Immunohistochemical Detection of CTGF in the Human Eye

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**ABSTRACT**

**Purpose/Aim of the study:** Connective tissue growth factor (CTGF) is a key player in the control of extracellular matrix remodeling, fibrosis, and angiogenesis. It is also involved in the modification of the trabecular meshwork, thus potentially modulating outflow facility and intraocular pressure (IOP). As a consequence, CTGF might be relevant for the development of elevated IOP, a major risk factor in glaucoma-pathogenesis. While comprehensive information on the origins of CTGF in the human eye is not available, the goal of this study is to identify ocular sources of CTGF using morphological methods.

**Materials and Methods:** Human donor eyes were prepared for immunohistochemical analysis of CTGF, α-smooth muscle-actin (ASMA), and CD31. Confocal laser scanning microscopy was used for documentation.

**Results:** In the cornea, CTGF-immunoreactivity (CTGF-IR) was detected in the epithelium, mainly in basal layers, stromal keratinocytes, and endothelial cells. Adjacent conjunctiva showed also CTGF-IR in epithelial cells. In the iris, both, the sphincter and dilator muscles displayed CTGF-IR, as did iris and ciliary body vessels, deriving at this location from the vascular endothelium, as detected with CD31, but not from vascular smooth muscle cells, as detected with ASMA. In the ciliary body, CTGF-IR was detected in smooth-muscle cells of the ciliary muscle and further in the non-pigmented epithelium. In the retina, CTGF-IR was detected in the NFL and weakly in the IPL/OPL. In the choroid, the choriocapillaris and blood vessels displayed CTGF-IR. Further, few cells in the optic nerve head and the lamina cribrosa were CTGF-positive.

**Conclusion:** CTGF was detected in various structures of the human eye. Since CTGF has been also described in aqueous humor, the identified structures might be the sources of CTGF in the aqueous humor. By means of aqueous flow, CTGF is transported into the trabecular meshwork, where it could change outflow facility and therefore affecting IOP homeostasis.

**INTRODUCTION**

Connective tissue growth factor (CTGF = CCN2) is a small protein consisting of 349 amino acids. It is actively secreted into the extracellular space and is involved in various cell signaling processes such as cell survival, proliferation, differentiation, migration, and extracellular matrix (ECM) synthesis.\(^1\) It is therefore termed a “secreted matricellular organizer”\(^2\) and widely expressed during embryogenesis.\(^3\),\(^4\) In pathological conditions, it is also a known downstream mediator of transforming growth factor beta (TGF-b), which in a complex cascade, leads to fibrosis in various organs.\(^5\) CTGF has therefore been recently named a “fibrogenic master switch.”\(^6\)

Regarding the healthy eye, CTGF has been detected in tear fluid,\(^7\)\(^8\) and conjunctiva,\(^9\)–\(^11\) but also intraocular in the ganglion cell layer of the retina (rat\(^12\)), retinal microglia (human\(^13\)), and retinal pigment epithelium (mouse\(^14\)).

In pathological conditions, CTGF has been detected in human choroidal neovascularization,\(^15\) diabetic retinopathy,\(^13,16\) and proliferative vitreoretinopathy.\(^17\)

Glaucoma is one of the leading causes of blindness worldwide, and elevated intraocular pressure (IOP) is a well-known risk factor for the development of glaucomatous optic neuropathy. It is generally accepted that this rise of intraocular pressure is, at least in part, related to the reduced outflow facility via the trabecular meshwork.\(^18\) Interestingly, in glaucomatous eyes a modification of the cytoskeleton of the trabecular meshwork cells by CTGF has been described recently.\(^18\) Although CTGF is considered a normal component of aqueous humor in the anterior chamber,\(^19\) elevated levels of CTGF were shown in patients with pseudoexfoliation glaucoma.\(^20\) However, detailed information on the origin of CTGF in the anterior segment of the human eye is lacking. This lack of knowledge prevents us from understanding the pathways by which CTGF might enter the aqueous humor.
possibly causing downstream alterations in the trabecular meshwork and its ECM.

It is the purpose of the present study to screen for sources of CTGF in the normal human eye with morphological methods.

