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From Cell Biology to Tissue Engineering

The presence of lysyl oxidase-like enzymes in human control and keratoconic corneas

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Summary. Purpose: Lysyl oxidases, a family comprising lysyl oxidase (LOX) and four LOX-like enzymes (LOXL1-4), catalyse the cross-linking of elastin and collagen fibrils. Keratoconus (KC) is characterized by progressive thinning leading to irregular astigmatism, resulting in significant visual impairment. Although the pathogenesis of KC remains unclear, one of the current hypotheses is based on alterations in the organization and structure of collagen fibrils. To extend existing general knowledge about cross-linking enzymes in the human cornea, in the present study we have focused on the detection of LOXL enzymes.

Method: The localization and distribution of LOXL1-4 were assessed in cryosections of 7 control donors (three males and three females; 25-68 years; mean age 46±17.6 years) and 8 KC corneas (5 males and 3 females; 25-46 years; mean age 31.3±7.5 years) using indirect fluorescent immunohistochemistry (IHC). The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200-1000x. Western blot analysis of 4 control and 4 KC corneas was performed for all tested enzymes.

Results: All four LOX-like enzymes were present in all layers of control corneas as well as in the limbus and conjunctiva. Almost no differences between control and pathological specimens were found for LOXL1. A lower staining intensity of LOXL2 was found using IHC and Western blot analysis in KC specimens. Decreases of the signal and small irregularities in the staining were found in the epithelium, keratocytes and extracellular matrix, where a gradual anterior-posterior weakening of the signal was observed. LOXL3 IHC staining was lower in the corneal stromal extracellular matrix and keratocytes of KC samples. No prominent differences were detected using IHC for LOXL4, but a slight decrease was observed in KC corneas using Western blot analysis.

Conclusion: We presume that the decrease of LOXL2 in KC corneas is more likely a consequence of the associated pathological processes (activation of stromal cells due to tissue weakening and consequent structural changes) than a direct cause leading to KC development. At this time, we are unable to provide a coherent explanation for the observed decrease of LOXL3 and LOXL4 in KC corneas.

Key words: Cornea, Lysyl oxidase-like enzymes, Keratoconus, Immunohistochemistry

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Introduction

Lysyl oxidases are extracellular copper-dependent enzymes that catalyse the formation of lysine- and hydroxylysine-derived cross-links in collagens and lysine-derived cross-links in elastin (Kagan and Li, 2003). These cross-links are essential for the tensile strength of collagens and the rubber-like properties of elastin; both are abundant extracellular matrix proteins

necessary for the structural integrity and function of connective tissues (Kagan and Li, 2003; Myllyharju and Kivirikko, 2004). In addition to lysyl oxidase (LOX) (Trackman et al., 1990; Hamalainen et al., 1991), four LOX-like (LOXL) proteins are currently known: LOXL1, LOXL2, LOXL3, and LOXL4 (Kenyon et al., 1993; Saito et al., 1997; Jourdan-Le Saux et al., 2001; Maki and Kivirikko, 2001). Up till now only LOX has been detected in healthy human corneas (Dudakova et al., 2012).

The molecular functions of LOX and the LOXLs and the degree to which their functions overlap remain up till now unsatisfactorily understood. The LOX-like enzymes are thought to act in the same way as the LOX enzyme, which is highly likely given the close homology of their catalytic domains at the C-terminal (Kagan and Li, 2003; Molnar et al., 2003). The primary structures of the LOX and LOXL1 polypeptides at the N-terminal of the protein differ dramatically from those of LOXL2, LOXL3, and LOXL4, suggesting that these structural differences may impact their functions or their location in the extracellular matrix (Table 1). LOXL1 is specifically required for tropoelastin cross-linking and for elastic fibre formation (Liu et al., 2004). The existence of a putative signal sequence and four scavenger receptor cysteine-rich region (SRCR) domains suggests that the LOXL2, LOXL3 and LOXL4 isoenzymes are extracellular proteins (Csiszar, 2001; Maki, 2009). The SRCR protein super family is involved in quite different functions, such as pathogen recognition, modulation of the immune response, epithelial homeostasis, stem cell biology and tumour development (Resnick et al., 1994; Yamada et al., 1998).

Keratoconus (KC) is a corneal thinning disorder, typically diagnosed in the patient's adolescent years, usually affecting both eyes. It is one of the leading causes of corneal transplantation in young adults in the Western world (Rabinowitz, 1998). KC corneas display both compositional and structural changes. A decrease in the number and undulating shape of collagen lamellae; reduced diameter and interfibrillar spacing of collagen fibrils together with an increase of proteoglycans density can be observed in the KC corneal stroma (Sawaguchi et al., 1991; Akhtar et al., 2008). Other structural stromal changes include a reduced number of keratocytes and a disorganization of the collagen fibre network (Radner et al., 1998; Meek et al., 2005).

