Expression of cellular fibronectin and tenascin in the rabbit cornea after excimer laser photorefractive keratectomy: a 12 month study

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Abstract

An indirect immunohistochemical technique was used to monitor the expression of cellular fibronectin (cFN) and tenascin (TN) in the rabbit cornea after photorefractive keratectomy (PRK) in a 1 year follow up study. Rabbits received a 5·0 D myopic PRK, and were killed 3 days, 1, 3, 6, or 12 months after the operation. In most corneas, secondary epithelial defects appeared after the primary healing (mean 6·3 (SD 1·2) days). Corneal haze appeared a few weeks after PRK and was observed throughout the follow up. Three days after wounding an immunoreaction for cFN was observed as a bright narrow subepithelial line, but no immunoreaction for TN could be seen in the anterior third of the corneal stroma. However, at 1–6 months a similar location of immunoreactions for both cFN and TN was observed. Both were found in the anterior stroma at depths of 30–50 μm. At 12 months, only a trace of cFN immunoreaction but no TN immunoreaction could be discerned. Our results suggest that subepithelial scar tissue contains both cFN and TN up to 12 months.

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Fibronectin (FN) is an adhesive glycoprotein which is also important in cell migration, differentiation, and growth.[1] It is a dimer composed of two 250 kD subunits. Soluble FN is found in plasma (plasma fibronectin, pFN), and an insoluble form is produced in tissues (cellular fibronectin, cFN).[1] FN has specific binding sites for fibrin, heparin, collagens, and various cell surface integrin α/β heterodimer complexes (for example, Arg-Gly-Asp (RGD) sequences).[1 2] More than 90% of the structure of the FN molecule consists of types I, II, and III amino acid sequence repeats. Type III contains the RGD sequences interacting with transmembrane integrins.[1]

Tenascin (TN) is an extracellular glycoprotein consisting of six identical subunits.[3] It was originally found in developing tissues.[4] TN is also expressed in tumours,[5] healing wounds,[6–9] and some inflammatory processes.[10–12] Interestingly, the TN molecule contains epidermal growth factor-like repeats[13] and fibronectin type III repeats.[13 14] However, the biological activities of TN are still largely unclear, but a modulating role in cell adhesion and migration has been suggested.[3]

After the development work of Trokel *et al*,[15] the 193 nm ArF excimer laser has become an attractive and widespread method of photorefractive (PRK) and phototherapeutic (PTK) keratectomy. The feasibility of the excimer laser for remodelling of the corneal curvature or for removing subepithelial opacities is based on minimal damage to the corneal stroma surrounding the photoablation area. Thus, corneal transparency is usually well preserved.[15] However, corneal haze and regression of the achieved refraction (myopic regression) after PRK are due to the wound healing process. Understanding the biology of corneal wound healing[16 17] is of crucial importance because technical reliability and predictability of excimer laser technology is at a high level.

Immunohistochemical studies with long term follow up after PRK are rare. Distributions of cFN and TN up to several weeks after PRK have been demonstrated.[9 18] To investigate long term changes of healing PRK wounds, we used indirect immunohistochemistry to demonstrate the duration and location of cFN and TN expression in the rabbit cornea in this 12 month follow up study.

Materials and methods

ANIMALS

This study conformed to the ARVO Resolution on the Use of Animals in Research and was accepted by the test animal committee of the Helsinki Central University Hospital. Altogether 14 adult NPHI (National Public Health Institute, Finland) Chinchilla bastard rabbits (28 eyes) of both sexes weighing 3–5 kg were used for this study. The animals were placed under deep intravenous ketamine-xylazine anaesthesia (Ketalar 10 mg/kg body weight, Parke-Davis, Barcelona, Spain; Rompun 13 mg/kg body weight, Bayer, Leverkusen, Germany). Topical oxibuprocaine (Oftan-Obucain, Leiras, Tampere, Finland) was applied to the corneas, and a bilateral central 6 mm epithelial abrasion was performed after demarcation with a marker. A VisX Twenty/Twenty Excimer Laser (VisX Co, Sunnyvale, CA, USA) was used to produce a −5·0 D PRK (diameter 5·5 mm); the depth of the ablation was approximately 54 μm (estimated by computer). Some corneas (n=9) received a shallower ablation of −2·5 D with a depth of 27 μm. The repetition rate was 5 Hz giving either 20 second (−2·5 D) or 40 second (−5·0 D) durations. The pulse energy was 160 mJ/cm^2. A single dose of indomethacin suppository (Indocid $^1/_4$×50 mg, MSD, Whitehouse, USA) was given after the PRK to

