PH.D. THESIS

PLASMINOGEN ACTIVATOR ACTIVITY IN TEARS AFTER EXCIMER LASER PHOTOREFRACTIVE KERATECTOMY

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1. INTRODUCTION

Excimer laser photorefractive keratectomy (PRK) is used for the correction of myopia, hyperopia and astigmatism. This laser removes through photoablative decomposition, in which incident photon energy is sufficient to break molecular bounds. 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 diopters (D) of that intended, although there is some variation in the refractive outcome may also be influenced by individual variability in wound healing and pharmacological intervention.

PRK complications include excessive myopic regression and disturbances in corneal transparency (haze, scarring). 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 3-6 months, and most cases disappears later.

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.

The second system is the *activated keratocyte system* which is involved in the replacement of damaged collagen by synthesizing new collagen and the collagen matrix of glycosaminoglycans.

This process is very important with respect to epithelial re-growth, however by activating the synthetic activity of keratocytes it can result in scar formation. The ulcerative mechanism with persistent epithelial defect is initiated if plasminogen activator is released with increased or prolonged activity.

Objectives:

1. To examine the change of plasminogen activator activity in tears after photorefractive keratectomy.
2. To find correlation between the change of urokinase type plasminogen activator (uPA) level and the development of corneal stromal haze.
3. To examine the role of low level uPA in haze development.
4. To examine the supressibility of the normal uPA level of the tear with serine protease inhibitor (SPI) in animals .
5. To create a new animal model which could provide a more extensive base for further biochemical, immunhistochemical and histological studies of the corneal stromal haze.

2. BACKGROUND

Seven decades ago the ocular was the symbol of education and wealth. Today it is a common accessory. We have the option choosing in between a pair of glasses or contact lenses in order to correct the refractive error of the eye. Contact lenses undoubtedly provide excellent optical results, however, in case of improper application it can carry a high risk of complications. Meanwhile wearing glasses or contact lenses provide only temporary vision improvement (duration of wear application), microsurgical methods obtains permanent results. Because of this reason the creation and application of the excimer laser in the field of ophthalmology has drawn great attention and fascination.

Few hours after the photorefractive keratectomy the epithelial regeneration begins and the ablated area will be covered within 1-3 days. In later phases of the wound healing process the epithelial could become hyperplastic by currently unknown reasons, results that the newly acquired refractive power of the eye diminishes. The Bowman membrane can not be regenerated after the surgery. Twenty four hours after the PRK surgery polymorphonuclear neutrophils start appearing in the corneal stroma from the tears. The level of plasminogen activator in the tear elevates and through the activation of the plasminogen activator plasmin system the improvement of the damaged collagen and extracellular matrix begins. Considering the regeneration of the epithelium the removal of the damaged cell particles are essential. At the same time the intensifying synthetic activity of the stromal keratocyts could result scar formation causing corneal stromal haze which can jeopardize the newly attained refractive power of the eye.

3. MATERIALS AND METHODS

Forty-two patients (26 female, 16 male) underwent PRK surgery between the ages of 17 and 51 years (mean, 27 ± 9 [SD] years). For a given patient, the PRK was performed on one eye at a time with an interval of 1 to 2 weeks between surgeries. Both PRK treated eyes were sampled except for seven patients, who volunteered for sampling from only one eye. Excluded from consideration were patients from whom a 15µl 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. 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).

Sixteen eyes of eight normal, healthy New Zealand rabbits (2–3.5 kg) underwent PRK surgery for spherical correction. Six eyes of 3 three rabbits used as a normal control and were not operated.

PRK treatments using the Schwind Keratom II ArF excimer laser (193nm) were performed by the same surgeon. For *human* surgery, the diameter of the ablation zone was 6mm, except for new, younger patients (6.5 mm) and a few older patients (5.5 mm). The mean ablation depth of the PRK surgery was 47 microns (SD: 20, range: 17 to 105 microns). The postoperative treatment included antibiotic eyedrops [Ciloxan (Ciprofloxacin HCL 0,3%, Alcon)] hourly on the first postoperative day, and five times daily during the next five days for each patient. After the first week the postoperative treatment included Flucon (Fluorometholone 0,1%, Alcon) and Tears Naturale II. (Dextran/Hydroxypropyl Methylcellulose, Alcon) five times daily during the first month, four times the second month and three times in the third one.

All patients received follow-up examinations at 1, 3 and 6 months following the PRK procedure.

Animals were handled and treated in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. De-epithelization was performed with blunt Keratome Blade knife after epithelial marking with 6.0-6.5 mm Hoffer trephine. The epithelium was scraped gently from periphery to center. Residual epithelial debris was removed with a steril microsponge.

Topical anesthetic (0.4% oxybuprocaine hydrochloride) eyedrops were administered twice before the surgery. General anesthesia was accomplished by intravenous injection of ketamine-xylazine (2.2:1 ratio, 10mg/kg).