We have previously demonstrated an irregular staining pattern of LOX in KC corneas compared to healthy controls and a lower total LOX activity (encompassing LOX and the LOXL enzymes) in the media of cultivated KC keratocytes (Dudakova et al., 2012). To date no report on the presence of LOXL enzymes in the human cornea has been published. The aim of the current study was to determine whether LOXL enzymes distributions are changed in KC corneas compared to control corneas.

Materials and methods

Specimen preparation

The study followed the standards of the Ethics Committee of the General Teaching Hospital and Charles University, Prague, and adhered to the tenets set out in the Declaration of Helsinki.

Seven control corneas from three male (in one case both eyes) and three female donors (25-68 years; mean age 46±17.6 years) and eight KC corneas (5 males and 3 females; 25-46 years; mean age 31.3±7.5 years) were dissected and snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature Compound and stored at -70° C. Four 7 μm thick cryosections were placed per slide and used for indirect fluorescent immunohistochemistry.

The clinical diagnosis of KC was based on the presence of typical corneal signs such as thinning, protrusion, Vogt striae, Fleischer ring and scarring. Corneal topography to validate the diagnosis was performed using an EyeMap EH-290 (Alcon Laboratories, Inc., Fort Worth, TX, USA) in three patients. In four patients the assessment of the anterior and posterior corneal surfaces and pachymetry were performed by Scheimpflug imaging (Pentacam, Oculus, Germany). None of the patients had any previous corneal surgery and their characteristics are shown in Table 2.

Control specimens, also without a history of previous corneal surgery, were obtained from the Bank of Biological Material (First Faculty of Medicine, Charles University in Prague and General Teaching Hospital in Prague) and processed within 4 hours after enucleation; pathological explants were obtained from the Department of Ophthalmology (First Faculty of

Table 1. A comparison of LOX family members.

Family member	Human chr.	mRNA and protein size	Greatest mRNA tissue distribution	Protein domains
LOX	5	4.4/5.2 kb; 187/417 AA	Lung, skeletal muscle, kidney, heart	AO
LOXL1	15	2.4 kb; 574 AA	Lung, heart, spleen, skeletal muscle, pancreas	AO
LOXL2	8	3.8 kb; 774 AA	Lung, thymus, skin, testis, ovary	4 SRCR, AO
LOXL3	2	3.0/3.3/3.7 kb; 392/608/753 AA	Heart, uterus, testis, ovary	4 SRCR, AO
LOXL4	10	3.7 kb; 756 AA	Skeletal muscle, testis, pancreas	4 SRCR, AO

AA: aminoacid; AO: Amine oxidase; chr: chromosome; SRCR: Scavenger Receptor Cysteine-Rich. Adapted from Hornstra et al., (2003).

Medicine, Charles University in Prague and General Teaching Hospital in Prague).

Indirect fluorescent immunohistochemistry

Three cryosections on each slide were stained with a single antibody. The fourth section (primary antibody omitted) served as a negative control. Three independent experiments were performed. The tissue was fixed with cold acetone for 10 min, rinsed in PBS and incubated with the primary antibody diluted in 1% bovine serum albumin (Sigma Aldrich Corporation, St. Louis, MO,

Table 2. Clinical characteristics of the keratoconic samples used in this study.

Sample No.	Age at surgery	Gender	TKC	Contact lens wear	Histopathological findings (Bowman breaks, scarring, hydrops etc.)
Kc1	28	F	2	Y Since 24 years	no scarring
Kc2*	27	M	4	N	thinning in centre of cornea, scarring, Vogt striae
Kc3	25	F	3	N	no scarring
Kc4	27	M	4	N	scarring, Vogt striae
Kc5	25	M	4	N	thinning in centre of cornea, scarring, Vogt striae
Kc6*	35	M	2	N	no scarring, Vogt striae
Kc7*	37	M	4	N	scarring
Kc8*	46	F	4	N	thinning in centre of cornea, scarring, Vogt striae

*: samples used in Western blot analysis; TKC: topographic keratoconus classification.

