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Original Article

Effect of Macrophage Migration Inhibitory Factor on Corneal Sensitivity after Laser In Situ Keratomileusis in Rabbit

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Purpose: To investigate the effect of macrophage migration inhibitory factor (MIF) on corneal sensitivity after laser in situ keratomileusis (LASIK) surgery.

Methods: New Zealand white rabbits were used in this study. A hinged corneal flap (160-μm thick) was created with a microkeratome, and -3.0 diopter excimer laser ablation was performed. Expressions of MIF mRNA in the corneal epithelial cells and surrounding inflammatory cells were analyzed using reverse transcription polymerase chain reaction at 48 hours after LASIK. After LASIK surgery, the rabbits were topically given either 1) a balanced salt solution (BSS), 2) MIF (100 ng/mL) alone, or 3) a combination of nerve growth factor (NGF, 100 ug/mL), neurotrophine-3 (NT-3, 100 ng/mL), interleukin-6 (IL-6, 5 ng/mL), and leukemia inhibitory factor (LIF, 5 ng/mL) four times a day for three days. Preoperative and postoperative corneal sensitivity at two weeks and at 10 weeks were assessed using the Cochet-Bonnet esthesiometer.

Results: Expression of MIF mRNA was 2.5-fold upregulated in the corneal epithelium and 1.5-fold upregulated in the surrounding inflammatory cells as compared with the control eyes. Preoperative baseline corneal sensitivity was 40.56 ± 2.36 mm. At two weeks after LASIK, corneal sensitivity was 9.17 ± 5.57 mm in the BSS treated group, 21.92 ± 2.44 mm in the MIF treated group, and 22.42 ± 1.59 mm in the neuronal growth factors-treated group (MIF vs. BSS, $p < 0.0001$; neuronal growth factors vs. BSS, $p < 0.0001$; MIF vs. neuronal growth factors, $p = 0.815$). At 10 weeks after LASIK, corneal sensitivity was 15.00 ± 9.65, 35.00 ± 5.48, and 29.58 ± 4.31 mm respectively (MIF vs. BSS, $p = 0.0001$; neuronal growth factors vs. BSS, $p = 0.002$; MIF vs. neuronal growth factors, $p = 0.192$). Treatment with MIF alone could achieve as much of an effect on recovery of corneal sensation as treatment with combination of NGF, NT-3, IL-6, and LIF.

Conclusions: Topically administered MIF plays a significant role in the early recovery of corneal sensitivity after LASIK in the experimental animal model.

Key Words: Corneal nerve regeneration, Corneal sensitivity, Laser in situ keratomileusis, Macrophage migration-inhibitory factors

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Laser in situ keratomileusis (LASIK) is among the most frequently performed ocular surgeries and is widely applied for the correction of refractive errors. The severing of the corneal nerves during the creation of the flap in the LASIK procedure affects corneal sensitivity, which is important for tear secretion and maintenance of the normal physiology of the cornea, including natural host defense mechanisms. Dry eye syndrome is the most commonly reported complication after LASIK variably affecting up to 80% of patients and is the most common reason for dissatisfied post-operative patients. Recovery of corneal sensation correlates with reinnervation of the corneal sub-basal nerve plexus. Although sub-basal nerve fiber bundles are known to regenerate following LASIK, their number after one year is less than half of that before the LASIK procedure [1].

There are several cytokines that promote nerve regeneration, such as neurotrophin, nerve growth factor, and IL-6. Theses cytokines are expressed during the neuronal regeneration process, and some reports have demonstrated that the exogenous application of these cytokines promotes nerve regeneration *in vivo* [2-4].

Macrophage migration inhibitory factor (MIF) was originally named as such because of its lymphokine activity in inhibiting the migration of macrophages from inflammatory loci [5]. However, MIF has since been shown to have various catalytic, cellular and immunological functions. It was found to be expressed in the central nervous system [6-8] and to have a protective role in neural tissues via a detoxification pathway for catecholamine products [9,10]. MIF has a potential role in peripheral nerve regeneration as well [11,12]. MIF was also found to be abundantly expressed in human corneal endothelial and epithelial cells [13], and is known to play a crucial role in wound healing of the ocular surface in a mice model of chemical burn [14].

Thus, we hypothesized that MIF could play a beneficial role in the recovery of corneal sensation after LASIK. The aim of this study was to investigate the expression of MIF in the cornea and the effect of the exogenous administration of MIF on corneal sensitivity after LASIK surgery.