Materials and methods

Anterior and posterior halves of 14 human eyes (of both sexes, 51–101 years of age; p.m. time 8–24 h) were investigated in this study. Study with human tissue was performed according to the Austrian Gene Technology Act. Experiments were performed in accordance with the Helsinki declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee being no clinical drug trial or epidemiological investigation. Furthermore, the study did not extend to examination of individual case records. The anonymity of the patients has been ensured. Human tissue samples were collected either from cornea donor eyes showing no signs of pathology or were obtained following enucleation due to melanoma of the posterior pole; in this case only the anterior segment of the eye has been investigated. Tissue was fixed via immersion in 4% paraformaldehyde and further processed for standard paraffin or cryo-embedding, followed by immunohistochemistry (both procedures resulted in identical findings). In detail, for paraffin embedding, 5 μm tissue sections were collected on adhesion slides, deparaffinized using a standard protocol and rehydrated via a series of graded alcohols (95%, 85%, 70%, 50%, for 3 minutes each at room temperature, RT). For cryo-sectioning, tissue samples were rinsed in phosphate buffered saline (PBS; 24–48 h) and transferred into PBS containing 15% sucrose (24 h at 4°C). Tissue was embedded in tissue embedding medium (NEG50, Fisher Scientific, Vienna, Austria) and frozen at −80°C by using liquid nitrogen-cooled methylbutane and stored at −20°C for further processing. 12 μm sections were obtained in a cryostat (HM 550, Microm, Walldorf, Germany) and collected on adhesion slides (Superfrost Plus; Thermo Scientific, Vienna, Austria) and air-dried for 1 h at RT.

For immunohistochemistry, slides were rinsed in tris-buffered saline (TBS; Roth, Karlsruhe, Germany, 5 min at RT) and incubated for 1 hour at RT in TBS containing 5% donkey serum (Sigma-Aldrich, Wien, Austria), 1% bovine serum albumin (BSA, Sigma-Aldrich, Vienna, Austria), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). After a 5 min rinse, slides were incubated overnight at RT with an anti-human connective tissue growth factor (CTGF; raised in goat, 1:100, AF660, R&D Systems, Minneapolis, MN, USA) in TBS, containing 1% BSA and 0.5% Triton X-100. Alternatively, also a different anti human CTGF has been used (raised in goat, 1:100, sc-14939, Santa Cruz Biotechnology, Heidelberg, Germany) and resulted in identical patterns of immunoreactivity. In double and triple labelling experiments, CTGF has been combined with α-smooth muscle actin (SMA, raised in mouse 1:1000, A2547, Sigma-Aldrich) or SMA and CD31 (CD31, raised in rabbit 1:50, PA5-16301, Thermo Scientific) in TBS, containing 1% BSA and 0.5% Triton X-100 over night at room temperature (RT). After a rinse in TBS (three times, 5 min), binding sites of primary antibodies were visualized by AF488, AF555, and AF647 tagged antisera (raised in donkey, 1:1000; Invitrogen, Karlsruhe, Germany) in TBS, containing 1% BSA and 0.5% Triton X-100 (1 hour at RT) followed by another rinse in TBS (three times, 5 min). Slides were further incubated 10 min with 4′,6-Diamidino-2 phenylindol dihydrochlorid (DAPI) (1:4000, stock 1 mg/ml, VWR, Vienna, Austria), rinsed three times 5 min in PBS and were embedded in TBS:glycerol (1:1 at pH 8.6).

Positive controls in cross-sections of human as well as rat skin revealed labelling in keratinocytes, in accordance with data provided by the human protein atlas (www.proteinatlas.org). Isotype controls with ASMA (raised in goat) revealed adequate labelling of the iris sphincter, while any cross-reactivity in the cornea was not detected. Further, pre-adsorption controls in human cornea revealed a clear decrease in immunoreactivity, while negative controls performed by omission of the primary antibodies resulted in absence of immunoreactivity.

Documentation was performed using a confocal laserscanning unit (Axio ObserverZ1 attached to LSM710, Zeiss, Göttingen, Germany; ×20 dry or ×40 and ×60 oil immersion objective lenses, with numeric apertures 0.8, 1.30, and 1.4, respectively; Zeiss). Sections were imaged using the appropriate filter settings for AF488 (488 nm excitation), AF555 (555 nm excitation), AF647 (647 nm excitation), and DAPI (345 nm excitation) and up to four channels were detected simultaneously. For better orientation, sites of tissue sampling in the human anterior eye are depicted in Figure 1.

Results

Cornea and conjunctiva

In the cornea, CTGF-immunoreactivity (CTGF-IR) was detected in the epithelium, mainly concentrated in basal layers, while in superficial stratified layers immunoreactivity was less prominent (Figure 2A). However, single superficial cells...
displayed intense CTGF-IR (Figure 2B). Immunoreactivity for CTGF was also detectable in stromal keratinocytes as well as in corneal endothelial cells (Figure 2C), but was absent in corresponding negative controls (Figure 2D).

