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Specific Role of Collagen Cross-linking Enzymes (Lysyl Oxidase and Tissue Transglutaminase) in Supramolecular Organization of Matrix in Chicken Embryonic Cornea and Tendon

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Specific Role of Collagen Cross-linking Enzymes (Lysyl Oxidase and Tissue Transglutaminase) in Supramolecular Organization of Matrix in Chicken Embryonic Cornea and Tendon

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ABBREVIATIONS

BMP-1	bone morphogenetic protein-1
BSA	bovine serum albumin
ßAPN	β-aminopropionitrile
cFN	cellular fibronectin
DAPI	4',6-diamidino-2-phenylindole
DEAE	Diethylaminoethyl
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	dimethylsulfoxide
ECM	extracellular matrix
EDTA	ethylendiamine tetra acetic acid
ER	endoplasmic reticulum
FACIT	fibril-associated collagens with interrupted triple helices
FCS	fetal calf serum
FGF-2	basic fibroblast growth factor
FN	fibronectin
GAG	glycosaminoglycan
GTP	guanosine triphosphate
LOX	lysyl oxidase
LOXL	lysyl oxidase like proteins
LRR	leucine-rich repeat
LTQ	lysine tyrosylquinone
MAGP	microfibril-associated glycoprotein
MARCO	macrophage receptors
MEM	minimum essential medium
P4H	prolyl-4-hydroxylase
PBS	phosphate buffered saline
PCP	procollagen C-proteinase
PDI	disulphide isomerase
pFN	plasma fibronectin

PPI	peptidylproline cis-trans isomerase
rhTG	recombinant human tissue transglutaminase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLRP	small leucine-rich proteoglycan
SP	signal peptide
TCA	trichloro acetic acid
TG	transglutaminase
TGF-ß	transforming-Growth-Factor-beta
Tris	tris(hydroxymethyl)aminomethane
tTG or TG2	tissue transglutaminase
VWA	von Willebrand type A repeats

1 ABSTRACT

The fibrillar organizations in cornea and tendon are vastly different although the tissues contain collagens I and V as their major collagenous components. Mature cornea consists of orthogonally stacked lamellae formed by uniformly thin collagen fibrils evenly spaced in parallel. Mature tendons contain fibrils with heterogeneous diameters arranged into parallel fibrous bundles. In both tissues, collagens must be cross-linked to exhibit the normal physical properties. However, they show distinctive patterns of cross-linking chemistry. In the extracellular matrix (ECM), cross-linking can result from the enzymatic activity of lysyl oxidase (LOX) and tissue transglutaminase (TG). In this present study, the relationship between tissue-specific fibril formation and cross-links in the ECM of chicken embryonic cornea and tendon has been investigated.

Here, we have studied the suprastructural organization of matrices deposited by keratocytes and tenocytes cultures with or without cross-link formation by transmission electron microscopy. Fibril diameter distribution has also been analyzed. In addition, *in-vitro* fibrillogenesis with corneal collagen mixtures with and without activated TG were investigated by turbidity measurements and analysis of reconstitution products by electron microscopy. Furthermore, in cell culture experiments LOX-activity was measured by a tritium-release assay and TG-activity by addition of fluorescent-labeled cadaverine. Keratocyte cultures were metabolic labeled with ^{14}C-proline for the characterization of cross-links. Some matrix-involved components were investigated with immunofluorescence labeling.

We found that typical sheets of orthogonally arranged collagen fibrils were formed by keratocyte cell cultures. Such lamellae were not apparent when aldehyde-derived cross-link formation was inhibited. On the other hand, the organization of parallel-arranged fibrils in tenocyte cell cultures also depends on the aldehyde-derived cross-links. These observations suggested that the formation of cross-links is crucial for the tissue-specific matrix organization in chicken cornea and tendon. Further studies on keratocyte cultures showed that the orthogonal arrangement of fibrils was not strongly affected with the TG inhibitor. However, the collagen fibrils formed were thicker with a clearly visible banding pattern. Interestingly, the presence of both

cross-link leads to striking effects on matrix formation visible by loss of matrix organization and an irregular fibril diameter distribution. We also found in our *in-vitro* experiments that fibrils reconstituted from soluble corneal collagens were thinner and formed networks in the presence of activated TG and proteoglycan components. Such networks were absent in controls without TG. We presume that the stabilization of aggregates formed early is essential for the formation of the typical matrix organization of chicken cornea. Moreover, it has been shown that the deposition of decorin into matrix seemed to be delayed when cross-link formation was inhibited.

2 INTRODUCTIONS

2.1 COLLAGEN

Collagens are ECM-components and the most abundant proteins in mammals. Collagens play structural roles and contribute to the molecular architecture, shape, and mechanical properties of tissue such as the tensile strength in skin and the resistance to traction in ligaments (Kadler, 1995, Ricard-Blum et al., 2000). Furthermore, collagens interact with cells through several receptor families and regulate the cell growth, differentiation and migration.

All members of the collagen family comprise three polypeptide α-chains and contain at least one domain composed of repeating Gly-X-Y sequences in each chain. In some collagens all three α-chains are identical (homotrimers) while in other collagens, there are two or three different chains (heterotrimers). Each of the three α-chains within the molecule coiled into a left-handed helix with a pitch of 3 amino acids per turn (Hofmann et al., 1978), are then super coiled around a central axis in a right-handed manner to form a triple helix (Fraser et al., 1979). The smallest amino acid, the glycine residue, in every third position of the polypeptide chains, is a structural prerequisite for the assembly into a triple helix. The X and Y position can be occupied by any amino acid, but often is proline and hydroxyproline, respectively (van der Rest and Garrone, 1991). In the triple-helix structure all glycine residue are positioned in the center while the more bulky side chains of other amino acids occupy the outer positions on the surface of the molecule. This allows a close packaging along the central axis of the molecule, polymerization of collagens per self-assembly into precisely structures. Therefore, the triple helix is rigid and this conformation is important for the biological properties of collagens.

The collagen superfamily comprises 28 different collagen types in vertebrates (I-XXVIII). There are also other proteins containing triple-helical domains such as the subcomponent C1q of complement, mannose-binding protein C, three macrophage receptors (MARCO), acetyl-choline esterase, and three ficolins. Based on their structure and supramolecular organization, collagens can be divided into several subfamilies: fibril-forming collagens, fibril-associated collagens with interrupted triple

helices (FACIT), network-forming collagens, membrane collagens, multiplexing, and others with unique functions.

Collagens I-XXVIII have been reviewed according to α-chains and molecular species (Table. 2-1). The different collagen types are characterized by their structural diversity, variants of non-helical domains, assembly and functions. The domain composition and structural organization of different collagen types are listed in figure 2-1. The fibril-forming collagens are rod-like collagens with a large triple helical domain (approximately 300 nm). These collagens co-assemble into banded fibrils in tissues including bone, dentin, tendons, cartilage, dermis, sclera, cornea, and the interstitial connective tissues in and around many organs (Birk and Bruckner, 2005). Collagen fibrils are made of collagens II, XI and IX in cartilage, of collagens I, III and V in skin (Keene et al., 1997), and of collagens I and V in cornea (Bruckner, 2010). Furthermore, collagen fibrils can be considered as macromolecular alloys of collagens and non-collagenous proteins or proteoglycans. Collagen XXIV is a novel collagen with structural features of invertebrate collagens (Koch et al., 2003). The α1-chain of collagen XXIV contains an amino-terminal domain closely related to those of the types V and XI collagen subunits. Collagen XXVII forms thin nonstriated fibrils (10 nm in diameter) that are distinct from the classical collagen fibrils (Plumb et al., 2007).

The FACITs do not form fibrils by themselves, but they are associated to the surface of collagen fibrils. Collagen IX is covalently linked to the surface of cartilage collagen fibrils mostly containing collagen II (Olsen et al., 1997), and collagens XII and XIV are associated to collagen I-containing fibrils. They can be integral parts and important organizers of the overall fibril structure rather than optional additions to preexisting aggregates (Birk and Bruckner, 2005). Collagen IV, with a more flexible triple helix, is the most important structural component of basement membranes integrating laminins, nidogens and other components into 2-dimensional supramolecular aggregate. The collagen VI microfibril is highly disulfide cross-linked and contributes to a network of beaded filaments interwoven with other collagen fibrils (von der Mark et al., 1984). Collagen X and VIII are both structurally related short-chain collagens and assemble to form hexagonal networks in tissues (Kühn, 1986; Sawada et al., 1990; Yamaguchi et al., 1991). Types XIII, XVII, XXIII, and XXV collagens are transmembrane collagens which contain a single-pass hydrophobic transmembrane

domain (for review see Peltonen et al., 1999; Hashimoto et al., 2002; Banyard et al., 2003; Franzke et al., 2003).

Corresponding to the high diversity of the collagen superfamily, some genetic and acquired diseases of collagens have been discovered. Several autoimmune disorders involve autoantibodies directed against collagens, such as epidermolysis bullosa acquisita (Ishii et al., 2010), skin blistering disease bullous pemphigoid (Franzke et al., 2005), Goodpasture syndrome (Khoshnoodi et al., 2008) and Bronchiolitis obliterans syndrome (Burlingham et al., 2007). Otherwise, many disorders are caused by mutations in the genes coding for collagen α-chains, is listed in the human collagen mutation database (Dalgleish, 1998), COLdb database (Bodian and Klein, 2009) and osteogenesis imperfecte (Marini et al., 2007). The mutations affect the ECM by decreasing the amount of secreted collagen(s), impairing molecular and supramolecular assembly through the secretion of mutant collagens, or by inducing endoplasmic reticulum stress and the unfolded protein response (Bateman et al., 2009).

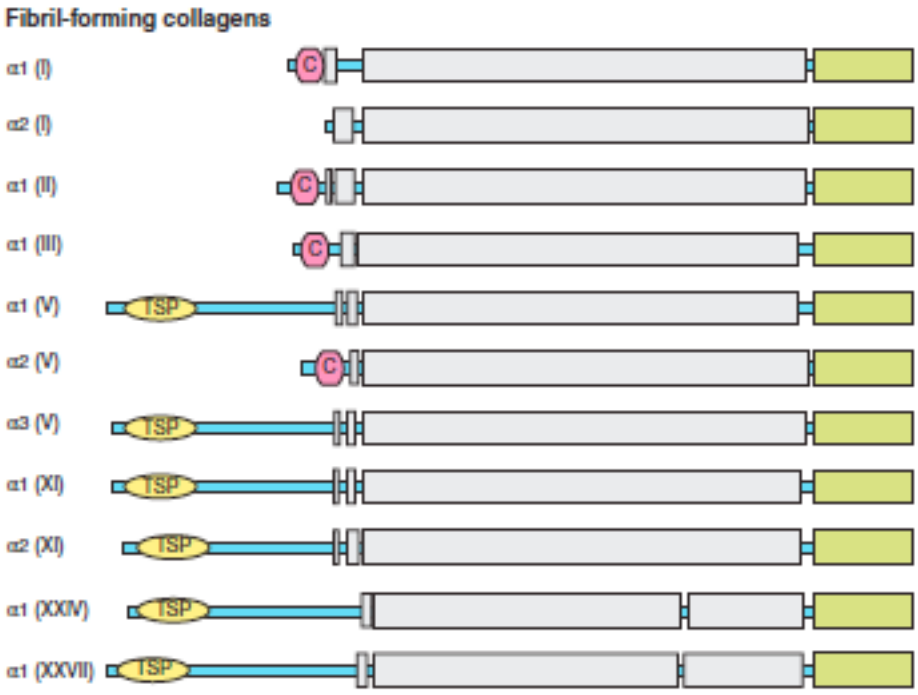
Table 2-1. The collagen family (reviewed by Ricard-Blum, 2011).

Collagen type	A Chains	Molecular species
Collagen I	α1(I), α2(I)	$[\alpha1(I)]_2$, α2(I) $[\alpha1(I)]_3$
Collagen II	α1(II)	$[\alpha1(II)]_3$
Collagen III	α1(III)	$[\alpha1(III)]_3$
Collagen IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), α6(IV)	$[\alpha1(IV)]_2$, α2(IV) α3(IV)α4(IV)α5(IV) $[\alpha5(IV)]_2$, α6(IV)
Collagen V	α1(V), α2(V), α3(V), α4(V)[a]	$[\alpha1(V)]_2$, α2(V) $[\alpha1(V)]_3$ $[\alpha1(V)]_2$, α4(V) α1(XI)α1(V)α3(XI)
Collagen VI	α1(VI), α2(VI), α3(VI), α4(VI)[b], α5(VI)[c], α6(VI)	
Collagen VII	α1(VII)	$[\alpha1(VII)]_3$
Collagen VIII	α1(VIII)	$[\alpha1(VIII)]_2$, α2(VIII) α1(VIII), $[\alpha2(VIII)]_2$ $[\alpha1(VIII)]_3$ $[\alpha2(VIII)]_3$
Collagen IX[e]	α1(IX), α2(IX), α3(IX)	α1(IX), α2(IX), α3(IX)
Collagen X	α1(X)	$[\alpha1(X)]_3$
Collagen XI	α1(XI), α2(XI), α3(XI)[d]	α1(XI)α2(XI)α3(XI) α1(XI)α1(V)α3(XI)

Collagen XII[e]	α1(XII)	$[α1(XII)]_3$
Collagen XIII	α1(XIII)	$[α1(XIII)]_3$
Collagen XIV[e]	α1(XIV)	$[α1(XIV)]_3$
Collagen XV	α1(XV)	$[α1(XV)]_3$
Collagen XVI[e]	α1(XVI)	$[α1(XVI)]_3$
Collagen XVII	α1(XVII)	$[α1(XVII)]_3$
Collagen XVIII	α1(XVIII)	$[α1(XVIII)]_3$
Collagen XIX[e]	α1(XIX)	$[α1(XIX)]_3$
Collagen XX[e]	α1(XX)	$[α1(XX)]_3$
Collagen XXI[e]	α1(XXI)	$[α1(XXI)]_3$
Collagen XXII[e]	α1(XXII)	$[α1(XXII)]_3$
Collagen XXIII	α1(XXIII)	$[α1(XXIII)]_3$
Collagen XXIV	α1(XXIV)	$[α1(XXIV)]_3$
Collagen XXV	α1(XXV)	$[α1(XXV)]_3$
Collagen XXVI	α1(XXVI)	$[α1(XXVI)]_3$
Collagen XXVII	α1(XXVII)	$[α1(XXVII)]_3$
Collagen XXVIII	α1(XXVIII)	$[α1(XXVIII)]_3$

Individual α chains, molecular species, and supramolecular assemblies of collagen types.

[a]The α4(V) chain is solely synthesized by Schwann cells; [b]The α4(VI) chain does not exist in humans; [c]The α5(VI) has been designated as α1(XXIX); [d]The α3(XI) chain has the same sequence as the α1(II) chain but differs in its posttranslational processing and cross-linking; [e]FACIT, Fibril-Associated Collagens with Interrupted Triple helices.



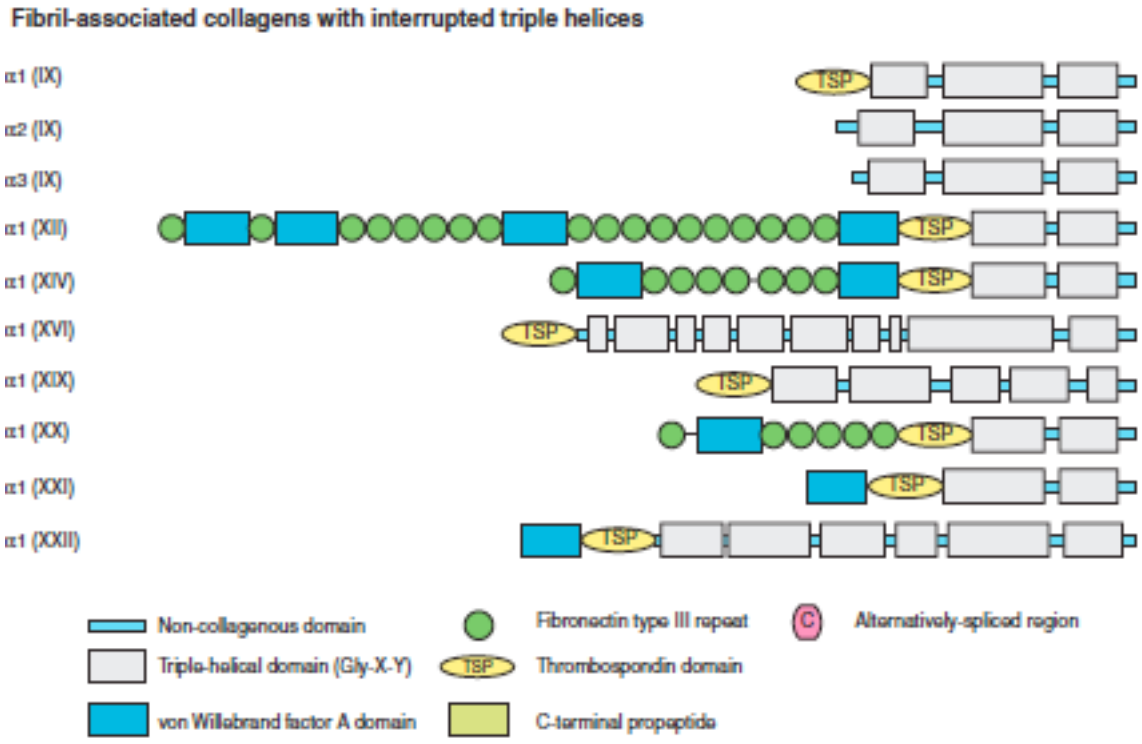


Figure 2-1. Domain composition and supramolecular assemblies of collagens. COL: Collagenous, triple-helical, domains; NC: Non-collagenous, nontriple-helical, domains. They are numbered from the carboxy- to the amino-terminus, except for collagen VII (reviewed by Ricard-Blum, 2011).

2.2 FIBRIL-FORMING COLLAGENS AND FIBRIL FORMATION

The classical fibril-forming collagens include collagen types I, II, III, V, and XI. Procollagen chains are synthesized in the endoplasmic reticulum (ER), are brought together by interactions between the C-propeptides and fold to form a rod-like triple-helical domain flanked by globular N- and C-propeptides. The C-propeptides play an important role in procollagen folding because they ensure association between monomeric procollagen chains and determine chain selectivity (Bulleid et al., 1997; Lees et al., 1997). Association between procollagen chains is preceeded by folding and disulphide bond formation within the individual C-propeptides (Doege and Fessler, 1986). A large number of post-translational modifications then occur in the ER, in which a number of enzymes and molecular chaperones assist the folding and trimerization of procollagen, such as the protein disulphide isomerase (PDI) (Bulleid et al., 1996), peptidylproline cis-trans isomerase (PPI) (Galat et al., 1995; Bachinger et al., 1987; Davis et al., 1989), prolyl-4-hydroxylase (P4H) (Kivirikko and Myllyharju, 1998; Myllyharju, 2003), prolyl-3-hydroxylase (Vranka et al., 2004), a family of lysyl hydroxylases (Kellokumpu et al., 1994; Valtavaara et al., 1998; Wang et al., 2002) and the collagen-specific chaperone HSP47 (Nagata, 1998 and 2003). Removal of

the N- and C-propeptides from fully folded procollagen by the procollagen N- and C-proteinases (Leung et al., 1979) occurs partly after transport of procollagen across the Golgi stacks in ECM and partly inside tendon fibroblasts (Canty et al., 2004). The resulting collagen molecules are then able to assemble into fibrils. Covalent crosslinks occur within and between triple-helical collagen molecules in fibrils. An overview of the fibril formation by fibroblasts was in figure 2-2 illustrated.

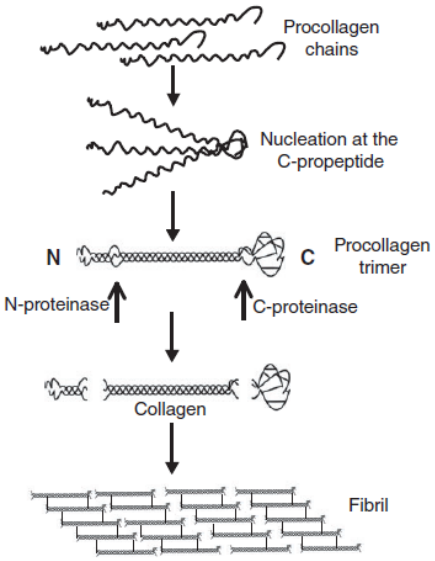


Figure 2-2. Overview of the steps involved in the production of collagen fibrils by fibroblasts (Canty and Kadler, 2005).

Collagen molecules are arranged in longitudinally staggered arrays resulting a tissue-specific length of staggers between adjacent fibrillar collagen molecules. Therefore, a gap occurs sequentially between neighboring molecules giving rise to a gap-overlap structure in the collagen fibrils to represent a banding pattern with a periodicity (D-period) of 64-67 nm (Birk and Bruckner, 2005; Bruckner, 2010). Collagen fibrils range in diameter from approximately 15 nm up to 500 nm or more depending on the tissue type (Kadler et al., 2007; Bruckner, 2010). The structure of a generic collagen fibril is illustrated in figure 2-3.