The postoperative treatment included antibiotic eyedrops, Ciloxan (Alcon), twelve times (hourly) on day 1 and five times (every two hours) on five additional days (postoperative days 2-5 and 7). Flucon (Alcon) and Tears Naturale (Alcon) were given 5 times daily during the first postoperative month, reduced to 4 times daily for the second month and 3 times daily for the third month. All rabbits received follow-up examinations at 1, 3 and 6 months following the PRK procedure.

In addition, the left eye of each rabbit received 1 drop of 20.000 IU/ml serine protease inhibitor, aprotinin (Gordox, Richter Gedeon Rt., Budapest) twelve times on day 1 and five times on five additional days. This was designated as „with SPI" group. The right eyes were not treated with SPI and were designated as „No-SPI" group. After the seventh day, identical treatment was used on both eyes. No other treatment was used during the six month follow-up period.

Determination of haze was made without any knowledge of the plasminogen activator levels for any of the rabbits. The haze grading system of Hanna was adopted.

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. Tear samples were collected with glass capillaries under slit lamp illumination from the lower tear meniscus (a horizontal thickening of the precorneal tear film by the lower tear margin) at the lateral canthus. 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 secretion rate was calculated in microliters per minute, dividing the obtained tear volume by the time of sample collection. Sample used in this investigation had secretion rates of 5 to 15 µl/min for both the PRK eyes and the contralateral controls. Samples were centrifuged (1800 rmp) for 8 to 10 minutes right after sample collection, and supernatants were deep-frozen at-80°C and were thawed only once for measurements.

In our animal experiments considering the low tear secretion rate of the rabbits we have taken samples as we described in human studies only after injecting 5 mg/kg pilocarpin. Before and after the PRK procedure we have been collecting tear samples as follows: in the beginning phase of the corneal wound healing (one week) every day and than throughout the following three month every fourth day.

Plasminogen activator activity was measured in the samples by a spectrophotometric method using human plasminogen and plasmin-specific chromogenic peptide substrate, D-valin-l-leucil-l-lizin-pNA (S-2251). This assay is predominantly sensitive to urokinase like plasminogen activator. Plasminogen activator activity was measured as described by Shimada. 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-test 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.

4. RESULTS

Human experiments:

During the first five days following PRK, there were no clinical features that distinguished any of the thirty-seven patients. However, six of the PRK treated eyes (five patients) developed a central corneal haze (Hanna grade 1-2) between the third and sixth month, accompanied simultaneously by a slight decrease in visual acuity. The results for these six eyes have been identified as a „complicated" cases. The remaining cases did not exhibited visual complications and are designated „normal" cases. It should be noted that two of the patients had one „complicated" eye and one „normal" eye.

With respect to the plasminogen activator measurements, the „**normal cases**" preoperative PAA level were 0.259 (± 0.082) IU/ml and decreased to 0.027 (± 0.029) IU/ml right after the PRK treatment. On the third postoperative day the PAA level increased up to 0.366 (± 0.109) IU/ml, which decreased by 0.269 (± 0.085) IU/ml for the fifth postoperative day.

There was not a significant difference ($p=0.14$) between the preoperative mean plasminogen activator activity and the value on the fifth postoperative day. However, the plasminogen activator values on the third postoperative day were significantly greater ($p< 10^{-9}$) than the preoperative and five-day postoperative plasminogen activator activity.

With respect to the plasminogen activator measurements, the „**complicated cases**" preoperative PAA level were 0.188 (± 0.045) IU/ml and decreased to 0.043 (± 0.015) IU/ml right after the PRK treatment. On the third postoperative day the PAA level increased up to 0.046 (± 0.041) IU/ml which increased up to 0.218 (± 0.056) IU/ml for the fifth postoperative day.

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 value 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 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.

Comparing this PAA change with the clinical observations we have found that stromal haze developed only among those where change in PAA did not appear on the third postoperative day. These cases we have designated as "complicated group"($n=6$) and the rest as a "normal group" ($n=71$).

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.

On the fifth postoperative day, there was no significant difference ($p>0.15$) between the plasminogen activator mean value for the complicated eyes and that for the normal eyes.

Among the normal group, the mean PAA of the control (non operated eye) has not shown a significant difference compared with the preoperative values.

Among the complicated group the mean PAA has been also unchanged in case of the non operated eye. The preoperative PAA mean of the non operated eye among the complicated group proved to be significantly lower than the preoperative PAA mean of the normal group under the same conditions.

Animal experiments:

In each case the postoperative procedure on the *right eye* of the animals has been carried out as it was described in our human studies. The corneas remained clear in these eyes over the six-month follow up period. The PAA change of the tears in these samples has shown the same pattern as it was observed among the human normal group.

