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Transglutaminase Activity in the Eye: Cross-linking in Epithelia and Connective Tissue Structures

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PURPOSE. To assess the distribution of transglutaminase (TGase) activity in ocular tissues and the target structures for cross-linking.

METHODS. Cryosections from human and cynomolgus monkey eyes were incubated with the biotinylated amine donor substrate cadaverine (biotC), which was subsequently visualized with streptavidin-peroxidase. Confocal laser scanning was used to colocalize biotC and fibrillin, a major component of elastic microfibrils and the zonular fibers in particular. Cryosections and isolated bovine zonules were treated with purified TGase 2 and biotC. The distribution of different TGases (1, 2, 3, and factor XIII) was confirmed immunohistochemically.

RESULTS. Virtually all ocular tissues showed TGase activity with a remarkable preponderance for the ciliary body, zonular fibers, and blood vessel walls. Confocal laser scanning revealed fibrillin-containing microfibrils as a major target for TGase activity, in particular the ciliary zonules. Conical epithelium and basement membrane showed a TGase cross-linking pattern similar to skin. Treatment of cryosections and isolated bovine zonular fibers with purified TGase 2 led to additional incorporation of biotC into extracellular matrix, particularly zonular fibers. The immunohistochemically predominant TGase 2 was associated with epithelia and particularly with connective tissue fibers. TGase 1 was restricted to the corneal epithelium, whereas factor XIII was found to be associated only with blood vessels. TGase 3 was absent.

CONCLUSIONS. TGase 2 appears to be an important cross-linker and thus stabilizer of ocular connective tissue. In particular, the zonular fibers are a major target for TGase 2. This is of relevance in hereditary microfibrillopathies such as Marfan syndrome, which exhibits distinct ocular manifestations such as elongated bulbus, retinal detachment, and subluxation of the lens. Purified or recombinant TGase might be of therapeutic use in the future. (Invest Ophthalmol Vis Sci. 1999;40:2780–2787)

Transglutaminases (TGases; EC 2.3.2.13) form a family of enzymes that stabilizes protein assemblies by γ-glutamyl-ε-lysine cross-links.1–2 Seven different genes are currently known in higher vertebrates.3,4 Obviously, the individual TGases have different substrate specificities—that is, factor XIIIa stabilizes the fibrin clot in hemostasis, whereas TGase 1 and 3 cross-link different intracellular proteins of the cornified envelope in differentiating epidermis.5 Accordingly, congenital deficiency of factor XIII causes reduced blood clot stability,6 whereas mutations in the TGase 1 gene underlie one form of autosomal recessive lamellar ichthyosis.6,7 Less is understood concerning the physiological function of tissue-type TGase (TGase 2), which is expressed widely in vertebrates.5,8,9 Besides a role in guanosine triphosphate (GTP)–binding in receptor signaling,10 and apoptosis,11 cross-linking of extracellular matrix proteins has been proposed.5,12 In fact, γ-glutamyl-ε-lysine cross-links have recently been demonstrated in osteonectin,8,9 fibronectin,13 and heterotypic collagen V/XI fibrils.14 Recently, anchoring fibrils of the basement membrane zones of skin and the cornea and their major component, collagen VII, were identified as substrate for TGase 2.15 Because this points to an important role of TGase 2 in the stabilization of tissue and maintenance of epithelial-mesenchymal cohesion, we studied the distribution of TGase activity in the human and cynomolgus monkey eye.

MATERIALS AND METHODS

TGase Substrates and Inhibitors

A 10-mM stock solution of biotinyl-5-(N-biotinyloamino-hexanoyl-pentylamine) (biotin-X-cadaverine, or biotC; Molecular Probes Europe, Leiden, The Netherlands) was freshly prepared by dissolving 7 mg in 300 µl 0.1 M HCl and subsequent addition of 1700 µl distilled water. A 100-mM stock solution of putrescine (diaminobutane; Fluka, Buchs, Switzerland) was

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made in water. A 200-mM stock solution of EDTA was made in water and the pH adjusted to 7.5 with sodium hydroxide.