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prevent postoperative pain. Gentamicin 0·5% (Garamycin, Schering-Plough, Kenilworth, USA) ophthalmic antibiotic ointment was applied immediately after the operation and twice daily during the next 3 days whereafter it was replaced by tobramycin eye drops (Tobrex, Alcon, Fort Worth, USA) applied twice daily until the epithelium had healed. Slit-lamp examination was performed daily beginning on the second postoperative day until the epithelial wound had closed. After primary healing the rabbits were examined weekly. The animals were killed 3 days (n=3), 1 month (n=3), 3 months (n=3), 6 months (n=3), or 12 months (n=2) after the PRK with an intravenous overdose of the ketamine-xylazine mixture. One cornea of an unoperated rabbit and areas from treated rabbits not exposed to excimer laser photoablation served as controls.

IMMUNOHISTOCHEMICAL PROCEDURE

For indirect immunohistochemistry the corneas were excised, but into two to four pieces and immersed in 96% ethanol for 2 hours at +4°C and then rinsed overnight in phosphate buffered saline (PBS), containing 25% sucrose. Sections of 7 μm were cut on a cryostat, washed in PBS and preincubated in 4% bovine serum albumin dissolved in PBS to prevent non-specific labelling. The specimens were incubated overnight at +4°C with the primary antibodies at appropriate dilutions. The following monoclonal antibodies (Mabs) were used: 52 DH1 to detect cFN-immunoreactivity[19] and 100 EB2 to detect TN immunoreactivity.[5] FITC conjugated goat anti-mouse IgG (Cappel, Organon Teknika, PA, USA) served as a secondary antibody. The samples were mounted in sodium veronal-glycerol buffer (1:1, pH 8·4) and viewed with a Leitz Diaplan 20 fluorescence microscope. A constant exposure time was used when the samples were photographed. In the control sections the primary or secondary antibodies were omitted, and no immunostaining was detected.

Results

CLINICAL OBSERVATIONS

Primary closure of the wounds occurred at a mean of 6·3 (SD 1·2) days. Six of the eyes did not heal before killing of the rabbits 3 days after the operation. The corneal haze was discernible throughout the study in all the eyes beginning from the second postoperative week and was maximal approximately 2–3 months after the operation. Secondary epithelial defects occurred in the PRK area in 17 out of 22 eyes, usually several times (two to six). Most secondary defects appeared at the beginning of the follow up and were very small. Towards 6 months they were very rare. The recurrences were distributed evenly among the two groups with the different ablation depths. Neither immunohistochemical result nor appearance of secondary defects showed observable difference between different ablation depths.

CELLULAR FIBRONECTIN

In the untreated cornea and in areas not exposed to the photoablation in PRK corneas, immunoreaction for cFN was observed in short scattered stromal bands, as well as in distinct layers on each side of the Descemet's membrane. A very weak but discernible subepithelial layer at the level of the basement membrane was observed in control areas of two specimens (Figs 1A, B).

A thin bright line of immunoreaction was observed underneath the migrating epithelium 3 days after PRK (Fig 1D). Neither the unepithelialised stroma in the middle of the wound nor the stroma beneath the newly healed epithelium showed any change in cFN immunoreactivity. Intense anterior stromal labelling (depth approximately 30–50 μm from the basement membrane) was observed under the wound area for 1–6 months (Figs 1, 2). The size of the rather sharply demarcated immunofluorescent area seemed to decrease with time. At 12 months the anterior stromal immunoreaction was noticeably diminished, with only a weak shallow zone of superficial stromal immunofluorescence (depth <5 μm) at a restricted area (Fig 2C). The presence of recurrent erosions did not seem to affect the anterior stromal immunofluorescence.

TENASCIN

In the control sections immunostaining for TN could be observed throughout the epithelium (Fig 1C). A faint immunoreactivity was seen in the stroma, but no labelling was detectable in the Descemet's membrane. Three days after PRK, the anterior stroma (75–100 μm) was completely negative, but immunofluorescence was slightly increased in the middle part (Fig 1E). At 1–6 months intense anterior stromal immunoreactivity similar to the cFN reaction was observed (Figs 1, 2). At 12 months the immunoreaction was similar to the control areas disregarding of the ablation depth. The occurrence of the secondary erosions did not seem to affect the result (Fig 2D).

