

Plasminogen Activator Activity in Tears after Excimer Laser Photorefractive Keratectomy

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PURPOSE. To quantify changes of plasminogen activator activity in tear fluid during corneal re-epithelialization after excimer laser photorefractive keratectomy (PRK).

METHODS. Tear samples were collected with glass capillaries from 77 eyes of 42 patients immediately before and immediately after PRK treatment and on postoperative days 3 and 5. In 20 patients, the contralateral eye was similarly sampled to serve as control. Plasminogen activator activity in the tear samples was measured by a spectrophotometric method using human plasminogen and chromogenic peptide substrate, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251).

RESULTS. In tears of all eyes that underwent PRK, the plasminogen activator activities were lower immediately after PRK than were the preoperative values. For patient eyes with normal wound healing, tear plasminogen activator activities were significantly elevated above the preoperative level on the third postoperative day and then returned to the preoperative level by the fifth postoperative day. In contrast, tear plasminogen activator activities remained low through the third postoperative day in all (six) eyes in which haze developed after 3 to 6 months. The contralateral control eyes showed no appreciable change in plasminogen activator activity over the 5-day period.

CONCLUSIONS. Plasminogen activator activity levels measured in tears of excimer laser PRK-treated eyes may serve as a predictor of wound healing. Extended low levels of plasminogen activator activity through the third postoperative day correlate with the development of corneal healing abnormalities (haze). The low plasminogen activator activity could be not only an accompanying sign but also a cause of defective corneal wound healing. (*Invest Ophthalmol Vis Sci.* 2000;41:3743-3747)

Excimer laser photorefractive keratectomy (PRK) is used for the correction of myopia, hyperopia, and astigmatism. The excimer laser removes tissue through photoblastic decomposition by delivering incident photon energy that is sufficient to break molecular bonds. Selective removal of tissue across the anterior surface results in a change in anterior corneal curvature. In the majority of cases the refractive outcome is within ± 0.5 D of that intended, although there is some variation in the refractive outcome, depending on the preoperative refractive error. The surgical outcome may also be influenced by individual variability in wound healing and pharmacologic intervention.¹

PRK complications include excessive myopic regression and disturbances in corneal transparency (haze, scarring).^{2,3} Histologically, this is due to the presence of unstructured collagen fibers excreted by activated keratocytes and affected

extracellular matrix. When present, haze usually appears in the first month, its intensity being highest in the postoperative 3 to 6 months. In most cases it disappears later. Although complete re-epithelialization usually occurs in human corneas at 2 to 4 days after surgery, normal epithelial thickness is not observed until 6 months after surgery.⁴ Scarring is caused by activated stromal keratocytes and inflammatory cells that invade the stroma after re-epithelialization. The activated keratocytes secrete collagen fibers that have different structures from normal collagen. As a result of this hyperplastic response, the thickness of the stroma increases, the corneal refractive power subsequently increases, and a myopic shift occurs. Regression can be explained not only by the stromal hyperplastic reaction but by epithelial hyperplasia as well.^{5,6}

Wound healing is regulated by two major systems that are controlled by activators and inhibitors. The first system is the plasminogen activator-plasmin system, which is involved in the degradation and removal of damaged extracellular matrix.^{7,8} The second system is the activated keratocyte system,⁸⁻¹³ which is involved in the replacement of damaged collagen by synthesizing new collagen⁹⁻¹² and the collagen matrix of glycosaminoglycans.¹⁰⁻¹³ This process is very important in epithelial regrowth, but by activating the synthetic activity of keratocytes, it can cause scar formation. The ulcerative mechanism with persistent epithelial defect¹⁴⁻¹⁶ is initiated if plasminogen activator is released with increased or prolonged activity.^{14,15}

Normal corneal epithelial cells do not release plasminogen activator, but damaged corneal epithelium causes keratocytes

