Expression of Tenascin and Cellular Fibronectin in the Rabbit Cornea After Anterior Keratectomy

Immunohistochemical Study of Wound Healing Dynamics

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Anterior keratectomy (AKE) was done on rabbits, and the appearance of immunohistochemically demonstrable tenascin (TN) or cellular fibronectin (cFN) was studied at different times (5 min to 14 months) after the operation. The substance TN was first observed 12 hr after wounding in the posterior stroma; cFN appeared with the same localization 12 hr later. During postoperative week 1, both TN and cFN immunoreactions shifted to more anterior parts of the cornea, and 9 days after wounding, they were localized in the most anterior part of the stroma only. Thereafter the reactions gradually decreased in intensity but still were visible 3 months after AKE. No reaction for TN or cFN was present 14 months postoperatively.


Wounding of the cornea induces production of several extracellular matrix (ECM) proteins such as fibrin and fibronectin (FN).1,2 The latter is an adhesive glycoprotein present in soluble form in body fluids and, in insoluble form, in both interstitial connective tissue and many basement membranes.3,4 The term cellular FN (cFN) generally is used for the insoluble tissue or ECM form of the protein.5 This substance was identified beneath the migrating corneal epithelium,6 and it was suggested as being synthesized by the corneal keratocytes.7

Tenascin (TN), a novel ECM glycoprotein, also called myotendinous antigen, cytotactin, or brachionectin, was described first by Chiquet and Fambrrouch.8 It is a component of the secretory proteins released from fibroblasts and muscle cells in vitro.9 During embryogenesis, it is distributed selectively around budding epithelia in developing organs.9 In ocular tissue, TN is expressed in the corneal stroma after keratectomy10 and also is present in normal human limbus.11 However, there is little information concerning the role of TN in corneal wound healing in adult animals.

In this study, we compared the immunohistochemical dynamics of the appearance of TN and cFN at the site of corneal lesion after anterior keratectomy (AKE).

Materials and Methods

Animals

Sixteen adult New Zealand rabbits were used for this study in accordance with the ARVO Resolution on the Use of Animals in Research. During general anesthesia with a mixture of ketamine (Ketalar; Parke-Davis, Barcelona, Spain) and xylazine (Rompun; Bayer, Leverkusen, FRG), AKE 250–350-μm deep was done with a 6-mm trephine bilaterally. Topical anesthetics also were applied (Oftan-Obucain; Star, Tampere, Finland). The rabbits were killed with an overdose of ketamine and xylazine at various times (5 min to 14 months after AKE). Eyes from normal rabbits served as controls.

Immunohistochemical Procedure

The corneas were excised immediately after the death of the animals and immersed for 1–2 hr in 96%
ethanol at 4°C. Subsequently, the corneas were rehydrated in a descending series of ethanol and rinsed in phosphate-buffered saline, containing 25% sucrose. Cryostat sections (7 μm) were cut and placed on glass slides. After this the samples were processed for the indirect immunofluorescence method.

For demonstration of TN, the sections were incubated with the monoclonal mouse hybridoma antibody (Mab 100 EB2; Locus-Genex, Helsinki, Finland) against human TN for 48 hr at 4°C. The monoclonal antibody Mab 100 EB2 detects two of the TN polypeptides (250 kilodaltons [kD] and 180 kD) in western blot testing and does not bind to purified FN isolated from human plasma or cell cultures. Preabsorption of the antibody by TN, but not by cFN, completely abolishes its immunoreactivity.

For demonstration of cFN, the sections were incubated with the monoclonal mouse hybridoma antibody (Mab 52 DH1; Locus-Genex). After preincubation of the Mab 52 DH1 with FN, the immunohistochemical reaction was blocked. The specificity of this antibody was described recently. Preincubation with TN did not block the reaction. Fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G (Cappel, Malvern, PA) was used as a secondary antibody. As an additional control, tissue sections were incubated with the secondary antibody alone.

**Results**

In both wounded and control corneas, immunoreaction for TN could be seen throughout the epithelium (Fig. 1A). The reaction also was found in the newly grown epithelium in wounded areas. No reaction was seen in corneas incubated with the secondary antibody alone (Fig. 1B).

Changes in the pattern for TN and cFN immunoreactivity were restricted to the wound area itself, its edge, and the underlying stroma; they began 12 hr after wounding. At that time, a weak immunoreaction for TN could be seen for the first time in the posterior stroma (Fig. 2A). No reaction for cFN was observed.

One day after AKE, TN immunoreactivity was observed in the posterior and central stroma. Weak labeling could be seen for the first time at the wound edge region in the anterior part of the stroma (Fig. 2B). The anterior stroma under the wound area was negative (Fig. 2B). By that time, cFN also was observed; a reaction of moderate intensity was located in the posterior and central stroma. However, at the wound edge, the reaction also reached the anterior stroma (Fig. 3A).

Two days after AKE, the intensity of the TN immunoreaction increased in the posterior and central stroma, but the anterior stroma in the middle of the wound still was negative. The intensity of the cFN labeling also increased (Figs. 3B–C), and the anterior stroma in the middle of the wound was negative (Fig. 3C).

Three days after wounding, the TN immunoreaction had almost disappeared from the posterior stroma. The reaction seemed to move to the anterior stroma. Only the most anterior part of the stroma was negative (Fig. 2C). The cFN labeling showed a similar tendency toward a more anterior location. A subepithelial reaction now was observed under the newly grown epithelium (Fig. 3D). This reaction seemed to be absent under the leading edge of the epithelium (Fig. 3E).

