patient applying for refractive surgical procedure.

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