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to release plasminogen activator, which converts plasminogen, already present in the stroma, to plasmin. The plasmin in turn feeds back to fibroblast cells, inducing them to secrete more plasminogen activators; activates the latent procollagenases to collagenases, which results in collagen molecule degradation; and degrades the fibronectin in the extracellular matrix. In addition, plasmin might stimulate the replication of endothelial cells, promoting angiogenesis, which has been shown to be an important factor in tissue repair.¹⁷ Plasmin might generate chemotactic factors for polymorphonuclear neutrophils from the complement system and cause the formation of vasoactive kinins, which result in the increased entrance of serum anti-proteases into the corneal stroma.¹⁴

Plasminogen activator plays a pivotal role in the plasminogen activator-plasmin wound-healing system. Plasminogen activator is a normal component of tear fluid, whose concentration is influenced by biochemical transformations in the cornea. Thus, the progression of corneal wound healing may be followed by determining the plasminogen activator levels in the tear fluid.¹⁸ The purpose of this study was to quantify the changes of plasminogen activator activity in tear fluid during corneal re-epithelialization after excimer laser PRK.

MATERIALS AND METHODS

Forty-two patients (26 female, 16 male) who underwent PRK surgery between the ages of 17 and 51 years (mean, 27 ± 9 [SD] years), were selected for this study after obtaining informed consent in adherence to the Declaration of Helsinki. For a given patient, the PRK was performed one eye at a time with an interval of 1 to 2 weeks between surgeries. Both PRK eyes were sampled except for seven patients (three female, four male), who volunteered for sampling from only one eye. Excluded from consideration were patients from whom a 15- μ l tear sample could not be obtained within 3 minutes. For 20 of the surgeries for which additional informed consent could be obtained, the contralateral eye was sampled as a control eye. This provided a sample population of 77 eyes (41 right, 36 left) and 20 contralateral control eyes. Data are means \pm SD. Preoperative mean refractive error was -3.0 ± 3.0 D (range, 5.0 to -10.0 D). Eighteen of the eyes had preoperative astigmatism (mean, -1.5 ± 0.6 cylinder; range, -1.0 to -2.75 cylinder). Seventeen of the patients had previous usage of contact lenses (mean usage, 4 ± 2 years; range, 1-9 years).

PRK treatments with an excimer laser (Keratome II ArF; Schwind, Kleinostheim, Germany; [193 nm]) were performed by the same surgeon at Vital-Laser LLC, Department of Ophthalmology, University Medical School of Debrecen, Hungary. De-epithelialization was performed with a blunt keratome blade knife after epithelial marking with a 6.0- to 6.5-mm Hoffer trephine for spherical correction and 7.5 to 8.0 mm for astigmatic correction centered over the pupil. The epithelium was scraped gently from periphery to center, with care taken to avoid damaging the surface of Bowman's layer. Residual epithelial debris was removed with a sterile micro sponge. Epithelial anesthesia was induced using 0.4% oxybuprocaine hydrochloride eyedrops. The diameter of the ablation zone was 6.1 ± 0.2 mm (range, 6.0-6.5 mm) for patients without astigmatism. For those with astigmatism, the diameter of the astigmatic ablation mask was 7.5 ± 0.6 mm (range, 6.0-8.1 mm), and the spherical ablation mask was 5.7 ± 0.1 mm (range,

5.3-6.0 mm). The mean ablation depth of the PRK surgery was 48 ± 22 μ m (range, 12-120 μ m). The mean ablation depth (47 μ m) of the subgroup without astigmatism was not statistically significantly different ($P > 0.66$) from the mean ablation depth (50 μ m) of the astigmatism subgroup.

The postoperative treatment included antibiotic eyedrops (Ciloxan; Alcon, Fort Worth, TX), hourly on the first postoperative day and five times daily during the next 5 days for each patient. The eyedrops were withdrawn for at least 8 hours before tear sampling to avoid the possibility of tear sample dilution. After the 5-day period, Flucon and Tears Naturale (both from Alcon) were administered 5 times daily during the first month, reduced to four times daily for the second month, and to three times daily for the third month. No other treatment was used during this period. All patients underwent follow-up examinations at 1, 3, and 6 months after the PRK procedure.

