Effects of transforming growth factor β on corneal epithelial and stromal cell function in a rat wound healing model after excimer laser keratectomy

Abstract ● Background: Transforming growth factor β (TGF-β) regulates extracellular matrix deposition, cell proliferation, and migration, and is expressed in cornea. TGF-β is thought to be involved in the corneal wound healing process.

● Methods: The central corneal area (3 mm in diameter) of Lewis rats was ablated using PTK mode excimer laser and the wound healing process was observed at 12 and 24 h and 2, 5, 10, and 30 days after treatment. The expression of TGF-β1, -β2 and -β3, TGF-β type I and type II receptors, α3, α5, β4 integrin subunits, laminin and fibronectin was studied immunohistochemically. Antibody neutralizing TGF-β1, -β2 and -β3 was administered intraperitoneally, 50 μg daily, for 5 days after the laser treatment to investigate the effects of TGF-β function blockade.

● Results: At the leading edge of the regenerating epithelium, no TGF-β type I and type II receptors and β4 integrin subunits were expressed after 24 h. Regenerating epithelium covered the ablated area after 2 days. An abnormal fibrotic layer was formed in the subepithelial area. This layer contained round-shaped cells in the stroma in the early stage (2–5 days after laser ablation) and spindle-shaped fibroblast-like keratocytes after 10 days. Laminin and fibronectin expression increased in the fibrotic layer. The increased stromal cells expressed TGF-β isoforms and TGF-β receptors. Neutralizing TGF-β inhibited the stromal cell increase in the laser ablated area after 5 days.

● Conclusion: TGF-β may be involved in epithelial cell migration and stromal cell reaction during the corneal wound healing process after excimer laser ablation in rat models.

Introduction

Excimer laser keratectomy is a useful surgical treatment for myopia. However, during the process of corneal wound healing, a stromal scar is formed under the regenerating corneal epithelium and causes corneal haze [1–3, 6, 9, 10, 25, 33, 38]. Clinically, corneal haze sometimes causes visual disturbances [20, 33]. According to previous reports, subepithelial stromal scar is composed of abnormal fibrous tissue and appears just under the ablated zone [2, 3, 33, 38]. After photo-ablation, quiescent keratocytes become activated and proliferate, and extracellular matrix, including collagen, fibronectin, laminin and tenasin, is produced [2, 3, 33, 38]. Many cytokines and growth factors are involved in corneal wound healing, namely epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), fibroblast growth factor-1 (FGF-1=acidic FGF), FGF-2 (basic FGF), vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), and TGF-β [7, 8, 15, 19, 32, 34, 39]. TGF-β is a multifunctional protein, regulating cell migration, proliferation, differentiation, apoptosis of various cells including corneal epithelial cells, stromal cells and endothelial cells [23]. TGF-β also regulates extracellular matrix deposition, cell proliferation, and migration, and is expressed in cornea.
matrix deposition and the expression of integrin family adhesion molecules [23]. In addition, TGF-β has been reported to regulate the cutaneous wound healing processes [5, 21, 23, 39]. TGF-β is detected in tear and corneal tissue, and TGF-β receptors are expressed in component cells of cornea [16, 24, 26]. These observations suggest that TGF-β is also involved in corneal wound healing after excimer laser ablation. To clarify the involvement of TGF-β, we observed the expression of TGF-β family members and their specific receptors and correlated them with wound healing in rat cornea models. To evaluate the functional significance of TGF-β in corneal wound healing, the effects of blocking the TGF-β functions were also examined.

**Materials and methods**

**Animals**

Thirty Lewis rats (10 weeks of age, weighing 250–310 g) were used in this study and treated according to “Principles of laboratory animal care” (NIH publication no. 86-23, revised 1985). Rats were anesthetized with ketamine hydrochloride (4.7 mg/100 g body weight; Sankyo, Tokyo, Japan) and 2% xylazine hydrochloride (0.46 mg/100 g body weight; Bayer, Leverkusen, Germany), and topical anesthetic drug 0.4% oxypropine hydrochloride (Santen, Osaka, Japan) was used just before the laser exposure in the right eye. The left eyes were untreated during examination to serve as normal controls.

**Laser treatment**

The Compak 200 excimer laser (Laser Sight Technology, Orlando, Fla., USA) was used as a source of 193-nm argon fluoride excimer laser light. Using the laser in PTK mode, an area of the central cornea of each right eye was ablated: diameter 3 mm, depth 90 μm, 0.8 mJ/pulse, 100 Hz.