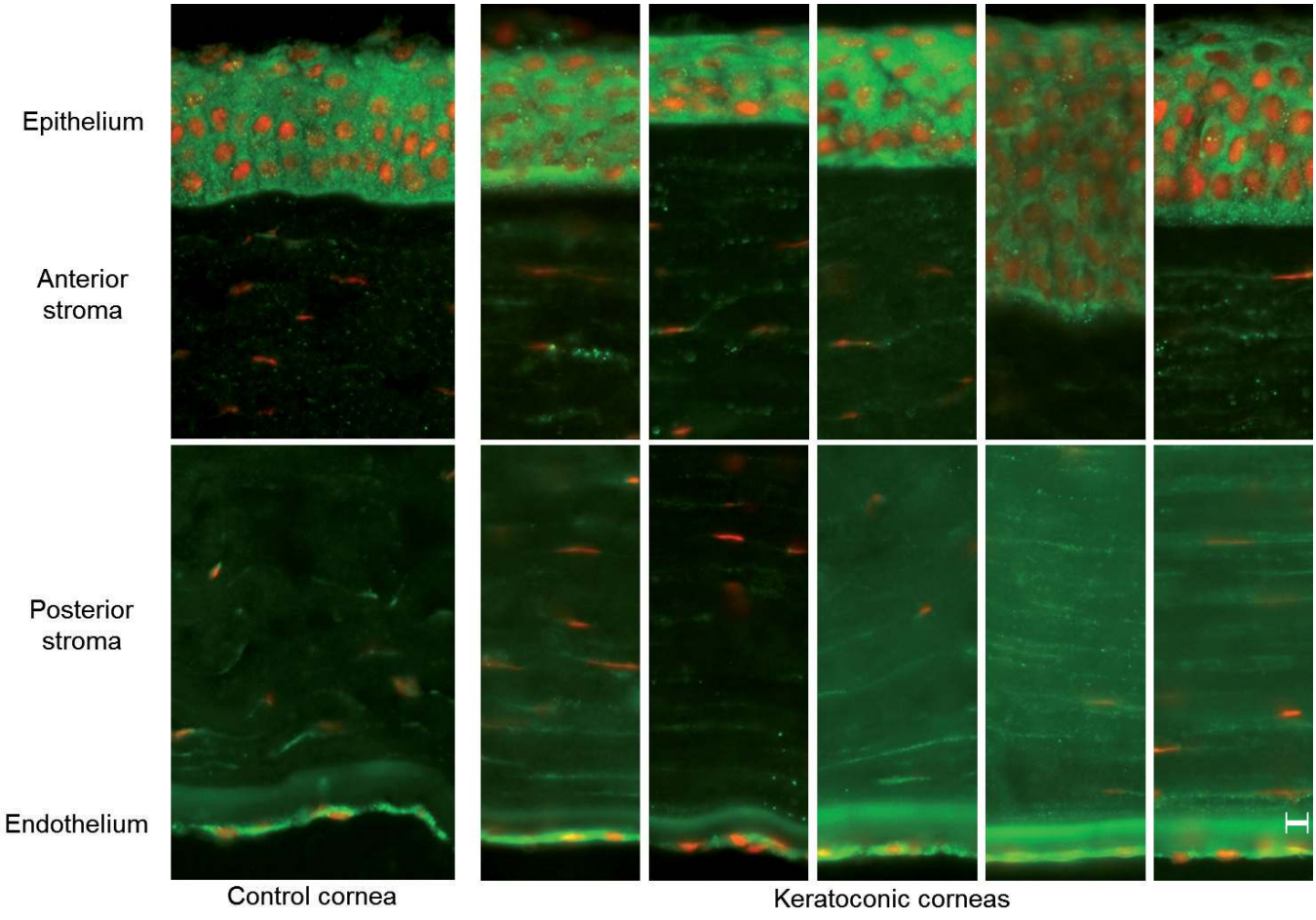

Fig. 1. LOXL1 staining in control and keratoconic (KC1-5) corneas. No differences were observed between control and keratoconic specimens. Scale bar: 10 μm.

USA) in phosphate-buffered saline (PBS) for 1 h at room temperature. The expression of LOX-like enzymes was detected using the following antibodies: rabbit polyclonal anti-LOXL1, anti-LOXL3 (sc-68939, Santa Cruz Biotechnology, Heidelberg, Germany), and anti-LOXL4 (ab88186, Abcam, Cambridge, UK) and mouse monoclonal anti-LOXL2 (ab60753, Abcam). Anti-LOXL1 was raised against recombinant human LOXL1 propeptide expressed in *E. coli* (Sasaki, unpublished data). Following incubation with the primary antibody, the specimens were washed three times in PBS and incubated with the secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. After rinsing in PBS the slices were mounted with Vectashield-propidium iodide (Vector Laboratories, Inc., Burlingame, CA, USA) to counterstain nuclear DNA.

The specimens were examined using an Olympus BX51 microscope (Olympus Co., Tokyo, Japan) at a magnification of 200-1000x. Images were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Germany) and JENOPTIK ProgRes C12plus camera (Jenoptik, Laser, Optik, Systeme GmbH, Jena, Germany). The cells in the epithelium, stroma and endothelium of KC corneas as well in the limbus and conjunctiva in the case of control corneas were examined. At least two hundred epithelial cells and keratocytes per section (600/slide) and one hundred endothelial cells per section (300/slide), directly connected to Descemet's membrane, were examined, and the percentage of positive cells was calculated. The intensity of cell staining was graded using a 0-4 scale as follows: 0: no discernible staining, 1: weak, 2: moderate, 3: intense, and 4: very intense staining.