On the 6th postoperative day, the entire wound was covered by new epithelium. Only a weak reaction for TN was seen in the central stroma. The wound edge, however, showed intense subepithelial labeling for TN under the epithelium (Fig. 2D). Similarly the intensity of the cFN immunoreaction began to decrease in the posterior and central stroma but was pronounced in the keratocyte-like structures under the newly healed epithelium in the wound edge (Fig. 3F). Although the most anterior part of the stroma was

![Fig. 1. (A) TN immunoreaction in the corneal epithelium of a control rabbit. (B) No immunoreaction can be observed in sections incubated with the secondary antibody alone (×520).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933382/ on 05/31/2017)
Fig. 2. (A) TN in the posterior stroma in the middle of the wound 12 hr after AKE. The anterior and central stroma are negative (asterisk). Open arrows indicate the anterior surface of the stroma (original magnification ×230). (B) TN in the wound edge region 1 day after wounding. The negative area under the newly grown epithelium is marked with open arrows (original magnification ×320). (C) TN 3 days after AKE in the middle of the wound. The most anterior part of stroma is still negative (open arrows). No reaction can be seen in the posterior stroma (original magnification ×230). (D) TN 6 days after wounding in the wound edge area and (E) 9 days after wounding in the same area (original magnifications ×320).
negative, an intense subepithelial reaction was observed at the level of the epithelial basement membrane.

Nine days after AKE, TN was not observed in the posterior or central stroma. The wound edge reaction decreased in intensity (Fig. 2E). Beneath the new epithelium at the wound area, a moderate reaction for posterior or central stroma. The wound edge reaction corresponded to that of TN (Fig. 3G), although the reaction for cFN was more intense with a clearer subepithelial distribution. Moreover, it was more restricted to cellular elements of the stroma.

During the following 2–10 weeks, the location of both TN and cFN were unchanged, but the intensity of the reaction gradually decreased. Three months after AKE, immunoreaction to both cFN and TN was still visible in the anterior stroma. The reaction was absent 14 months postoperatively.

Discussion

The FN immunoreactivity detected in tissues using antibodies recognizing both the soluble plasma FN and cFN may be caused by a deposited plasma form of the protein. The monoclonal cFN antibody used in this study recognizes the extradomain (ED) sequence of the FN molecule. The ED sequence does not occur in plasma FN. The ED-containing FN seems to be a transient component of embryonic tissues, but it is reexpressed in reactive connective tissues in adults. After showing TN in corneal wounds, we wished to compare the localization of TN with cFN and to monitor it during corneal wound healing. This study confirms our previous findings that cFN and TN are involved in corneal wound healing and demonstrates that wounding of the cornea leads to synthesis of these two ECM proteins, also known to occur during development. There was a difference in the histochemical location of cFN and TN. The former was more restricted to keratocyte-like cellular structures, whereas TN seemed to be located diffusely, both in the layers of stromal collagen lamellae and the epithelium.

Immunoreactivity to both TN and cFN first appeared in the posterior and central stroma at the site of the lesion. At the wound area, the superficial corneal stroma was negative until the regenerated epithelium covered the wound (approximately 4 days). The absence of both TN and cFN in the newly keratectomized superficial stroma might result from the degeneration of keratocytes. Corneal abrasion was found to induce degenerative changes in keratocytes of the superficial stroma. This author also showed that, at the time of epithelial wound closure, keratocyte numbers reached the level of normal corneas. The retardation of the appearance of cFN and TN in corneas after AKE might be a result of their dependence on the presence of immigrating or otherwise activated keratocytes. Unlike cFN, TN was present in the corneal epithelium. This reaction does not seem to be a specific response to the wounding because it was observed throughout the epithelium and in the control corneas. The normal presence of TN in epithelium suggests its involvement in the normal turnover of corneal epithelial cells. A different spatial and temporal localization of cFN and TN also was reported recently in the healing of cutaneous wounds in rats, in rabbit cornea, and in human limbus.

However, the presence of both cFN and TN as late as 3 months after the primary wounding suggests that these ECM glycoproteins might have functions other than supporting epithelial cell movement and adhesion. Activation of the proteolytic machinery in the subepithelial granulation tissue during late stages of wound healing, suggests initiation of degradation of these ECM proteins and termination of the healing process. These phenomena apparently correlate with maturation and rearrangement of the healing stroma. Certain growth factors, proteolytic systems, and ECM proteins seem to form a complex, interregulated system, activated during wound healing. The procedure AKE, for example, was shown to induce a brief elevation of plasmin activity and a decrease in plasminogen activator activity in the tear fluid. Epidermal growth factor (EGF) is a natural component of the tear fluid. Reflex tearing, eg, caused by wounding of the ocular surface, leads to decreased concentrations of EGF in the tear fluid and to an increase in the absolute amount of EGF released into the tear fluid. Other growth factors such as transforming growth factor-β (TGF-β) can be activated by plasmin, also known to activate latent collagenase and stromelysin. In turn, both FN and TN are important for the adhesion and spreading of epithelial cells over wound areas. Synthesis of FN and collagens and their accumulation around cells is increased by TGF-β. Moreover, FN was shown to bind TGF-β, which, in turn, was reported to increase the density of receptors for adhesive proteins (integrins) on cell surface.

This system is complex and requires further investigation, especially because current knowledge has produced new therapeutic approaches to improving wound healing. The use of aprotinin for continuously elevated plasmin activity detected in tears of patients.
with slowly healing corneal ulcers, for example,\textsuperscript{29,30} was reported to be beneficial. Several investigations done with topical EGF\textsuperscript{31,32} suggest that this growth factor also might improve corneal healing. Similarly, FN preparations have been used, with variable results.\textsuperscript{33-35} All these approaches probably interact with a single system. Clarification of its components and their interrelation therefore would result in immediate benefits for patients.

**Key words:** tenasin, fibronectin, anterior keratotomy, corneal wounds, wound healing

**References**