Materials and Methods

Animals

New Zealand white adult female rabbits (3.5 to 4.5 kg of body weight) underwent LASIK surgery on the right eye. All animals were treated according to the ARVO Regulations for the Use of Animals in Research and the Guidelines for the Use of Animals in Neuroscience Research.

Laser in situ keratomileusis procedure

Intramuscular ketamine (30 mg/kg body weight; Ketaject, Phoenix Pharmaceutical, St. Joseph, MD, USA) and intramuscular xylazine (5 mg/kg body weight; Xyla-ject, Phoenix Pharmaceutical) were used to induce anesthesia. A Barraquer-style speculum was placed between the lids and the eye was rinsed with balanced salt solution (BSS; Alcon Laboratories, Fort Worth, TX, USA). A pararadial linear mark with a gentian violet pencil was applied to the corneal surface. After placement of the suction ring, the intraocular pressure was verified to be greater than 65 mmHg, using a Barraquer tonometer. A nasal-based, 160-μm-thick and 8.5-mm-wide hinged corneal flap was created using an automated microkeratome (SKBM microkeratome; Summit Technologies, Cork, Ireland). Subsequently, the microkeratome and the suction ring were removed from the eye and the corneal flap was lifted and retracted against the peripheral cornea.

Excimer laser photoablation was performed on the stromal bed, using the Summit Apex Plus excimer laser (Summit Technologies). A single zone approach (laser zone diameter, 6.0 mm), was used in all LASIK eyes. A myopic correction of -3.0 diopter was performed in all eyes for an approximate ablation depth of 36 μm. After the photoablation, the corneal flap was carefully repositioned. A temporary tarsorrhaphy was then performed by suturing of the upper and lower eyelids using a 6-0 black silk at the lateral two-thirds of the lids in order to keep the lids closed for the first 1 week. Antibiotic (Ocuflox 0.3%; Allergan, Irvine, CA, USA) and corticosteroid (Fluorometholone 0.1%, Allergan) eye drops were instilled four times a day for the first seven days.

Recombinant migration inhibitory factor

Recombinant MIF was produced as described earlier [9]. Reverse transcription (RT) polymerase chain reaction (PCR) of rabbit corneal RNA was used to amplify the coding sequence of rabbit MIF using the primers TCC GCC CAT ATG CCT ATG TTC ATC GTG AAC ACC (5' primer) and AGC GGT GGA TCC AAG TGG GGC CAG GAC

TCA AGC (3' primer), which incorporated *Nde*I (5') and *Bam*HI (3') restriction sites. The cDNA was first cloned into the pCRII vector (Invitrogen, Carlsbad, CA, USA) and sequenced and then subcloned into the *Nde*I and *Bam*HI sites of the PET 17b vector (Novagen, Madison, WI, USA). Following the manufacturer's protocol, the protein was expressed in BL21 (DE3) pLysS cells and induced with 0.4 mM IPTG for 3 hours at 25°C. The induced cells were washed, and the cells were stored as frozen pellets at -70°C. The cells were lysed by thawing and sonication with a microtip at a high output setting for two 10-s pulses and centrifuged, and the supernatant (soluble fraction) was dialyzed overnight. The dialyzed sample was centrifuged and concentrated. From the concentrated sample, MIF was purified in the AKTA FPLC system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by ion exchange chromatography on a Q-Sepharose high performance column (Amersham Pharmacia Biotech), followed by gel filtration on a Superdex 75 pg column (Amersham Pharmacia Biotech).

Real-time reverse transcription polymerase chain reaction

Five rabbits underwent LASIK as described above. Forty-eight hours after LASIK surgery, rabbits were sacrificed by an overdose of anesthetics and the corneal epithelium above the LASIK flap and the cells under the LASIK flap (from both flap and stromal sides) were collected by gentle debridement using a crescent blade. The left eyes of the same rabbits served as the control, and the central epithelial cells and the cells from the superficial stromal bed were collected. The expression of MIF in corneal epithelial cells and the surrounding cells was determined using a real-time PCR technique [15]. Total RNA from the corneal tissues was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). Random primers and dNTP mix used for the first-strand cDNA synthesis was purchased from Invitrogen. For real-time PCR analysis, a Light Cycler FastStart DNA Master SYBR Green kit (Roche Diagnostics, Penzberg, Germany) and an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) were used. Primer sets for MIF were: sense-CTG TCG GAG CTC ACC CAG C and antisense-CGA TGC TGT GCA GGC TGC. Hypoxanthine phosphoribosyl transferase (HPRT) [16] was used as an internal control and the HPRT primers were: sense-GGG AGG CCA TCA CAT TGT G and antisense-TCC AGC AGG TCA GCA AAG AAC. SYBR green was incorporated into the reaction mixture to facilitate measurement of the product. Real-time PCR values were determined by reference to a standard curve that was generated by real-time PCR amplification of serially diluted cDNAs using MIF and HPRT primers. Values obtained for the levels of MIF were normalized to the levels of MIF mRNA.

