

# Increased release of tumour necrosis factor- $\alpha$ in human tear fluid after excimer laser induced corneal wound

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## Abstract

**Aims**—To measure the pre- and postoperative tear fluid tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) concentration and release in patients undergoing excimer laser photorefractive keratectomy (PRK).

**Methods**—Tear fluid samples from 18 PRK patients were collected with scaled microcapillary tubes preoperatively (day 0), on the second (day 2), and on the seventh (day 7) postoperative days. The TNF- $\alpha$  concentration was measured using a double antibody radioimmunoassay, and the TNF- $\alpha$  release was calculated by multiplying the concentration by the tear fluid flow in the collection capillary.

**Results**—The mean tear fluid flow in the capillary was 22.5  $\mu\text{l}/\text{min}$  (range 1.5–93.2) on day 0, 80.7  $\mu\text{l}/\text{min}$  (3.0–219,  $p = 0.0002$ ) on day 2, and 14.6  $\mu\text{l}/\text{min}$  (1.8–41.7, NS) on day 7. The mean TNF- $\alpha$  concentration and release values were: day 0, 358 ng/l (110–680) and 9.5 pg/min (0.2–37.5, NS); day 2, 417 ng/l ( $< 5$ –750, NS) and 28.6 pg/min (0.6–81.5;  $p = 0.003$ ); and day 7, 320 ng/l ( $< 5$ –735, NS) and 4.8 pg/min (0–25.4, NS), respectively.

**Conclusion**—TNF- $\alpha$  appears to be a component of normal tear fluid. In spite of hypersecretion caused by the corneal wound, TNF- $\alpha$  concentrations remain constant during wound healing. TNF- $\alpha$  release increases significantly during the 2 postoperative days following PRK, suggesting a role in corneal wound healing.

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Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a proinflammatory cytokine, produced by activated macrophages and monocytes, lymphocytes, neutrophils, endothelial cells, and smooth muscle cells.<sup>1</sup> The active human TNF- $\alpha$  consists of three non-covalently bound 157 amino acid subunits (each 17 kDa), produced as 233 amino acid propeptides and activated by cleavage of 76 amino acid signal peptides.<sup>2</sup> Originally TNF- $\alpha$  was considered an anti-tumour agent, but later its role in the regulation of inflammation has become obvious. It has multiple biological functions including induction of fibroblast proliferation.<sup>3</sup> Only recently it has been shown that fibroblasts cultured from wound sites are capable of secreting TNF- $\alpha$ .<sup>4</sup> Ayliffe *et al* suggested that corneal cells produced TNF- $\alpha$  in response to injury, based on the finding that TNF- $\alpha$

mRNA was expressed in the cornea after wounding.<sup>5</sup> On the other hand, in normal lacrimal gland TNF- $\alpha$  mRNA has been demonstrated in both acinar cells and macrophages. Moreover, expression of TNF- $\alpha$  is enhanced after corneal wounding.<sup>6</sup> TNF- $\alpha$  is also a potential factor contributing to scar formation<sup>7</sup> and, hence, development of postoperative haze. Based on these findings the aim of our study was to assess whether TNF- $\alpha$  is present in human tear fluid and whether its concentration and release change after an excimer laser induced photorefractive keratectomy (PRK) wound.

## Materials and methods

### SUBJECTS

The present study was performed according to the Declaration of Helsinki, and was approved by the ethics review committee of Helsinki University Eye Hospital. Informed consent was obtained from each patient. Eighteen patients (10 females, eight males, mean age 31.6 years, range 21–49 years) were studied for the presence and release of TNF- $\alpha$  in tear fluid before PRK (day 0,  $n = 18$ ), on the second (day 2,  $n = 18$ ), and on the seventh (day 7,  $n = 12$ ) postoperative day. Six patients who lived a long way from the hospital consulted their local private ophthalmologists at 1 week, so their tear fluid samples were not available at that time. Clinical investigations before PRK showed no signs of ocular inflammation or allergy. The patients were also advised to stop wearing contact lenses 2 weeks before PRK. After PRK each eye was pressure patched for 3 days. Post-surgical medication included chloramphenicol ointment (Oftan Chlora; Leiras, Tampere, Finland) twice a day for 4 days and fluorometholone drops (FML; Allergan, Irvine, CA, USA) starting on day 4 three times a day for 1–3 months. In addition, the patients received oral diclofenac 25 mg (Voltaren; Ciba-Geigy, Basel, Switzerland) just before the operation and two to three times a day for the first days after PRK. The patients also had oral benzodiazepine medication (5–10 mg; Diapam; Orion, Helsinki, Finland) for the first 2 postoperative nights.