Tear samples for plasminogen activator analyses were obtained immediately before and immediately after the PRK treatment and on the third and fifth postoperative days from the PRK-treated eye and the contralateral eye where it was used. Samples consisted of tears collected with glass capillaries^{16,19} under slit lamp illumination from the lower tear meniscus (a horizontal thickening of the precorneal tear film by the lower margin) at the lateral canthus.¹⁶ Care was taken not to touch the conjunctiva. We used the same collection method throughout the study. The duration of the sampling time was recorded, and the secretion rate was calculated in microliters per minute, dividing the obtained tear volume by the time of sample collection. Samples used in this investigation had secretion rates of 5 to 15 μ l/min for both the PRK eyes and the contralateral controls. Samples were centrifuged (1800 rpm) for 8 to 10 minutes right after sample collection, and supernatants were deep-frozen at -80°C and were thawed only once for measurements.

Plasminogen activator activity was measured in the sample tears by a spectrophotometric method using human plasminogen and a plasmin-specific chromogenic peptide substrate, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251).²⁰ This assay is sensitive predominantly to urokinase-like plasminogen activator.¹⁶ Plasminogen and the S-2251 were purchased from Chromogenix (Mölnådal, Sweden). Urokinase standard was purchased from Choay (Paris, France). This assay is suitable to measure plasmin activity but can also be used for determining plasminogen activator activity by adding plasminogen to the reagents. Plasminogen activator activity was measured as described by Shimada et al.²⁰ with the following modifications according to Tözsér et al.¹⁶ and Tözsér and Berta²¹: 5 μ l tear, or standard urokinase, or plasmin was incubated in 100 μ l of 0.05 Tris buffer (pH 7.4) at 37°C in the presence of 0.5 mM chromogenic substrate S-2251 and 1 μ M human plasminogen in wells of microtiter plates. After 4 hours' incubation, the reaction was terminated by the addition of 500 μ l of 8 M acetic acid. The absorption was measured at 405 nm with a spectrophotometer (Multiscan MS; Labsystem, Helsinki, Finland). Plasminogen independent amidolytic activity was measured similarly, but plasminogen was omitted from the incubation mixture. The absorption difference between the values obtained with and without plasminogen was considered to be due to the plasminogen activator activity in tear, whereas the absorbance value obtained without plasminogen was considered to be plasminogen-independent amidolytic activity. Based

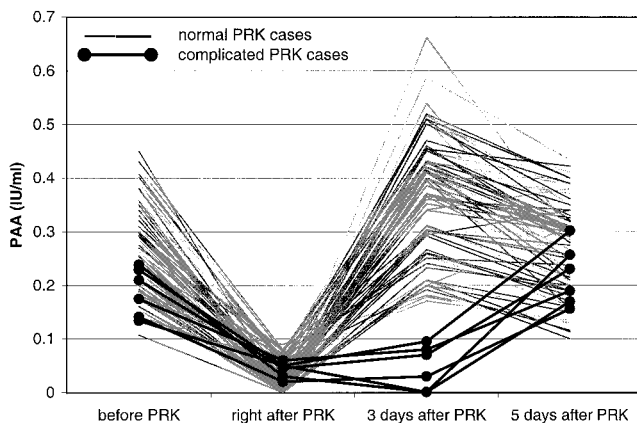


FIGURE 1. Plasminogen activator activities before and after PRK showing the cluster of individual normal values compared with the cluster of individual complicated cases.