Western blot analysis

Samples were thawed and homogenized in a lysis buffer (0.2% Triton X-100, 10% glycerol, 0.5 mM

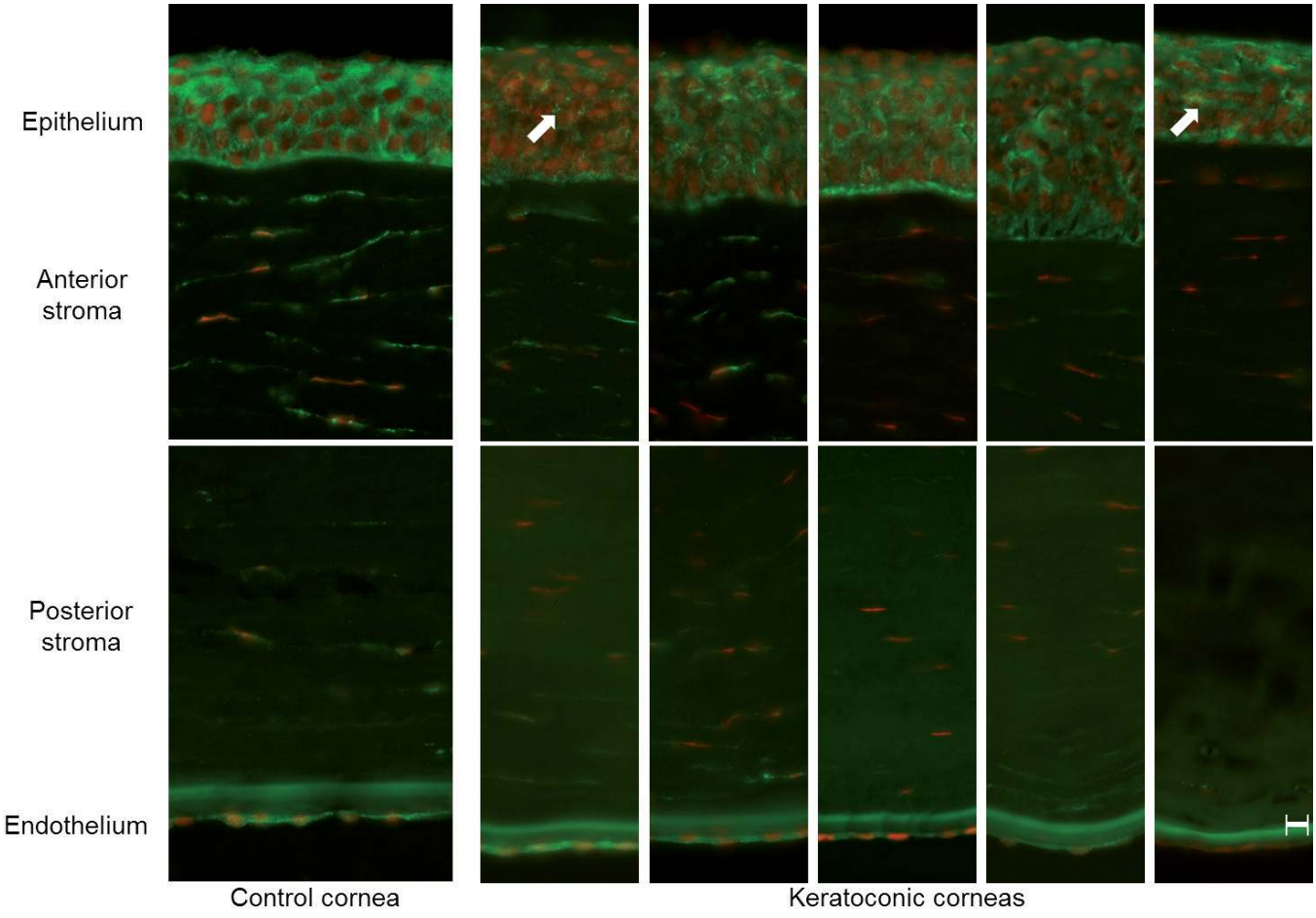

Fig. 2. LOXL2 staining in control and keratoconic (KC1-5) corneas. Note the decrease of the signal and the staining irregularities in the epithelium of keratoconic corneas (arrows). Scale bar: 10 μm.

EDTA, 1 mM dithiothreitol and protease inhibitors in PBS). Subsequently, all samples underwent protein extraction for two days at 4°C, followed by centrifugation for 30 minutes at 10,000× g. The supernatants were removed and frozen at -20°C.

The protein concentration was determined with a commercial kit (BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL, USA). Equal volumes of protein extract and sample buffer were mixed, reduced by 5% β-mercaptoethanol, and fractionated on 10% SDS polyacrylamide gels. After electrophoresis was completed, the proteins were transferred to nitrocellulose membranes (Serva Electroforesis GmbH, Heidelberg, Germany) and blocked with 5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBS-T) at 4°C overnight.