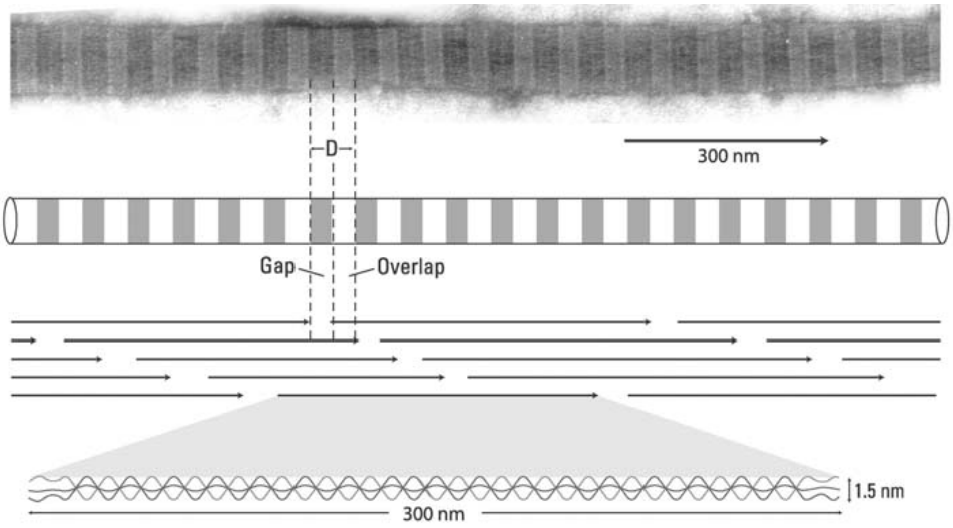


Figure 2-3. Structure of a generic collagen fibril. A D-periodic collagen fibril from tendon is presented at the top of the panel. The negative stained fibril has a characteristic alternating light/dark pattern representing the gap (dark) and overlap (light) regions of the fibril. The diagram represents the staggered pattern of collagen molecules giving rise to this D-periodic repeat. The collagen molecules (arrows) are staggered N to C. The fibrillar collagen molecule is approximately 300 nm (4.4 D) in length and 1.5 nm in diameter (Birk and Bruckner, 2005).

2.3 MOLECULAR CONSTITUENTS OF CORNEA

2.3.1 Collagens

2.3.1.1 Type I collagen

Type I collagen is the most abundant fibrillar collagen. It forms more than 90% of the organic mass of bone and is the major collagen of tendons, skin, ligaments, cornea, and many interstitial connective tissues with the exception of very few tissues such as hyaline cartilage, brain, and vitreous body. The collagen I triple helix is usually formed as a heterotrimer $[\alpha1(I)]_2\alpha2(I)$. *In vivo* the triple helix are mostly incorporated into composite fibres containing either type III collagen in skin and reticular fibres (Fleischmajer et al., 1990) or type V collagen in cornea, bone and tendon (Niyibizi et al., 1989). In most organs collagen I provides tensile stiffness and load bearing. However, the homotrimers consisting of three α1(I) chains, $[\alpha1(I)_3]$ (Bornstein and Sage, 1980; Deak et al., 1983; Kadler et al., 2007; Kielty et al., 1993) have been shown to be present during embryogenesis (Jimenez et al., 1977), in tumors (Moro and Smith, 1977; Rupard et al., 1988; Makareeva et al., 2010), fibrotic tissues (Rojkind et al., 1979; Narayanan et al., 1980; Ehrlich et al., 1982; Philips et al., 2002) and in stressed mesangial cells (Haralson et al., 1987). Recent finding revealed that the type I collagen homotrimers are much more resistant to degradation by matrix

metalloproteinase (MMPs) than the heterotrimers (Makareeva et al., 2010; Han et al., 2010).

2.3.1.2 Type V collagen

Type V collagen is a quantitatively minor fibrillar collagen present in tissues where type I collagen is expressed. There are several type V collagen isoforms that differ in chain composition. The most common isoform found in cornea is $[\alpha1(V)]_2\alpha2(V)$ (Birk et al., 1988; Birk and Linsenmayer, 1994). The native, completely-processed type V collagen molecule, as extracted from tissues, retains a large pepsin-sensitive, globular domain at the NH_2-terminal end of its α1(V) chain (Bächinger et al., 1982; Broek et al., 1985; Fessler et al., 1985, 1987; Kumamoto et al; 1981). When co-assembled along with type I collagen into heterotypic fibrils, collagen V serves to regulate fibril diameter via partially processed NH_2-terminal globular sequences. Because of the high content (15-20% of the fibrillar collagens) of corneal collagen fibrils, collagen V is considered to be responsible for the thin, uniform diameter (Birk et al., 1990; Marchant et al., 1996). However, an $[\alpha1(V)]_3$ homotrimer as well as an [α1(V)α2(V)α3(V)] form have been reported in human placenta (Sage and Bornstein, 1979; Madri et al., 1982; Niyibizi et al., 1984). Type V collagen α chains also form heterotypic molecules with type XI collagen α chains. The α1(V) and α2(V) has been detected additionally to α1(XI) in bovine bone (Niyibizi and Eyre, 1989), whereas, in the bovine vitreous, the α2(V) coexist with the α1(XI) chain (Mayne et al., 1993). The formation of heterotypic $[\alpha1(XI)]_2\alpha2(V)$ molecules were demonstrated to occur in the human rhabdomyosarcoma cell line A204 (Kleman et al., 1992). A novel α4(V) chain is solely synthesized by Schwann cells and the α4(V)-collagen exhibits particular high affinity for the heparan sulfate transmembrane proteoglycan syndecan-3 via a heparin binding site located in the variable region of the N-propeptide (Mechanic et al., 1987).

2.3.1.3 Type VI collagen

Type VI collagen is a nonfibrillar collagen present as a network throughout many tissues including the chick embryo secondary corneal stroma. It is initially defined as a large collagenous glycoprotein composed of three different α-chains, the α1(VI), α2(VI), and α3(VI) chains (Kielty et al., 2002; Chu et al., 1987). Collagen VI monomers have a relatively short triple helical domain flanked by N- and C-terminal

globular domains which contain varying numbers of “von Willebrand type A repeats” (VWA). The structural heterogeneity of collagen VI is introduced by alternative splicing of domains, primarily of the α3(VI) N-terminal domain containing a maximum of ten type A repeats. It is now known that three additional chains, α4(VI),α5(VI),α6(VI), exist in mouse. All three are similar to the α3(VI) chain. Orthologs of α5(VI) and α6(VI) have been found in human (Fitzgerald et al., 2008; Gara et al., 2008). The α4 chain forms trimers far more frequently than the α5 and α6 chains in mouse whereas it does not exist in humans. In human the α5 is equivalent to the α4 in mouse (Gordon, 2010).

Collagen VI has a ubiquitous distribution in connective tissues. The assembly of collagen VI monomers is initiated in the lumen of intracellular compartments. Tetramers are secreted and are the building blocks that assemble extracellularly into the tissue forms of type VI collagen. In the ECM, tetramers associate end-to-end forming beaded filaments, which laterally associate to form beaded microfibrils (Bruns et al., 1986; Furthmayr et al., 1983; Baldock et al., 2003).

Collagen VI interacts with a large number of ECM molecules including collagen I, II, IV, XIV, microfibril-associated glycoprotein (MAGP-1), perlecan, decorin, biglycan, hyaluronan, heparin and fibronectin as well as integrins and the cell-surface proteoglycan NG2. Supramolecular aggregates of collagen VI are composite structures with other integrated molecules modulating the functional properties of the collagen VI-containing suprastructure (Birk and Bruckner, 2005). Studies on corneal fibroblasts showed that collagen VI is associated with cells and between striated fibrils forming a network around fibrils and close to corneal fibroblasts (Doane et al., 1992). It was suggested that type VI collagen plays a role in cell-matrix interactions during corneal stroma development.

2.3.2 Keratin

Keratins are a group of water-insoluble proteins that form 10 nm intermediate filaments in all epithelial cells. Approximately 30 different keratin molecules have been identified (Moll et al., 1982; Cooper et al., 1996), with a range of molecular masses between 40 and 80 kDa (Franke et al., 1981). The keratins can be divided into two subfamilies: the acidic or type I and the basic or type II (Schiller et al., 1982).

Each acidic keratin is preferentially co-expressed with a particular basic keratin, forming a keratin pair. Different pairs are expressed in a tissue-specific, differentiation-related and developmentally regulated fashion (Kurpakus et al., 1992; Liu et al., 1993; Moll et al., 1982; Fuchs et al., 1978). Expression of the keratin 3/keratin 12 pair has been found in human, bovine, guinea pig, rabbit, and chicken cornea and is regarded as a biochemical marker for an advanced stage of "corneal-type" epithelial differentiation (Cooper et al., 1996; Chaloin-Dufau et al., 1990; Schermer et al., 1986; Sun et al., 1984).

2.3.3 Fibronectin

Fibronectin (FN) is a ubiquitous component of the ECM and has been well characterized as an ECM glycoprotein that can regulate many cellular functions such as proliferation, differentiation, migration, adhesion and apoptosis (Pankov and Yamada, 2002; Magnusson and Mosher, 1998; Wierzbicka-Patynowski and Schwarzbauer, 2003; Mosher, 1989; Hynes, 1990). FN is secreted as a large dimeric glycoprotein with subunits that range in size from 230-270 kDa depending primarily on alternative splicing (Hynes, 1990; Mosher, 1989). It was categorized as either soluble plasma FN (pFN) or insoluble cellular FN (cFN) (Pankow and Yamada, 2002; ffrench-Constant, 1995; Kosmehl et al., 1996). The cellular FN is the major form and abundant in the fibrillar matrices of most tissues. FN is composed of three different types of modules termed type I, II and III repeats (Petersen et al., 1983; Hynes, 1990). The domain organization and isoforms of FN were reviewed in Figure 2-4.

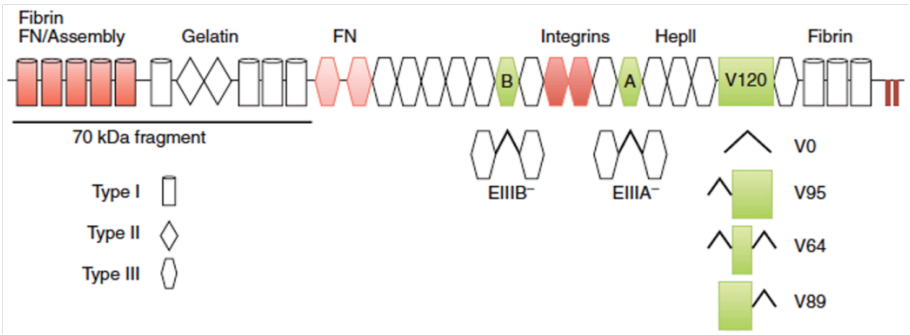


Figure 2-4. Fibronectin (FN) domain organization and isoforms. Each FN monomer has a modular structure consisting of 12 type I repeats (cylinders), 2 type II repeats (diamonds), and 15 constitutive type III repeats (hexagons). Two additional type III repeats (EIIIA and EIIIB, green) are included or omitted by alternative splicing. The third region of alternative splicing, the Vregion (green box), is included (V120), excluded (V0), or partially included (V95, V64, V89). Sets of modules comprise domains for binding to other extracellular

molecules as indicated. Domains that are required for fibrillogenesis in red: the assembly domain (repeats I_{1-5}) binds FN, III_{9-10} contains the RGD and synergy sequences for integrin binding, and the carboxy-terminal cysteines for the disulfide bonded FN dimer (▮▮). The III_{1-2} domain (light red) has two FN binding sites that are important for fibrillogenesis. The amino-terminal 70 kDa fragment contains assembly and gelatin-binding domains and is routinely used in FN binding and matrix assembly studies (Schwarzbauer and DeSimone, 2011).

FN accomplishes diverse functions through interactions with a large variety of proteins including collagens, fibrin, cell surface integrins, heparan sulfate proteoglycans, and tenascin-C. In tissues, activated FN is deposited and organized into a polymeric matrix which is associated with collagen fibers. FN matrix was proposed to act as a scaffold to regulate the deposition and assembly of type I and III collagens (Velling et al., 2002; Sottile et al., 2002). In the context of the findings of the most performed in this PhD-project, the following observations are of particular interest. It has been demonstrated previously that lysyl oxidase (LOX) has a high binding affinity for cellular FN. FN acts as a scaffold for enzymatically active 30-kDa LOX. The co-localization of LOX and FN in cultured fibroblasts and normal human tissues has been revealed. Additionally, the proteolytic activation of LOX is much reduced in cultures of FN-null mouse embryo fibroblasts (Fogelgren et al., 2005). A recent study showed that FN binds bone morphogenetic protein-1 like (BMP1-like) proteinases *in vivo* and it is an important determinant of the *in vivo* activity levels of BMP1-like proteinases (Huang et al., 2009). This conclusion implies a crucial regular function of FN on LOX activity because BMP1 is required for LOX activation. As fibrin polymerizes, factor XIII transglutaminase covalently cross-links glutamine residues near the amino terminus of FN to fibrin α chains (Corbett et al., 1997; Mosher, 1975). The gelatin/collagen-binding domain, which composed of type I and II modules, binds to tissue transglutaminase (Radek et al., 1993) and fibrillin-1 (Sabatier et al., 2009).

2.3.4 Decorin

Decorin, a member of a family of proteins with leucine-rich repeat (LRR) motifs, is a ubiquitous small ECM proteoglycan. It is composed of a ~40 kDa core protein containing 10 leucine-rich-repeats flanked by cysteine loops on both sides and contains N-linked glycosylation sites (Krusius and Ruoslahti, 1986) (Fig. 2-5). Furthermore, at the N-terminus decorin is covalently linked with a glycosaminoglycan (GAG) chain of chondroitin/dermatan sulfate type depending on the tissue in which it is expressed (Seidler and Dreier, 2008).

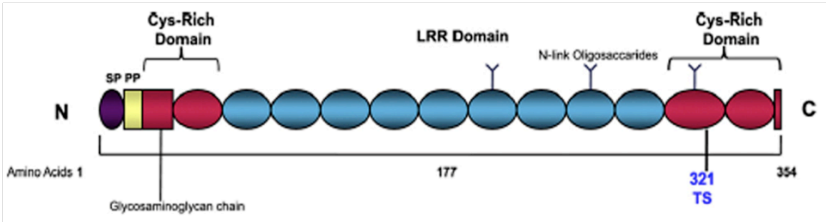


Figure 2-5. Domain structure of the decorin protein core. From N- to C-terminus: signal peptide (SP); propeptide (PP); the glycosaminoglycan attachment site at a serine residue in the N-terminal Cys-rich domain; central LRR repeats; C-terminal Cys-rich domain. There are 3 N-linked oligosaccharide attachment sites in the LRR domains (Chen and Birk, 2011).

Decorin is thought to be responsible for the structure, tissue organization and surface properties of fibrils. It was shown to associate with collagen fibrils in a number of connective tissues (Pringle et al., 1990; Fleischmajer et al., 1991; Scott et al., 1992). It was given the name decorin since it decorates the surface of fibrils at the “d” and “e” bands with its GAGs extending into the interfibrillar space (Pringle and Dodd, 1990). Decorin binds to collagens I and II via its core protein and inhibits collagen fibril formation *in vitro* (Vogel et al., 1984; Hedbom et al., 1989). The influence of decorin on collagen I fibrillogenesis *in vitro* was assigned to a delayed initial assembly of collagen molecules and a decreased fibril diameter (Vogel and Trotter, 1987). This interaction occurs mainly via the leucine-rich repeats 4-5 of the decorin core protein (Svensson el al., 1995). Furthermore, decorin bridges type VI collagen filaments to fibrils (Bidanset et al., 1992) and also acts as a bridging molecule between type I and type VI collagen, interacting with them via different binding sites (Nareyeck et al., 2004). In addition, decorin interacts with collagen types XII and XIV through its GAG chains and mediates the interaction of tenascin with collagen (Font et al., 1993, 1996). All of these interactions are possibly involved in the corneal stroma organization. In addition to the closely related class I small leucine-rich proteoglycan (SLRP), biglycan, data indicate that decorin is the dominant corneal class I SLRP and a major regulator of corneal fibrillogenesis (Zhang el al., 2009).

Decorin has also multiple non-structural functions: it binds to growth factors such as TGF-ß and FGF-2, and sequesters them in the ECM or presents them to receptors. It also modulates the TGF-ß signal pathway through the LRP1 receptor, inhibiting cell growth and increasing ECM deposition (Winnemoller et al., 1992). Moreover, decorin binds fibronectin and thrombospondin modulating cell adhesion and migration (Hildebrand et al., 1994; Iozzo et al., 1999). Also, decorin can modulates cell

immunity via binding of the complement, factor C1q, scavenger receptor-A or surfactant-associated protein-D. These interactions are consistent with decorin´s involvement in diverse pathological processes (Schaefer and Iozzo, 2008).

2.3.5 Hydroxyproline

Hydroxyproline is a non-proteinogenic amino acid, which is produced by post-translational hydroxylation of the amino acid proline. In contrast to other mammalian proteins, collagen contains a high concentration of hydroxyproline, which is thought to be confined exclusively to the connective tissue seleroproteins, collagen and elastin (Gross and Piez, 1960). Therefore, hydroxyproline has been widely used as an indicator of both the presence and the metabolism of collagen.

2.4 COLLAGEN CROSS-LINKING

Collagens must be cross-linked to exhibit the normal physical properties of tensile strength and different tissue types showed distinctive pattern of cross-linking chemistry. ECM cross-linking can result from the enzymatic activity of lysyl oxidase, tissue transglutaminase (Aeschlimann and Paulsson, 1991), or nonenzymatic glycation (Girton et al., 1999).

2.4.1 Lysyl oxidase

Lysyl oxidase (LOX) is a copper-dependent amine oxidase. It is responsible for the oxidative deamination of key lysine residues in collagen and elastin prior to cross-link formation, and plays a pivotal role in the formation of a stable, insoluble ECM. LOX catalyzes the formation of ε-aldehydes in collagen and elastin from certain lysyl and hydroxylysyl residues (Siegel and Martin, 1970; Rodriguez et al., 2008) (Figure 2-6). These aldehydes, allysine and hydroxyallysine, are intermediates in formation of intra- and intermolecular cross-links. The LOX-catalyzed crosslinks occur in various connective tissues within the body, including bone, cartilage, skin, lung etc., and are believed to be a major source of mechanical strength in tissues. LOX is secreted from fibrogenic cells as a 50 kDa, N-glycosylated pro-enzyme, and is processed in the extracellular environment to produce the active, mature 32-kDa enzyme and an 18 kD propeptide (Trackman et al., 1992). The pathway of LOX synthesis and its intra- and extracellular functions are illustrated in figure 2-7. The sequence of the proteolytic processing site in pro-lysyl oxidase resembles that of the fibrillar

procollagen C-terminal pro-peptide processing sites cleaved by procollagen C-proteinase (PCP) (Cronshaw et. al., 1995), whose activity is provided by products of the bmp1 (Bone Morphogenetic Protein 1) gene (Kessler et al., 1996). Moreover, preparations highly enriched in PCP activity have been shown to process pro-lysyl oxidase at the correct physiological site (Panchenko et al., 1996).

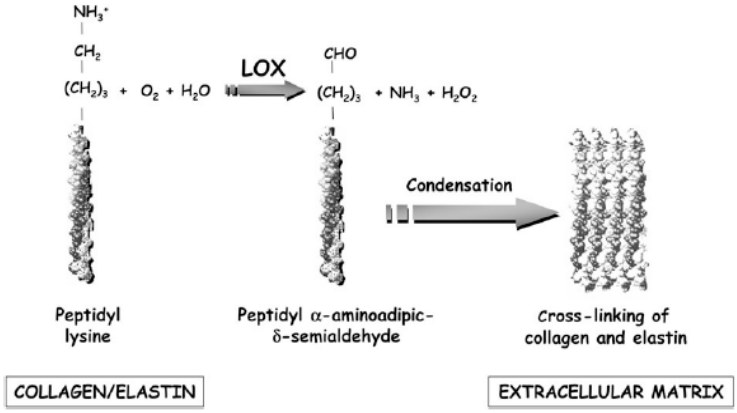


Figure 2-6. Reaction catalysed by lysyl oxidase (LOX). LOX oxidizes primary amines on collagen and elastin substrates to reactive semialdehydes that condense to form covalent cross-linkages (Rodriguez et al., 2008).

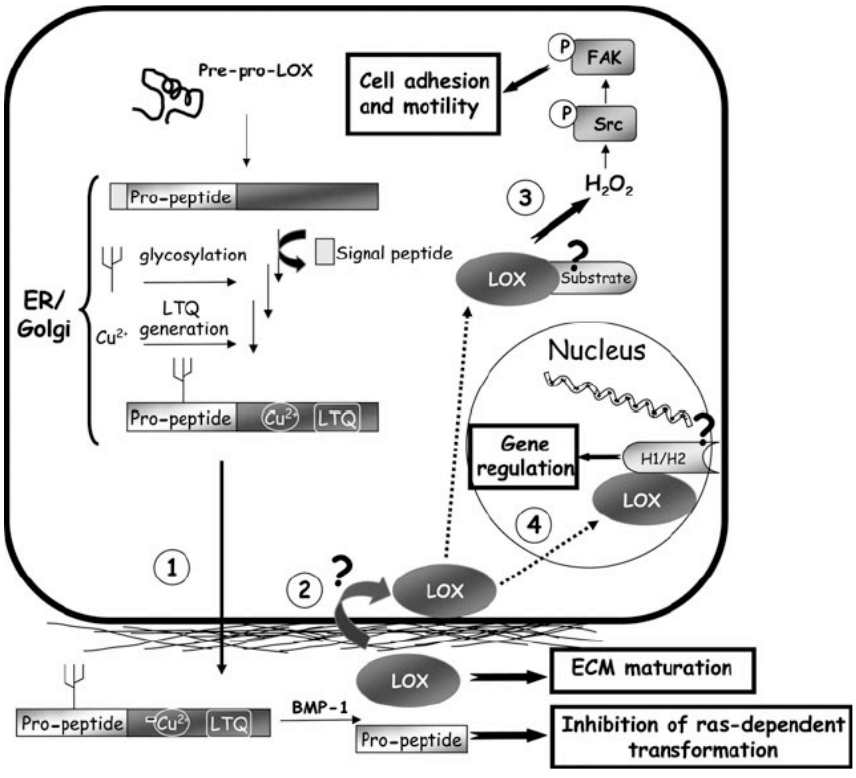


Figure 2-7. Lysyl oxidase (LOX) synthesis and intra- and extracellular functions. LOX is synthesized as a pre-protein, and after signal peptide hydrolysis, enzyme glycosylation,

copper incorporation, and lysine tyrosylquinone (LTQ) generation, the enzyme is released into the extracellular space (1). Then, BMP-1 processes LOX yielding the mature LOX form and its pro-peptide. Whereas LOX participates in ECM stabilization, its propeptide induces phenotypic reversion of ras-transformed cells. Extracellular LOX could translocate from the extracellular environment to intracellular compartments (2). In tumour breast cells, cytosolic LOX controls cell adhesion and motility (3). Nuclear forms of lysyl oxidase control gene expression (4). Histones H1 and H2 could be nuclear substrates of this enzyme (Rodriguez et al., 2008).