**Visualization of Endogenous TGase Activity in Ocular Tissues**

The eyes of a female cynomolgus monkey (Macaca fascicularis) were snap frozen in toto in melting isopentane. Cryosections were examined of anterior and posterior segment tissue from five normal human eyes (age range, 46–82 years) obtained at autopsy and snap frozen in liquid nitrogen-isopentane 2.5 to 8 hours after death. The eyes had no history or morphologic evidence of ocular disease. Ten- to 12-μm cryostat sections of whole eyes (cynomolgus monkey) or 7-μm cryosections of pretrimmed tissue blocks (human eyes) were incubated with 1% bovine serum albumin in 0.1 M Tris/HCl (pH 8.2) for 30 minutes at room temperature to block nonspecific binding. TGase activity was detected by subsequent incubation in the same buffer containing 100 μM biotC and 10 mM CaCl₂. Control sections were incubated with the same biotC-supplemented buffer containing in additional 2 mM putrescine or 10 mM EDTA, instead of CaCl₂. The enzyme reaction was allowed to proceed for 2 hours at room temperature, was stopped by washing the slides for 5 minutes in phosphate-buffered saline (PBS) containing 10 mM EDTA, and was followed by two further washings in plain PBS. Light microscopic visualization of transamidated ocular structures was performed with streptavidin-peroxidase (Jackson Immunoresearch, West Grove, PA), and the chromogenic reaction was performed with aminothiolcarbazol with a kit from Dako (Glostrup, Denmark).

In this case sections were lightly counterstained with hemalum and embedded in gelatin. For double immunofluorescence the polyclonal antibody PF2 against the peptic fragment 2 of fibrillin (1:100 in PBS) was used. BiotC was visualized using dichlorotrizaminofluorescein-conjugated streptavidin (1:100; Jackson Immunoresearch), the fibrillin antibody with a Texas red-coupled goat anti-rabbit IgG (1:100; Jackson Immunoresearch). Preparations were mounted in Mowiol (Hoechst, Frankfurt am Main, Germany) in Tris/HCl (pH 8.6) and examined using an inverted confocal laser scanning microscope (LSM 410; Carl Zeiss, Oberkochen, Germany) combined with two HeNe lasers (543 and 633 nm) and an argon laser (488 nm) for multicolor fluorescence.

**Immunogold Electron Microscopy of Isolated Bovine Zonular Fibrils**

Bovine eyes were obtained from animals 6 to 8 months old (PelFreez, Rogers, AR) and shipped overnight on ice. Zonules were dissected and washed in 0.01 M PBS plus protease inhibitors (0.002 M EDTA, 0.01 M N-ethylmaleimide, and 0.001 M phenylmethylsulfonyl fluoride). One milligram zonular fibrils was sedimented at 14,000 g for 30 minutes and redispersed in 500 μl 100 mM Tris buffer (pH 8.3) containing 10 mM CaCl₂, 200 μM biotC, and 12 μg purified guinea pig liver TGase 2 and incubated overnight at 25°C. Electron cryosections were treated under the same conditions. In control experiments TGase was omitted or the enzymatic activity inhibited by the addition of 30 mM EDTA. Zonular fibrils were washed three times in PBS, and aliquots were adsorbed for 2 minutes onto Formvar (Sigma, St. Louis, MO) carbon-coated copper grids. The grids were washed with PBS and were treated for 30 minutes with 2% (wt/vol) dried skim milk in PBS. The adsorbed material was then allowed to react for 2 hours at room temperature with rabbit anti-fibrillin antibody PF2 (1:50 in 0.2% wt/vol) dried skim milk and PBS. After the grids were washed five times for 2 minutes with PBS, they were incubated for 2 hours with a suspension of 6 nm colloidal gold particles coated with streptavidin (Amersham Buchler, Braunschweig, Germany) and 12 nm colloidal gold particles coated with IgG against rabbit immunoglobulins (Dianova, Hamburg, Germany). Finally, the grids were washed with PBS and negatively stained with 2% uranyl acetate. Micrographs were taken at 80 kV with an electron microscope (CM 10; Philips, Eindhoven, The Netherlands). Cryosections were processed as has been described.

**Immunohistochemical Studies**

The following antibodies and dilutions in PBS were used and applied for 16 hours at room temperature: goat anti-TGase 1 (1:200),7 rabbit anti-human TGase 3 (1:100; a generous gift of Peter Steinert, National Institutes of Health, Bethesda, MD), monoclonal mouse antibody CUB 7402 (NeoMarkers, Union City, CA) against TGase 2 (1:50) and against human factor XIII (1:50; Enzyme Research, South Lafayette, IN). The goat antiserum was visualized using biotinylated donkey anti-goat anti-serum 1:100 (Jackson Immunoresearch) for 1 hour and subsequent incubation with streptavidin-DTAF (1:100; Jackson Immunoresearch). Bound rabbit IgG was detected using swine anti-rabbit fluorescein-isothiocyanate (1:20; Dako), and the monoclonal mouse antibodies were visualized using goat anti-mouse Texas red (1:50). Blocking of nonspecific binding sites was achieved by pretreatment of the sections using 10% normal sera of the appropriate species in PBS.