After washing in PBS-T, membranes were probed with rabbit and mouse antibodies against LOXL1-4 (1:1,000, same as used in immunohistochemistry experiments) and β-actin (1:2,000; ab8226, Abcam) for 2 hours at room temperature. After another wash in PBS-T, the membranes were incubated with the appropriate peroxidase conjugated anti-mouse (P0260, Dako-Cytomation, Glostrup, Denmark) and anti-rabbit antibodies (P0399, Dako) diluted 1:8,000 in 1% BSA for 45 minutes at room temperature and washed with PBS-T.

Positive reactions were visualized with an enhanced chemiluminescence technique (SuperSignalWest Femto Maximum Sensitivity Substrate kit; Pierce Biotechnology) for 5 minutes and examined with a membrane documentation system (Syngene Chemigenius-Q and the GeneSnap program; Synoptics Ltd., Cambridge, UK).

Results

Moderate to intense staining was detected for LOXL1 in all parts of the cornea in the control as well as in the KC samples (Fig. 1).

Using LOXL2 antibody, moderate to intense staining was observed in the epithelium, stroma and endothelium of all control corneas. Staining irregularities in the epithelium (a decrease of staining and a clump-like

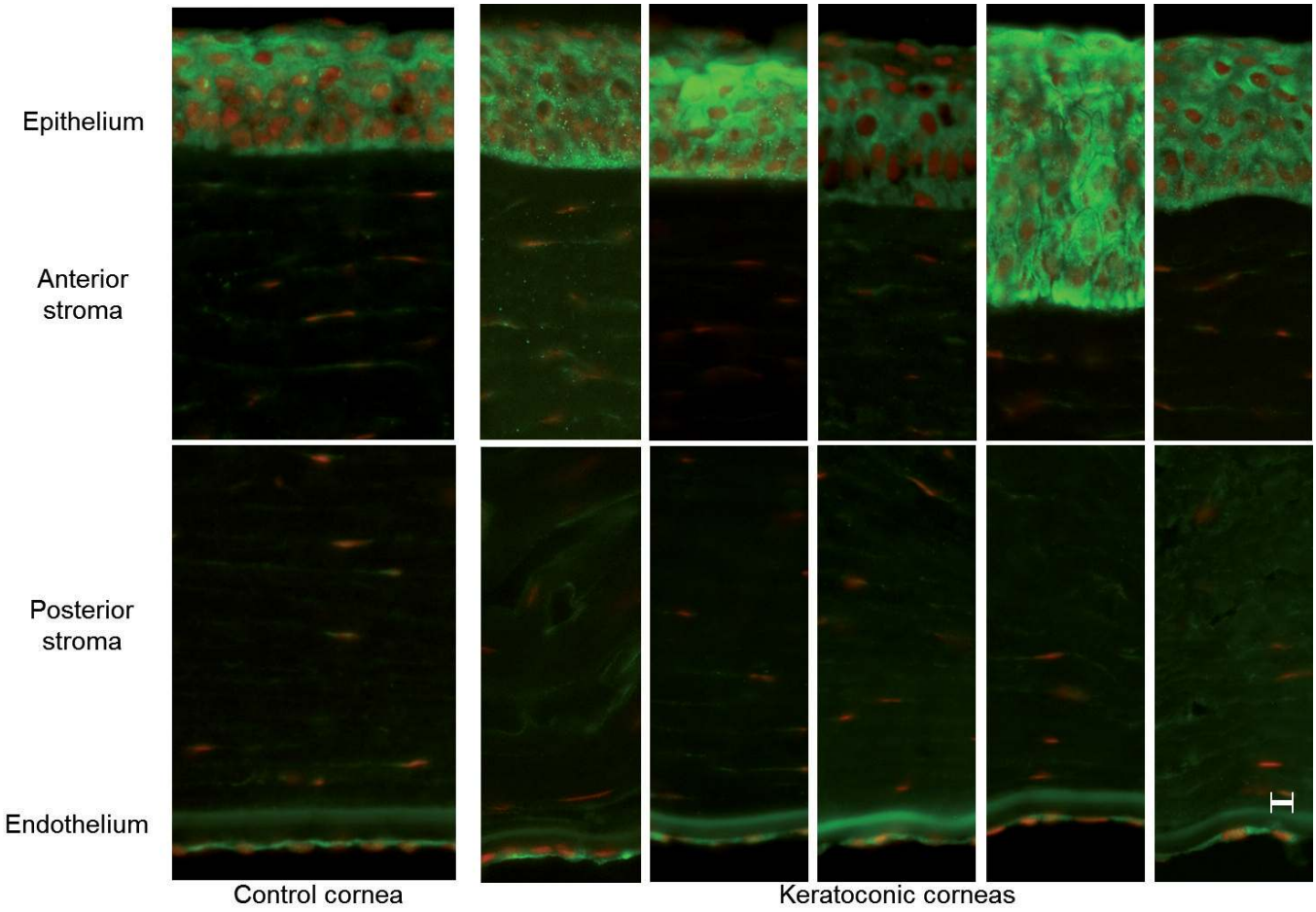

Fig. 3. LOXL3 staining in control and keratoconic (KC1-5) corneas. Note the decreased staining in the stroma and local increase of signal in epithelium of keratoconic corneas. Scale bar: 10 μm.

pattern) and a gradual anterior-posterior weakening of the signal from moderate to weak were observed in the stroma of KC corneas (Fig. 2).