Transforming growth factor-ß (TGF-ß), platelet-derived growth factor, angiotensin II, retinoic acid, fibroblast growth factor, altered serum conditions, and shear stress is among the effectors or conditions that regulate LOX expression. Besides its role in connective tissues, LOX enzyme promotes growth, migration, invasion and metastasis of tumor cells, especially in response to hypoxia (Erler et al., 2006). Moreover, LOX has also been involved in non-tumoral processes, including the control of cell adhesion, gene regulation (Giampuzzi et al., 2003) and differentiation, within various cell types such as osteoblasts, adipocytes and keratinocytes (Huang et al., 2009).

The lysyl oxidase gene family comprises five members acting as extracellular modulating enzymes: LOX, LOXL1, LOXL2, LOXL3 and LOXL4. The LOXL1-4 (lysyl oxidase like proteins) have significant sequence identity with mature LOX, but have no similarity to the propeptide region of LOX (Csiszar et al., 2001). The first identified isoform is LOX. LOX activity modulation induces multiple effects on the structure and major characteristics of the ECM. For example, LOX is essential in maintaining the characteristics of blood vessels and arteries (Rodriguez et al., 2002). The down-regulation of LOX is correlated to many connective tissue disorders seen in Ehler-Danlos syndrome (Kuivaniemi et al., 1985), cutis laxa (Khakoo et al., 1997), and Menke´s syndrome (Pinnel et al., 1982). In tumors, LOX up-regulation is found in the stromal reaction observed around tumor foci in ductal breast carcinomas and in bronchopulmonary carcinomas (Peyrol et al., 2000). LOX-null animals die late in gestation or soon after birth and exhibit a wide range of connective tissue abnormalities (Maki et al., 2002; Hornstra et al., 2003).

LOX activity was originally detected by a tritium release assay in which chick aorta elastin labeled with [6-^{3}H]lysine in organ culture was used as a substrate (Pinnell and Martin, 1968) (Fig. 2-8). Tritium was released as aldehydes formed under the

influence of LOX. This reaction was inhibited by micromolar concentrations of β-aminopropionitrile (ßAPN). It has been mentioned that LOX activity with collagen substrates was significantly increased when collagen was precipitated from solution as reconstituted fibrils (Siegel et al., 1974). It also has been shown that the enzyme binds to the triple-helical portion of collagen melecules and binds predominantly to the fibrillar surface. Other data indicated that LOX initiates crosslink formation at an early stage in collagen fibrillogenesis (Cronlund et al., 1985).

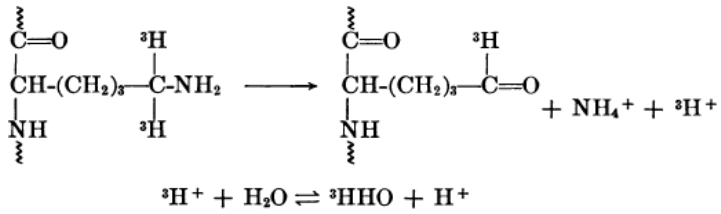


Figure 2-8. Measurement of LOX activity: release of tritium during allysine formation (Pinnell and Martin, 1968).

The β-aminopropionitrile (ßAPN) (Fig. 2-9) was initially found and identified in *Lathyrus odoratus* peas and it caused osteolathyrism in sheep (Geiger et al., 1933; McKay et al., 1954). In 1961 it was understood that ßAPN inhibited collagen and elastin cross-linking in some way (Martin et al., 1961); and Pinnell and Martin later reported that ßAPN inhibited an enzyme that catalyzed oxidative deamination of lysine residues in elastin, and named it lysyl oxidase (Pinnell and Martin, 1968).

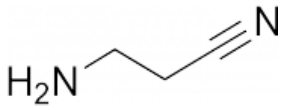


Figure 2-9. Chemical structure of β-aminopropionitrile.

2.4.2 Tissue transglutaminase

Tissue transglutaminase (TG), also known as TG2, belongs to a family of structurally and functionally related enzymes (Aeschlimann et al., 1994) which catalyze Ca^{2+}-dependent acyl-transfer reactions between the γ-carboxamide group of specific peptide-bound glutamine residues and primary amines, including the ε-amino group of peptide-bound lysine. These reactions lead to the formation of the γ-glutamyl-ε-lysine cross-link which is stable and resistant to proteolysis, thereby increasing the

resistance of tissue to chemical, enzymatic and mechanical disruption. TG catalyzed cross-linking is a physiological mechanism for the stabilization of basement membranes and cartilage matrix (Aeschlimann et al., 1994; 1995). Several collagen types (II, V/XI, VII, the N-propeptide of procollagen III) and a number of other extracellular proteins (fibronectin, nidogen, osteonectin, osteopontin, vitronectin and microfibril associated glycoprotein) are glutaminyl substrates of TG *in vitro* and/or *in vivo* (reviewed in Aeschlimann et al., 1994; Brown-Augsburger et al., 1994; Kleman et al., 1995; Raghunath et al., 1996). There are currently eight described transglutaminase enzymes in mammals, all of them require Ca^{2+} for activity, some also require proteolytic cleavage of propeptides, and three of them (TG2, TG3 and TG5) are inhibited by GTP (R.J. Collighan and M. Griffin, 2009). In vertebrates nine evolutionary related genes have evolved: encoding blood coagulation Factor XIII-a, TG1-7 and the inactive epb42 (Grenard et al., 2001). TG2 is predominantly a cytoplasmic protein, but it is also found in the nucleus and mitochondria, on the plasma membrane and the extracellular cell surface, and in the ECM (Park et al., 2010).

Due to low-GTP and high-calcium concentrations in the extracellular space, it is conceivable that extracellular TG2 is an active TGase. Data suggest that the TGase activity is involved in remodeling of the ECM under normal and pathological circumstances. Besides its classical protein cross-linking activity, TG2 possesses several other biochemical functions at various cellular locations (Fig. 2-10). It can function as a G protein (high-molecular weight GTP-binding protein) (Nakaoka et al., 1994), also has protein disulfide isomerase (PDI) (Hasegawa et al., 2003), protein kinase (Mishra et al. 2007) and DNA nuclease activities (Takeuchi et al., 1998). This multifunctional protein is expressed ubiquitously and abundantly, and is implicated in a variety of cellular processes, such as differentiation, cell death, inflammation, cell migration, and wound healing (reviewed in Fesus and Piacentini, 2002; Lorand and Graham, 2003; Fesus and Szondy, 2005; Ientile et al., 2007; Collighan and Griffin, 2009; Sarang et al., 2009). Beyond its functional diversity, TG2 has both pro-and anti-apoptotic functions (Fesus and Szondy, 2005). Although it has been initially studied as an intracellular enzyme, TG2 is secreted into the extracellular space or onto the cell surface (Verderio et al., 1998; Gaudry et al., 1999). Cell-surface TG2 is proposed to be an important tissue-stabilizing enzyme that is active during wound

healing (Haroon et al., 1999). Cell surface TG2 is also involved in cell adhesion via its tight interaction with fibronectin and the resultant intracellular signaling effects are reported to be mediated by various β1 and β3 integrins (Gaudry et al., 1999). Since the assembly of fibronectin is the initiator for a number of other ECM structures such as fibrillin1, TG2 has the potential to play a major role in initial assembly of the ECM, not just in modification of the existing ECM. TG2, once deposited into the matrix and in complex with fibronectin, can also bind to the heparan sulphate chains of syndecan 4 on the cell surface (Verderio et al., 2003). Therefore, TG2 may mediate cell-ECM interactions.

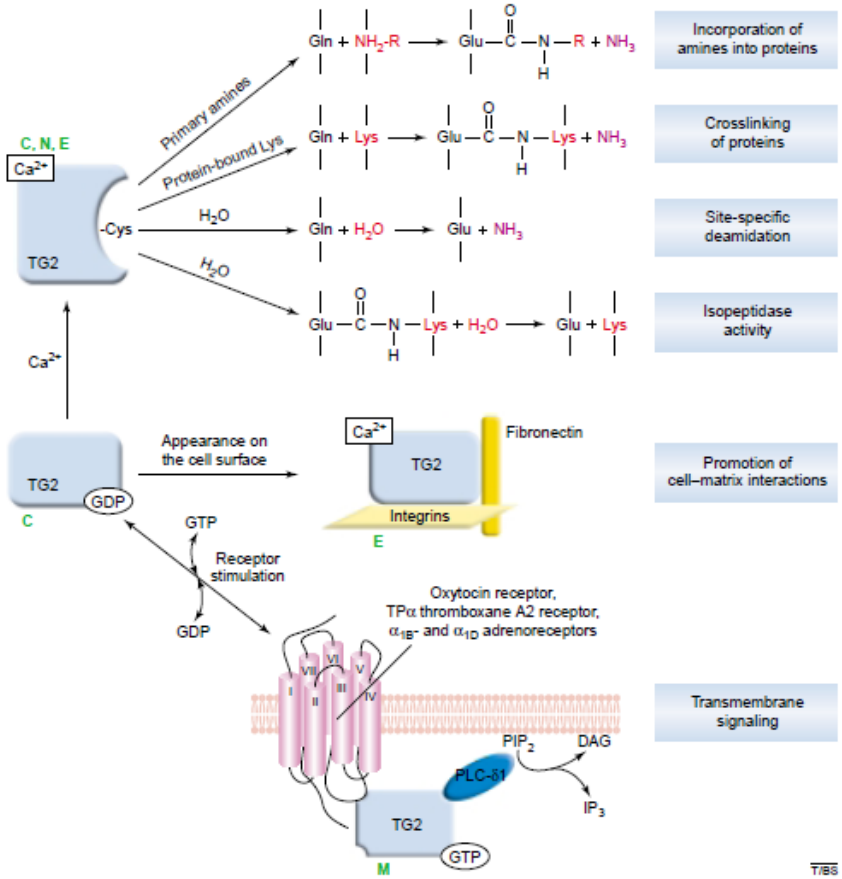


Figure 2-10. Biochemical activities of TG2. TG2 catalyzes Ca^{2+}-dependent acyl-transfer reaction between γ-carboxamide group of a specific protein-bound glutamine and either the ε-amino group of a distinct protein-bound lysine residue (covalent protein crosslinking; the principal in vivo activity) or primary amines such as polyamines and histamine. Water can replace amine donor substrates, leading to deamidation of the recognized glutamines. TG2, similar to factor XIIIa, has Ca^{2+}-dependent isopeptidase activity and at least under test tube conditions, can hydrolyse γ:ε isopeptides. TG2 can be exposed on the external leaflet of the plasma membrane. The presence of TG2 outside the cell has been proposed to depend on its interaction with fibronectin and integrins. TG2 binds and thereby activates phospholipase C following stimulation of several kinds of cell surface receptors; its endogenous GTPase activity ensures proper regulation of transmembrane signalling through these receptors.

Functions of TG2 are performed in the cytosol (C), the nucleus (N), at the cell membrane (M) and in the extracellular space (E). Except for its isopeptidase activity, all other functions have been shown to occur in intact cells and/or tissues (Fesus and Piacentini, 2002).

The Boc-DON-Gln-lle-Val-OMe, a synthetic modified polypeptide produced by ZEDIRA GmbH, is a potent, active site directed inhibitor of tissue transglutaminase (Fig. 2-11). The reaction results in an alkylation of active site cysteine which irreversibly inhibits the activity of TG2. It has been proven that this peptide showed a selective reaction with the cysteine residue in the active site of TG2.

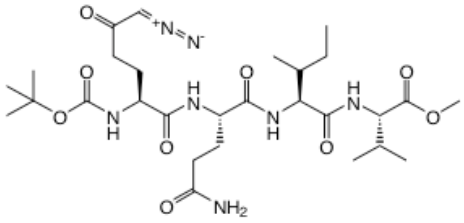


Figure 2-11. Chemical structure of Boc-DON-Gln-lle-Val-OMe.

2.4.3 Factor XIII

Factor XIII, also known as fibrin-stabilizing factor, is a plasma TGase, and circulates in blood as a pro-transglutaminase. It consists of two potentially active a- subunits and two inhibitor/carrier b-subunits. XIII-a is synthesized in cells of bone marrow origin, whereas XIII-b is produced in the liver by the hepatocytes. In normal conditions, all FXIII-a present in the plasma is in a complex, whereas FXIII-b is in excess, and about 50% of it circulates as a free, uncomplexed protein (Karimi et al., 2009). The enzyme precursor factor XIII can be activated by thrombin and Ca^{2+} in the final phase of the clotting cascade. The activation that immediately follows fibrin formation is a process to unmask the active site of factor XIII-a, a ε-lysyl-γ-glutamyl aminoacyl transferase. It is capable of cross-linking a growing list of proteins, not only within plasma, but also proteins within the vascular matrix, platelets, endothelial cells and monocytes (Hsieh and Nugent, 2008). A number of other proteins are known to be substrates of factor XIII: plasminogen-activator inhibitor type-2, osteopontin, lipoprotein, platelet vinculin, factor V, thrombospondin, etc., are also reported to become substrates for activated XIII *in vitro* (Ichinose, 2005).

Accordingly, the main hemostatic functions of FXIII-A are to form fibrin γ-chain dimers, to cross-link its α-chains into high-molecular-weight polymers, and to attach α2 plasmin inhibitor to fibrin α-chains. In this process, FXIIIa mechanically stabilizes the fibrin clot and protects it from shear stresses and from the prompt degradation by the fibrinolytic system. A recent review provides further details on the key regulatory role of FXIII in fibrinolysis (Muszbek et al., 2008). In addition to the above functions, FXIII is also involved in wound healing (Duckert et al., 1960), angiogenesis (Dardik et al., 2006), and is essential for maintaining pregnancy (Koseki-Kuno et al., 2003). The enzyme promotes clot stability by forming covalent bonds between fibrin molecules and also by cross-linking fibrin with several proteins including α2-plasmin inhibitor, fibronectin, and collagen. These reactions lead to an increase in the mechanical strength, elasticity and resistance to degradation by plasmin of fibrin clots, and promotion of wound healing by providing a scaffold for fibroblasts to proliferate and spread.

2.5 TISSUE-SPECIFIC FIBRIL ORGANIZATION

The characteristics of different extracellular matrices are determined by the synthesis, assembly and deposition of collagen molecules and their organization into unique macromolecular structures. Fibrillar collagen molecules are organized into fibrils, which are organized into tissue-specific macroaggregates such as regular layers in cornea and bone, cables in tendon and ligaments, or irregular layers or wickerwork in dermis. At each level of this hierarchy the collagen fibrils have tissue specific characteristics, i.e. fibril diameter, packing and organization.

2.5.1 Cornea

The cornea is the transparent front part of the eye that comprises a highly structured, membrane bound, transparent collagenous tissue, which joins the more disorganized and opaque sclera at the limbus. The native cornea provides three fundamental functional attributes to the ocular optical system: protection, transmission and refraction of the incident light to the retina. It consists primarily of three layers: an outer layer containing an epithelium, a middle stromal layer consisting of a collagen-rich ECM interspersed with keratocytes and an inner layer of endothelial cells (Fig. 2-12). The Bowman's membrane is a thin acellular matrix, which lies subjacent to the corneal epithelium and its basement membrane. It contains also fibrils thinner than

those of the stroma (Hay and Revel, 1969), and shows strong immunofluorescence for type V collagen without unmasking (Linsenmayer et al., 1983, 1984; Birk et al., 1986). The Descemet's membrane is a thin acellular layer that serves as the modified basement membrane of the corneal endothelium, is composed mainly of collagen type IV-containing networks. At the heart of the cornea is the stromal tissue that comprises 90% of the total thickness of cornea. As the major protein component of the stroma, collagens comprise 71% of the dry weight (Newsome et al., 1981). The corneal stroma is unique in having a homogeneous distribution of small diameter (25-30 nm) fibrils that are regularly packed within orthogonal layers, called lamellae. This lattice-like structure produces minimal light scattering to provide transparency (Maurice, 1957; Benedek, 1971).

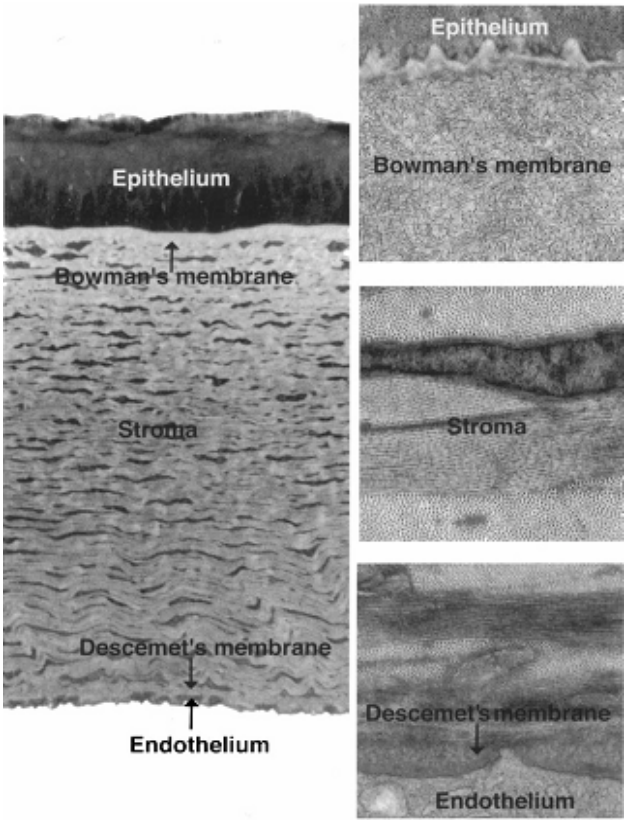


Figure 2-12. The left panel is a light micrograph of a developmentally mature cornea. The right panels are electron micrographs of portions of Bowman´s membrane (top), the stroma proper (middle), and Descemet´s membrane (bottom) (Linsenmayer et al., 1998).

The ECM of the corneal stroma consists primarily of collagens with lesser amounts of proteoglycans. Type I collagen is the predominant collagen of the cornea, which assembles with collagen V (10-20% of the total collagen) to form heterotypic fibrils

(Birk et al., 1986). However, the heterotypic type I/V collagen fibrils of corneal stroma show different distribution of both collagen types within the fibrils. The triple helical domain of type I molecules occurs both within the fibril and at the fibril´s surface, whereas the triple helical domain of most type V collagen molecules is buried within the fibril´s interior. Moreover, type V molecules have a large NH_2-terminal domain (globular domain derived from α1(V) chains) extending outwards through a gap zone to the fibril´s surface (Linsenmayer et al., 1993). These collagen I/V fibrils interact with FACIT collagens, type XII and/or XIV depending on developmental stage. In addition, the small leucine rich proteoglycans, decorin, lumican, biglycan, keratocan and osteoglycine interact with the fibril surface (Fig. 2-13) (Birk and Bruckner, 2005).

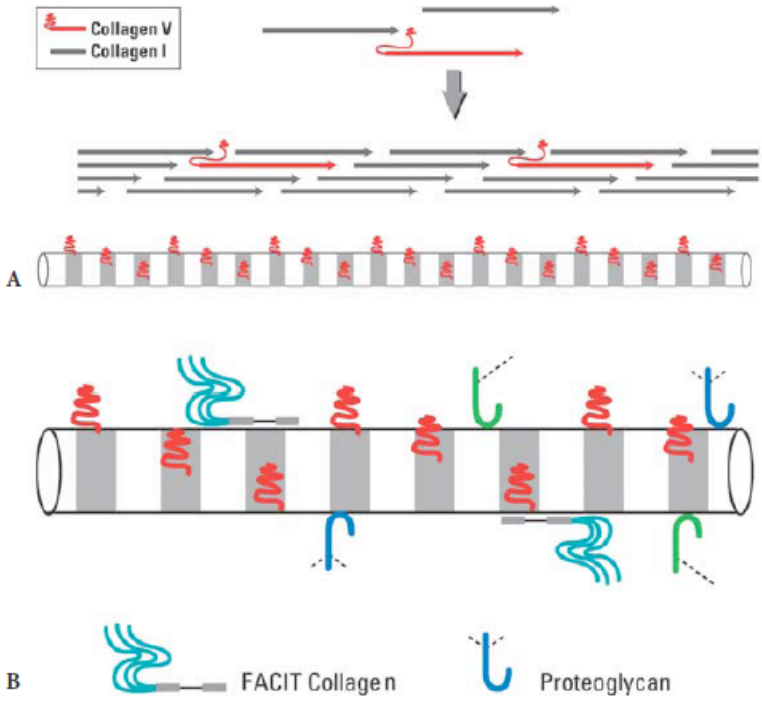


Figure 2-13. Corneal fibril: (A) corneal fibrils are heterotypic, co-assembled from collagens I and V. Collagen V is a quantitatively minor component of most collagen I-containing fibrils. It has a retained N-terminal, non-collagenous domain that must be in/on the gap region/fibril surface. The heterotypic interaction is involved in efficient initiation of fibril assembly; (B) the heterotypic alloy forms the core of a composite fibril with fibril-associated leucine-rich repeat proteoglycans and FACIT collagens bound to the surface. While the heterotypic composition is relatively constant, the fibril-associated macromolecules are more dynamic, changing temporally during development or repair and spatially in different tissues/tissue domains (Birk and Bruckner, 2005).

2.5.2 Tendon

Tendons are collagen-based fibrous tissues that connect and transmit forces from muscle to bone (Birk and Trelstad, 1986; Carvalho et al., 2000). They are composed

of closely packed parallel collagen fibre bundles, have high tensile strength and function to transmit forces and stabilize joint structures (Birk and Trelstad, 1986; Erksen et al., 2002; Edom-Vovard and Duprez, 2004). The fibril bundles together with the tendon fibroblasts are organized into fascicles, and the fascicles are bound together in a connective tissue sheath to form a tendon (Kastelic et al., 1978; Birk and Trelstad, 1986; Birk and Mayne, 1997; Birk et al., 1990) (Fig. 2-14).