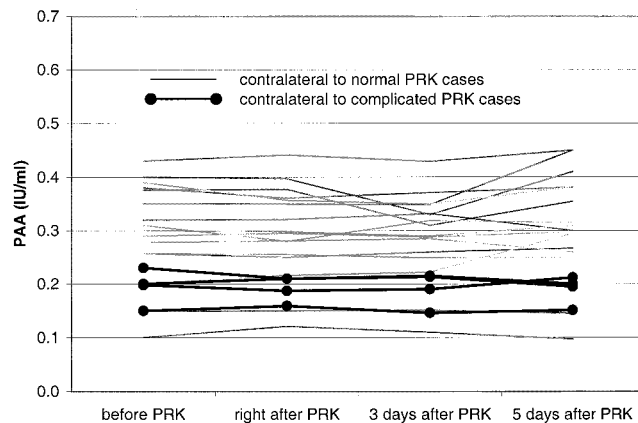


FIGURE 2. Plasminogen activator activities in the contralateral eye before and after PRK, showing the individual cases of contralateral-to-normal eyes and contralateral-to-complicated eyes.

on the absorption values gained in the same system with urokinase standard solutions with different concentrations we produced a calibration curve. The plasminogen activator activities of the measured samples were calculated with this calibration curve and were expressed in international units per milliliter urokinase equivalent values. Plasminogen-independent activity was found to be negligible in all the tear samples.

Determination of haze was made without any knowledge of the plasminogen activator levels for any of the patients. Therefore, there was no bias in the determination of haze or in the correlation of plasminogen activator activity with haze. The haze-grading system of Hanna was adopted.¹³

Standard statistical procedures were used to compare patient characteristics between different groups (*t*-test for means of correlated pairs). Plasminogen activator activities were compared between different groups using *t*-tests for means with equal variances. Comparisons with control eyes were performed using paired *t*-tests. Differences resulting in $P < 0.05$ were considered significant, and $P < 0.001$ was considered highly significant.

RESULTS

During the 5-day period of tear sampling for each eye, there were no clinical features that distinguished any of the eyes. However, six of the PRK-treated eyes (five patients: four female, one male) showed mild to marked subepithelial corneal haze (grade 1-2)¹³ between the third and sixth month, accompanied simultaneously by a slight decrease in visual acuity. These six eyes were retrospectively labeled complicated cases and represent an 8% (6/77) frequency of occurrence. The remaining eyes did not exhibit visual complications and were retrospectively designated normal cases. Three of the patients had one complicated and one normal eye; one patient had two complicated eyes; and one of the patients with a complicated eye had volunteered only one eye for sampling.

Individual values of the plasminogen activator activities for each eye of the normal and complicated groups are shown in Figure 1. The plasminogen activator activities of the two groups are distinctly separated on the third postoperative day and overlap at the other three measurement times. Figure 2

shows the individual values of the plasminogen activator activities for all the contralateral eyes.

Patient characteristics are provided in Table 1, and visual and surgical characteristics of the eyes are given in Table 2. There are no statistically significant differences in age, length of contact lens wear, prior refractive correction, or extent of astigmatism between the two groups.

For the plasminogen activator measurements, the normal cases established a pattern as tabulated in Table 3. There was a decrease in the mean value of plasminogen activator to approximately 11% of the preoperative mean level immediately after the PRK treatment, an increase of 41% above the preoperative level by the third postoperative day, and a return to within 4% of the preoperative level by the fifth postoperative day. There was not a significant difference ($P = 0.15$) between the preoperative mean plasminogen activator activity and the value on the fifth postoperative day. However, the mean plasminogen activator values immediately after and on the third postoperative day were significantly lower and higher, respectively ($P < 0.001$), than the preoperative and 5-day postoperative mean plasminogen activator activity.

A different pattern of plasminogen activator values is seen in Table 3 for the complicated cases. During the postoperative period for the six complicated eyes, the mean plasminogen activator value decreased to 23% of the preoperative mean value immediately after the PRK treatment and remained at the 25% level through the third postoperative day. By the fifth day, the mean plasminogen activator level was 16% above the preoperative mean value. The mean plasminogen activator values immediately after PRK and on the third postoperative day were not significantly different ($P = 0.81$) from one another, and each was significantly less ($P < 0.001$) than the preoperative

TABLE 1. Characteristics of Patients

	Age (y)	Contact Lenses (y)
Normal (<i>n</i>)	27 ± 9 (40)	4 ± 2 (15)
Complicated (<i>n</i>)	23 ± 5 (5)	5 ± 2 (4)
<i>P</i>	0.38	0.35

P (normal vs. complicated eyes) by Student's *t*-test. Data are means ± SD.