In the conjunctiva, a structure unequivocally identified by the presence of goblet cells, CTGF-IR was detectable throughout all epithelial layers (Figure 2E), while it was clearly absent in corresponding negative controls (Figure 2F). Further, in the trabecular meshwork and Schlemm’s canal, a clear CTGF-IR was not detectable (Figure 2G), and revealed an identical staining pattern compared to corresponding negative controls (Figure 2H).

**Anterior uvea**

In the iris CTGF-IR was detectable in anterior superficial layers of the iris and further in the iris sphincter and dilator,
as identified with α-smooth muscle actin (ASMA; Figure 3A). CTGF-IR was not detectable in the corresponding negative control (Figure 3B).

Iris and ciliary body vessels also showed immunoreactivity for CTGF. To further clarify the source of this CTGF-IR, triple experiments with ASMA for the identification of the muscularis layer and CD31 for the identification of vascular endothelium were performed (Figure 3C). While an overlap of CTGF and ASMA was not detected, an association of CTGF and CD31 was obvious thereby indicating an endothelial origin of CTGF (Figure 3C). Corresponding negative controls revealed absence of immunoreactivity (Figure 3D).

In the ciliary body, weak CTGF-IR was present in smooth muscle fibers of the ciliary muscle (Figure 3E), but was lacking in the corresponding negative control (Figure 3F). In the ciliary body epithelium, CTGF-IR was present in the non-pigmented epithelium (Figure 3G). Corresponding negative controls were devoid of CTGF-IR; however, an auto-fluorescent signal is present in the non-pigmented part that needs to be considered (Figure 3H).

Besides anterior uvea, CTGF-IR was also identified in superficial fibers of the lens, but was absent in the lens epithelium (Figure 3I), when compared with the corresponding negative control (Figure 3J).

**Posterior eye**

In the retina, a clear CTGF-immunoreactivity was detectable in the nerve fiber layer (NFL), and further a weak signal was present in the inner and outer plexiform layer (IPL/OPL) (Figure 4A), while CTGF was not unequivocally identified in the photoreceptor layer (PRL) due to the rather high auto-fluorescence, as compared to the corresponding negative controls (Figure 4B).

In the choroid, CTGF was present in the choriocapillaris as well as in blood vessels of the choroidal stroma (Figure 4C), and aforementioned immunoreactive signals were clearly absent in corresponding negative controls (Figure 4D). In the optic nerve head and lamina cribrosa, endothelial cells of the central retinal artery and also smaller blood vessels displayed CTGF-immunoreactivity (Figure 4E). CTGF was further also present in few cells embedded in connective tissue strands of the optic nerve (Figure 4F), while other portions were lacking CTGF immunoreactivity.

**Discussion**

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family, including five members. CCN proteins function as matricellular proteins that can bind to matrix and modulate cellular functions.\(^{21-23}\) In this context, CTGF represents a key player in the control of the ECM and is responsible for a multitude of processes in tissue regulation. CTGF is significantly up-regulated in experimental models of fibrosis associated with aberrant ECM deposition (reviewed in\(^{24}\)). While CTGF has been detected in various animal studies analyzing ocular structures, to the best of our knowledge a thorough investigation in healthy human ocular tissue has not yet been published. This study describes possible origins of CTGF in the human anterior eye using morphological methods.

**CTGF in tear fluid – Secretion of corneally expressed CTGF?**

CTGF-IR has been detected in human corneal epithelium, mainly in the basal layers. While an intercellular space is not developed in this tissue and therefore the ECM is rather scarce, a secretion to the surface can be postulated, and indeed CTGF has been detected in tear fluid.\(^{7,8}\) On the other hand, in vitro experiments of human corneal fibroblasts revealed that CTGF is required for the re-epithelialization via cell migration,\(^ {25}\) which is an important process during the healing of superficial corneal trauma. In addition CTGF is also involved in the formation of cell-cell junctions within the corneal epithelium,\(^ {26}\) as demonstrated by in vitro studies. This is also supported by the presence of CTGF in basal layers of the epithelium, as demonstrated in Figure 1A and B.

**CTGF in tear fluid – Secretion of CTGF expressed in the conjunctiva?**

Besides lacrimal glands\(^ {7}\) the conjunctiva might be an alternative source for tear-derived CTGF, and indeed CTGF-immunoreactivity has been detected in conjunctival tissue, as presented here. While conjunctiva-derived CTGF has already been described earlier in humans,\(^ {11}\) others failed to detect CTGF in normal human conjunctiva.\(^ {27}\) As also seen for fibrotic processes in other systems, e.g., skin,\(^ {27}\) or kidney,\(^ {28}\) CTGF is also upregulated in the conjunctiva of ocular cicatricial pemphigoid.\(^ {41}\) However, CTGF was not detected in conjunctiva of trachoma patients,\(^ {27}\) and it was found to be decreased in ulcerated eyes in an animal study (most likely due to dilution effects as a result of increased tear production in that case).\(^ {7}\) Summarizing aforementioned reports, two possible actions of conjunctival CTGF are postulated; first via secretion into the tear fluid and second as a mediator into deeper layers of the conjunctiva.