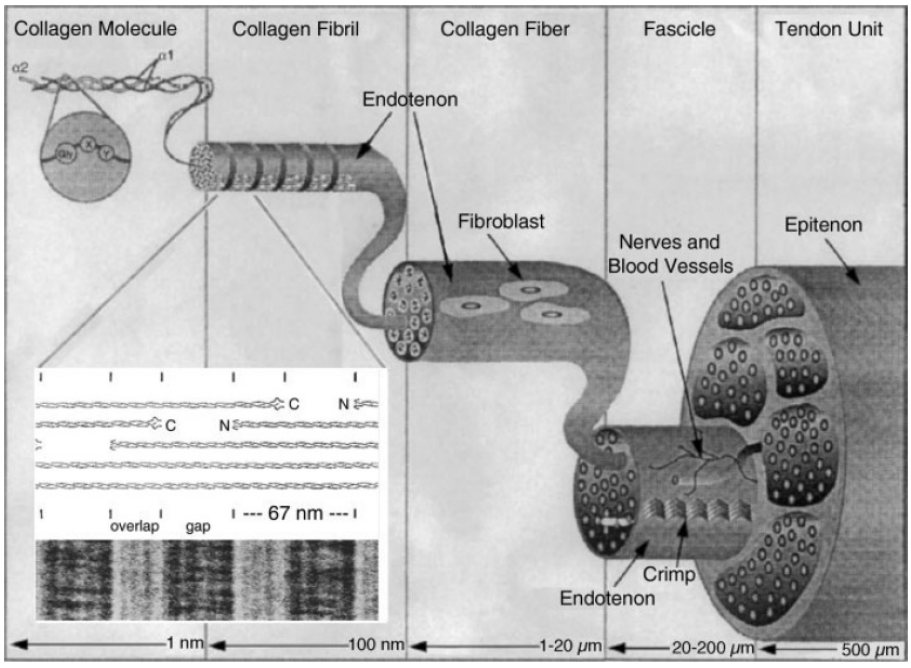


Figure 2-14. Structural hierarchy of tendon: collagen molecules aggregate to form fibrils; fibrils group together to form fibers; fibers bundle together to form fascicles; fascicles group together to form tertiary fiber bundles which act as the primary tendon unit. Connective tissue called endotenon surrounds the bundles and fascicles. Although the diagram does not show fibril subunits, collagen fibrils appear to be self-assembled from intermediates that may be integrated within the fibril (modified from Silver et al., 2003).

Tendon consists predominantly of collagen type I, and to a lesser degree other fibrillar (type III and V) and non-fibrillar collagens (type XII and XIV), proteoglycans and glycoproteins. Both collagen V and III form heterotypic alloys with collagen I and have the retained/slowly processed N-terminal domains typical of the regulatory fibril-forming collagens. During tendon development, there are at least three distinct steps in fibrillogenesis: molecular assembly of collagens, linear growth step; and lateral growth step (Fig. 2-15). Tissue-specific fibrillogenesis would involve the independent regulation of each step. During tendon development there are changing expression patterns for FACIT collagens and the leucine-rich proteoglycans (Ezura et al., 2000;

Young et al., 2000; Zhang et al., 2003). For example, the expression level of collagen XIV, biglycan and lumican changed drastic from early development to maturation of tendon. In the absence of any of the four proteoglycans the regulation of fibril growth in tendon is abnormal (Danielson et al., 1997; Jepsen et al., 2002; Young et al., 2000).

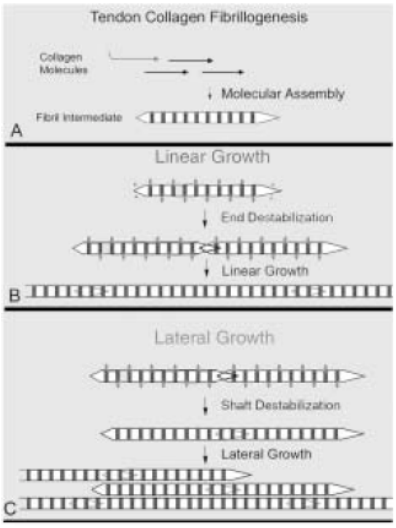


Figure 2-15. Tendon fibrillogenesis. (A) Molecular assembly of type I and III and/or V collagens generates the fibril intermediate. It can be hypothesized that heterotypic interactions are a mechanism regulating this assembly step. (B) In the linear growth step, the intermediates in (A) grow by end-to-end growth to generate longer fibrils. Alterations in molecular interactions, at the α and β ends of the intermediate, initiate and control this growth. (C) In the lateral growth step, there is a lateral association and growth of the developing fibrils. Alterations in interactions mediated by fibril-associated molecules (ovals) along the main fibril shaft regulate this step (Zhang etl al., 2005).

In tendons, collagen fibrils are dense, well organized and aligned in parallel along the main axis of tension (Kastelic et al., 1978). In contrast to the cornea, the collagen fibrils in tendon have significant increases in diameter during development and growth, which supplying stability and tensility of tendon. Particularly, the parallelism of tendon fibril formation is determined by the late secretory pathway and interaction of adjacent membrane protrusions to form extracellular channels called fibripositors. There are two models described about fibripositor formation during collagen fibrillogenesis in tendon according to the Birk model and Kadler model (Birk and Trelstad, 1986; Canty et al., 2004; Richardson et al., 2007). Moreover, it has been shown that actin filaments are required for fibripositor-mediated collagen fibril alignment in tendon (Canty et al., 2006)

3. AIM OF THE PRESENT STUDY

This present study has the aim to understand the relationship between cross-link formation and collagen fibril organization in chicken embryonic cornea and tendon. The fibrillar organizations in cornea and tendon are vastly different although both tissues contain collagens I and V as their major collagenous components. Collagens have to be cross-linked to exhibit the normal physical properties, in which all the fibril-forming collagens (types I, II, III, V, and XI) and the fibril-associated type IX collagen rely on this mechanism of cross-linking to provide tissue structural integrity and material function (Eyre et al., 2005). Therefore, it is interesting to investigate the collagen fibril organization in embryonic chicken at the tissue level, employing a 3D cell culture system with and without cross-link formation using cross-link-specific enzyme inhibitors. The objectives of this study were the following:

(i) To prepare 3D cell cultures of fibroblasts isolated from chicken embryonic cornea or tendon with or without cross-link formation.

(ii) To obtain collagenous matrices from 3D culture.

(iii) To check the suprastructural organization and macromolecular components of matrices with or without cross-link formation by transmission- and immuno-gold electron microscopy.

(iv) To determine the existence of cross-link as well as the relevant enzyme activity during ECM formation.

(v) To supplement the data from cell-culture-system with *in-vitro* fibrillogenesis experiments.

4 MATERIALS AND METHODS

4.1 CHEMICALS AND ANTIBODIES

4.1.1 Chemicals

Name	Supplier
L-proline, [^{14}C(U)]- 0.1 mC$_i$/ml	Hartmann Analytic, Germany
2-propanol	Roth, Karlsruhe, Germany
Acetic acid	Merck, Darmstadt, Germany
Alexa Fluor® 555 cadaverine, disodium salt	Invitrogen
Boc-DON-Gln-Ile-Val-OMe Tissue transglutaminase inhibitor	ZEDIRA GmbH
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim
Calcium chloride	Merck, Darmstadt, Germany
CF626 radioactive molecular marker	Amersham Biosciences, UK
Coagulation factor XIII from human plasma Approximates 1,000 µg / 5,000 µg factor XIII	ZEDIRA Gmbh
Collagenase B 0.191 U/mg lyo. from Clostridium histolyticum	Roche Diagnostics GmbH, Germany
Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany
2,5-diphenyloxazol	Sigma-Aldrich, Steinheim
Dry skim milk	Fluka Chemie GmbH, Switzerland
Ethanol	AppliChem, Darmstadt, Germany
Ethylendiamine tetra acetic acid (EDTA)	Fluka Chemie GmbH, Switzerland
Fluoromount G	Southern Biotech, USA
Formaldehyde 8%	Polysciences, Inc., Eppelheim, Germany
Glutaradehyde 8%	Polysciences, Inc., Eppelheim, Germany
Glycerol	Roth, Karlsruhe, Switzerland
L-Cysteine Hydrochloride Monohydrate	Sigma-Aldrich, Steinheim, Germany
L-Lysine, [4,5-^{3}H(N)]- 1.0 mC$_i$/ml; 3.3 µg/ml	Hartmann Analytic, Germany
L(+)-Ascorbic acid	Merck, Darmstadt, Germany
LR-White	Agar Scientific, Stansted, UK
Lumasafe Plus	LUMAC. LSC, Holland
MEM Eagle w/Earle's, L-Glutamine w/o Leucine, Lysine, Methionine (powder)	USBiological
Methanol	AppliChem, Darmstadt, Germany
Paraffin	Roth, Karlsruhe, Germany

Penicillin / Streptomycin	PAA Laboratories GmbH, Pasching
Pepsin	Serva, Heidelberg, Germany
Polyacrylamide	Roth, Karlsruhe, Germany
Recombinant human tissue transglutaminase (His_6-rhTG2), 250 µg / 1 mg dry substance	ZEDIRA GmbH
Roti-Histol	Roth, Karlsruhe, Germany
Sodium pyruvate	Fluka Chemie GmbH, Switzerland
Sodium chloride	AppliChem, Darmstadt, Germany
Sodium di-hydrogen phosphate dihydrate	Merck, Darmstadt, Germany
Sodium dodecyl sulphate (SDS)	Serva, Heidelberg, Germany
ß-Aminopropionitrile fumarate salt (BAPN)	Sigma-Aldrich, Steinheim, Germany
Thrombin, human plasma	Calbiochem®, Germany
Trichloro acetic acid (TCA)	Merck, Darmstadt, Germany
Tris (hydroxymethyl)-aminomethan	MP Biomedicals, Eschwege, Germany
Trypsin-EDTA	PAA Laboratories GmbH, Pasching
Uranyl acetate	Merck, Darmstadt, Germany

4.1.2 Antibodies

4.1.2.1 Primary antibodies

Protein	Antibody Name/clone	Immunofluorescence (IF), Western blot (WB), Immuno gold (EM), Immunohistochemistry (ICH)	Donor aminal	Reference/ Source
Collagen I	AB752P	IF, WB, EM, ICH	rb	Chemicon
Collagen VI	39	IF, WB, EM	ms	Developmental Studies Hybridoma Bank
Fibronectin	B3/D6	IF, WB, EM	ms	Developmental Studies Hybridoma Bank
Cytokeratin	AE1/AE3	IF, ICH	ms	Acris Antibodies
Elastin	MAB2503	IF, WB	ms	Chemicon
Decorin	CB-1-c	IF, WB, EM	ms	Developmental Studies Hybridoma Bank

4.1.2.2 Secondary antibodies

Name	Reference/Source
Alexa Fluor® 488, goat anti-rabbit IgG (H+L)	Invitrogen
Cy™3-conjugated AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch
Colloidal Gold-AffiniPure Goat Anti-Rabbit IgG (H+L) (18nm)	Jackson ImmunoResearch
Colloidal Gold-AffiniPure Goat Anti-Mouse IgG (H+L) (12nm)	Jackson ImmunoResearch

4.2 CELL CULTURE

4.2.1 3D cell culture

Fibroblasts were isolated from 17-day-old chicken cornea or tendon. After the dissection of the central parts of cornea, the tissues were washed 3 times in Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 2 mM $CaCl_2$, 10 mM glucose, 200 µM sodium pyruvate and 10 mM Hepes, pH 7.4). Subsequently, epithelial cells were removed from the tissue fragments by digestion in 0.25% (w/v) trypsin / 2mM EDTA solution in Krebs buffer for 15 min in Petri dishes. After 3 futher washing steps, the tissues were minced and matrix-free fibroblasts were obtained by incubation with 1mg/ml collagenase B (0.191 U/mg lyo. from Clostridium histolyticum, lyophilized, Roche, Germany) in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin, added to avoid bacterial contamination. The dishes were kept overnight in a humidified-atmosphere incubator at 37°C and with 5% CO_2. The cells then required 3 washing steps with DMEM and centrifugation (1000 rpm, 5 min, S4180, Beckman), and were resuspended in DMEM (10% FCS), counted in a Neubauer chamber, and distributed in a 24-well-plate (Nunc, Roskilde, Denmark) with a culture surface about 1.9 cm^2 pro well. The cell density was about 2×10^5 pro well. Cells were cultured overnight and then supplemented with 0.14 mM L(+)-ascorbic acid, 1 mM sodium pyruvate and 1 mM L-cysteine. The crosslink inhibitors were added to give final concentrations of 0.2 mM ßAPN and / or 5 µM TG-inhibitor (Boc-DON-Gln-Ile-Val-OMe, ZEDIRA GmbH). After 14 days of cultivation the fibroblasts formed multilayer cell matrices which were removed from the wells with a spatula.

4.2.2 Immunofluorescence of cell culture in µ-slides (ibidi)

The ibidi µ-Slide $VI^{0.4}$ is a 6-channel µ-slide, which is suitable for small-scale cell cultivation (30 µl channel volume) and multiple immunofluorescence staining (work principle see Fig. 4-1). Fibroblasts were taken up in 1 ml DMEM medium containing

10% FCS, 1% penicillin-streptomycin and counted using a Neubauer chamber. 30 µl cells were seeded into each channel at a density of 3×10^5 cells/ml and the slides were incubated at 37°C and with 5% CO_2 for 2-3h. For each channel 90 µl cell-free medium (supplemented with 0.14 mM L(+)-ascorbic acid, 1 mM sodium pyruvate, 1 mM L-cysteine, 0.2 mM ßAPN or / and 5 µM TG-inhibitor) was filled, for a further cultivation for 2-7 days until cells grew dense in each channel.

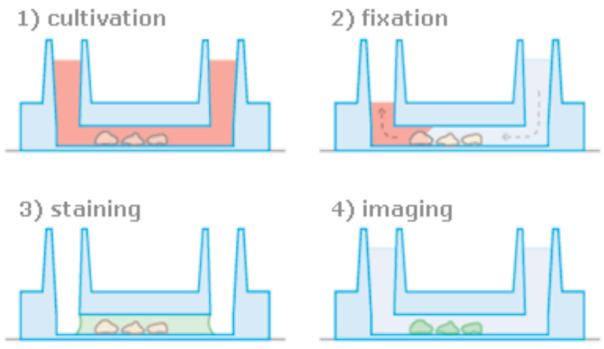


Figure 4-1. Work principle of ibidi-slide for immunofluorescence staining.

4.3 PROTEIN CHEMISTRY

4.3.1 Collagen purification

An overview of the procedures used for collagen purification is shown in Figure 4-2. 17-day-old embryonic chicken corneas were washed 3 times in PBS (20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl). All of the following steps were performed at 4°C. Collagens were extracted in 15 volumes of 0.5 M acetic acid by stirring overnight. Supernatants (S1) were recovered by centrifugation (10,000 rpm, 30 min, JLA10.500, Beckman). This step was repeated and the supernatants (S2) were combined. The whole supernatants (S1+S2) were again centrifugated (14,000 rpm, 30 min, JA10.50, Beckman) and pellets were discarded. Subsequently, 25% [w/v] solid NaCl was dissolved in 0.5 M acetic acid (V = 25%(S1+S2)) and added into the crude mixture of collagens very slowly by stirring overnight. Total collagens (A) were obtained by centrifugation (10,000 rpm, 1.5 h, JLA10.500, Beckman) and dissolved in 0.05 M Tris-HCl, pH 7.4 containing 2 M urea, 0.2 M NaCl. Here, total collagens (A) were mixture of collagens and proteoglycans.

For chromatographic separation, the crude collagen pellets were dissolved in 0.05 M Tris-HCl, pH 7.4, containing 2 M urea, 0.2 M NaCl. After dialysis against the same buffer the crude mixture was passed over a DEAE-cellulose column (3.5×21 cm, 200

ml, DE52; Whatman Ltd.), equilibrated with the same buffer. Total collagens (B) were recovered from the breakthrough fraction while other proteins containing proteoglycans ("rest") were bound to the column. The column was eluted with buffer containing 1 M NaCl in order to discard the "rest" proteins from the column. The product was a mixture of total collagens (B) without proteoglycans.

The total collagens (A) and (B) were dialyzed extensively against 100 mM Tris-HCl, pH 7.4, containing 1 M NaCl, and were precipitated by adding solid NaCl to a final concentration of 4.5 M. After centrifugation, pelleted precipitates were redissolved in storage buffer (0.1 M Tris-HCl, pH 7.4, containing 0.4 M NaCl) at appropriate concentrations and were clarified by centrifugation. The purified and concentrated collagen mixtures with or without proteoglycans were used for *in-vitro* fibrillogenesis.

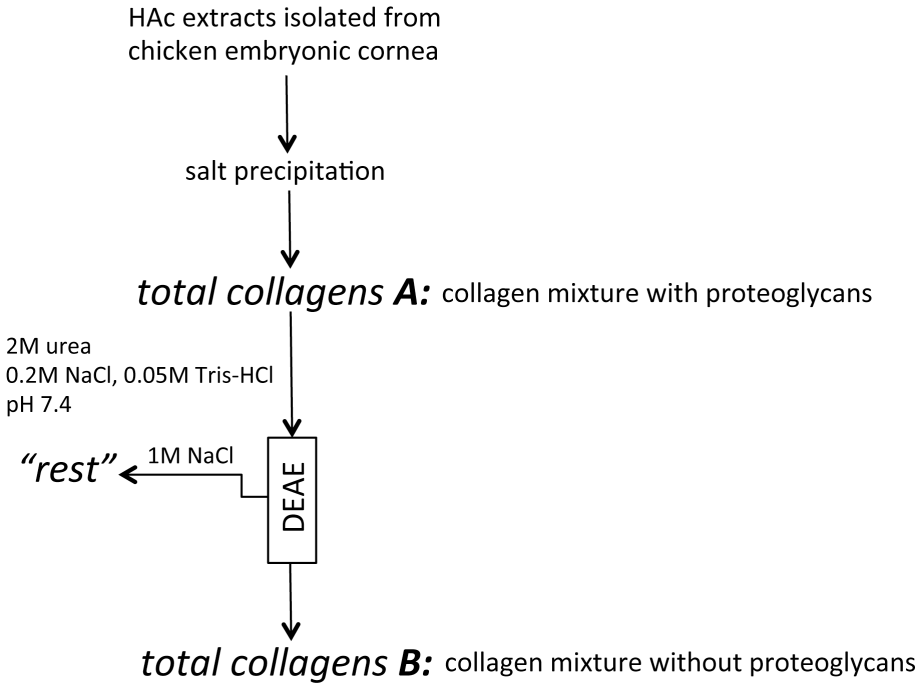


Figure 4-2. Flowchart representation of the protocol for purification of corneal collagen mixtures with or without proteoglycans from embryonic chicken cornea.

4.3.2 SDS-PAGE

Samples were pooled and prepared for electrophoresis by precipitation with three volumes of cold ethanol. The precipitates were resuspended in SDS sample buffer (10 mM Tris-HCl, pH 6.8, containing 10% [v/v] glycerol, 0.03% bromphenol blue, 2% sodium dodecyl sulphate, 0.5 M urea) and boiled at 95°C for 5 min, then loaded on

4.5-8% polyacrylamide gradient gels under non-reducing conditions. After electrophoretic separation, the gels were dehydrated for further analysis by fluorography (see 4.7).

4.4 *IN-VITRO* FIBRILLOGENESIS

An overview of the procedures used for *in-vitro* fibrillogenesis is shown in Figure 4-3. The crude mixture of corneal collagens from embryonic chicken (see 4.3.1) in storage buffer was degassed under vacuum. *In-vitro* fibrillogenesis was carried out in a microcuvette (Multicell, light path, 1 cm, Beckman, Palo Alto, CA) and was initiated by diluting the collagen mixtures with an equal volume of distilled water. Where appropriate, tissue transglutaminase, $CaCl_2$, factor XIII, and thrombin were added to the mixtures directly after dilution. The kinetics of fibrillogenesis was monitored by measurement of the development of turbidity at 313 nm. The reconstitution products were examined by (immuno-) electron microscopy.

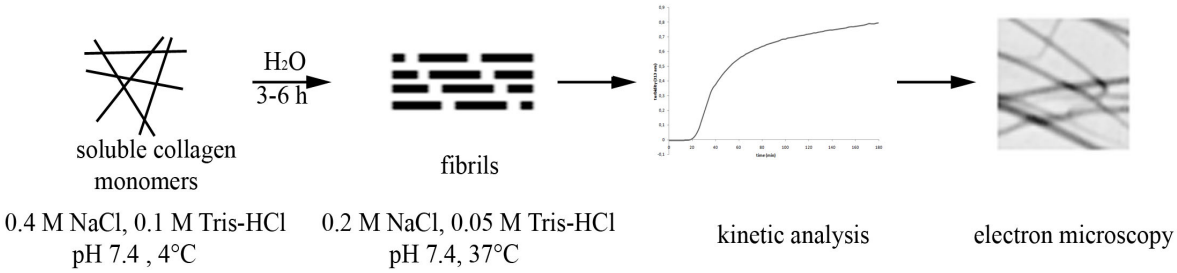


Figure 4-3. Procedure of *in-vitro* fibrillogenesis with purified collagens.