TABLE 2. Visual and Surgical Characteristics

	Pre-PRK Astigmatism (Cylinder)	Pre-PRK Refraction (D)	Ablation Depth (μ m)
Normal (<i>n</i>)	-1.6 \pm 0.6 (16)	-2.9 \pm 3.1 (71)	47 \pm 22 (71)
Complicated (<i>n</i>)	-1.0 \pm 0.0 (2)	-5.1 \pm 1.3 (6)	57 \pm 23 (6)
<i>P</i>	0.24	0.08	0.33

P (normal vs. complicated eyes) by Student's *t*-test. Data are means \pm SD.

mean plasminogen activator value. In addition, there was a significant increase ($P = 0.02$) in mean plasminogen activator activity on the fifth postoperative day compared with the preoperative mean value.

The plasminogen activator mean values for the normal and complicated cases are compared in Table 3. The preoperative mean value is significantly lower ($P = 0.04$) for the complicated cases than the corresponding normal plasminogen activator mean value. However, the plasminogen activator mean values immediately after PRK and 3 days after surgery for the complicated eyes were not significantly different from the immediate postoperative plasminogen activator mean value for normal eyes ($P > 0.14$). On the third postoperative day, there was a highly significant difference ($P < 0.001$) between the plasminogen activator mean value for the complicated eyes and that for the normal eyes.

The contralateral plasminogen activator mean levels are shown in Table 3. The mean plasminogen activator activity levels (normal group: 0.299 \pm 0.087 IU/ml; complicated group: 0.192 \pm 0.026 IU/ml) remained steady (Fig. 2) over the 5-day measurement period (mean change in value, normal group: 0.019 \pm 0.023 IU/ml; complicated group: 0.011 \pm 0.007 IU/ml). The mean plasminogen activator levels in the contralateral complicated cases were significantly lower ($P < 0.001$) than the corresponding mean levels of the contralateral normal eyes. Levels in both contralateral groups differed significantly from the PRK-treated eyes immediately after and 3 days after surgery.

DISCUSSION

The interesting observation in this study is that normal and complicated corneal healing after PRK surgery were accompanied by different patterns of plasminogen activator values and

specifically a significant difference on the third postoperative day (Fig. 1). The low plasminogen activator values for the complicated cases were sufficiently separated from the higher normal group values on the third postoperative day that there was some promise of distinguishability (Fig. 1). Therefore, determination of plasminogen activator activity and comparisons with preoperative and normal values at the third postoperative day may serve predictive and diagnostic purposes in the identification of patients who are prone to abnormal corneal wound healing. Six of six retrospectively identified eyes showed the effect, and all 71 remaining eyes showed no ensuing haze: this corresponds to a sensitivity of 1.0 and a specificity of 1.0. If low (below 0.1 IU/ml) plasminogen activator activity on postoperative day 3 were used to prospectively identify (predict) eyes in which wound healing abnormalities (haze) might develop, six of six eyes would have given a valid prediction. Although six eyes were sufficient to provide statistical meaning, additional complicated corneal healing cases should be investigated to increase the statistical validity of the results.²²

When the contralateral control eye was measured, the tear fluid flow and plasminogen activator activities remained essentially constant (Fig. 2). This implies that PRK surgery and tear sampling have little collateral effect on changes in tearing or fibrinolytic activity in the contralateral eye. The preoperative plasminogen activator mean values for the complicated and corresponding contralateral eyes are significantly lower than the mean plasminogen activator levels in the normal groups (Table 3). This may indicate a predisposing factor for the complicated eyes. Moreover, for each of the three patients in the complicated group who had one normal and one complicated eye, the preoperative plasminogen activator value was lower in the eye that became complicated. However, the ranges of plasminogen activator activity for the normal and complicated groups overlapped on the preoperative measurement (Figs. 1 and 2). Therefore, the normal and complicated eyes were not distinguishable on the basis of the preoperative plasminogen activator activity level.