**CTGF in the corneal stroma – Fibrotic processes**

CTGF has been detected in keratinocytes of the stroma, presented here for the first time in human specimens. These results are in line with CTGF-expression studies in healthy mice and rabbit eyes,\(^ {29,30}\) and also with rat in vitro studies.\(^ {30}\) In these cells, CTGF is involved in corneal fibrotic and subsequent scaring processes. Furthermore, an in vitro downregulation of CTGF as well as TGFβ1 and TGFβ2 revealed a reduction in mRNA expression of downstream “scaring” genes (e.g., α-SMA), thus highlighting the importance of CTGF in corneal fibrosis.\(^ {31}\) The function of CTGF in fibrosis is connected to its ability to induce epithelial-to-mesenchymal transition and ECM synthesis.

CTGF has been detected in human corneal endothelial cells. Again, this feature has been described earlier in
Figure 3. (A, B) In the iris, CTGF-immunoreactivity (A, red) was present in anterior layers of the iris (arrows) as well as muscle fibers of the iris sphincter (arrowheads), as detected with alpha-smooth-muscle actin (green), but was absent in the corresponding negative control (B). DAPI: white. (C, D) In C, iris vessels displayed immunoreactivity for CTGF (red) in the vascular endothelium (blue, CD31), as seen by an association of both signals (purple color), while an overlap of CTGF with vascular smooth muscle cells (green, ASMA) was not observed, as seen by absence of yellow-mixed color. Immunoreactivity was absent in corresponding negative controls (D). Asterisks indicates vessel lumen; DAPI: white. (E, F) CTGF-immunoreactivity (E, red) was present in the muscle fibers of the ciliary body (asterisk), as detected with alpha-smooth muscle actin (green), but was absent in the corresponding negative control (E). DAPI: white. (G, H) CTGF-immunoreactivity was present in the non-pigmented ciliary epithelium (G, arrowheads), but was absent in the corresponding negative control (H). DAPI: white. (I, J) CTGF-immunoreactivity (red) in the lens was absent in the lens epithelium (I, arrowheads), but present in superficial layers of the lens (J, asterisk), while it was absent in the corresponding negative control (J, asterisk). DAPI: blue.
healthy mouse and rabbit eyes and most likely contributes to a basal secretion of CTGF into the corneal stroma or into aqueous humor of the anterior chamber. Indeed, CTGF in the aqueous humor of the anterior chamber has been considered its normal component. Earlier functional studies revealed that PGF2α up-regulates CTGF-mRNA expression in cat iris smooth muscle and human ciliary smooth muscle cells in vitro, while in a porcine anterior chamber perfusion model without an analysis of morphology, CTGF was not altered after administration of TGF-b2. Considering these observations, and keeping in mind that CTGF release is known from airway smooth muscle or has been detected in human myometrium, it seems plausible that substantial CTGF-activity derives also from smooth muscle cells of the iris and ciliary body of human donor eyes as shown in Figure 2A and E. Both, iris as well as ciliary body could be a source of contribution to CTGF found in the aqueous humor and in the vitreous body.

CTGF in iris, ciliary body, and lens

In mice and rabbits, the presence of CTGF in various cells of the iris has been reported without further details. Earlier functional studies revealed that PGF2α up-regulates CTGF-mRNA expression in cat iris smooth muscle and human ciliary smooth muscle cells in vitro, while in a porcine anterior chamber perfusion model without an analysis of morphology, CTGF was not altered after administration of TGF-b2. Considering these observations, and keeping in mind that CTGF release is known from airway smooth muscle or has been detected in human myometrium, it seems plausible that substantial CTGF-activity derives also from smooth muscle cells of the iris and ciliary body of human donor eyes as shown in Figure 2A and E. Both, iris as well as ciliary body could be a source of contribution to CTGF found in the aqueous humor and in the vitreous body. As yet, the scientific literature lacks evidence on possible local effects within the tissue of origin by paracrine secretion of CTGF.