4.5 HISTOCHEMISTRY

4.5.1 Preparation of paraffin-embedded matrices

Matrices deposited during 14-days of 3D-culture by corneal fibroblasts were gently detached from cell culture wells and washed 3 times in PBS, and were fixed in 4% paraformaldehyde overnight at 4°C. The fixed cell cultures were washed in PBS again and folded carefully in OBI specimen paper. The packed cells were put in an embedding-cassette and washed in floating distilled water for at least 1h; then dehydrated in the following solvents under continuous stirring:

1) 2h 50% ethanol at 4°C
2) 2h 70% ethanol at 4°C
3) overnight 96% ethanol (I) at 4°C
4) 4h 96% ethanol (II) at 4°C

5) 2h 2-propanol (I) at 4°C
6) 2h 2-propanol (II) at 4°C

Afterwards cassettes were sequentially put into inter-medium containing 50% 2-propanol and 50% paraffin mild for 24h at 60°C, in paraffin mild (I) for 12h at 60°C, in paraffin mild (II) overnight at 60°C, in paraffin hard for 12h at 60°C. Finally, the treated cells were cast in paraplast. Paraffin blocks were cut with a microtome (MICROM Cool-Cut HM 355), the sections were picked up on glass slides, and dried overnight at room temperature.

4.5.2 Immunofluorescence staining of paraffin sections

Sections were incubated in a drying oven for at least 30 min at 60°C. Deparaffinization and rehydration were performed with following procedures:

1) 2 times washes of Roti-Histol for 5 min
2) 50%, 70%, 90%, 100% ethanol for 5 min each
3) sections rinsed in distilled water for 5 min

All following steps were carried out in a wet and light-tight chamber to prevent drying and fluorochrome fading. Sections were washed in PBS for 5 min and encircled with DakoPen. Isolated sections were washed again in PBS for 3-4 times and blocked with 2% BSA (in PBS) for 45 min at room temperature. Subsequently they were incubated with primary antibody solution containing 1% BSA in PBS at 4°C overnight. Sections were rinsed 3 times in PBS for 5 min and incubated with secondary antibody solution containing 1% BSA and DAPI (1:10,000 in PBS) for 1h at 37°. Finally, sections were rinsed in PBS for 3 times again and covered with Fluoromount G (Southern Biotech). Slides were examined using a Zeiss Axio Imager microscopy equipped with fluorescent optics and documented using a Hamamatsu ORCA ER camera. Images were analyzed using Volocity 4.2 software (Improvision).

4.5.3 Immunofluorescence staining of cells

Immunofluorescence staining of cells was carried out directly in µ-slides (ibidi), which had a confluent cell density after 3-7 days growth. Cells were washed 3 times in PBS and then fixed with cold methanol for 10 min at -20°C. After another 3 washing steps, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room

temperature. Cells were washed in PBS, again, and blocked with 2% BSA in PBS for 45 min at room temperature. Afterwards cells were incubated with primary antibody solution containing 1% BSA in PBS overnight at 4°C. Bound primary antibodies were visualized using Cy3- or Alexa 488 conjugated secondary antibodies (Jackson Laboratories & Molecular Probes) containing 1% BSA and DAPI (1:10,000 in PBS) by 1h incubation at room temperature. Cells were washed again and covered with fluoromount G. Slides were examined using a Zeiss Axio Imager microscopy equipped with fluorescent optics and documented using a Hamamatsu ORCA ER camera and with a Zeiss confocal laser scanning system LSM 510 meta. Images were analyzed using Volocity 4.2 software (Improvision).

4.6 IMMUNO-GOLD ELECTRON MICROSCOPY

4.6.1 Preparation of ultrathin sections

14-days-cultured cornea or tendon fibroblasts were gently detached from cell culture wells and then fixed in fix buffer overnight (0.1M cacodylate buffer in distilled water, pH 7.4, containing 2% [v/v] formaldehyde and 2.5% [v/v] glutaraldehyde). After washing 3 times in PBS over 1h, specimens were dehydrated with an ethanol series from 30% to 70%. Subsequently, the specimens were taken into LR White (Agar Scientific, Stansted, UK) / 70% ethanol (2:1) overnight at 4°C before embedding in pure LR-White. Pure LR-White was changed for 5 times within 2–5 days until the samples were ready for embedding. Samples were then embedded in LR-White using a plastic template (TED RELLA INC bedding CA) and were exposed to UV-light for 24-48h for complete polymerization. Blocks were cut on an Ultramicrotome (Reichert-Jung, Austria) using a diamond knife (ultra 35° MT7239, DiATOME, histo HI7900 or ultra 35° MT7239). Ultrathin sections were picked up on nickel grids (200 square mesh), covered with formvar and coated with carbon, and dried at room temperature.

4.6.2 Immuno-gold electron microscopy

All following steps were carried out at room temperature. Grids with ultrathin sections were floated on drops of 100 mM glycerol in PBS for 30 seconds in order to reduce electrostatic charges. Alternatively, 20 µl reconstituted products from *in-vitro collagen* fibrillogenesis was spotted onto sheets of parafilm. Nickel grids were floated on the drops for 10 min to allow adsorption of fibril fragments. Thereafter, grids were dried

on filter paper, then washed once with PBS, and treated for 30 min with 2% [w/v] dried skim milk in PBS (2% blocking solution) which was centrifuged twice at 14,000 rpm for 10 min. Primary antibodies were diluted to appropriate concentrations with 0.2% blocking solution and the grids were allowed to react for 2h. After washing 4 times with PBS, grids were incubated for 2h with 0.2% blocking solution containing secondary antibodies (12 or 18 nm colloidal gold particles coated with goat anti-rabbit or anti-mouse immunoglobulins, diluted 1:30). The grids were then washed 4 times with distilled water and negatively stained with 2% [w/v] uranyl acetate for 7-10 min. Finally, grids were washed again with distilled water and dried in a grid box. For double-labeling experiments, a mixture of gold particles of two different sizes was used. In negative controls, grids were treated with blocking buffer instead of primary antibody solutions. Electron micrographs were taken at 80 kV using a transmission electron microscope (Philips EM410). The imaging plates were scanned with a digital film scanner (Micron imaging plate scanner, Ditabis, Pforzheim, 6000 × 5000 pixel).

4.7 FLUOROGRAPHY OF SDS-PAGE

Collagens newly synthesized by corneal fibroblasts and separated by SDS-PAGE were visualized by fluorography. Cells were cultured for 24h or 6 days in the presence of 1 µCi/ml ^{14}C-proline (Hartmann analytic, Braunschweig, Germany), L(+)-Ascorbic acid, sodium pyruvate, L-cysteine, and crosslink inhibitors (TG-inhibitor / ßAPN: -/-, +/-, -/+ or +/+ respectively). Media were removed and cell layers were harvested in 1.5 ml HAc, containing 1 mg/ml pepsin. Proteins were digested under stirring by rotation for 2h at room temperature. Salt-precipitation was performed by adding 1.2 M NaCl and stirring overnight at 4°C. After centrifugation at 14,000 rpm for 30 min, cell pellets were resuspended in 100 mM Tris-HCl, 150 mM NaCl, pH 8.6, and were precipitated again by adding three volumes of cold ethanol for at least 2h. Cells were centrifuged again and resuspended in 100 µl of distilled water. Ethanol precipitation was repeated and pellets were dried. Cell lysates digested with pepsin were resuspended in 50 µl of 5-fold concentrated sample buffer (details described in 4.3.2) without reducing agents and heated to 95°C for 5 min. 2 µl aliquots of the labeled proteins or 2 µl molecular marker solution (CF626, Amersham Biosciences / GE Healthcare, Chalfont St Giles, UK) were diluted in 2 ml “Lumasafe Plus” (Lumac LSC, Groningen, Netherlands), and were counted in a “scintillation counter” (Beckman, Fullerton, USA). Samples were loaded with same volume (luminescence

"counts" varies from approximately 8000 to 25000) on 4.5-8% polyacrylamide gradient gels. After electrophoretic separation, the gels were dehydrated 3 times in DMSO and incubated in 20% (w/v) diphenyloxazol in DMSO for at least 3h. After 45 min of rehydration in distilled water, gels were dried on a gel dryer (Bio-Rad, Hercules, USA), exposed on a X-ray film (Thermo Fisher Scientific Inc, USA) for 7-14 days at -80°C, resulting in visualization of band patterns of radioactively labeled proteins. The films were developed and were scanned with a HP scanjet 7400C. Band intensities were analyzed with ImageJ 1.44 software.

4.8 TOTAL PROTEIN ASSAY

The total protein assay is based on the detection of hydroxyproline which is a collagen-specific amino acid in mammals. The amount of hydroxyproline in hydrolysates of conditioned cell extracts can be used as a direct measure of total collagen in ECM. The measurement is started by complete hydrolysis of corneal fibroblast cell layers in 6M HCl at 110°C for 20h. In the hydrolysate, hydroxyproline residues were quantified using the QuickZyme total collagen assay kit (QuickZyme biosciences, work principle sees Fig. 4-4). Collagen I from chicken embryonic tendon or bovine serum albumin (BSA) was used as standards or as negative controls, respectively.

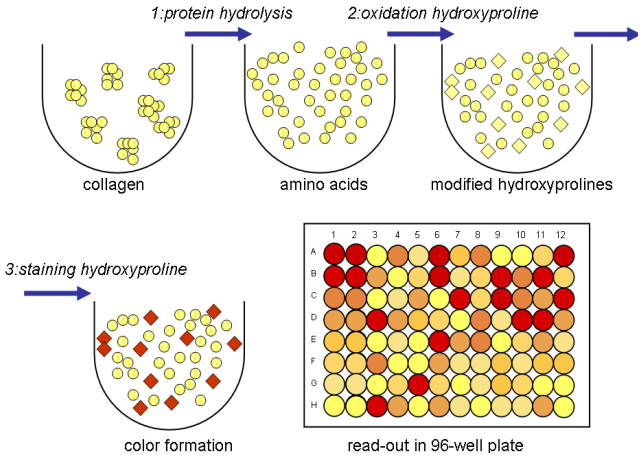


Figure 4-4. Work principle of total protein assay

4.9 MEASUREMENT OF LOX ACTIVITY: ^{3}H-RELEASE

4.9.1 Preparation of substrate

18 aortas from 17-day-old embryonic chickens were incubated in a 50-ml Erlenmeyer flask with 10 ml Eagle´s MEM with Earle's salts lacking free L-lysine (USBiological)

but containing 200 µCi L-[4,5-^{3}H]lysine (Hartmann Analytic) and 20 µg/ml ßAPN. Flasks were incubated at 37°C and 5% CO_2 for 24 h. After incubation, the aortas were rinsed with distilled water and dried in a SpeedVac® concentrator (Savant). Dry aortas were homogenized in phosphate-buffered saline (0.15 M NaCl, 0.1 M Na_2HPO_4, pH 7.8) and centrifuged (11,000 g, 30 min, S4180, Beckman). Supernatants were discarded; the pellets were rehomogenized in saline and recoverd twice more by centrifugation. Finally, the substrates were suspended in 0.01M HCl, collected by centrifugation and dried again in SpeedVac® concentrator. In general, substrates equivalent to the pellet from two aortas containing approximately 500,000 cpm^{3}H were used per incubation.

4.9.2 Assay of lysyl oxidase

Cell layers from corneal fibroblasts cultured for 14 days were homogenized in PBS with Polytron® (KINEMATICA AG), then centrifuged at 4800 rpm for 3 min (S4180, Beckman). The supernatants were applied for activity assay. The enzymic reaction was carried out for 4h at 37°C in a volume of 1ml containing 300,000-500,000 cpm of ^{3}H-labeled substrate. Controls were done with radioactive substrates and buffer. 50 µl of 2% BSA was added to each assay tube and precipitated by TCA together with the substrate proteins. 2 µl supernatants containing tritiated water formed by enzyme-catalyzed reaction were diluted in 2 ml “Lumasafe Plus” (Lumac LSC, Groningen, Netherlands), and were counted by liquid scintillation as described above.

4.10 DEMONSTRATION OF TG ACTIVITY VIA FLUORESCENCE-LABELED CADAVERINE

Corneal fibroblasts were cultured in µ-slides (ibidi) as described in 4.2.2. At day 2, 0.1 mM Alexa Fluor® 555 (Invitrogen)-conjugated cadaverine was added to cell culture and slide was kept in incubator at 37°C and with 5% CO_2 for further 12h in the presence or absence of TG-inhibitor (Boc-DON-Gln-Ile-Val-OMe, ZEDIRA). Afterwards, cells were washed 3 times in PBS and covered with fluoromount G. Slides were examined using a Zeiss AxioImager microscopy equipped with fluorescent optics and documented using a Hamamatsu ORCA ER camera and with a Zeiss confocal laser scanning system LSM 510 meta. Images were analyzed using Volocity 4.2 software (Improvision).

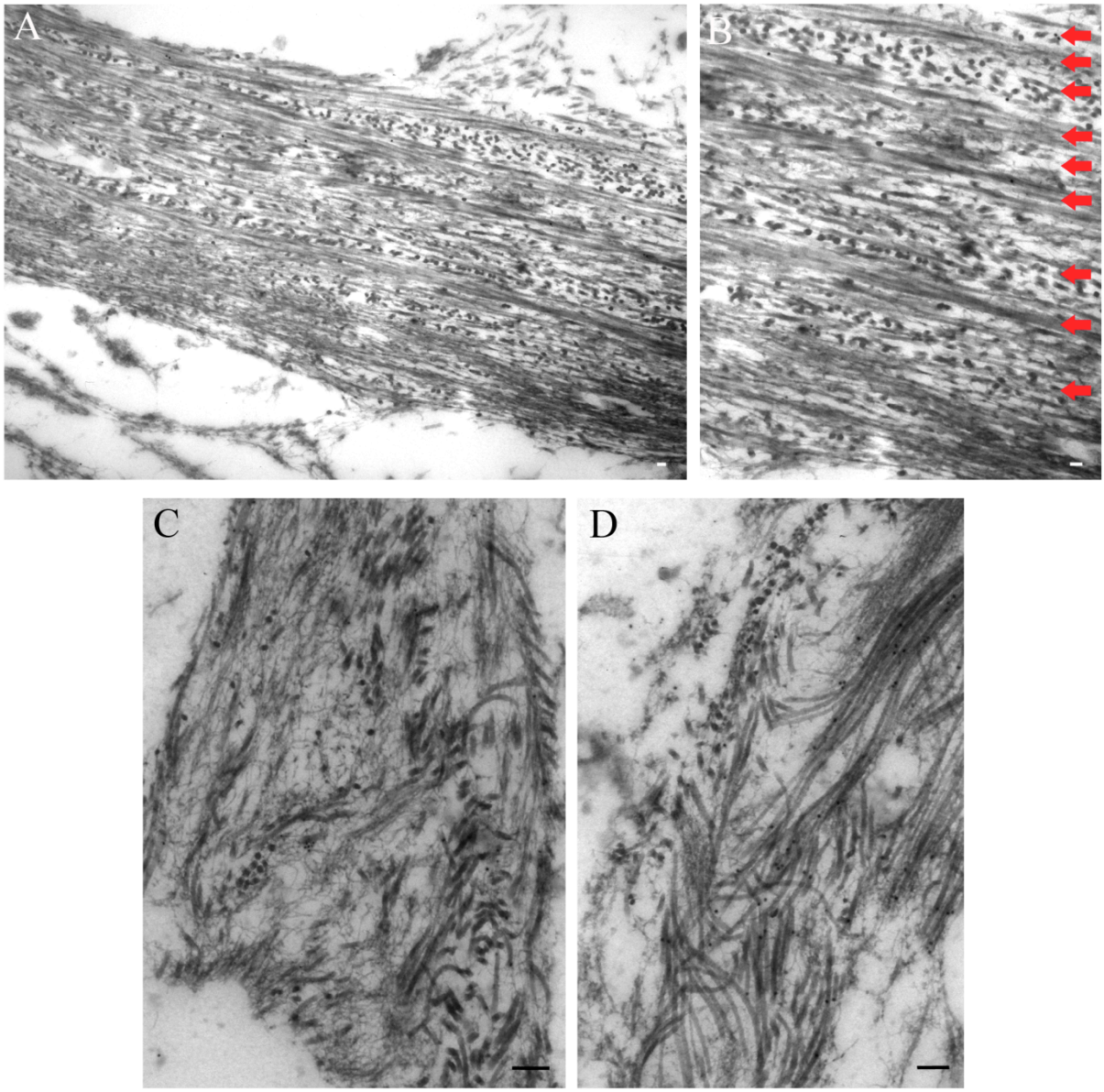
5 RESULTS

5.1 The tissue-specific matrix organization in chicken cornea is crucially influenced by cross-link formation.

In early studies, Ruberti and co-workers have shown that human corneal fibroblasts produced lamellae of fibrils with resemblance to those in the developing mammalian cornea (Guo et al., 2007). In his work, primary fibroblasts were allowed to emigrate from tissue fragments and were subsequently subjected to cell culture. In this thesis project, matrix-free keratocytes were isolated from 17-day-old chicken embryonic cornea by digestion with bacterial collagenase. The collagenous matrices with and without cross-links were obtained from keratocytes cultivated in 3D-cultures for 14 days. The arrangement of the collagen fibrils and the fibril diameter distribution depended on cross-link formation and were analyzed on ultrathin sections of 3D matrices by electron microscopy.

Electron micrographs revealed orthogonal arrays of fibrils deposited by the keratocytes under conditions allowing the formation of LOX- and TG-derived cross-links, i.e. in the absence of enzyme inhibitors (Fig.5-1 A). Orthogonal stacks were formed of arrays of uniformly thin collagen fibrils. Equidistantly spaced in parallel to yield so-called “lamellae” construct. Although the arrangement of lamellae is compact, at least nine lamellae (red arrows) were found with a change in collagen orientation (cross section – longitudinal section). Panel B shows a high magnification section of the micrograph shown in panel A. The authentic corneal stroma is composed of collagen fibrils of a uniform diameter, embedded in a highly hydrated matrix made up mostly of proteoglycans (Quantock et al., 2001; Meek et al., 2003). The authentic corneal fibrils are remarkable in the uniformity of their diameters and the regularity of their spacing. We found that the lamellae composed of homogeneous fibrils in our cell cultures had an organization highly similar to that of authentic mature corneal fibrils. However, formation of these lamellae was abrogated in the presence of the LOX inhibitor ßAPN. The fibrils were disorganized and thin, network-like structures between normal collagen I containing fibrils (C, D) were apparent. Such thin fibrils were not labeled with antibodies to collagen I or collagen VI. By contrast, in the presence of a TG inhibitor, the collagen fibrils were thicker, but the organization into lamellae was less affected (E, F). At high magnification (inset in panel E), thicker

fibrils were revealed with clear banding pattern (D-period) which was not seen under other conditions of cross-link formation. The diameters of fibrils formed in the presence of TG-inhibitor were also very homogeneous. Interestingly, the presence of both cross-link inhibitors leads to striking effects on matrix formation: the organization of fibrils in lamellae is lost (G-J), and the fibrils themselves were abnormal in that they had a bimodal and broad diameter distribution (G, H). Fibrils were very thin (diameter 15 nm - 30 nm) in one region and much thicker, but disrupted in structure in a neighboring region (diameter 45 nm – 150 nm). Such “abnormal” fibrils could be labeled with collagen I antibody gold conjugates (small gold particles indicated by red arrows in micrograph H), proving their collagenous nature. In some cases there are very few fibrils formed close to keratocytes (J) and exhibited weak or diffuse banding patterns (I).



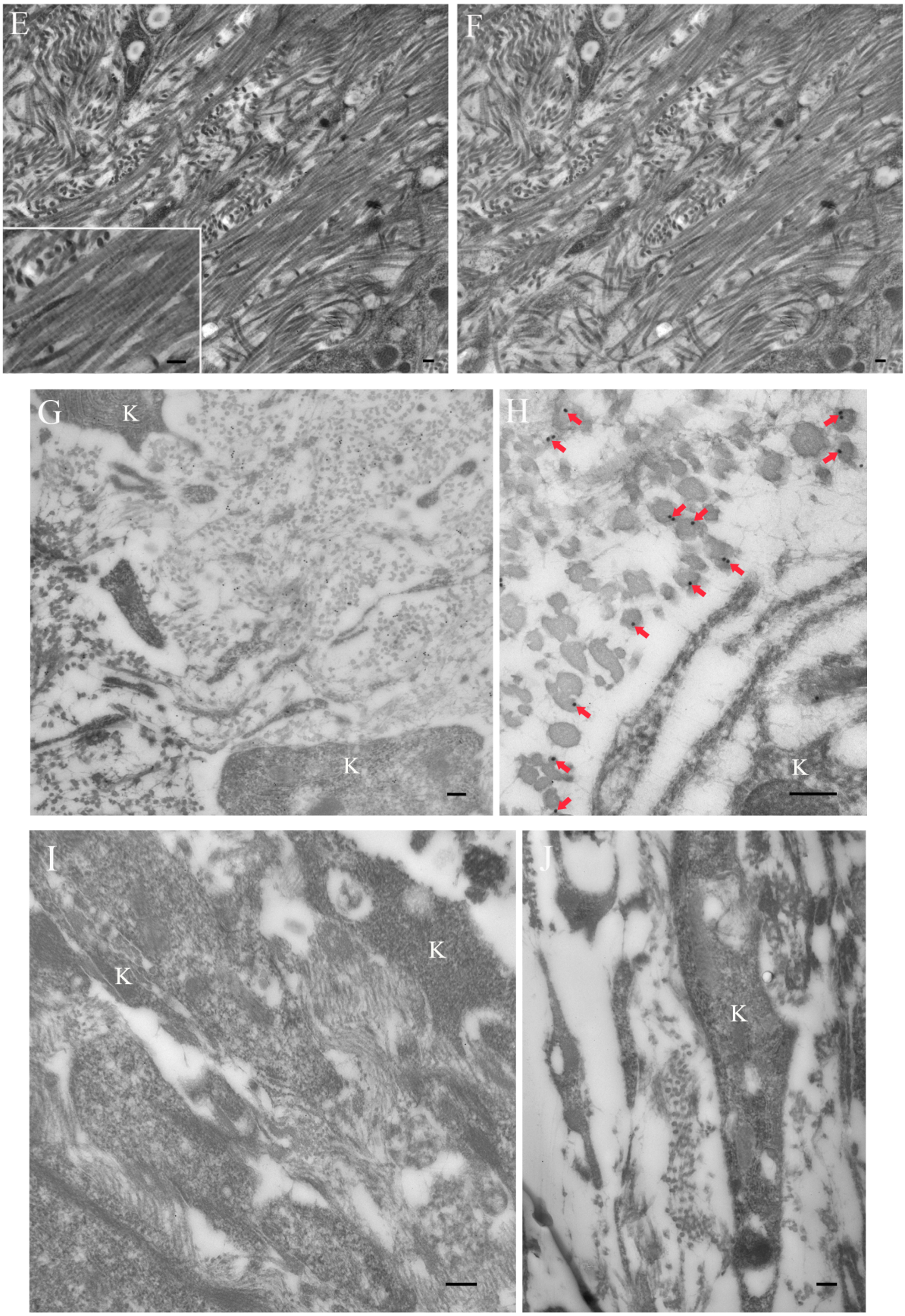


Figure 5-1. Suprastructures of matrices deposited by keratocytes. Control condition allowing for LOX- and TG-derived cross-link formation (A, B); in the presence of LOX-inhibitor ßAPN (C, D); or in the presence of a TG-inhibitor (Boc-DON-Gln-Ile-Val-OMe) (E, F); or with both

inhibitors (G-J). Small gold particles (red arrows) indicate the localization of collagen I (H). Keratocyte cells were marked with “K”. Bars: 200 nm.