**Immunohistochemical procedures**

Groups of three to five rats were killed by intraperitoneal injection of an overdose of ketamine hydrochloride just after the excimer laser ablation and at 12 and 24 h and 2.5, 10, and 30 days after ablation. The eyes were enucleated and immediately frozen in Tissue-Tek OCT compound (Miles Laboratories, Naperville, Ill.). Tissue sections 6 μm thick were mounted on 3-aminopropyl triethoxysilane-coated slides. The frozen sections were fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or cold acetone for 10 min and then rinsed in phosphate-buffered saline (PBS) (pH 7.4). The sections were stained with Carazzi’s hematoxylin (Wako, Osaka, Japan) for histological observation, and thereafter the expressions of TGF-β type I and type II receptors, TGF-β isoforms (TGF-β1, β2, β3), integrin subunits (α3, α5, β4 integrin subunits), and components of extracellular matrix (laminin and fibronectin) were observed immunohistochemically. The α3 integrin subunit is a component of α3β1 integrin, a receptor for laminin. The α5 subunit is a component of α5β1, a receptor for fibronectin. The β4 integrin subunit forms a complex with α6 integrin subunit, a component of hemidesmosome and a receptor for laminin [40]. The immunohistochemical observation was performed as follows. The sections were treated with 3% hydrogen peroxide in PBS for 15 min to block the endogenous peroxidase activity and then rinsed in PBS, followed by the incubation with normal goat serum for 20 min at room temperature (RT) to avoid non-specific binding of the antibodies. Sections were then incubated with the primary antibodies overnight at 4°C. Immunoreactivity was detected by means of the streptavidin-biotin-peroxidase method using Histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to the manufacturer’s protocol. Sections were incubated with biotinylated anti-rabbit or mouse goat serum for 15 min at RT and then rinsed in PBS. Subsequently, sections were incubated with streptavidin-biotin-peroxidase (SABC) complex for 10 min at RT. The localization of SABC complex was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB). The sections were counterstained with hematoxylin. The experiments were repeated three times.

A negative control, samples were treated with PBS or non-immunized IgG in place of the primary antibody.

**Antibodies**

Antisera against TGF-β type I and type II receptors and TGF-β1, β2, β3 were raised against peptides in rabbits.

As for the receptors, the expression of TGF-β type I and type II receptors was observed; both types are necessary for TGF-β signal transduction [41]. As specific antibody detecting TGF-β type I receptor or type II receptor rabbit polyclonal antiserum was made against the synthetic peptide, and has been confirmed not to cross-react with the other TGF-β superfamily receptors [4, 26, 37]. TGF-β isoforms are produced and secreted from various cells as the latent TGF-β complex, which is composed of mature TGF-β and latency-associated peptide (LAP) [23]. To detect the expression of TGF-β isoforms avoiding cross-reactivity the antibodies against the COOH-terminal part of the LAPs were used, because the LAPs show very low similarities in amino acid sequence level among three TGF-β isoforms, while mature TGF-β shows high sequence similarities (70–80%) [23, 24, 28]. The antisera raised against TGF-β type I and type II receptors, TGF-β1, β2, α3, α5, β4 were affinity purified using immobilized peptides for histochemical use. Affinity-purified antibodies were diluted to the final concentration of about 3 μg/ml with PBS (pH 7.4) containing 1% bovine serum albumin and used as the primary antibodies.

Antibodies against α3, α5, β4 integrin subunits were purchased from Chemicon International (Temecula, Calif., USA). The α3 integrin antibody was rabbit anti-human integrin α3 polyclonal antiserum (AB1920), the α5 integrin antibody was rabbit anti-human integrin α5 subunit polyclonal antibody (AB1928), and the β4 integrin antibody was mouse anti-human integrin β4 monoclonal antibody (MAB1964). Anti-laminin and anti-fibronectin antibodies were purchased from Cosmo Bio (Tokyo, Japan): the anti-laminin antibody was rabbit anti-mouse laminin polyclonal antiserum (LBN1013), while the anti-fibronectin antibody was rabbit anti-bovine fibronectin polyclonal antiserum (LB1027). Antibodies were diluted to 1:1000 with PBS (pH 7.4) containing 1% bovine serum albumin and used as the primary antibodies.

**Treatment of neutralizing antibodies to TGF-β during the wound healing process**

To investigate the functions of TGF-β, neutralizing antibody to TGF-β, Pan-specific TGF-β Neutralizing Antibody (R&D Systems, Minneapolis, Minn., USA) was administered intraperitoneally. The antibody was produced in rabbits immunized with a mixture of purified, recombinant human TGF-β1, porcine TGF-β1.2, porcine TGF-β2, and recombinant amphibian TGF-β3. Total IgG was purified by protein A affinity chromatography. This antibody to TGF-β blocks the functions of TGF-β1, β2, and β3. The antibody was administered at a daily dose of 50 μg for 5 days after the laser treatment. As the control, the physiological saline was administered intraperitoneally. The corneas were observed histologically just after the cessation of antibody administration.