CTGF is detected in endothelial cells of various vessels in normal tissues in agreement with the CTGF-IR in vascular endothelial cells of both iris and ciliary body and is most likely attributed to endothelial cell adhesion. Nevertheless, expression patterns in vascular endothelial cells change depending on the vessel site, and moreover in states of disease, e.g., diabetic retinopathy.

Regarding CTGF results in the lens, several in vitro studies reported the presence or induction of CTGF in lens epithelial cells, and CTGF was increased in lens epithelial cells in anterior polar cataract. In situ hybridization of anterior subcapsular cataract revealed CTGF-mRNA in the cataractous plaques and transformed lens epithelial cells, but CTGF was absent in either lens fiber cells or non-transformed lens...
CTGF in the posterior eye

CTGF expression in the human posterior eye is associated with many pathological conditions. For example, high levels have been detected in choroidal neovascularizations in the course of age-related macular degeneration (AMD), high myopia and angiod streaks, and in these conditions ECM production by fibroblasts and subsequent angiogenesis could be promoted by CTGF. Furthermore, CTGF has been recently shown in synovial fibroblasts to increase vascular endothelial growth factor, a master controller in angiogenesis. In line with this, CTGF expression is reported in endothelial cells and retinal pigment epithelial cells of choroidal neovascular membranes surgically excised from AMD patients. Additionally, in vitro studies reported a CTGF up-regulation in response to angiogenic growth factors using isolated RPE and choroidal endothelial cells from these patients, thus further supporting a possible role of CTGF in choroidal neovascularization. While up to now CTGF expression has not been reported in healthy choroid, our here observed CTGF-IR in choroidal vessels and the choriocapillaris, as well as in endothelial cells of the central retinal artery, could easily contribute to aforementioned pathologies. The same applies to the here detected CTGF-positive cells in the lamina cribrosa, and their involvement in ECM remodeling in this part of the eye.

Less clear is the role of CTGF-origin in the retina. The CTGF-signal detected in theNFL most likely derives from astrocytes as seen in our cross-sections close to the optic nerve head, but could also originate from microvascular pericytes. Although CTGF expression in microglia and pericytes has been investigated in diabetic retinopathy, the contribution ofNFL-derived CTGF and its role in distinct ocular pathological conditions, especially of the posterior pole, needs to be clarified in upcoming studies. The same pertains to the weak signal observed in the IPL/OPL, that could derive from Mueller glial cells, which in a functional proteomics assay has been identified as a potential survival factor for photoreceptor cells.

CTGF and glaucoma

As aqueous humor is mainly drained via the chamber angle and the trabecular meshwork (TM), increased resistance in this tissue may result in elevated IOP. Indeed, in glaucoma patients, an increase in extracellular matrix in the TM has been reported in several studies; for review see. Interestingly, CTGF, as a downstream mediator of TGF-b2-signaling, is associated with the up-regulation of ECM production. Further, enhanced ECM production and deposition by CTGF was reported in human TM cells, and this fibrotic effect was reduced/ blocked by inhibiting CTGF signaling via the prostaglandin f2α-analog fluprostenol. In mice and rats, CTGF overexpression caused a decrease in outflow facility with a corresponding increase in IOP and subsequent optic nerve damage while a disruption of the CTGF-signaling pathway in these CTGF over-expressing mice, using rho-kinase inhibitors, resulted in an IOP reduction. Taken together, CTGF seems to play an important role in the modification of the TM and potentially also in the development of some types of glaucoma. First studies were already able to introduce a possible new therapeutic approach for glaucoma therapy: TM and lamina cribrosa cells were stimulated by aqueous humor from the patients with pseudoexfoliation and primary open angle glaucoma but showed a reduced expression of profibrotic genes and proteins after anti-CTGF immunotherapy. Besides local effects, systemic effects due to elevated plasma CTGF also need to be considered in TM-alterations. This might represent a link for a systemic disease or syndrome acting on the TM as a locus minoris resistitiae that possibly leads to glaucoma. However, in this sense it should be mentioned that part of the aqueous humor leaves the eye through the non-conventional or alternative outflow pathway via the iris root and the sclera. CTGF has the potential to alter the ECM also along this exit route thus resulting in an increased outflow resistance in this alternative pathway, an effect extensively described for activation of metalloproteinases by prostaglandin analogues.

Conclusion

In conclusion, this study shows that the anterior and posterior human eye contains several significant sources of CTGF which all may contribute to the concentration of CTGF in anterior chamber fluid. It is well known that cyclodestructive procedures used in glaucoma surgery lower intraocular pressure by decreased aqueous humor production. A decrease in the CTGF-level in the aqueous humor might also contribute to this effect, which will have to be proven by a future clinical study looking at intraoperatively collected samples.

Declaration of interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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