Treatment

Eighteen New Zealand white adult female rabbits underwent LASIK surgery as described above and were randomly assigned to three groups. MIF (100 ng/mL) alone (n = 6), combination of nerve growth factor (NGF; 100 ug/mL, Invitrogen), neurotrophine-3 (NT-3; 100 ng/mL; Chemicon International, Temecula, CA, USA), interleukin-6 (IL-6; 5 ng/mL, Chemicon International), and leukemia inhibitory factor (LIF; 5 ng/mL, Chemicon International) (n = 6), or balanced salt solution (BSS, n = 6) were topically given to the rabbit cornea four times a day for three days after LASIK in each group. The concentration of each growth factor was determined based on either our previous work [17] or literature reviews regarding their activity.

Corneal sensitivity measurements

Basic ocular surface examination was performed using a portable slit-lamp to exclude other ocular pathology. Corneal sensitivity was measured with the Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres, France) as previously described by Joo et al. [17]. The diameter of the nylon filament was 0.12 mm, and its length could be varied from 0 to 60 mm. The pressure applied to the cornea thus ranged from 0.4 to 15.9 g/mm^2. Under direct visual control, the nylon filament of the Cochet-Bonnet instrument approached the center of the cornea smoothly and perpendicularly, until the slightest bend of the filament was observed. Care was taken not to touch the lid or cilia. Stimulus by the filament was applied a minimum of six times, and the corneal sensitivity was taken as the length of the filament in millimeters that gave a 50% positive corneal reflex (blinking reflex) response. Corneal sensitivities were checked twice at two weeks and at 10 weeks after LASIK surgery, and the mean of the two measured sensitivities were used as the corneal sensitivity.

Statistical analysis

Corneal sensitivity in each treatment group was compared by the Kruskal-Wallis test using PASW Statistics ver. 17 (SPSS Inc., Chicago, IL, USA). A *p*-value was calculated with the Bonferroni method.

Results

Migration inhibitory factor expression

The expression of MIF mRNA was upregulated 2.5 fold in the corneal epithelium and upregulated 1.5 fold in the cells from the LASIK interface at 48 hours after LASIK surgery as compared with the control eyes (Fig. 1).

Corneal sensitivity

Preoperative baseline corneal sensitivity was 40.56 ± 2.36 mm. There was no significant difference in preoperative corneal sensitivity among the three groups. In the BSS-treated group, corneal sensitivity decreased down to 9.17 ± 5.57 mm at two weeks postoperative and slightly recovered to 15.00 ± 9.65 mm at 10 weeks postoperative. With MIF treatment, corneal sensitivity was recovered up to 21.92 ± 2.44 mm at two weeks postoperative and 35.00 ± 5.48 mm at 10 weeks postoperative. Treatment with the combination of NGF, NT-3, IL-6, and LIF increased corneal sensitivity to 22.42 ± 1.59 mm at two weeks postoperative and 29.58 ± 4.31 mm at 10 weeks postoperative (Table 1). Treatment with either MIF or neuronal growth promoting factors following LASIK surgery significantly enhanced the recovery of corneal sensitivity compared to the BSS treatment at two weeks postoperative (MIF vs. BSS, $p < 0.0001$; neuronal growth factors vs. BSS, $p < 0.0001$) and at 10 weeks postoperative (MIF vs. BSS, $p = 0.0001$; neuronal growth factors vs. BSS, $p = 0.002$). There was no statistical significance in corneal sensitivity between the group treated with MIF and the group treated with a combination of the neuronal growth promoting factor either at