### TEAR FLUID COLLECTION

Tear fluid samples (minimum 10  $\mu\text{l}$ ) were collected by MV with a scaled 5 or 25  $\mu\text{l}$  microcapillary tube from the lower conjunctival sac as previously described.<sup>8</sup> They were immediately transferred to Eppendorf tubes

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and centrifuged at 9000 *g* for 2 minutes, after which the supernatants were placed on dry ice and kept at  $-70^{\circ}\text{C}$  until assessed. In the morning of the second postoperative day the patch was removed and the lids were gently patted with a paper wipe. After waiting for about 30 seconds the tear fluid sample was collected. Then the ointment was applied and the eye was repatched. The tear fluid flow in the collection capillary was calculated by dividing the volume of the tear fluid sample by the tear fluid collection time. We also calculated TNF- $\alpha$  release, as previously described, by multiplying its concentration in the sample by the tear fluid flow in the collection capillary.<sup>9</sup> The capillary method of collecting tears, although performed with special attention to the technique, contained several possible sources of error. Firstly, both the residual tears of the conjunctival fornix and the newly secreted tears were collected. The tear fluid flow in the capillary was thus considerably higher than the actual tear fluid secretion rate. Secondly, reflex tearing was easily stimulated during preoperative tear fluid collection. Most patients were not familiar with tear fluid collection, and in spite of gentle aspiration technique with fire polished capillaries some of them complained having a sense of a foreign body during tear collection. During the first postoperative days, on the other hand, as the corneal epithelial wound stimulated overflow of tears, it was sometimes difficult to collect all the tears secreted. Finally, on day 7 when the epithelial defect had healed the tear fluid secretion rate returned to the preoperative level. Despite the inaccuracy of the capillary tube method, we consider it a suitable technique for collecting tears for studying the release of various proteins in tear fluid, once the limitations of the technique are realised.<sup>9-12</sup>

#### PHOTOREFRACTIVE KERATECTOMY

PRK was performed after surgical abrasion of the epithelium using a Beaver eye blade (Becton Dickinson, Franklin Lakes, NJ, USA). PRKs, 6 mm wide and of varying ablation depths (16–125  $\mu\text{m}$ ), were performed without nitrogen blow using a Visx 20/20 excimer laser (Visx Co, Sunnyvale, CA, USA) equipped with 4.02 software.

#### IMMUNOASSAY

TNF- $\alpha$  concentrations in tear fluid were determined by a double antibody radioimmunoassay developed for measuring of serum TNF as previously described.<sup>13</sup> A volume of 10  $\mu\text{l}$  of tear fluid samples was first diluted by adding assay buffer to reach a volume of 100  $\mu\text{l}$ . TNF- $\alpha$  from tears competed with a fixed amount of  $^{125}\text{I}$  labelled TNF- $\alpha$  (10 000 counts/min for 50  $\mu\text{l}$ ) for the binding sites of 30 000-fold diluted specific rabbit antibodies. The bound TNF- $\alpha$  was precipitated with Sepharose bound antirabbit IgG and then centrifuged and the radioactivity of the pellets was counted. *Escherichia coli* derived recombinant human TNF- $\alpha$  (Code TNF-H, Genzyme Diagnostics, Cambridge, USA) was used as standard. It had a molecular weight of 36 kD,

and a specific activity  $\geq 1 \times 10^7$  U/mg of protein, as measured by bioassay with mouse L 929 cells. Rabbit antiserum to human TNF- $\alpha$  (Code P-300A, Endogen, MA, USA) showed  $< 1\%$  cross reaction with lymphotoxins. The detection limit of the assay was 5 ng/l. Within assay measurement variabilities were 10–12% for the TNF- $\alpha$  concentration of 5 ng/l and 6.3% for 92 ng/l. The values from day to day remained within 10% of variation. In calculations the values  $< 5$  ng/l were regarded as 5 ng/l. In all determinations we routinely run two positive controls with TNF- $\alpha$  levels of 92 ng/l and 272 ng/l (pooled serum with added TNF- $\alpha$ , divided in aliquots and stored at  $-35^{\circ}\text{C}$ ).