**RESULTS**

**Incorporation of Biot C into Ocular Tissue by Endogenous TGase Activity**

**Human Eye.** In the cornea, biotC incorporation was detected in the epithelium, predominantly within the intercellular spaces but also in the cytoplasm of the epithelial cells and along their basement membranes. Keratocytes in the superficial layers of the corneal stroma were also labeled (Fig. 1A). TGase activity was visualized in the conjunctival epithelium, especially within intercellular spaces (Fig. 1B). Further cross-linking of biotC was detected in the walls of stromal vessels. In the sclera the presence of TGase activity was restricted to the endothelial lining of intra- and episcleral blood vessels (Fig. 1C) and single scattered cells, presumably fibroblasts, between the collagen lamellae. In the trabecular meshwork TGase activity was found in association with the trabecular endothelial cells, particularly in the posterior part of the meshwork (Fig. 2A) and in association with the endothelial cells lining Schlemm’s canal, collector channels, and intrascleral aqueous veins. In the iris biotC incorporation was prominent in the smooth muscle cells of the dilator and sphincter muscles, in the vascular endothelial cells of stromal vessels, and along delicate fibrillar structures in the stroma, which were particularly concentrated in the anterior borderer layer (Fig. 1D). Extensive cross-linking was apparent along most ciliary body structures, especially along the zonular fibers covering the surface of the ciliary epithelium, and also in the ciliary muscle cells and the outer limiting membrane (i.e., the basement membrane of the pigmented ciliary epithelium; Figs. 2A through 2D).
limiting membrane (the basement membrane of the nonpigmented epithelial layer) expressed some TGase activity in the pars plana region only. The nonpigmented epithelial layer was essentially negative in the pars plana area. The stromal connective tissue of the ciliary body was characterized by TGase activity in stromal cells (presumably fibrocytes), in vascular endothelial cells, and along extracellular fibrillar strands. In the lens, biotC incorporation was strong in the zonula lamella on the surfaces of the lens capsule and in the zonular fibers adhering to it and to a minor extent in the lens epithelial cells. Some TGase activity was also detected in the equatorial portions of the lens capsule (not shown). The choroid expressed high-level TGase activity within cells and fibrous strands of the connective tissue, within Bruch’s membrane, and within vascular endothelial cells of the choriocapillaris (Fig. 3A). TGase activity in the retina was strictly confined to the endothelial

FIGURE 1. Visualization of endogenous TGase activity in human ocular tissues (A) Cornea: TGase activity was present in the corneal epithelium, its basement membrane, and keratocytes of the superficial stroma. (B) Conjunctiva: BiotC incorporation was prominent in the conjunctival epithelium, particularly along the intercellular spaces. (C) Sclera: BiotC was predominantly incorporated into episcleral vessel walls. (D) Iris: TGase activity was present in the dilator muscle, in the walls of stromal blood vessels, and along delicate fibrillar structures in the stroma, particularly in the anterior border layer. Bars, 100 μm.

FIGURE 2. Visualization of TGase activity in human ocular tissues. (A) Ciliary body and trabecular meshwork: Prominent biotC incorporation can be observed in the ciliary muscle and also in the ciliary stroma, the nonpigmented ciliary epithelium, and the trabecular meshwork. (B) Incubation with EDTA inhibited incorporation of biotC in ciliary body structures. (C) Pars plicata region of the ciliary body: TGase activity was demonstrated in zonular fibers, nonpigmented ciliary epithelium, outer limiting membrane, stromal connective tissue, and ciliary muscle cells. (D) Pars plana region of the ciliary body: BiotC was incorporated into zonular fibers, the inner and outer limiting membranes, vascular walls in the connective tissue layer, and extensions of the ciliary muscle. Bars, 100 μm.
lining of retinal capillaries (Fig. 3A). Activity in the optic nerve was localized to capillaries within the glial columns of the prelaminar portion, to the cribriform plates of connective tissue in the laminar portion, and to the connective tissue septa including their vasculature in longitudinal sections of the postlaminar part. In the presence of EDTA, biotC incorporation into the optic nerve was prevented. Bars, 100 μm.