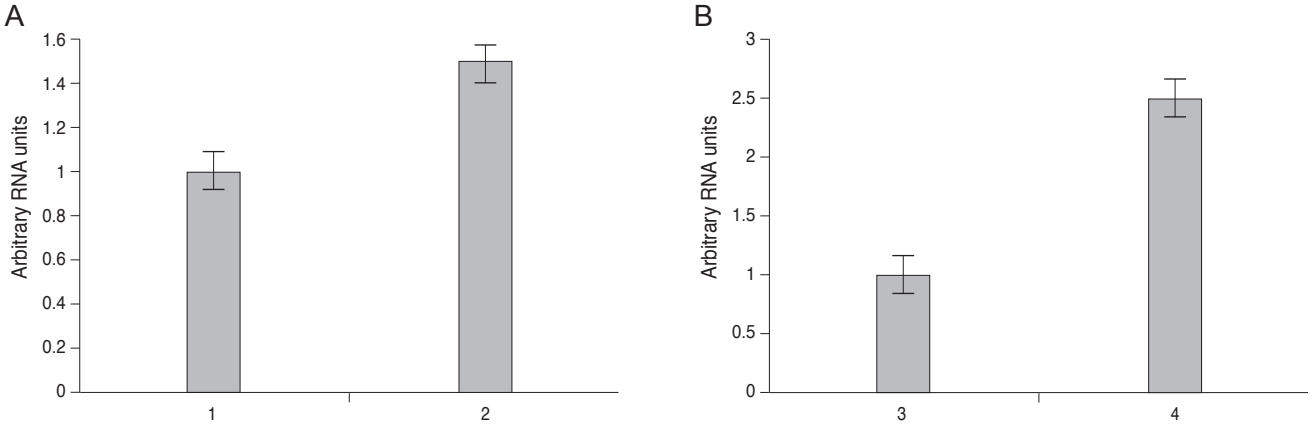

Fig. 1. Real time reverse transcription polymerase chain reaction (PCR) of migration inhibitory factor amplification from 1) cells from the superficial stromal bed of a normal rabbit eye and 2) the cells at the flap interface from the rabbit eye following laser in situ keratomileusis (LASIK) (A), 3) corneal epithelium from the control eyes and 4) corneal epithelium from the rabbit eye following LASIK (B). RNA was isolated at 48 hours after LASIK. All real time PCR values were normalized to hypoxanthine phosphoribosyl transferase as the internal control. Vertical lines represent standard deviations from the mean.

Table 1. Corneal sensitivity following LASIK surgery in the rabbit eye (mm, mean ± SE)

	Preoperative	2 wk postoperative	10 wk postoperative	*p*-value*
Control (BSS) (n = 6)	40.83 ± 2.58	9.17 ± 5.57	15.00 ± 9.65	0.009
MIF (n = 6)	40.00 ± 2.24	21.92 ± 2.44	35.00 ± 5.48	0.006
NGF + NT-3 + IL-6 + LIF (n = 6)	40.83 ± 2.58	22.42 ± 1.59	29.58 ± 4.31	0.003

LASIK = laser in situ keratomileusis; BSS = balanced salt solution; MIF = macrophage migration inhibitory factor; NGF = nerve growth factor; NT-3 = neurotrophine-3; IL-6 = interleukin-6; LIF = leukemia inhibitory factor.
*Friedman test.