#### STATISTICAL ANALYSIS

The non-parametric Wilcoxon signed rank test (two group paired test) was used to obtain the probability (*p*) values to determine the significance of changes in tear fluid flow, TNF- $\alpha$  concentration, and release in tears during healing of the PRK wound. The factors measured on day 2 and day 7 were compared with the preoperative levels (day 0) of the same patients. The results were presented as means and standard deviations. Also medians and ranges were given. Probability values of *p*  $< 0.05$  were considered significant.

#### Results

The mean tear fluid flow in the collection capillary was 22.5  $\mu\text{l}/\text{min}$  (SD 24.9, range 1.5–93.2) on day 0, 80.7  $\mu\text{l}/\text{min}$  (55.1, 3.0–219; *p* = 0.0002) on day 2, and 14.6  $\mu\text{l}/\text{min}$  (11.4, 1.8–41.7; NS) on day 7. The mean TNF- $\alpha$  concentrations and release values were 358 ng/l (170, 110–680) and 9.5 pg/min (11.2, 0.2–37.5) on day 0, 417 ng/l (207,  $< 5$ –750, NS) and 28.6 pg/min (24.8, 0.6–81.5; *p* = 0.003) on day 2, and 320 ng/l (246,  $< 5$ –735, NS) and 4.8 pg/min (7.2, 0–25.4, NS) on day 7. The pre- and postoperative tear fluid flows in the capillary, TNF- $\alpha$  concentrations and TNF- $\alpha$  release values are shown in Table 1 and Figure 1.

The TNF- $\alpha$  concentration of nine patients increased from day 0 to day 2 (range 1.2–4.8-fold), and  $\geq 2$ -fold increase was seen in the tear fluid samples of six patients. Three patients sustained their TNF- $\alpha$  concentrations during the 2 postoperative days, and in six patients the level decreased. The TNF- $\alpha$  release of 14 patients increased from day 0 to day 2 (range 1.8–45-fold), and seven patients showed an increase  $\geq 10$ -fold.

One patient showed TNF- $\alpha$  concentrations below the detection limit on days 2 and 7, although the preoperative concentration was as high as 680 ng/l and release 14.6 pg/min. Another patient presented with a preoperative TNF- $\alpha$  concentration of 345 ng/l and release of 6.2 pg/min, but on day 2 the TNF- $\alpha$  was unmeasurable. Unfortunately, the patient was unable to give a tear fluid sample on day 7. The reason for this decrease in TNF- $\alpha$  concentration is not known.

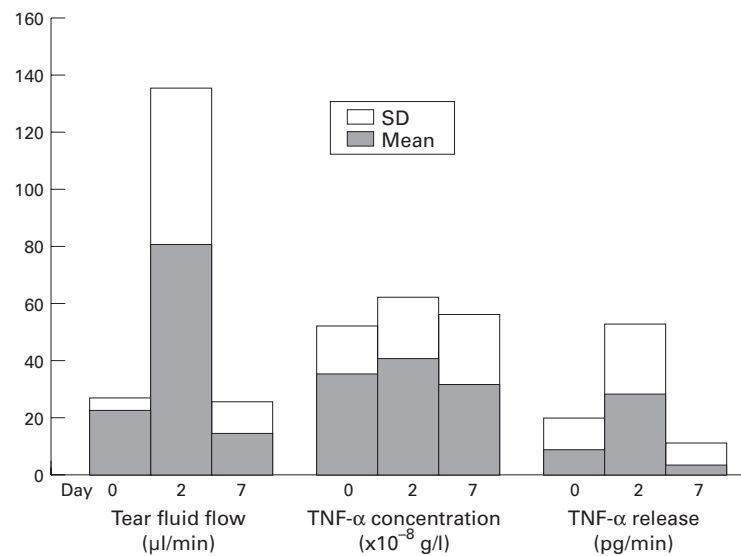


Figure 1 Mean preoperative (day 0) and postoperative (days 2 and 7) tear fluid flow in collection capillary ( $\mu\text{l}/\text{min}$ ), TNF- $\alpha$  concentration (the values have to be multiplied by 10 to get ng/l), and TNF- $\alpha$  release (pg/min). Standard deviation (SD) is also shown.