Discussion

This study was designed to investigate the location of the immunofluorescence for both cFN and TN expressed as a response to PRK over a period of 12 months, which is known to be a time of disappearance of most of the corneal haze.

The biological activity of cFN under the migrating epithelium might offer a suitable adherent surface in the absence of hemidesmosomes, basement membrane, or anchoring fibrils during the first days after wounding.[9 18 20–24] At the same time, TN was completely absent from the anterior stroma, a finding that agrees with the degeneration of keratocytes subjacent to the wounded area as described earlier.[8 9 23 25 26] The presence of cFN and TN in the anterior stroma 1–6 months after PRK primarily underlines their roles in the stromal healing and remodelling processes. The immunoreactions for both cFN

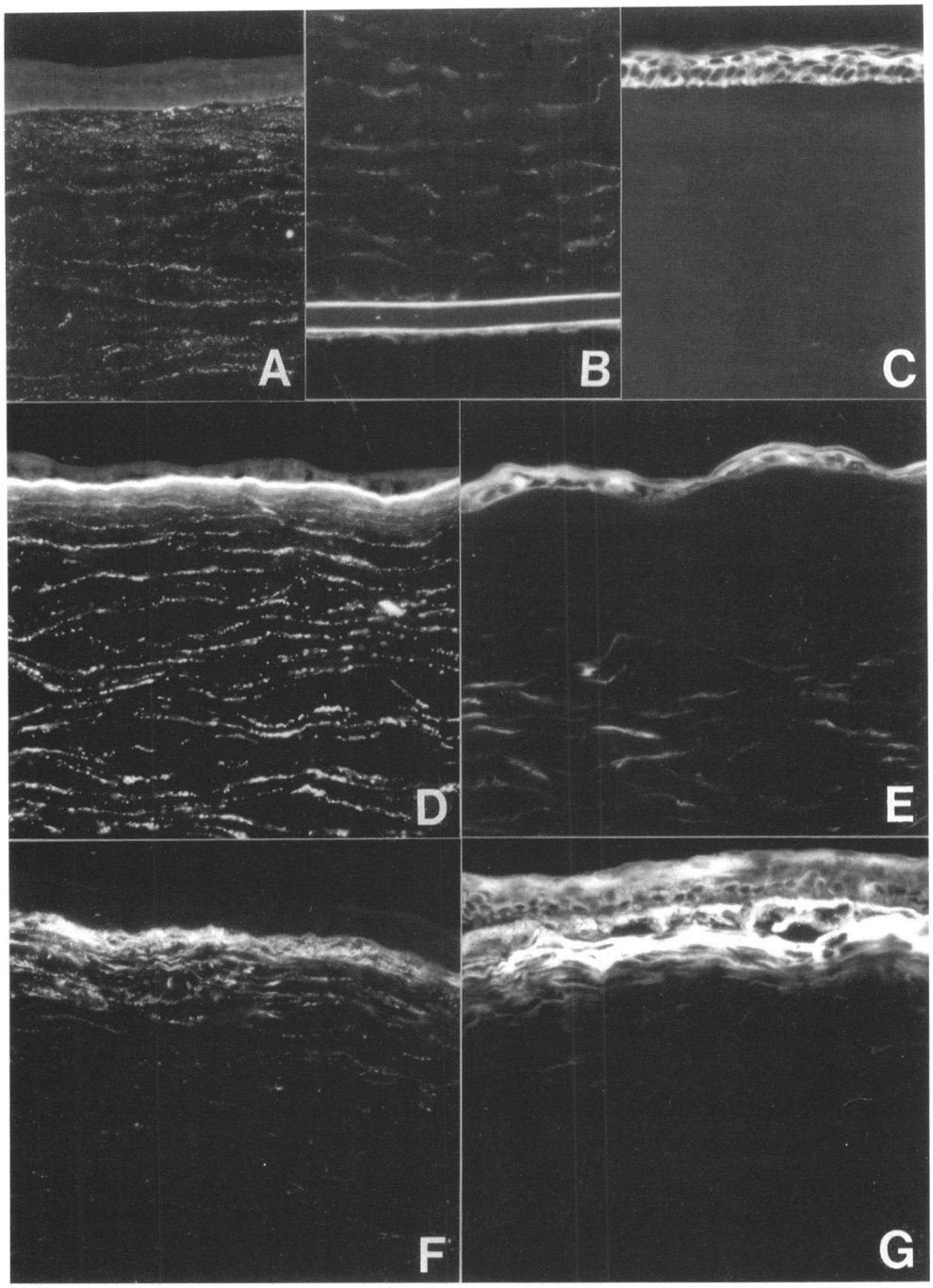

Figure 1 Normal distributions as indicated in text: cellular fibronectin (cFN) epithelial side (A) and endothelial side (B) and tenascin (TN) (C). Immunoreaction for cFN (D) and TN (E) 3 days after −2·5 D photorefractive keratectomy (PRK). A bright zone of immunoreaction for cFN was seen under the epithelium. Immunoreaction for TN appeared negative in the anterior stroma. However, specific immunoreactivity was observed in the mid stroma. One month after −2·5 D PRK, cFN (F) and TN (G) were present in the anterior stroma (depth 30–40 μm from basement membrane). Magnification ×250.

and TN were observed not only subepithelially, but also clearly in the stromal side (at about 30–50 μm deep).

Unlike in the data reported by SundarRaj *et al*,[18] cFN was detectable in the anterior stroma 6 and even 12 months after PRK. On the other hand, the specificities of the antibodies used were different. We used Mab 52 DH1,[19] which binds to the extradomain A, whereas SundarRaj *et al* used an antibody against the gelatin binding region of the FN molecule.[1]

The corneal scar forming process involves the presence of abnormal proteoglycans[27] and hyaluronic acid[28] both of which may be related to the disturbance of corneal transparency. Our results indicate that subepithelial scar tissue is also rich in cFN and TN. The extent of the immunoreactions agrees with the depth of the disorganisation of collagen lamellae reported earlier.[29] The simultaneous disappearance of the clinically observable corneal haze,[30–32] and the disappearance of immunoreactions for both cFN and TN also suggest their involvement in the corneal wound healing process.

Interesting speculation is possible on the involvement of TN in the healing process of the corneal stroma. The structure of the TN molecule contains epidermal growth factor-like sequences.[13] Other growth factors may also be involved in the modulation of corneal wound healing. Transforming growth factor