**Monk Eye.** BiotC incorporation into whole monkey eye sections highly resembled the pattern obtained for human eyes. Endogenous TGase activity was detected in the corneal epithelium and its basement membrane and in superficial stromal keratocytes, in the trabecular endothelial cells in low amounts, and in the dilator and sphincter muscles of the iris and vessel walls of the iris stroma. Extensive TGase cross-linking sites were again observed in the ciliary body (Figs. 4A, 4B), particularly in the zonular fibers on the surface, in the ciliary muscle, the outer limiting membrane, the inner limiting membrane in the pars plana region, and the stromal connective tissue including vessel walls. The nonpigmented ciliary epithelium was only weakly labeled in the region of the pars plicata. BiotC incorporation was further localized to the lens epithelium, the zonular lamella on the surface of the lens capsule, and equatorial portions of the lens capsule proper. TGase activity was also apparent in retinal capillaries (Figs. 4C, 4D), in stroma and vasculature of the choroid, and in episcleral vessels. In addition, biotC incorporation was prominent in the sclera in association with fibrillar structures (Fig. 4D). In the conjunctiva, biotC cross-linking was found in the conjunctival epithelium, in its basement membrane, and in association with vessel walls and fibrilar structures in the stroma, which appeared to be particularly concentrated subjacent to the epithelial layer (Figs. 4E, 4F). A characteristic pattern of cross-linking surrounded all individual muscle fibers of extraocular muscle tissue, which was not included in the human specimens (Fig. 4D). The optic nerve was not included in the monkey eye sections.

**TGase Activity in Fibrillin-Containing Microfibrils**

The observed distribution of fibrillin, the major component of 10- to 12-nm microfibrils was highly comparable in both species and was in very good accordance with earlier findings in human eyes. In particular, the epithelial basement membrane region consistently exhibited fibrillin staining, which was most prominent in the peripheral cornea (Fig. 5A) but faint in the central cornea (not shown). The sclera showed interlamellar fibrillin staining, predominantly in the anterior part (Fig. 5b). In the retina, fibrillin was present only in the walls of retinal vessels (Fig. 5C). Fibrillin in the optic nerve was localized to capillaries within the glial columns of the prelaminar portion, to the cribriform plates of connective tissue in the laminar portion, and to the connective tissue septa including their vasculature in longitudinal sections of the postlaminar portion (Fig. 5D). Fibrillin was also present in the optic nerve meninges, particularly the pia mater and arachnoid (not shown). A particularly strong signal for fibrillin was obtained with ciliary zonules (Fig. 5E), a moderate signal in the adventitia of conjunctival blood vessels (Fig. 5F) and endomysial sheets in eye muscle (data obtained only in monkey eyes; not shown). TGase activity showed a broader distribution than that of fibrillin; however, there was extensive colocalization of fibrillin and TGase activity, which became evident after superimposition of images (Fig. 5).

**Transamidation of Isolated Zonules with Purified TGase 2**

In the presence of exogenous TGase 2, the pattern of biotC incorporation was not significantly altered, except for an increased reaction in the iris stroma (both cell-bound and along
thin, delicate fibrillar structures), in the trabecular meshwork (cell-bound), in the equatorial portions of the lens capsule, and in the photoreceptor layer (inner segments) of the retina. This demonstrates that these contained substrate sites for the enzyme that were not cross-linked under normal conditions (data not shown). The zonular fibers again appeared as a prominent target structure, which led us to treat isolated bovine zonules in suspension with purified guinea pig liver TGase 2. This led to incorporation of biotC, which was not evenly distributed along the entire zonules but largely clustered, probably because of masked or saturated cross-linking sites (Fig. 6).

**Distribution of Various TGases in the Eye: Predominance of TGase 2**

Immunohistochemically, we could demonstrate TGase 2 in comparison with TGases 1 and 3 and factor XIII as the predominant enzyme in ocular tissue. TGase 2 was associated extracellularly with fibers in the stromata of the ciliary body (Fig. 7A), iris (Fig. 7B), and conjunctiva (not shown). Cell-associated TGase 2 was noted within corneal epithelium (not shown), endothelia, and single cells in the iris stroma (Fig. 7A, 7B). There was also moderate staining of endomysial sheets of rectus muscle (not shown). The ciliary zonules were negative in human eyes and only faintly positive in the cynomolgus monkey eye (not shown). The other TGases were not found in association with connective tissue structures. TGase 1 was identified exclusively in the corneal epithelium, mostly in suprabasal cells (Fig. 8A). Factor XIII was associated with the endothelium of arterioles in the conjunctival stroma, mostly in suprabasal cells (Fig. 8B) and capillaries (Fig. 8C). TGase 3 was not absent in the eye but was visualized in human skin as a positive control in the cytoplasm of the granular layer (not shown).