**Discussion**

Several modulators coming from tears, inflammatory cells, extracellular matrix, nerve cells, corneal epithelial cells, or stromal fibroblasts can regulate the complex biological process of wound healing. Researchers in our group are engaged in studying the presence of these modulators in tear fluid after PRK. In our earlier studies we have shown increased release of plasmin, cellular fibronectin, tenascin, and neuropeptide calcitonin gene related peptide into tears following excimer laser keratectomy.<sup>9-12</sup>

Growth factors and cytokines in the maintenance of ocular surface and wound healing constitute an interesting and expanding field of research. At least two growth controlling factors are synthesised in lacrimal gland: epidermal growth factor (EGF) primarily by

ductal cells and its homologue transforming growth factor- $\alpha$  (TGF- $\alpha$ ) primarily by acinar cells.<sup>14,15</sup> Both these growth factors are also secreted into human tear fluid.<sup>8,16</sup> Furthermore, EGF mRNA expression in the lacrimal gland appears to be enhanced after corneal wounding.<sup>17</sup> Several other growth factors potentially involved in corneal wound healing—for example, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), platelet derived growth factor (PDGF), and vasoendothelial growth factor (VEGF) are expressed in the lacrimal gland or are present in tears.<sup>6,18-20</sup> TNF- $\alpha$  mRNA is also present in the lacrimal gland and its expression is increased 2- to 4-fold 8 hours after corneal wounding in a manner similar to EGF mRNA expression, suggesting activation of a neural pathway from wounded corneal surface to lacrimal gland.<sup>21</sup>

In the present study we showed that TNF- $\alpha$  is present in human tear fluid at a mean concentration of 358 ng/l. As all patients showed measurable TNF- $\alpha$  concentrations preoperatively, we consider TNF- $\alpha$  to be a normal component of human tear fluid. The mean TNF- $\alpha$  concentration increased to 417 ng/l on day 2, but the increase was not statistically significant. Reflex tearing seems to be essential for wound healing, and a PRK wound clearly induces considerable hypersecretion of tears during the first postoperative days. Although the actual concentration of TNF- $\alpha$  in tears remained the same after PRK, its release (the absolute amount released over a fixed period) was significantly higher during the first postoperative days after PRK. This might contribute to an enhanced bioavailability of TNF- $\alpha$  to its receptor on the ocular surface immediately after PRK. On the other hand, the tear outflow and, hence, clearance of TNF- $\alpha$ , is also likely to increase. In an earlier study using the same method TNF- $\alpha$  was detected in 21 serum samples (52%) from 40 healthy

Table 1 Pre- and postoperative flows in the collection capillary, TNF- $\alpha$  concentrations, and TNF- $\alpha$  releases

No	Sex/age	Abl depth	Tear fluid flow ( $\mu\text{l}/\text{min}$ )			TNF concentration (ng/l)			TNF release (pg/min)		
			Day 0	Day 2	Day 7	Day 0	Day 2	Day 7	Day 0	Day 2	Day 7
1	M/23	63	93.2	138.9	ND	285	350	ND	26.6	48.6	ND
2	F/37	70	23.6	51.7	26.1	350	350	<5	8.3	18.1	0.1
3	M/21	43	62.5	78.1	15.5	600	450	735	37.5	35.2	11.4
4	M/27	70	21.4	219.3	24.2	680	<5	<5	14.6	1.1	0.1
5	F/49	63	1.5	3.0	1.8	130	630	315	0.2	1.9	0.6
6	F/32	51	19.0	67.0	13.2	400	265	430	7.6	17.8	5.7
7	F/39	63	9.9	50.0	11.2	195	600	235	1.9	30.0	2.6
8	F/36	63	61.5	67.1	ND	460	330	ND	28.3	22.2	ND
9	F/37	81	4.6	100.0	9.3	365	365	220	1.7	36.5	2.1
10	F/37	85	8.6	25.6	15.6	260	540	195	2.2	13.9	3.1
11	F/21	125	14.3	43.0	5.8	375	385	260	5.4	16.6	1.5
12	M/21	50	17.9	111.1	ND	345	<5	ND	6.2	0.6	ND
13	M/21	63	9.3	166.7	ND	180	450	ND	1.7	75.0	ND
14	M/46	16	9.5	108.7	7.5	450	750	685	4.3	81.5	5.1
15	M/36	88	29.4	111.1	ND	680	620	ND	20.0	68.9	ND
16	F/21	73	8.1	37.7	41.7	110	385	610	0.9	14.5	25.4
17	F/31	88	7.1	24.5	ND	350	715	ND	2.5	17.5	ND
18	M/21	33	2.7	49.0	3.2	220	315	145	0.6	15.4	0.5
N*	18	18	18	18	12	18	18	12	18	18	12
Mean	31	66	22.5	80.7	14.6	358	417	320	9.5	28.6	4.8
Median	32	63	12.1	67.1	12.2	350	385	248	4.9	18.0	2.4
SD	9	24	24.9	55.1	11.4	170	207	246	11.2	24.8	7.2
Range	21-49	16-125	1.5-93.2	3.0-219	1.8-41.7	110-680	<5-750	<5-735	0.2-37.5	0.6-81.5	0.1-25.4
p Value†				0.0002	NS		NS	NS		0.003	NS