LOXL3 antibody revealed moderate to intense staining in the epithelium and endothelium of both control and KC samples. In KC samples, we observed a local increase of staining (Fig. 3). The staining of the ECM was moderate and intense for keratocytes in controls but weak or almost absent in most of the KC specimens (Fig. 3).

Using LOXL4 antibody, the epithelium and endothelium of both control and KC samples showed a moderate to intense signal, while a moderate signal with a punctate-like pattern was observed in the stroma (Fig. 4).

All LOX-like enzymes were present in the limbus and conjunctiva of control samples. For LOXL1 we observed intense and for LOXL2, -3 and -4 moderate to intense staining (Fig. 5). LOXL1 and LOXL3 exhibited staining heterogeneities – cells adjacent to the superficial layer of the epithelium showed higher positivity compared to cells located in the deeper layers.

Using Western blot analysis we did not find differences between control and KC samples when staining with anti-LOXL1 antibody. We observed a decrease of LOXL2 and 4 in KC samples compared to controls (Fig. 6), while a slight increase of LOXL3 was observed in KC corneas.

Discussion

In this study we show for the first time the distribution of LOXL1-4 in the normal human cornea, limbus and conjunctiva as well as in corneal buttons obtained from patients with KC.

Previous studies indicated a disorganized collagen fibre network in KC corneas (Radner et al., 1998; Meek et al., 2005) suggesting that these abnormalities may contribute to the mechanical weakness of KC corneas, hence leading to their conical shape. Thinning of the collagen lamellae has been attributed to a decrease in the number of cross-links (bonds between and within