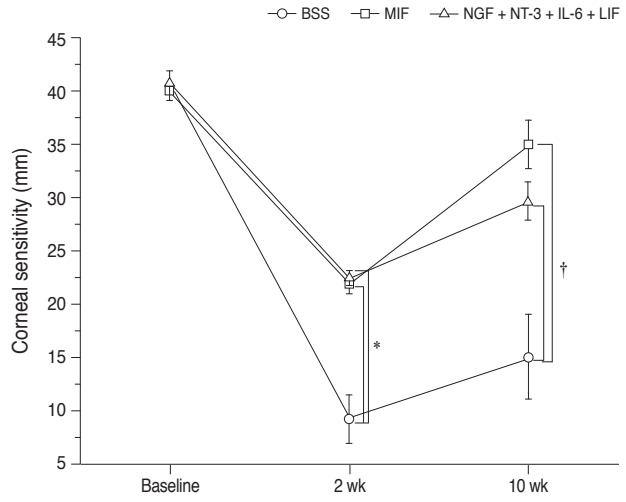

Fig. 2. Corneal sensitivity after laser in situ keratomileusis (LASIK) surgery in the rabbit eye. Preoperative baseline corneal sensitivity was 40.83 ± 2.58 mm in the balanced salt solution (BSS)-treated group, 40.00 ± 2.24 mm in the migration inhibitory factor (MIF)-treated group, and 40.83 ± 2.58 mm in the neuronal growth factors-treated group. At two weeks after LASIK, corneal sensitivity was 9.17 ± 5.57 mm in the BSS-treated group, 21.92 ± 2.44 mm in the MIF-treated group, and 22.42 ± 1.59 mm in the neuronal growth factors-treated group. At 10 weeks after LASIK, corneal sensitivity was 15.00 ± 9.65 mm, 35.00 ± 5.48 mm, and 29.58 ± 4.31 mm, respectively. NGF = nerve growth factor; NT-3 = neurotrophine-3; IL-6 = interleukin-6; LIF = leukemia inhibitory factor. *Kruskal-Wallis test, *p*-value with the Bonferroni method, MIF vs. BSS, $p < 0.0001$; neuronal growth factors vs. BSS, $p < 0.0001$; †Kruskal-Wallis test, *p*-value with the Bonferroni method, MIF vs. BSS, $p = 0.0001$; neuronal growth factors vs. BSS, $p = 0.002$.

two weeks postoperative ($p = 0.815$) or at 10 weeks postoperative ($p = 0.192$) (Fig. 2).

Discussion

Our results showed that MIF mRNA expression in the corneal epithelium was upregulated 2.5-fold as compared with the control eyes at 48 hours after LASIK, as determined by real-time PCR. Cells from the LASIK interface, which are assumed to be inflammatory cells or keratocytes, also showed upregulation in the expression of MIF mRNA by a factor of 1.5 as compared with the control eyes. These data suggest that the source of MIF in the wound healing process after LASIK could be either the corneal epithelium, keratocytes or inflammatory cells. Expression of MIF in cornea wound healing has been studied in a rat model, and Matsuda et al. [18] have shown that MIF was released within three hours from the corneal epithelial cells after a penetrating linear incision. They reported that the MIF mRNA level of the injured cornea increased from 6 to 48 hours after injury and then diminished. The upregulation of MIF expression implies that MIF could have an important role in the wound healing process.

The present study demonstrates that topically-administered MIF showed a beneficial effect on the recovery of corneal sensitivity following LASIK surgery. Dry eye after LASIK results from severing of the corneal nerves and subsequent loss of corneal sensation. Decreased corneal sensitivity may trigger a cascade of events that degrade the corneal integrity by reducing the protective blinking reflex, delaying epithelial wound healing, and decreasing aqueous tear layer production [19-21]. Recovery of corneal sensation occurs in three to seven months, but a morphological study showed that recovery of corneal innervation would take more than 12 months after LASIK [1]. It has been postulated that various neuronal growth promoting factors such as a glial cell line-derived neurotrophic factor, opioid growth factor, and ciliary neurotrophic factor might enhance the recovery of corneal reinnervation after LASIK [22-24]. Our previous study has shown that nerve growth factor induced an earlier recovery in corneal sensitivity after LASIK [17]. We used a combination of NGF, NT-3, IL-6, and LIF as a positive control in this study. Our results demonstrate that the treatment with MIF alone could achieve as much of an effect on the recovery of corneal sensation as treatment with a combination of NGF, NT-3, IL-6, and LIF. The detailed mechanism of MIF on the regeneration of the corneal nerve has not been investigated in this study. It has been reported that MIF exerts an enzymatic activity to catalyze the conversion of toxic quinone products of catecholamine neurotransmitters to indoledihydroxy derivatives [9] and this has raised the possibility that MIF could have a protective effect on nervous tissue by converting toxic products of catecholamine metabolism. A preliminary study has also suggested that MIF could rescue neuronal cells from catecholamine-induced cell death (data not shown). Nishio et al. [11] also reported that MIF mRNA was up-regulated in peripheral nerves after axotomy and blocking MIF with anti-MIF antibody resulted in delayed nerve regeneration and more apoptosis in rat peripheral nerves [12]. Thus, up-regulation of MIF in the early period of the wound healing process may protect against neuronal damage. Our study suggests that exoge-

nous application of MIF during the early period of wound healing may further facilitate nerve recovery.

Limitations of this study would include the small numbers of animals, and also the short follow-up period. This study also did not include histological evidence of corneal nerve regeneration. However, rabbits assigned to a topical treatment with MIF showed a faster recovery of corneal sensation in the early postoperative period after LASIK. This beneficial effect on earlier corneal regeneration may interrupt the cascade events that lead to deterioration of the ocular surface and may reduce dry eye symptoms in patients undergoing LASIK [25].

The results of this study suggest that the topical application of MIF plays a potential role in reinnervation of damaged corneal nerves after LASIK. Further studies are needed to elucidate the detailed mechanism of MIF on corneal nerve regeneration.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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