\*N is the number of patients. †The p values refer to comparisons of postoperative and preoperative samples of the same patients (Wilcoxon signed rank two group paired test).

individuals, and the median concentration was 9 ng/l (range <7–40 ng/l).<sup>13</sup> The preoperative tear fluid TNF- $\alpha$  concentration was thus about 40-fold compared with that of serum, indicating that tear fluid TNF- $\alpha$  most probably is derived from other sources than serum—for example, lacrimal gland, corneal epithelial and stromal cells, conjunctival cells, or inflammatory cells. According to Thompson *et al*, corneal wounding regulates the TNF- $\alpha$  mRNA production of the lacrimal gland.<sup>6</sup> Transcription of the message was seen in acinar cells as well as in local macrophages. Whether the lacrimal gland also produces TNF- $\alpha$  protein is not known.

In intact cornea the epithelial cells are anchored to each other by tight junctions, so it is unlikely that preoperative TNF- $\alpha$  would derive from deep epithelium or stroma. During the first 2 postoperative days the breakdown of this barrier would, however, permit stromal fibroblast or epithelial cell contribution to its production. At least dermal wound fibroblasts are capable of synthesising and secreting TNF- $\alpha$ , in contrast with normal skin fibroblasts.<sup>4</sup> TNF- $\alpha$  mRNA has been detected in normal rat cornea and, furthermore, its expression immediately after wounding seemed to localise in the epithelium, in contrast with its later appearance in the stroma 24 hours after surgery.<sup>3</sup> Also, normal epibulbar conjunctival epithelium and extracellular substantia propria as well as inflammatory cells in conjunctival stroma have shown clear staining with anti-TNF- $\alpha$  antibody.<sup>22</sup> To avoid conjunctival TNF- $\alpha$  contamination of the sample the tear fluid collection capillaries were fire polished to diminish conjunctival irritation, and the tear fluid samples were centrifuged immediately after tear collection to get rid of the cellular debris. The contribution of conjunctival TNF- $\alpha$  to the total amount cannot, however, be totally excluded. In healthy cornea there are low numbers of immunocompetent cells capable of secreting cytokines.<sup>23</sup> In wounded cornea the necrotic keratocytes and stromal and subepithelial nerves have been shown to be removed by invading macrophage-like cells and other inflammatory cells during the first 3 days.<sup>24</sup> This renders inflammatory cells a possible source of postoperative tear fluid TNF- $\alpha$ . TNF- $\alpha$  is capable of binding to extracellular matrix proteins, such as collagen or fibronectin<sup>25</sup> which, in theory, could serve as a reservoir for TNF- $\alpha$  in the tissue, independent of the origin of TNF- $\alpha$ .

What is the possible function of tear fluid TNF- $\alpha$ ? TNF- $\alpha$ , among other proinflammatory cytokines, has been shown to stimulate fibronectin induced migration of corneal epithelial cells<sup>26</sup> and fibroblast proliferation.<sup>3</sup> It is capable of inhibiting extracellular matrix protein production<sup>27</sup> and stimulating collagenase and prostaglandin E<sub>2</sub> secretion.<sup>28</sup> According to Castagnoli *et al*, hypertrophic scarring of skin may be partially caused by a low amount of TNF- $\alpha$  in tissue,<sup>7</sup> which leads to the question of the role of TNF- $\alpha$  in the haze formation after PRK. TNF- $\alpha$  also regulates cell–matrix interaction by increasing integrin expression

and adhesion of fibroblasts to collagen.<sup>29</sup> Furthermore, it is chemotactic for neutrophils and macrophages,<sup>30</sup> and induces production of other cytokines and growth factors in corneal cell cultures.<sup>31,32</sup> In any case, TNF- $\alpha$  binding sites have been located on both epithelial cells and corneal fibroblasts.<sup>32</sup> Theoretically, TNF- $\alpha$  could modulate both epithelial and stromal wound healing. The next step of assessing the effects of TNF- $\alpha$  on corneal healing would be exogenous application after wounding *in vivo*. Contradictory results have been achieved in studies on skin wound healing, as local administration of TNF- $\alpha$  improved wound disruption strength in one study,<sup>33</sup> and decreased it in the other.<sup>34</sup>

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