5.2 Addition of cross-link inhibitors changed fibril diameter distribution in keratocytes.

The analysis of fibril diameter distributions unraveled further morphological details of collagen fibrils formed in keratocytes. All diameters of collagen fibrils were measured on the basis of ultrathin sections, either of 17-day-old embryonic chicken cornea (Fig. 5-2 A) or of 3D matrices from 14-days chicken cultures of keratocytes by electron microscopy (B, C, D, E).

Fibrils formed by keratocytes under control conditions were only slightly thicker (diameter distribution B: 45.60 ± 12.16 nm, n = 758) than those in corneal tissue (A: 35.48 ±10.81 nm, n = 714). In the presence of ßAPN, the fibrils had a broader distribution and larger diameters (C: 59.01 ± 25.36 nm, n = 918). Interestingly, fibrils formed in the presence of TG-inhibitor were obviously thicker. There was a large shift of diameter distributions towards higher values (D: 110.31 ± 15.75 nm, n = 593). This result is consistent with the electron microscopic observation that fibrils were generally thicker with clearly visible banding pattern. Under the culture conditions with both cross-link inhibitors, diameter distributions were bimodal (E: 59.97 ± 26.88 nm, n = 1198): there was a population with small diameter (peak at about 20-25 nm), while the other at 50-55 nm of diameter. When the formation of both cross-links was prevented, a loss of matrix organization and heterogeneous fibril distribution was observed which clearly differed from the fibril populations when only one type of cross-link was inhibited.

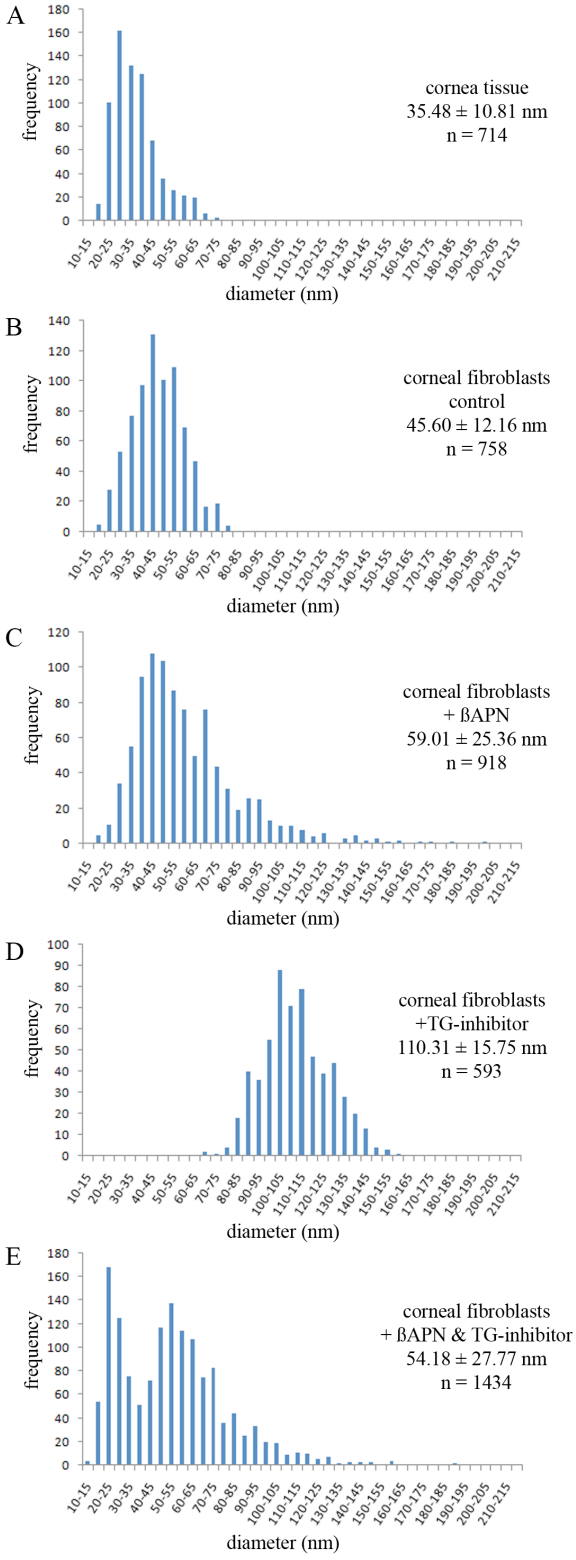


Figure 5-2. Analysis of fibril diameter distribution: The diameter of collagen fibrils were measured on ultrathin sections of authentic tissue (A) or of 3D matrices from chick cultures of keratocytes from cornea by electron microscopy (B-E).

5.3 Cross-links derive from both lysyl oxidase and transglutaminase in chicken keratocytes.

Collagen-fluorography of ^{14}C-proline labeled keratocytes was analyzed with respect to cross-linked components under different inhibitor conditions and at different time points of culture. The 3D-cultured keratocytes were metabolically labeled with ^{14}C-proline for 6 days or 24 hours. The harvested cell matrices were pepsin-solubilized, collagens were salt-precipitated, and SDS-PAGE was run under non-reducing conditions. The result from cell layers labeled for 6 days is shown in figure 5-3 A. Four lanes indicated four different conditions of cross-link formation, specified at the bottom of each lane. The cross-link products were classified into five groups:

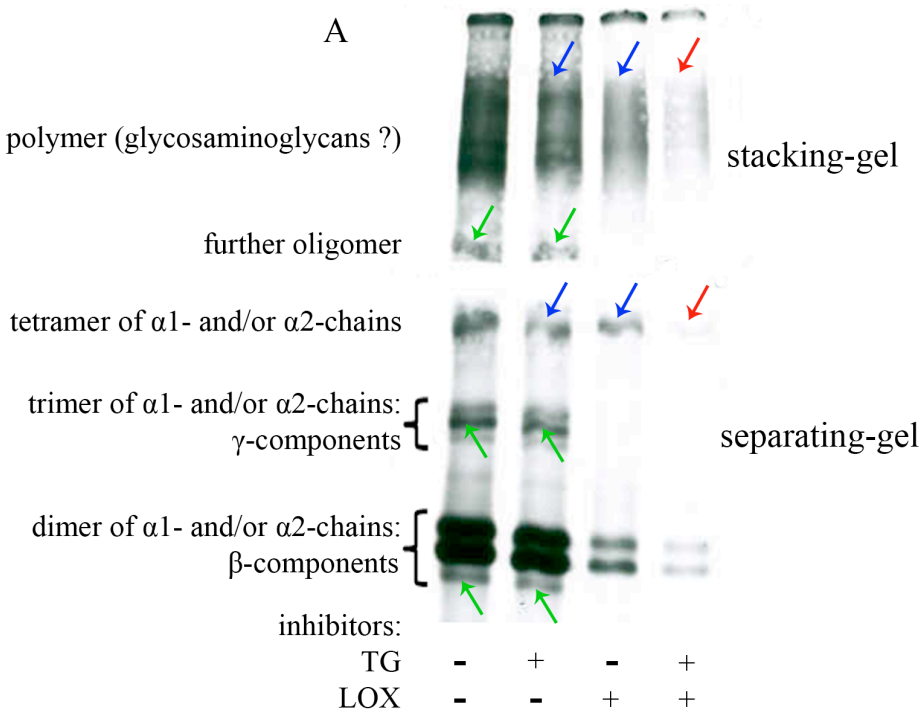
i) dimer of α1- and/or α2-chains (β-components);
ii) trimer of α1- and/or α2-chains (γ-components);
iii) tetramer of α1- and/or α2-chains;
iv) further oligomer;
v) potential polymers containing non-collagenous proteins substituted by GAG chains.

Some products resulted from exclusive LOX-dependent cross-linking. These were the fastest migrating band of the β-components, all γ-components, as well as further oligomers which appeared only when LOX activity was intact (green arrows). These cross-links could be essential for the generation of lamellar structures, but less necessary for the regulation of the fibril diameter. In addition, there are products of both LOX- and TG-dependent cross-linking. The tetramers are dependent on both cross-links. Each of the enzymes produced about half of the tetramers (blue arrows), since inhibition of both enzymes leads to absence of tetramers (red arrows). The further oligomers, whose formation is LOX-dependent, display similar patterns as trimers and dimers. The polymers presented dependence of cross-link products on the enzyme inhibitors similar to that of tetramers with each of the enzymes contributing about half of them. Solely TG-dependent cross-link products were not formed in these experiments.

Furthermore, in order to investigate the onset of cross-link formation, 24-hour metabolic labeling was done and the result is shown in figure 5-3 B. At the beginning of fibril formation and organization, LOX-dependent cross-links have partly formed

(green arrows near γ-components), but the fastest migrating band of β-components has not yet appeared (green arrows near ß-components). The LOX- and TG-dependent cross-link products of tetramers represent no difference in form, which implies delayed functions of TG-derived cross-link formation. The further oligomers and polymers were not yet formed within 24 hours.

To summarize all observations of collagen-fluorography, cross-links do derive from both LOX and TG in chicken keratocytes. LOX-derived cross-link formation is initiated at early stages of fibril formation and had a sustained influence at later stages of fibril organization, while TG-derived cross-links formed later. These observations suggest that LOX-derived cross-links are formed at the beginning of fibril organization and eventually led to the formation of lamellae. TG contributes to the adjustment of fibril diameters at later stages of fibril organization.



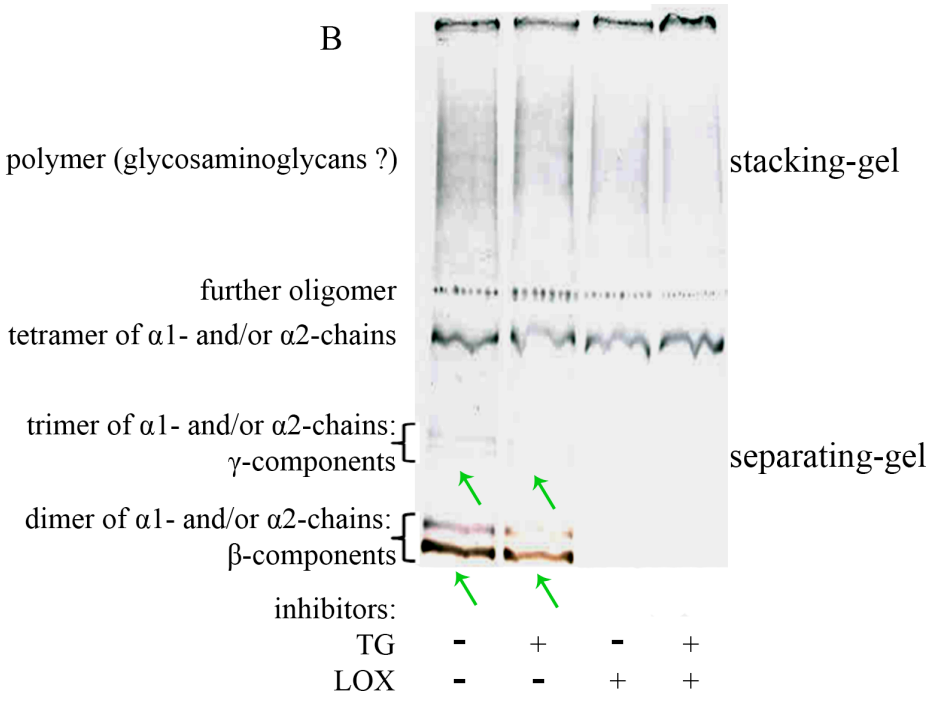


Figure 5-3. Cross-link analysis of cultured keratocytes labeled with ^{14}C-proline for 6 days (A) or 24 hours (B). The cell matrices of 3D-cultures were pepsin-solubilized, collagens were salt-precipitated and SDS-PAGE was run under non-reducing conditions. Gel exposures on a X-ray film finally. Green arrows: LOX-dependent cross-links; blue arrows: both enzyme-dependent cross-links; red arrow: absence of cross-link product.

5.4 Keratocytes produced less collagen V in the presence of LOX inhibitors, and more collagen V in the presence of TG inhibitors

Except for cross-link analysis, the relative amounts as well as proportion of collagen α-chains were studied. This densitometric analysis is based on the same gels of collagen-fluorography, which are described in section 5.3. The α-chains were derived from cultured keratocytes metabolically labeled with ^{14}C-proline for 6 days or 24 hours under different cross-linking conditions.

Three α-bands are visualized by fluorography: α1 and α2 bands of type I collagen, and one band of type V collagen (Fig. 5-4, left diagrams in A, B). In column diagrams, blue columns represent the ratio of α2 and α1 bands of collagen I. Red and green columns represent the intensity of the collagen V band compared with α1 or α2 band of collagen I, respectively. Keratocytes produced less collagen V in the presence of LOX-inhibitors after 6-day cultivation (A: plot 1 vs. 3). In cultures with TG-inhibitor, more collagen V was produced (A: plot 1 vs. 2). Inhibition of both enzymes led to a slight reduction of collagen V (A: plot 1 vs. 4). This change of collagen I/V proportions

could affect the formation and organization of corneal fibrils because the corneal fibrils are heterotypic I/V fibrils. By contrast, the proportions of α1 and α2 bands of collagen I were not clearly affected by cross-link formation (A: blue columns), which means that the chain composition of collagen I didn´t depend on cross-links formation as expected. In comparison with cultures labeled for 6-days, the relative amounts of collagen α-bands extracted after 24h of labeling are comparable whether or not LOX- or/and TG-derived cross-link formation was allowed (B). After 24h of cultivation only a small amount of collagen V was obtained. However, under control conditions, a maximum of collagen V was produced (B: plot 1). A minimum of collagen V appeared under the condition of LOX inhibition (B: plot 3).

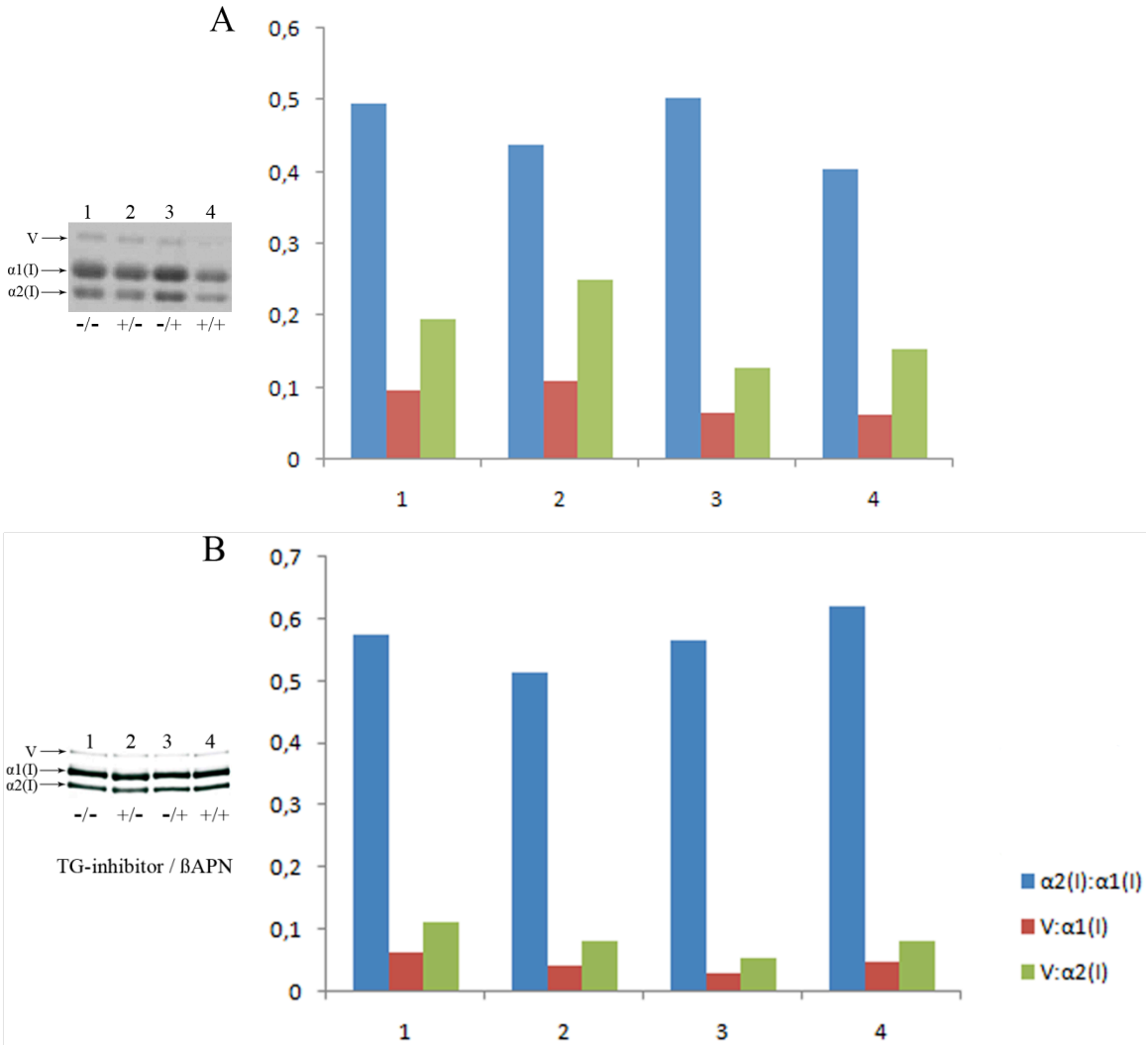


Figure 5-4. Densitometric analysis of collagen α-bands in cultured keratocytes labeled with ^{14}C-proline for 6 days (A) or 24 hours (B). The cell matrices of 3D-cultures were pepsin-solubilized, collagens were salt-precipitated and SDS-PAGE was run under non-reducing conditions.

5.5 Keratocytes predominate in preparations of corneal cells.

5.5.1 Cell preparations virtually exclusively contain corneal fibroblasts at early stages of fibril organization.

Since initial preparations of primary corneal cells not only contain keratocytes, but also epithelial cells, we were interested in the question of which cell type survived after prolonged culture. The cell types in our cultures were identified with a monoclonal antibody against cytokeratin (subtype CK3), which was described as a specific cell marker of corneal epithelial cells (Chaloin-Dufau et al., 1990; Cubitt et al., 1993). Corneal fibroblasts were isolated from 17-day-old chicken embryonic cornea and allowed to proliferate on µ-slides for 2 days without cross-link inhibitors. Thereafter, cells were stained by immunofluorescence with an antibody against cytokeratin. Nuclear DNA was visualized by DAPI staining.

In the cell slides, two types of areas with abundant cells can be found. Nevertheless, several cytokeratin-staining cells (red) can be found in area 1 but not 2 (Figure 5-5). However, area 2 corresponds to the majority of areas sampled from the slides, whereas area 1 appeared rarely. This experiment showed the coexistence of keratocytes and epithelial cells in corneal fibroblast at early stage of fibril organization, but the amount of keratocytes was much larger than that of epithelial cells.

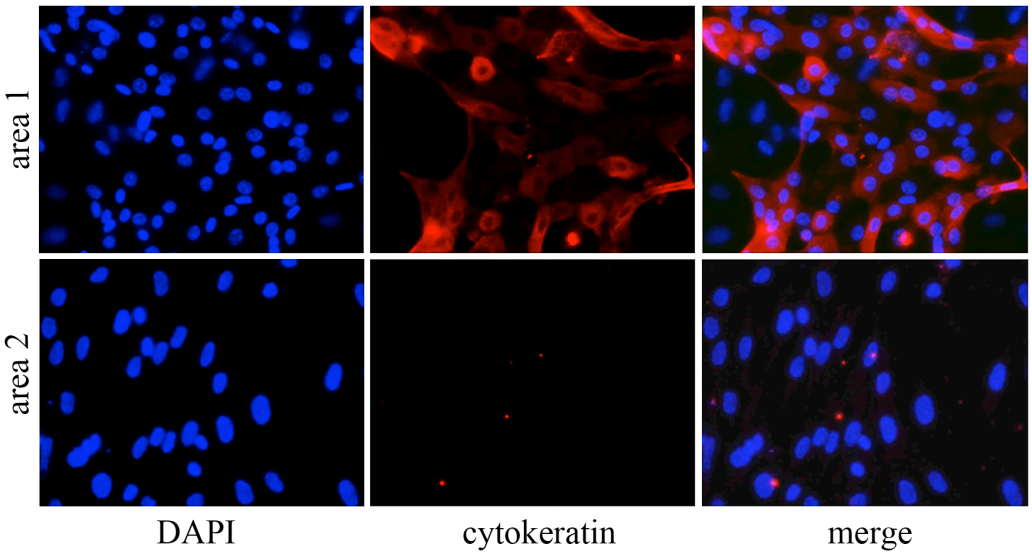


Figure 5-5. Immunofluorescence staining of cytokeratin in corneal fibroblast ECM at day 2 in µ-slides (ibidi). Ethanol-fixed cultures of corneal fibroblasts, permeabilized with Triton X-100, were immunostained with antibody against cytokeratin (red) and were stained with DAPI (blue) for localization of nuclear DNA.