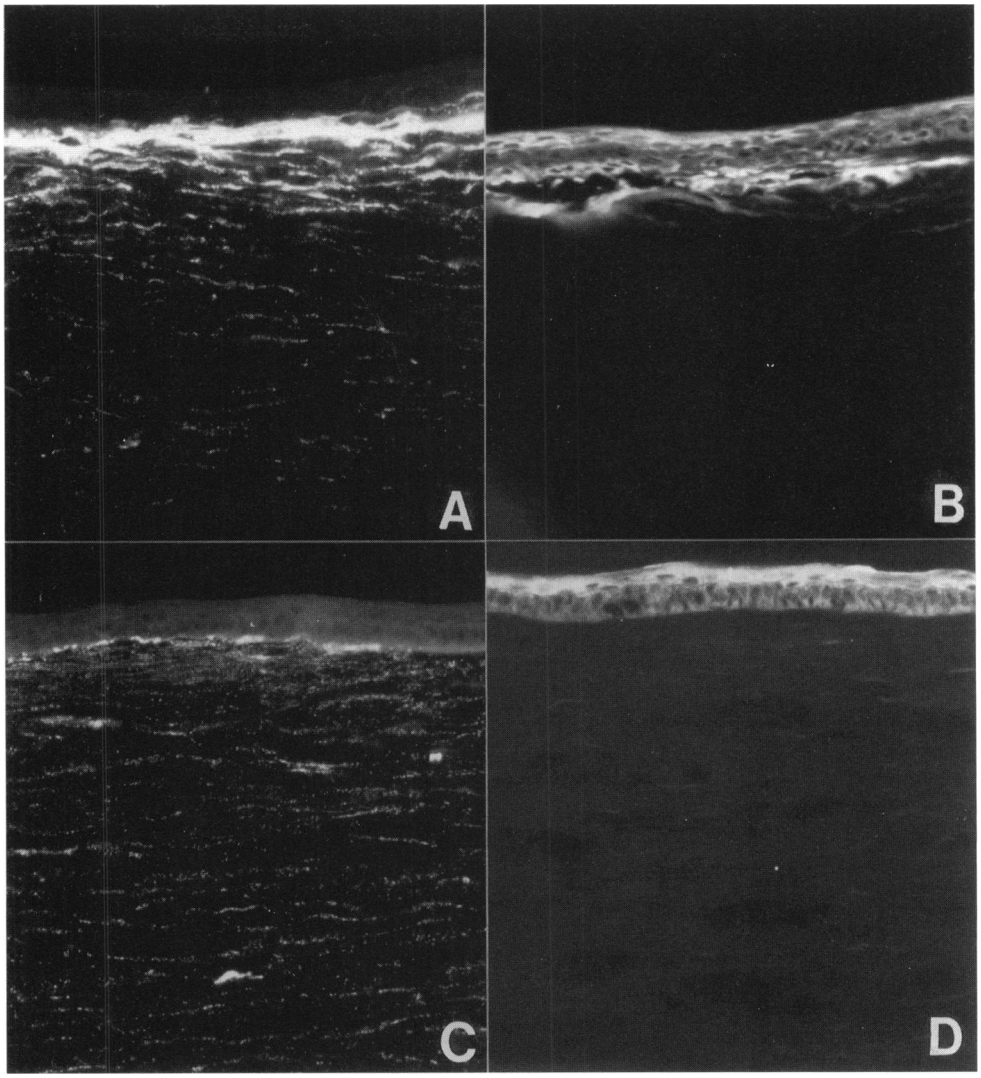

Figure 2 Expression of cellular fibronectin (cFN) (A) and tenascin (TN) (B) 6 months after −2·5 D photorefractive keratectomy (PRK). Towards 12 months only a faint immunoreaction for cFN (C) was observed, and expression of TN (D) was normalised. The latter cornea (C, D) had −5·0 D PRK with a recurrent erosion problem. A similar hardly discernible immunoreaction was observed in corneas with −2·5 D PRK and −5·0 D PRK without recurrent erosion problem. Magnification ×250.

beta (TGF-β) has been proved to induce TN synthesis.[33] On the other hand, neutralising antibodies against TGF-β have been shown to inhibit formation of scar tissue.[34] It remains to be clarified whereas neutralising antibodies have any effect on the intensity of haze. Recently, basic fibroblast growth factor (bFGF) has been shown to be an even more potent inducer of TN expression.[35] Corneal epithelial cells have previously been suggested to express their synthesis of mRNA coding for bFGF as a response to epithelial scrape wound.[36] These findings suggest that TN might have a role related to locally produced growth factors in corneal wound healing.

The correlation between the slow primary healing of the wounds and considerable amount of secondary defects and the expression of cFN and TN is unclear. Before this experiment we also tried chloramphenicol ointment to avoid potential inhibition of epithelial healing due to aminoglycoside epithelial toxicity[37] as well as aprotinin[38] (a serum proteinase inhibitor) eyedrops. This did not eliminate the occurrence of secondary epithelial defects. The tendency towards epithelial detachments was unexpected, because only one secondary epithelial defect has occurred in a series of 100 human PRKs and 50 PTKs performed by us. Other investigators have also reported that secondary defects are very rare in monkeys and humans.