The mean plasminogen activator activity value before surgery was not significantly different ($p=0.16$) than the 19-91 day equilibrium plasminogen activator activity level. The plasminogen activator activity values were significantly ($p=0.001$) lower (day 1) and higher (day 2 and day 3) than the equilibrium plasminogen activator activity level. The plasminogen activator activity on day 4 was significantly ($p=0.02$) higher than the equilibrium level, but from day 5 and beyond, there were no significant differences with the equilibrium value.

The *left eye* of the rabbits have been treated according to the same procedure as it was described on the right eye of the animal in additional to adding serine protease inhibitor by the following methods: 1 drop of 20.000 IU/ml serine protease inhibitor, aprotinin (Gordox), twelve times on day 1 and five times (every two hours) on five additional days (postoperative days 2-5 and 7). All 8 of these eyes developed corneal haze after two month.

The serine protease inhibitor suppressed the plasminogen activator activity to a mean value averaged over days 1-7 of 0.13 (± 0.15) IU/ml. The mean plasminogen activator activity prior to surgery was not significantly different

($p=0.18$) than the equilibrium plasminogen activator activity level of 2.88 (± 0.22) IU/ml, averaged over days 19-91. Plasminogen activator activity on days 1-7 were significantly ($p<0.001$) lower than the equilibrium plasminogen activator activity level. The mean plasminogen activator activity on day 11 remained significantly lower ($p=0.002$) than the equilibrium plasminogen activator activity level, but from day 15 onwards, there were no significant differences with the equilibrium plasminogen activator activity value.

The differences between the pre-surgical mean plasminogen activator activity for the two groups was not significant ($p=0.46$)nor was the equilibrium (days 19-91) mean plasminogen activator activity significantly different ($p=0.06$) for the two groups.

5. DISCUSSION

The interesting observation in this study is that normal and complicated corneal healing after PRK surgery are accompanied by a difference in the pattern of plasminogen activator values and specifically a significant difference on the third postoperative day. There are no distinguishing differences between the normal and complicated cases observable in patient characteristics. The preoperative mean plasminogen activator level of the group of all normal, however, the range of plasminogen activator values for the two groups are overlapping and therefore not distinguishable. In contrast, the low plasminogen activator values for the complicated cases are sufficiently separated from the higher normal group values on the third postoperative day that there exists some promise of distinguish ability. Therefore, determination of the plasminogen activator value at the third postoperative day might serve predictive and diagnostic purpose in the identification of patients that are prone to abnormal corneal wound healing.

The animal experiments have proven our observations of the human studies. In both studies (human and animal) after the procedure the prolonged low level of PAA correlated with the possible development of the corneal stromal haze in the following days.

Although the exact mechanism 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. Low plasminogen activator activity sustained over a three day period, evidenced in tear fluid, is an accompanying sign of the complicated excimer PRK cases observed in this study. Based on the known importance of the fibrinolytic system in the wound healing process, we conclude

that prolonged low plasminogen activator activity could also be one of the possible causes of defective corneal healing. The determination of the level of plasminogen activator activity on the third postoperative day could be a strong predictor to identify patients who are vulnerable to future visual abnormalities. The concept of treatment aimed at minimizing degradation and tissue removal due to the plasminogen activator-plasmin system could open a new area of investigation for the postoperative management of excimer laser photorefractive keratectomy.

6. SUMMARY OF THE CURRENT RESULTS AND THEIR POSSIBLE APPLICATIONS

1. In our human studies we have verified that after the photorefractive excimer laser procedure there is a significant change in the level of urokinase type plasminogen activator of the tear. In case of a healthy, normal wound healing we have detected that the PAA of the tear is significantly lower right after the surgery compared with the preoperative and the postoperative fifth day PAA measurements. The postoperative third day measurement has shown a significant increase, however.

2. In case of an irregular wound-healing which results in corneal stromal haze we have also detected a unique PAA change pattern. In these cases the PAA levels measured right after the photorefractive procedure and on the postoperative third day has shown no significant difference. However, the preoperative and the postoperative fifth day measurements have shown a significant drop.

3. Our animal experiments have supported the observations made in our human cases. The group which has not been treated with the serine protease inhibitor has shown a PAA change consistent with the one observed in those human cases where the wound healing was normal. The group treated with aprotinin has shown PAA change consistent with the one observed among humans with irregular wound healing, however. Through the process of re-epithelization lowering the PAA with serine protease inhibitor we have successfully created an arteficial corneal stromal haze.

4. Using serine protease inhibitor we have created a new model on animals which opens up new grounds for researching how to manipulate the possible development of corneal stromal haze after photorefractive excimer laser procedure.

5. Although the exact mechanisms underlying post-PRK 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. 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.

7. PUBLICATIONS

7.1 Research articles

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7.2 Abstracts

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