5.5.2 Even less epithelial cells exist after 14-days in cultures of corneal fibroblasts.

In order to demonstrate the cell distribution at later stages of fibril organization, matrices from 14-days corneal fibroblasts were embedded in paraffin and immunofluorescence stained with antibodies against cytokeratin and collagen I.

Large amounts of collagen I (green) were seen in the newly formed ECM whereas only very few cells labeled for cytokeratin (red) were found (Figure 5-6). These data indicate that the proliferation and / or the survival of epithelial cells was suppressed under culture conditions employed. Epithelial cells were in our cultures and are unlikely to have a meaningful influence on the formation of lamellae and regulation of diameters of collagen fibrils.

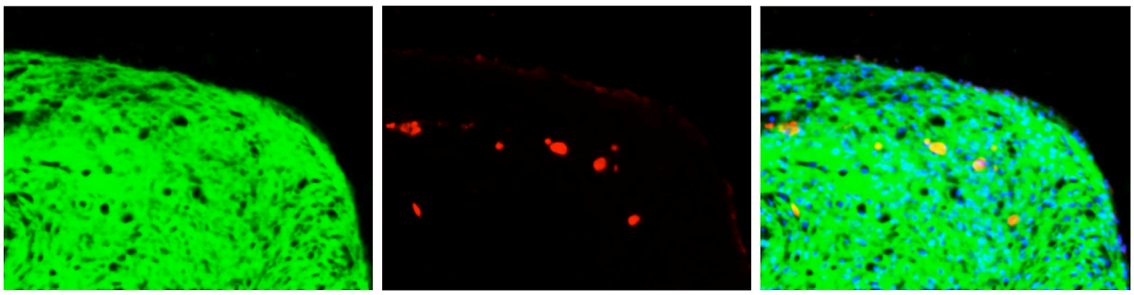


Figure 5-6. Immunofluorescence staining of cytokeratin and collagen I on paraffin section of embedded corneal fibroblast ECM after 14-days cultivation. Paraformaldehyde-fixed cultures of corneal fibroblasts were embedded in paraffin and cut into thin sections. After deparaffinization and rehydration the sections were immunostained with antibodies against cytokeratin (red) and collagen I (green), and co-stained with DAPI (blue) for localization of nuclear DNA.

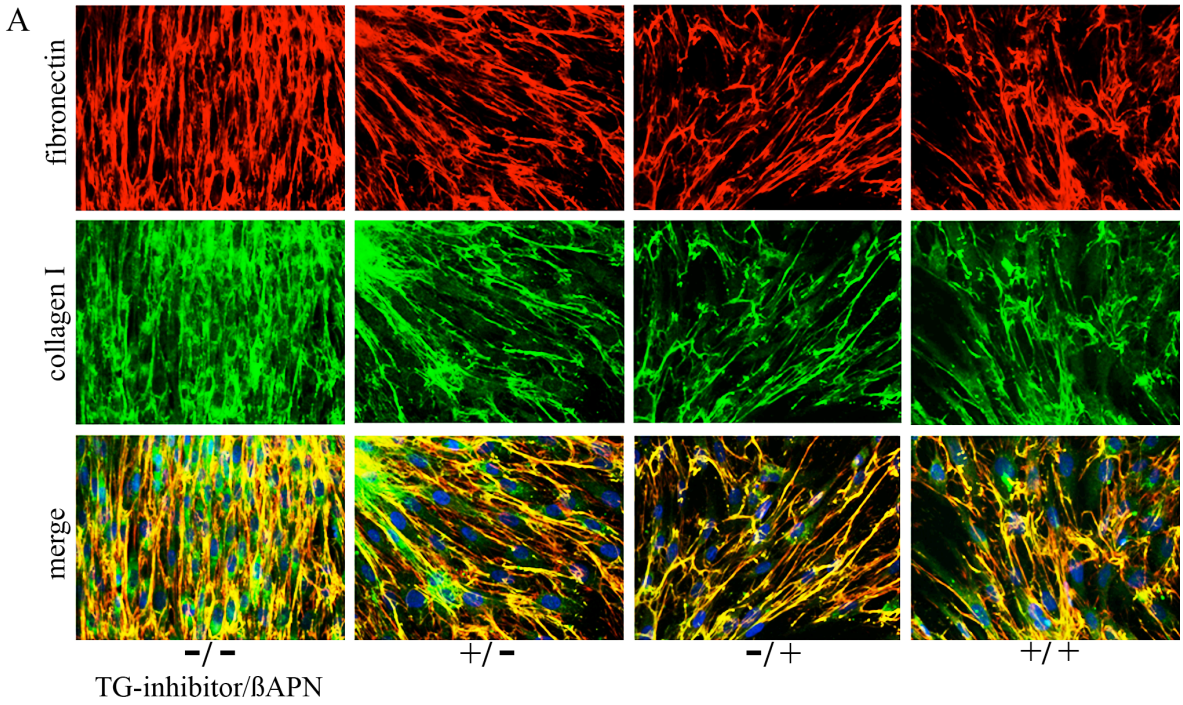
5.6 Matrix components of keratocyte cultures possibly involved in cross-link formation

5.6.1 Fibronectin

Fibronectin has been well characterized as an extracellular matrix glycoprotein that regulates many cellular functions. It has been reported that cellular fibronectin binds with high affinity to lysyl oxidase and is critical for proteolytic activation of LOX (Fogelgren et. al, 2005). Moreover, fibronectin binds to tissue transglutaminase and plays a role in the assembly of ECM (Gaudry et al., 1999). Therefore, the localization of fibronectin was checked in keratocyte cultures under different conditions of cross-

link formation. Keratocytes were cultured in µ-slides (ibidi) as described in 4.2.2 and were allowed to proliferate for 2 or 7 days. Cell layers were doubly labeled by immunofluorescence for fibronectin (red) and type I collagen (green). Nuclei were visualized by DAPI staining (blue).

Immunofluorescence micrographs (Fig. 5-7) show that the deposition of fibronectin in the ECM already occurred at the beginning of cell proliferation (A, red). At day 7 fibronectin was densely distributed over the whole ECM (B, red). There is no obvious difference in the distribution of fibronectin at day 2 and day 7 (A, B, red). The production and deposition of collagen I also seems to be the same and was not detectably influenced by cross-link inhibitors (A.B, green). Fibronectin co-distributed with collagen I at early phases of matrix formation already (A, merge, yellow). These results suggest that the deposition of fibronectin in the ECM is not affected by either LOX- or TG-derived cross-link formation.



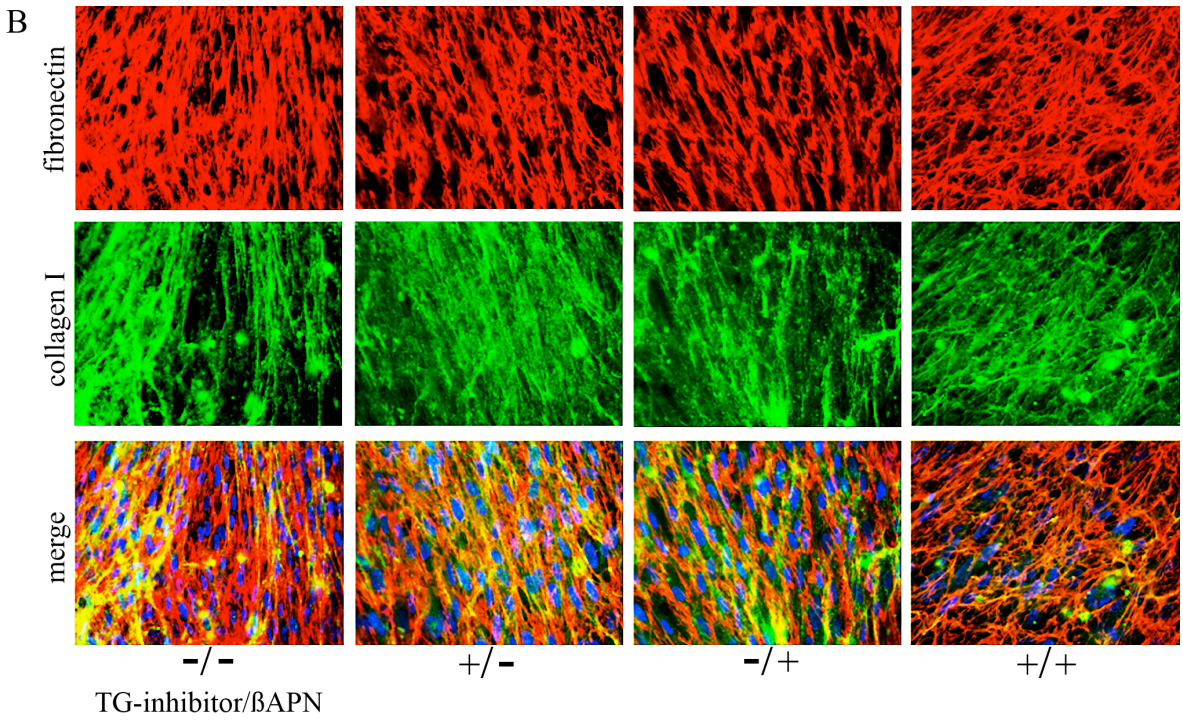


Figure 5-7. Immunofluorescence staining of keratocytes in µ-slides (ibidi). Cells were cultured under 4 conditions (control, -/-; in the presence of TG-inhibitor, +/-; in the presence of ßAPN, -/+; and with both inhibitors, +/+) for 2 (A) or 7 days (B). Ethanol-fixed cultures, permeabilized with Triton X-100, were immunostained with antibodies against fibronectin (red), collagen I (green) and with DAPI (blue, nuclei).

5.6.2 Decorin

Decorin is a proteoglycan widely distributed in the ECM and thought to be responsible for the structure, tissue organization, and surface properties of fibrils. Decorin has a primary role in regulating fibril assembly and show a coordinated regulation of collagen fibrillogenesis in the cornea together with biglycan. In decorin-null stroma, occasional abnormal fibrils were observed (Zhang et. al, 2009). Therefore, we were interested whether the cross-link inhibitors influence the deposition of decorin in the ECM. Keratocytes were cultured in µ-slides as described in 4.2.2 and allowed to proliferate for 2 or 7 days under different conditions of cross-link formation. Cell layers were doubly labeled by immunofluorescence for decorin (red) and type I collagen (green). Nuclei were visualized by DAPI staining (blue).

Decorin was deposited into extracellular space under control condition at day 2 (Fig. 5-8 A, -/-, red, extracellular staining indicated by white arrows) whereas it remained mostly intracellular in the presence of cross-link inhibitors (A, +/-, -/+ and +/+, red). After 7-day of cultivation, decorin was deposited into all of the extracellular space.

Moreover, micrographs suggest that ßAPN reduced the intensity of the decorin signal at day 7 (B, -/- -/+ versus -/+ +/+). But such differences of signal intensity may be caused by the mode of matrix deposition which can be demonstrated only at higher magnifications. The data suggest co-localization of collagen I and decorin at day 7 (B, merge, yellow).

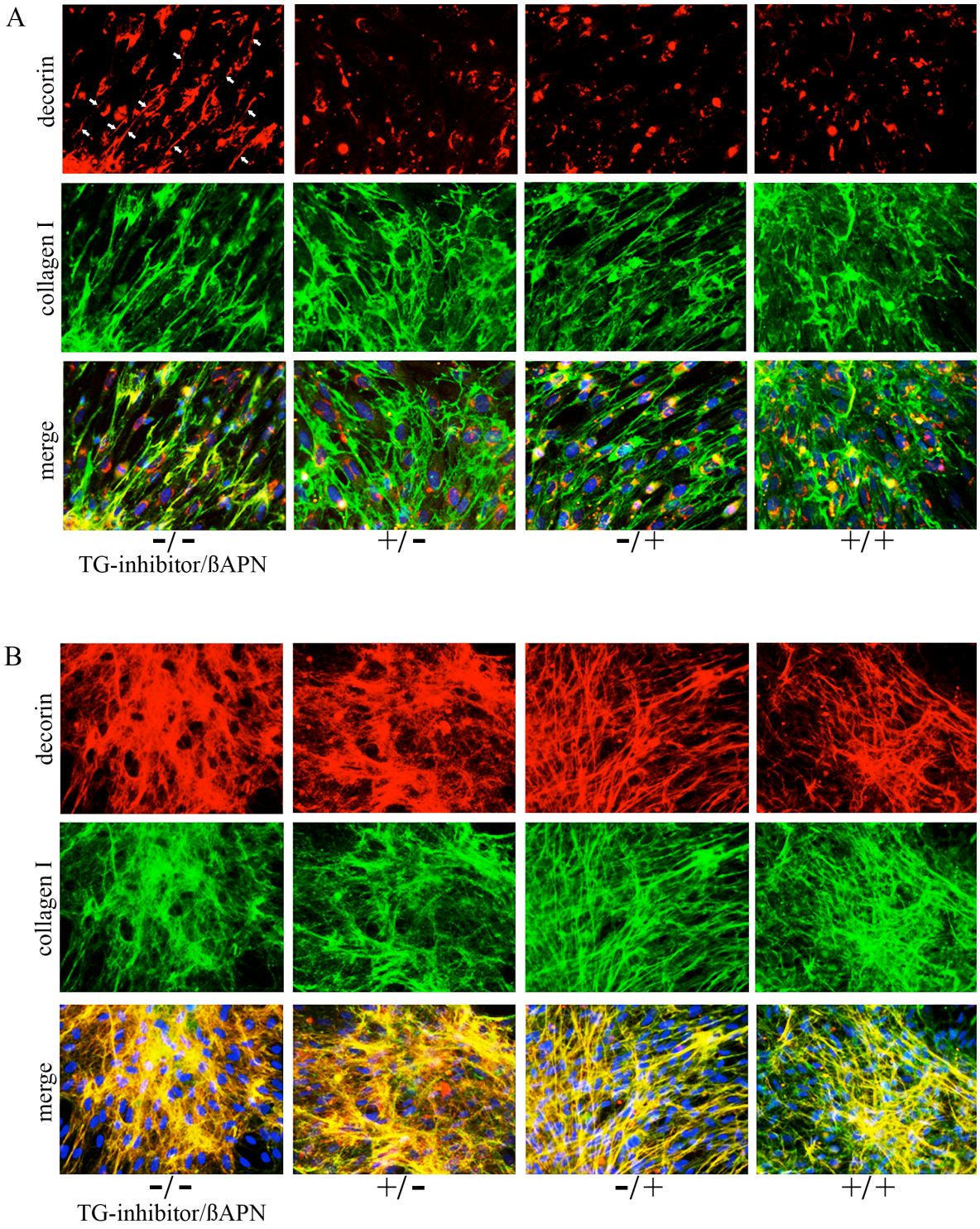


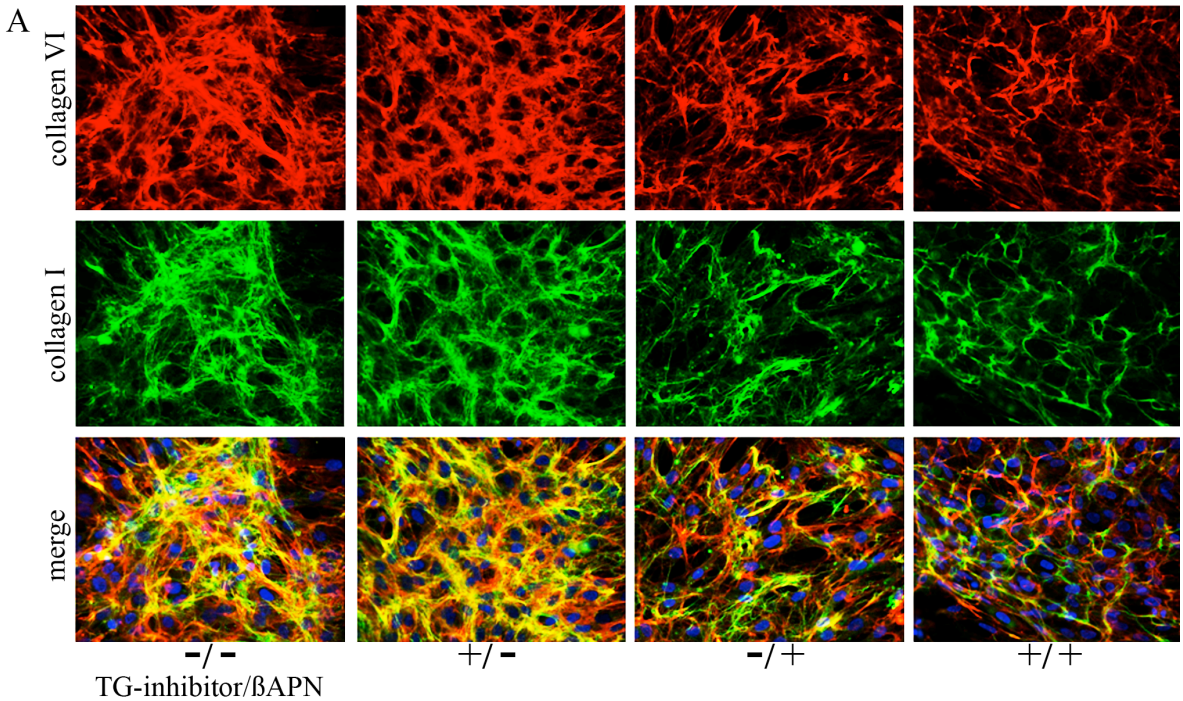
Figure 5-8. Immunofluorescence staining of keratocyte in µ-slides (ibidi). Cells were cultured under 4 conditions (control, -/-; in the presence of TG-inhibitor, +/-; in the presence of LOX-

inhibitor ßAPN, -/+; and with both inhibitors, +/+) for (A) or 7 days (B). Ethanol-fixed cultures, permeabilized with Triton X-100, were immonostained with antibodies against decorin (red), collagen I (green) and with DAPI (blue, nuclei).

5.6.3 Collagen type VI

Type VI collagen is a ubiquitous matrix protein. It is a major component in human corneal stroma and exists also in chicken cornea. The collagen VI microfibril is highly disulfide cross-linked and contributes to a network of beaded fiaments interwoven with other collagen fibrils (von der Mark et. al, 1984). Because of the ubiquitous distribution of collagen VI and its relationship with cross-links, the localization of collagen VI was investigated in keratocytes cultures. Cells were cultured in µ-slides as described in 4.2.2 and were allowed to proliferate for 2 or 7 days under different conditions of cross-link formation. Cell layers were doubly labeled by immunofluorescence for collagen VI (red) and type I collagen (green). Nuclei were visualized by DAPI staining (blue).

Immunofluorescence micrographs (Fig. 5-9) show that the deposition of collagen VI in ECM occurred at the beginning of cell proliferation and fibril organization (A, red). There is no apparent difference of the distribution of collagen VI at day 2 and day 7 (A,B, red), and also was not affected by LOX-and TG-derived cross-link formation. These data suggest that collagen VI was co-distributed with collagen I at early phase of matrix formation already (A, merge, yellow).



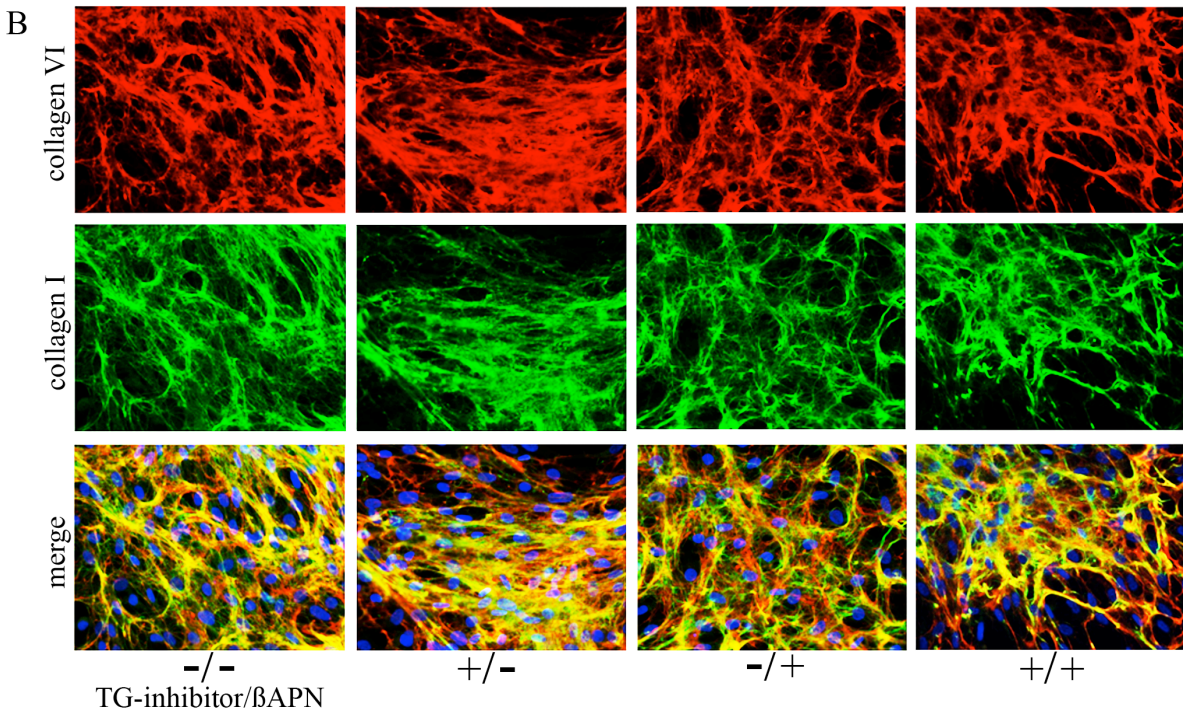


Figure 5-9. Immunofluorescence staining of keratocytes in µ-slides. Cells were cultured under 4 conditions (control, -/-; in the presence of TG-inhibitor, +/-; in the presence of LOX-inhibitor ßAPN, -/+; and with both inhibitors, +/+) for 2 (A) or 7 days (B). Ethanol-fixed cultures, permeabilized with Triton X-100, were immunostained with antibodies against collagen VI (red), collagen I (green) and with DAPI (blue, nuclei).