[18 29–32] However, data are often omitted or reported indirectly concerning the appearance of secondary defects in rabbits.[24 26–28 39 40] Gipson *et al*[22] have observed secondary epithelial defects in rabbit corneas after mechanical anterior keratectomy. We have observed, in another study with shorter follow up (unpublished), that younger rabbits also showed secondary epithelial defects but less frequently and for a shorter time. Consequently, we assume that a difference in healing of PRK wounds exists between humans and rabbits. Epithelial attachment complex (hemidesmosomes, basement membrane, and anchoring fibrils) have also been reported to show segmented patterns of morphology months after PRK,[18 23 29 41] which might be related to problems in epithelial adherence.[42] One explanation for the difference in healing rates might be the very low (4–6 blinks per hour[43] or 1–2 blinks per minute[44]) spontaneous blinking frequency of

normal rabbits compared with that of humans (15–20 blinks per minute[44]). There is also some evidence for the importance of tear fluid secretion rate in corneal wound healing.[45–47]

We have observed that cFN remains in the anterior stroma of the rabbit cornea for up to 12 months and TN for up to 6–12 months. The long lasting expression of these two extracellular matrix proteins probably contributes to the wound healing process and may also reflect changes contributing to the postoperative haze formation together with subepithelial hypercellularity,[23 24 26 29 41] epithelial hyperplasia,[26 29] abnormalities in collagens (for example, disorganisation of lamellae)[18 23 24 29 41] and proteoglycans,[27] hyaluronic acid,[28] basement membrane abnormalities[18 24 29 41] and tissue vacuolisation.[23 24 27 29]

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