A previous study investigated the tear fluid level of plasminogen activator activities and the plasmin level of seven rabbits (11 eyes) during wound healing after anterior keratectomy. In that study, plasmin and plasminogen activator activities were determined using radial caseinolysis procedures.¹⁸ A pattern of plasminogen activator activity was found in the rabbit after surgery that was qualitatively similar to the pattern of plasminogen activator over time found here in Figure 1 for

TABLE 3. Plasminogen Activator Activities

	Pre-PRK (IU/ml)	Post-PRK (IU/ml)	Post-PRK +3 Days	Post-PRK +5 Days
Normal (<i>n</i> = 71)	0.259 \pm 0.082	0.027 \pm 0.029	0.366 \pm 0.109	0.269 \pm 0.085
Complicated (<i>n</i> = 6)	0.188 \pm 0.045	0.043 \pm 0.015	0.046 \pm 0.041	0.218 \pm 0.056
Contralateral Normal (<i>n</i> = 16)	0.299 \pm 0.092	0.297 \pm 0.086	0.290 \pm 0.080	0.310 \pm 0.097
Contralateral Complicated (<i>n</i> = 4)	0.195 \pm 0.033	0.192 \pm 0.024	0.191 \pm 0.032	0.190 \pm 0.027
<i>P</i> (normal versus complicated)	0.04	0.21	<0.001	0.15
<i>P</i> (contralateral normal versus normal)	0.74	<0.001	0.002	0.87
<i>P</i> (contralateral complicated versus complicated)	0.49	<0.001	0.006	0.83

P by Student's *t*-test. Data are means \pm SD.

our normal cases. In particular, for 11 rabbit eyes, the mean (\pm SEM) preoperative plasminogen activator activity in the rabbit was 2.0 ± 0.6 IU/ml; this declined to 0.3 ± 0.1 IU/ml after surgery and then increased to 2.1 ± 0.3 IU/ml during wound healing.¹⁸ This agreement in the pattern of plasminogen activator values strengthens the notion that there is a normal pattern leading to normal, healthy wound healing.

In the previous rabbit study,¹⁸ tear fluid flow was measured in addition to plasminogen activator activity. The tear fluid flow was found to increase after anterior keratectomy by a factor of 2.3 compared with preoperative flow. Therefore, dilution of plasminogen activator was offered as a possible explanation for the decrease in postoperative plasminogen activator level.¹³ However, the plasminogen activator did not change by the dilution factor of 2.3: The level of plasminogen activator decreased after surgery by a factor of 6.7 compared with preoperative levels.¹⁸ Therefore, dilution alone is not a sufficient mechanism to explain the decrease in plasminogen activator level. In the present work, the tear fluid flow remained in the range of 5 to 15 μ l/min for all eyes over all sample times. Therefore, tear fluid flow levels cannot be responsible for the lowered plasminogen activator activities seen after PRK in this work.

Although the exact mechanisms underlying post-PRK corneal healing complications are unknown, it is generally suspected that individual variations in corneal wound healing play a significant role in post-PRK refractive regression and haze formation.²³ Low plasminogen activator activity sustained over a period of 3 days, evidenced in tear fluid, is an accompanying sign of the complicated PRK-treated eyes observed in this study. It is not possible from the present results to distinguish whether the low plasminogen activator activity is a primary phenomenon or a result of some other primary event. However, based on its known importance in the wound healing process, we conclude that prolonged low plasminogen activator activity could be a possible cause of defective corneal healing. The determination of plasminogen activator activity on postoperative day 3 could be a strong predictor to identify patients who are vulnerable to future visual abnormalities (haze). The concept of treatment intended to minimize degradation and tissue removal due to the plasminogen activator-plasmin system could open a new area of investigation for the postoperative management of PRK.

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