**DISCUSSION**

With the histochemical methods used here, we demonstrated substantial TGase activity in various ocular tissues in situ. There is only limited information in the literature on the expression pattern of TGases in different tissues of the eye, and that prompted us to conduct this study. Prior research focused primarily on the lens, because it had been suggested that TGase...
cross-linking may contribute to cataract formation, based on the finding that TGase activity and γ-glutamyl-ε-lysine cross-links are increased by an order of magnitude in senile cataract both in humans and in animal models.\textsuperscript{20,21} β-Crystallins,\textsuperscript{21–23} and more recently, vimentin\textsuperscript{24} have been identified as the target proteins for TGase cross-linking in lens. TGase 2 has been implicated in this process\textsuperscript{20} and shown to be expressed by the anterior lens epithelium,\textsuperscript{25} consistent with our results. An induction of TGase 2 expression has also been associated with programmed cell death in various cell types,\textsuperscript{11} and TGase 2 has recently been shown to be involved in apoptosis of retinal photoreceptor cells after both in photic injury and in the rat model for hereditary retinal dystrophy.\textsuperscript{26} Accordingly, we have not seen significant levels of TGase activity in retinal neurons in the normal eye.

There is also not much information available on the presence of other TGases in the eye. Band 4.2, a TGaselike molecule without cross-linking activity, has been detected in bovine and chicken eye lens and possibly participates in the architecture of the lens fiber cell membranes.\textsuperscript{27} TGase 1 was only recently investigated and found by in situ hybridization to be expressed in conjunctival epithelium of patients with Stevens-Johnson syndrome, but not in normal conjunctival epithelium.

Figure 5. Confocal laser scanning microscopic analysis of double immunofluorescence labeling of endogenous TGase activity and fibrillin in the monkey eye. Only the superimposed images for fibrillin immunostaining (red signal) and biotC incorporation (green signal) are shown. There was extensive colocalization of the microfibrillar protein fibrillin and TGase activity resulting in a bright yellow mixed color. (A) Cornea, (B) sclera, (C) retina close to the photoreceptor layer, (D) optic nerve, (E) zonular fibril, (F) conjunctival stroma. Bar, 25 μm.

Figure 6. Visualization of biotC after treatment of isolated bovine zonules with tissue TGase. Double immunogold visualization of fibrillin (15 nm gold) and incorporated biotC (6 nm gold). (A) Transamidation in the presence of EDTA. Zonules exhibited only 15-nm gold particles, indicating bound fibrillin antibodies. (B, C) Transamidation in the presence of CaCl\textsubscript{2} led to inhomogeneous incorporation of biotC into zonules, evidenced by the 6-nm gold particles (arrowheads). Fibrillin epitopes were labeled with 15-nm gold particles. Bars, 100 nm.

Figure 7. Immunofluorescence detection of TGase 2 in human eye. (A) Ciliary body TGase 2 was present in connective tissue fibers of the ciliary stroma and endothelia and associated extracellularly with fibers in the stromata of the ciliary body. (B) In the iris the enzyme appeared to be associated with endothelia and single cells in the iris stroma. Bar, 20 μm.
activity of TGases. If the enzymes stays in place, it may mask its own epitope for a monoclonal antibody.

However, other microfibrillar components may also be partners for fibrillin in TGase cross-links. Not much is known presently about the composition of microfibrils except for the microfibril-associated glycoprotein (MAGP)-1, which has recently been localized to the beaded domains of microfibrils from bovine zonules. Interestingly, MAGP has been shown to be a glutaminyl substrate for TGase 2. Other microfibril-associated proteins are latent transforming growth factor-binding proteins (LTBP)-1 and LTBP-2. Apparently, LTBP-1 is also a substrate for TGase 2 but appears to be immunohistochemically absent from zonular fibrils and cyanogen bromide preparations of isolated zonule fibrils.

Microfibrillar defects due to fibrillin mutations play a major role in Marfan syndrome and its ocular manifestations, such as ectopia lentis, dehiscences of suspensory ligaments, myopia, retinal detachment, presenile cataracts, glaucoma, iris abnormalities, and corneal flattening. Extensive ultrastructural studies of microfibrils extracted from fibroblast cultures of Marfan patients have consistently shown abnormalities. Remarkably, microfibrils formed in the presence of TGase inhibitors in normal fibroblast cultures also show considerable structural alterations (MR et al., unpublished data, July, 1996). It is therefore tempting to speculate that fibrillin mutations disrupting or deleting sites for TGase cross-linking can cause the microfibrillar instability that results in the ocular abnormalities found in the Marfan syndrome or related phenotypes. This may be particularly interesting for the pseudoxefoliation syndrome, in which excessive production and abnormal aggregation of fibrillin-containing microfibrils have been proposed and demonstrated. Finally, cataract research is currently focusing on inhibitors against the TGase-catalyzed cross-linking of lens proteins. In this regard our data underline that the area and place make TGase activity beneficial or disease causing.

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