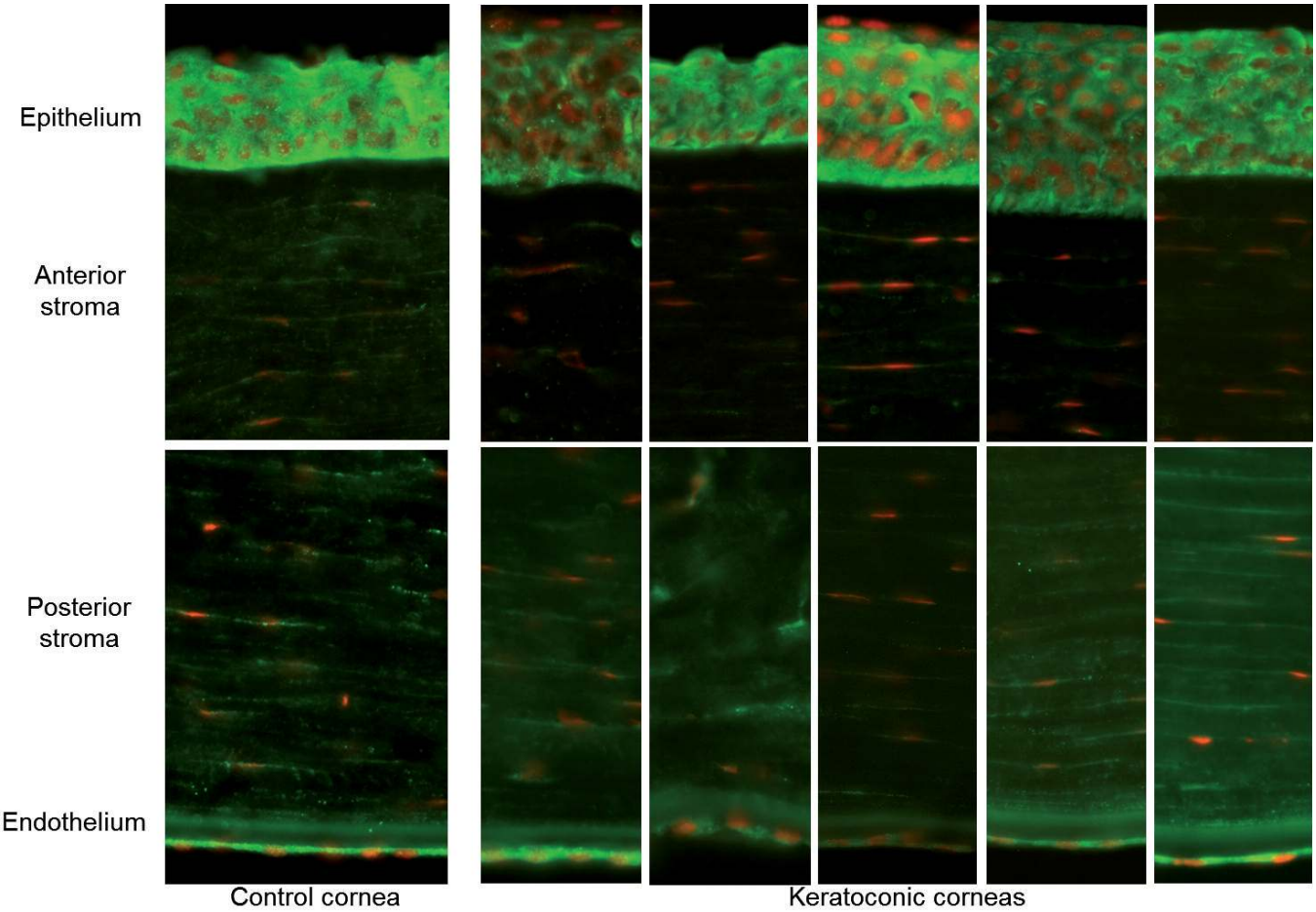

Fig. 4. LOXL4 staining in control and keratoconic (KC1-5) corneas. No prominent differences were observed between control and keratoconic specimens. Scale bar: 10 μm.

collagen fibrils) (Meek et al., 2005).

LOXL1 is important for elastic fibre formation (Liu et al., 2004). Elastin fibres are present mostly in the mid-posterior part of the peripheral human cornea (Kamma-Lorger et al., 2010), while the corneal thinning in KC occurs in the central part. Due to these facts and according to our results, we hypothesize that this enzyme is not directly involved in the disease pathogenesis.

Corneal keratocytes normally remain quiescent but during corneal wound healing they are activated and undergo transformation into corneal fibroblasts and myofibroblasts (West-Mays and Dwivedi, 2006). LOXL2 is abundantly expressed in senescent fibroblasts, cells with limited proliferation (Saito et al., 1997). The decrease of LOXL2 staining in KC corneas compared to controls could be caused by the transformation of keratocytes into myofibroblasts in KC corneas (Maatta et al., 2006; Bystrom et al., 2009; Dudakova et al., 2012). Additionally, we have observed a gradual anterior-posterior weakening of the LOXL2 signal which may be attributed to the fact that keratocytes in the posterior stroma are more likely to be activated (Hindman et al., 2010).

After transformation of keratocytes into myofibroblasts, these cells migrate to the wound site where they increase the synthesis of ECM components, proliferate and acquire contractile properties (West-Mays and Dwivedi, 2006). The increased expression of LOXL2 has been shown in several adherent tumour cell lines, while down-regulation has been observed in several non-adherent tumour cell lines. This suggests that LOXL2 may be involved in cell adhesion and that a loss of this protein may be associated with the loss of cell adhesion (Saito et al., 1997). The observed decrease of LOXL2 staining in KC corneas could enable the migration of activated corneal cells. Barker et al. (2013) have shown that cancer-associated fibroblasts express

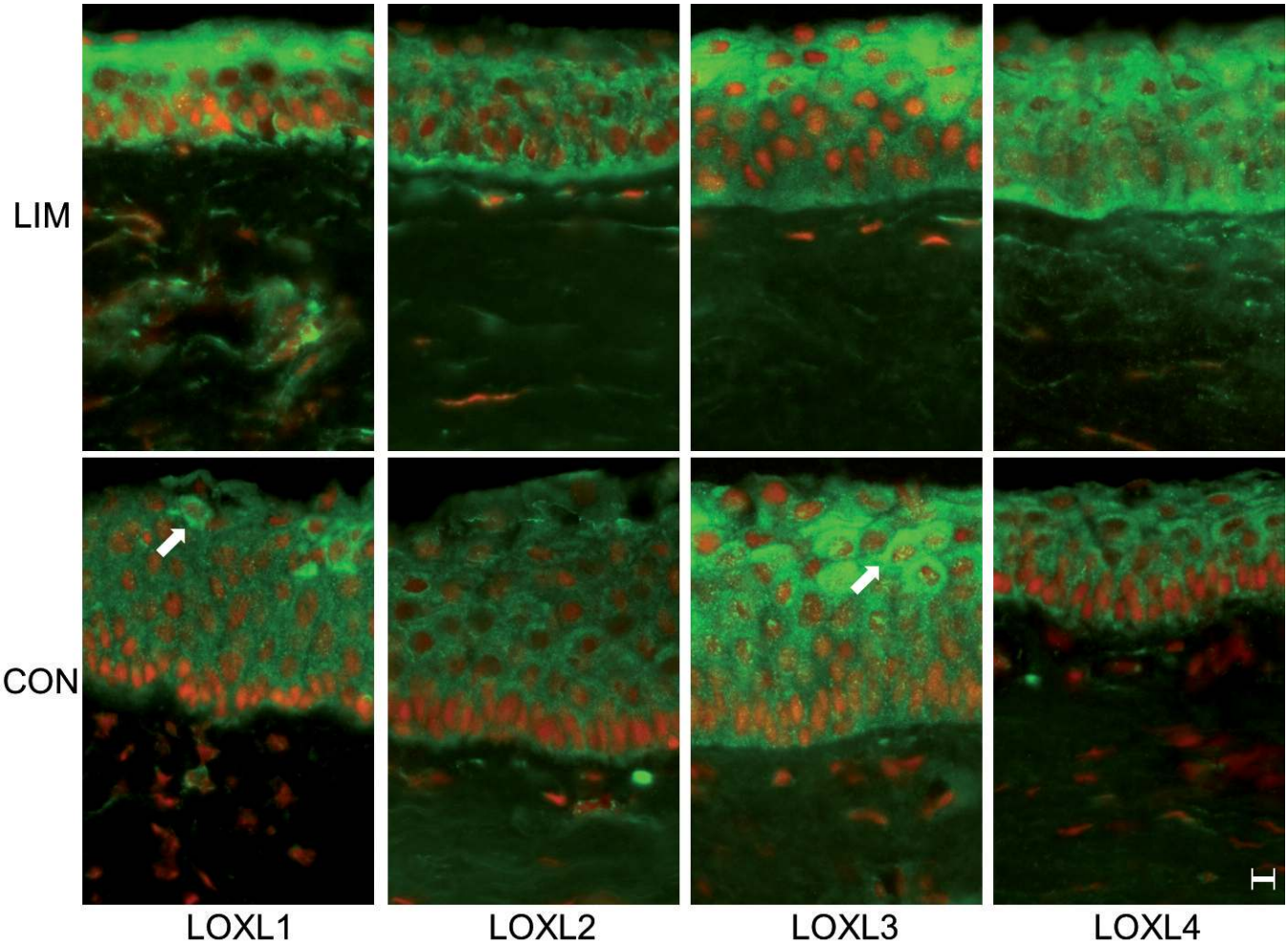

Fig. 5. Indirect fluorescent immunohistochemistry using antibodies against LOXL1-4 in the limbus (LIM) and conjunctiva (CON) of control samples. Note the increased staining of cells in the superficial epithelial layer (arrows). Scale bar: 10 μm.

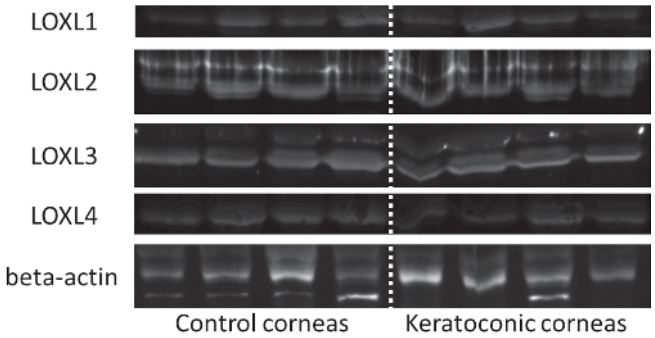

Fig. 6. Western blot analysis of control and keratoconic corneas. Note the slight decrease of the signal for LOXL2 and -4 and increase of LOXL3 in the keratoconic samples.

more LOXL2, further enhancing cancer progression. Treatment with LOXL2-specific inhibitors inhibits cell invasion and metastasis. In tumour cells, deregulation of LOXL2 expression may occur and an increased amount of this protein may lead to the persistent activation of cells in contrast to corneal cells, where after activation, LOXL2 presence diminished.

We presume that the decrease of LOXL2 in KC corneas is more likely a consequence of the associated pathological processes (activation of stromal cells due to tissue weakening and consequent structural changes) than a direct cause leading to KC development.

At this time, we are unable to provide a coherent explanation of the observed changes in LOXL3 and LOXL4 expression in KC corneas compared to the control tissue. The increase of the LOXL3 signal in the Western blot experiment could be caused by the local intensity increase observed in epithelial cells in the IHC experiments. Since LOXL enzymes are expressed in many tissues, it is difficult to investigate the functions and interpret the roles of individual LOXs in cellular processes in these tissues (Molnar et al., 2003). Different expression regulators, alternative splicing, structural and substrate specificities; all of these could contribute to their varied functions and their location in the ECM. A more detailed characterization of LOXL proteins will be necessary in the future to understand the diverse functions of this group of enzymes.

The therapeutic targeting of extracellular proteins is becoming hugely attractive in light of the evidence implicating the tumour microenvironment as pivotal in all aspects of cancer initiation and progression. Secretion of the LOX family members by tumours and their roles in tumorigenesis have been a subject of intense research (Barker et al., 2012). Much attention is focused on LOX and LOXL2 as their increased expression has been observed in aggressive cancers and has shown significant correlation with decreased survival in a number of clinical cancer studies (Barker et al., 2012). Both of these enzymes were found to be decreased in KC corneas (Dudakova et al., 2012 and this study). Therefore, studying the involvement of these enzymes in corneal pathology will help to understand their role in ECM remodelling in a broad context.

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