5.7 Effect of crosslink inhibitors

5.7.1 Tissue transglutaminase was effectively inhibited by TG-inhibitor in keratocytes

The activity of transglutaminase in cell culture was measured by addition of fluorescence-labeled cadaverine, a primary amine donor for detecting the endogenous substrates for active TG (Lajemi et al., 1997). Keratocytes were cultured in the presence of Alexa Fluor® 555 (Invitrogen)-conjugated cadaverine and visualized by immunofluorescence microscopy, as described in 4.10.

Under control conditions, strong signals of Alexa Fluor® 555 (red) documented the existence of TG activity in keratocyte cultures (A), while the matrix showed much weaker signals in the presence of 5 µM TG-inhibitor (B). These results validated the effective inhibition of TG activity in keratocytes by the addition of the TG-inhibitor.

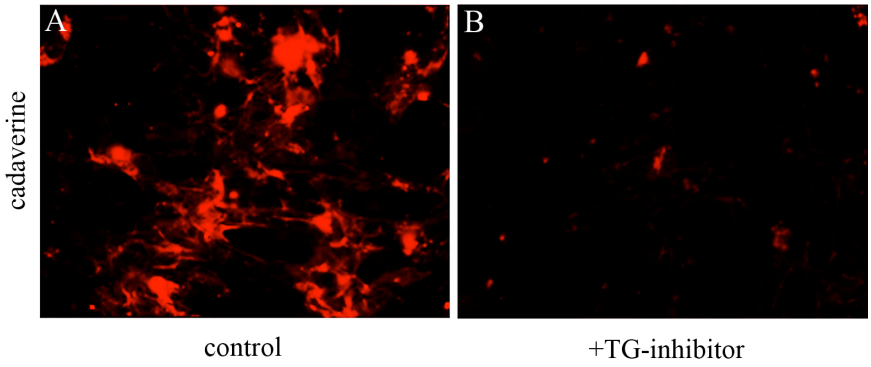


Figure 5-10. The activity of tissue-transglutaminase was proved in the cell cultures by addition of fluorescent-labelled cadaverine as a potential substrate.

5.7.2 Lysyl oxidase activity is detectable and varied during the process of keratocytes proliferation.

The activity of LOX in keratocytes was monitored during the cell growth by a tritium-release assay (see 4.9). The enzyme activity was tracked for 6 days. After 24h cultivation, LOX-activity was already detectable and was discontinuous over a 6-day culture period. At day 3 it reached a maximum, decreased subsequently to a minimum at day 4 and thereafter, increased at least until day 6 (Fig. 5-11). The changes of LOX-activity in keratocytes suggested that LOX had distinct effects on collagen fibril organization as time progressed in corneal fibroblast proliferation.

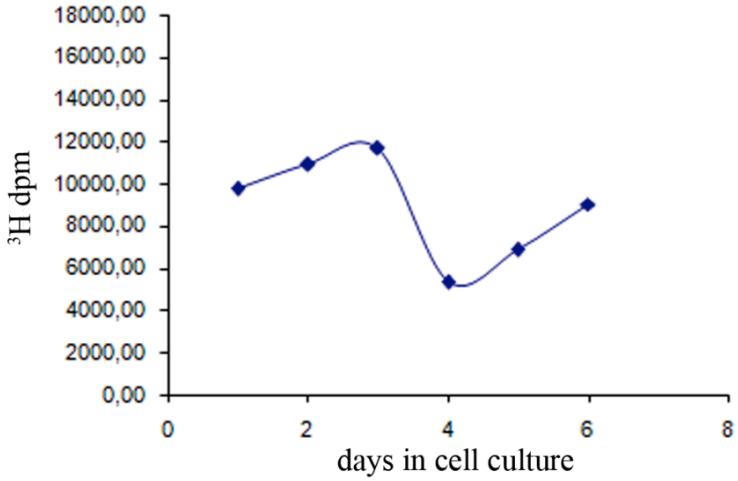


Figure 5-11. The activity of lysyl-oxidase in corneal fibroblast was measured per a tritium-release assay using L-[4, 5-^{3}H]lysine labeled chick aortas as elastin substrate.

5.8 Determination of absolute collagen amounts produced by keratocytes in culture

Total collagen was assessed in order to determine the absolute collagen amounts deposited in matrices of keratocytes. This assay is based on the detection of hydroxyproline (see 4.8).

The proteins were extracted from the 3D matrices deposited after 2-days or 14-days of culture under different conditions of cross-linking. At day 2, matrices under control conditions (-/-) represented highest collagen deposition whereas matrices in the presence of both cross-link inhibitors (+/+) had the lowest collagen amount. Matrices with TG- or LOX-inhibitor (+/-, -/+) contained intermediate amount of collagens in comparison to controls (Fig. 5-12, A). Similarly to the cultures at day 2, matrices at day 14 showed also a highest collagen level in controls (-/-) and lowest level when both inhibitors were applied (+/+). In comparison with ßAPN (-/+), the addition of TG-inhibitor led to small collagen quantities deposited into the matrices (+/-) (Fig. 5-12, B). Inhibition of LOX- or/and TG-derived cross-link formation does not lead to a drastic reduction of collagen deposition in matrices at day 2 and day 14. Additionally, purified tendon collagen I isolated from 17-day-old chicken embryos and bovine albumin (SERVA) were measured in parallel as positive and negative controls proving the specificity of this “total collagen assay” (Fig. 5-12, C).

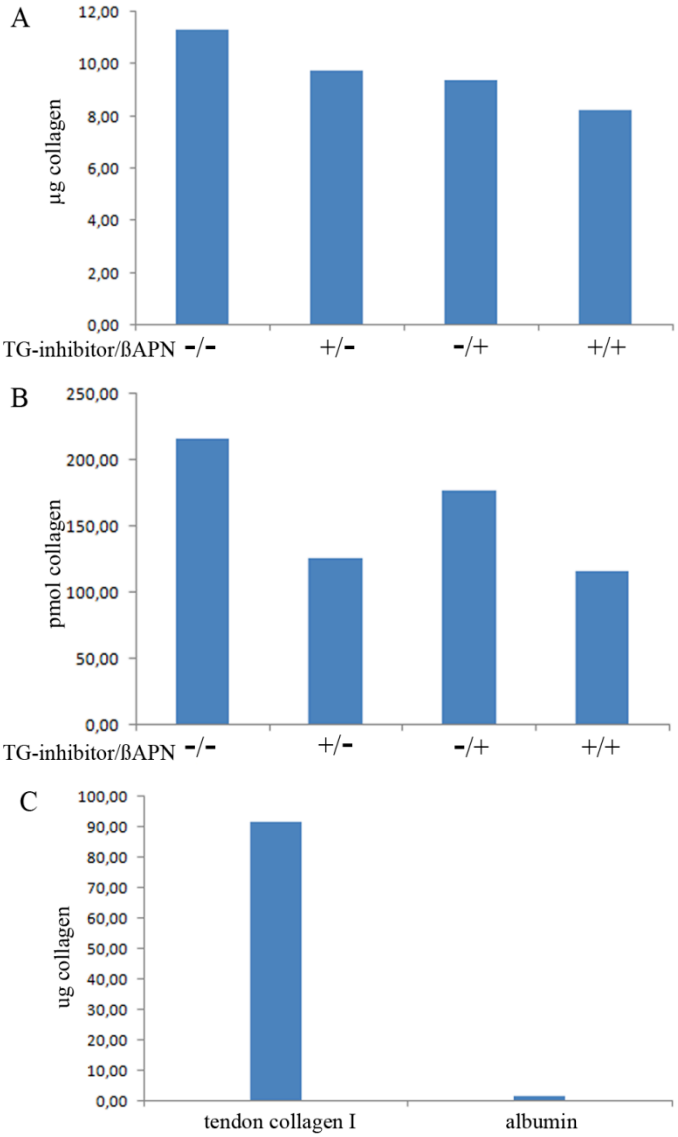


Figure 5-12. Total collagen assay, which based on hydroxyproline measurement, was performed of keratocytes at day 2 (A), day 14 (B). Tendon collagen I and bovine albumin were parallel measured as positive and negative controls (C).

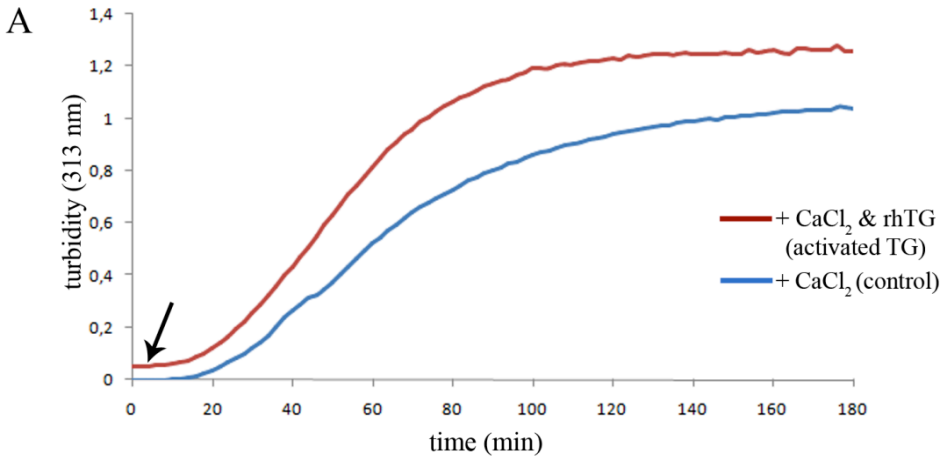
5.9 *In-vitro* experiments indicated that TG controls fibril diameter, whereas factor XIII does not.

5.9.1 Turbidity curves of *in-vitro* fibrillogenesis with crude corneal collagen mixture in the presence of TG or factor XIII

Factor XIII is a transglutaminase abundantly occurring in keratocyte cultures (component of FCS). The enzyme catalyzes cross-linking of proteins with the same isopeptide bond as tissue transglutaminases. Therefore, we investigated the effects of factor XIII on corneal collagen fibril organization. Crude collagen mixtures without proteoglycans were isolated from 17-days embryonic cornea. Fibrillogenesis was initiated by adjusting the solutions to appropriate conditions and was monitored by

turbidity measurements (4.3.1). Human recombinant human tissue transglutaminase (rhTG) and coagulation factor XIII from human plasma were added to the reconstitution mixtures direct after dilution with water. Both enzymes are commercially available from ZEDIRA and are Ca^{2+}-dependent. In addition, factor XIII must be activated by thrombin.

In the presence of activated rhTG (Fig. 5-13, A, red) the curve achieved higher plateau levels of turbidity than the controls (A, blue). It is noteworthy that a sudden increase of turbidity appeared at the onset of fibrillogenesis (A, red, black arrow). This phenomenon was not detectable in controls. The results may imply that small aggregates of collagens were formed at the initial phase of fibrillogenesis, which may be very important to subsequent fibril organization. On the other hand, in the presence of activated factor XIII (Fig. 5-13, B, red) we observed lower plateau levels of turbidity than in controls (B, blue). There are no obvious effects detectable at the onset phase of fibril formation. Additionally, a delayed lag phase in turbidity occurred with activated factor XIII (B, red) in comparison with the controls (B, blue).



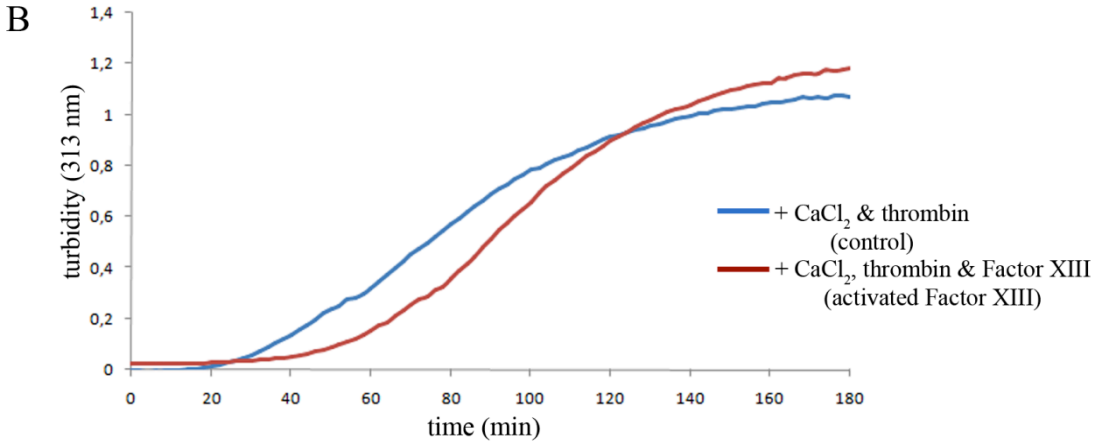


Figure 5-13. *In-vitro* fibrillogenesis of mixtures of crude cornea collagen in the presence of rhTG (A) or factor XIII (B), monitored by development of turbidity at 313 nm. In both cases blue curve indicates control condition while red curve indicates the activated condition. rhTG or factor XIII were added at the beginning of fibrillogenesis.

5.9.2 Diameter distribution of fibrils reconstituted in the presence of activated TG or factor XIII

In-vitro fibrillogenesis was performed with different amounts of enzymes. Again, the crude corneal collagen mixtures without proteoglycans were used (see 4.3.1). Reconstituted products were directly adsorbed to nickel grids, negatively stained with uranyl acetate, and observed with a transmission electron microscope. Fibril diameters were analyzed in all cases (Fig. 5-14). Fibrils reconstituted under control conditions (collagen mixtures with $CaCl_2$) represented a diameter distribution of 545.60 ± 142.29 nm, n = 215 (A). In the presence of activated rhTG, the fibrils became thinner in a dose-dependent manner. Addition of 2 µg rhTG led to a diameter distribution of 398.20 ± 104.11 nm, n = 226 (B), and 4 µg rhTG led to a distribution of 302.60 ± 101.72 nm, n = 115 (C). In the presence of activated factor XIII the diameter of reconstituted fibrils did not change in comparison with controls. They had a distribution of 553.74 ± 146.82 nm, n = 42 (25 µg factor XIII) or 610.51 ± 168.68 nm, n = 71 (50 ug factor XIII).

These results illustrate that tissue transglutaminase controls fibril diameters in "*in-vitro* systems", even when collagen preparations already containing cross-links were used as starting material. Factor XIII has no effect on diameter control of corneal collagen fibrils.

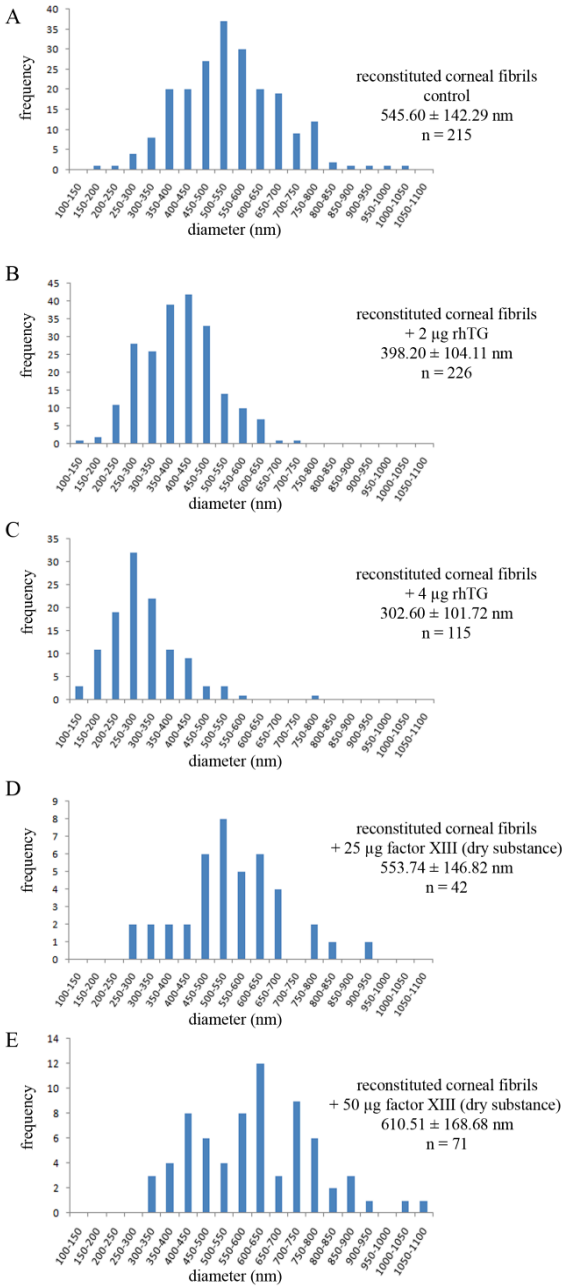


Figure 5-14. Analysis of fibril diameter distribution: The diameter of collagen fibrils were measured of reconstituted products from 3h-fibrillogenesis of crude mixture alone (A), in the presence of a recombinant human tissue transglutaminase (His_6-rhTG2) (B, C) or a coagulation factor XIII from human plasma (D, E) by electron microscopy.

5.9.3 Morphologic changes of fibrils

Although we have shown that the reconstituted fibrils became thinner in the presence of activated rhTG, the “thin fibrils” had a mean diameter around 300 nm. Not only the thickness, but also the morphology of fibrils was different from fibrils in authentic corneal stroma. Therefore, a corneal collagen mixture still containing proteoglycans was used as starting materials in further fibrillogenesis experiments (see 4.3.2). *In-*

vitro experiments were done as described in 5.9.1 and the reconstituted products were investigated by transmission electron microscopy.

Interestingly, network-like structures can be found only in the reconstituted products with activated rhTG (Fig. 5-15 B). High magnification showed that those network structures were labeled with antibodies to collagen I (gold particles highlighted with red arrows, Fig. 5-15 B inset). Such collagen fibril containing networks were not apparent under control condition (A). These results may imply that proteoglycan components (such as decorin, lumican, biglycan etc.), as well as activity of tissue transglutaminase contributes to formation of thin fibrils with diameters of about 45 nm, approaching the diameter of authentic corneal fibrils.

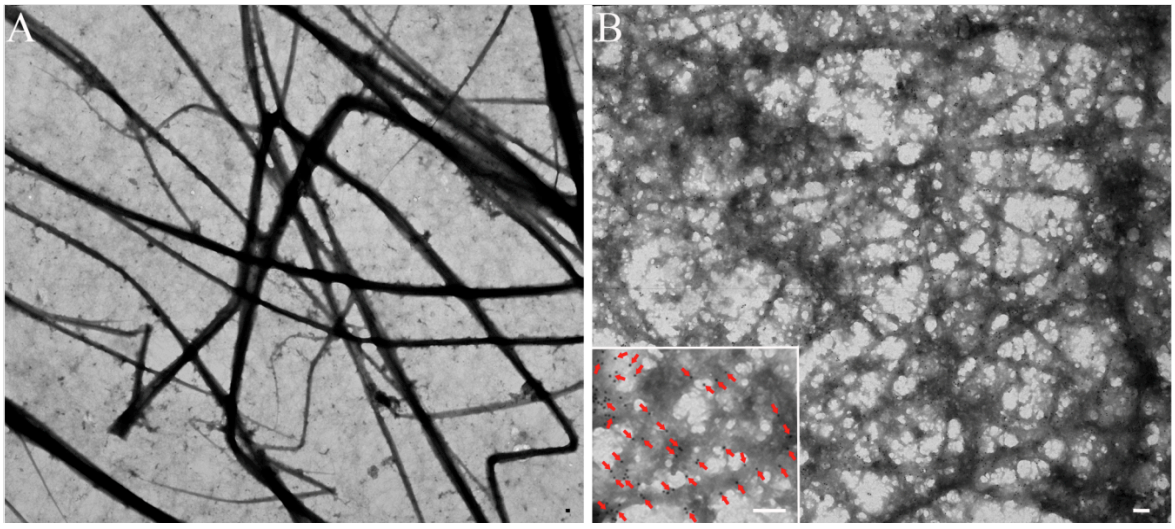


Figure 5-15. Fibrils reconstituted *in-vitro* from crude corneal collagen mixture with proteoglycan: electron micrograph of fibrils from *in-vitro* fibrillogenesis in the presence of not activated rhTG (A) or of activated rhTG (B). Collagen type I is heavily labeled with antibody gold conjugates indicated by red arrows in the inset of B. Bars: 200 nm.

5.10 The cross-link dependent collagen fibril formation in chicken embryonic tendon

The fibrillar organization in cornea and tendon are vastly different although both tissues contain collagens I and V as their major collagenous components. Mature cornea consists of orthogonally stacked lamellae formed by uniformly thin collagen fibrils evenly spaced in parallel whereas mature tendons contain fibrils with heterogeneous diameters arranged into parallel fibrous bundles. From this point of view, I was interested at the beginning of my study in the suprastructures of matrices

deposited by keratocytes or tenocytes from cornea or metatarsal tendons with and without cross-link formation. During the development of studies, cornea exhibited more important aspects to a cross-link-dependent fibril organization. Therefore, I focused my study mostly on cornea. However, some principles of “cross-link dependent collagen fibril formation in chicken embryonic tendon” were investigated in the studies described below.

5.10.1 Suprastructures of matrices deposited by tenocytes

Collagenous matrices with and without ßAPN were obtained from 3D-cultures of tenocytes isolated from metatarsal tendons of 17-day-old chick embryos and cultivated for 14 days. The arrangement of the collagen fibrils and the fibril diameter distribution with and without cross-link formation were analyzed on ultrathin sections of 3D matrices by electron microscopy.

In cultures of tenocytes, a small number of discrete bundles (Fig. 5-16 A) contained fibrils arranged in parallel arrays in the absence of ßAPN. Such structures of bundles were very similar to those of metatarsal tendons. Otherwise, irregular fibril arrangements and disorganized matrices were formed in the presence of ßAPN (D). This implies that LOX controls collagen organization also in embryonic tendon.

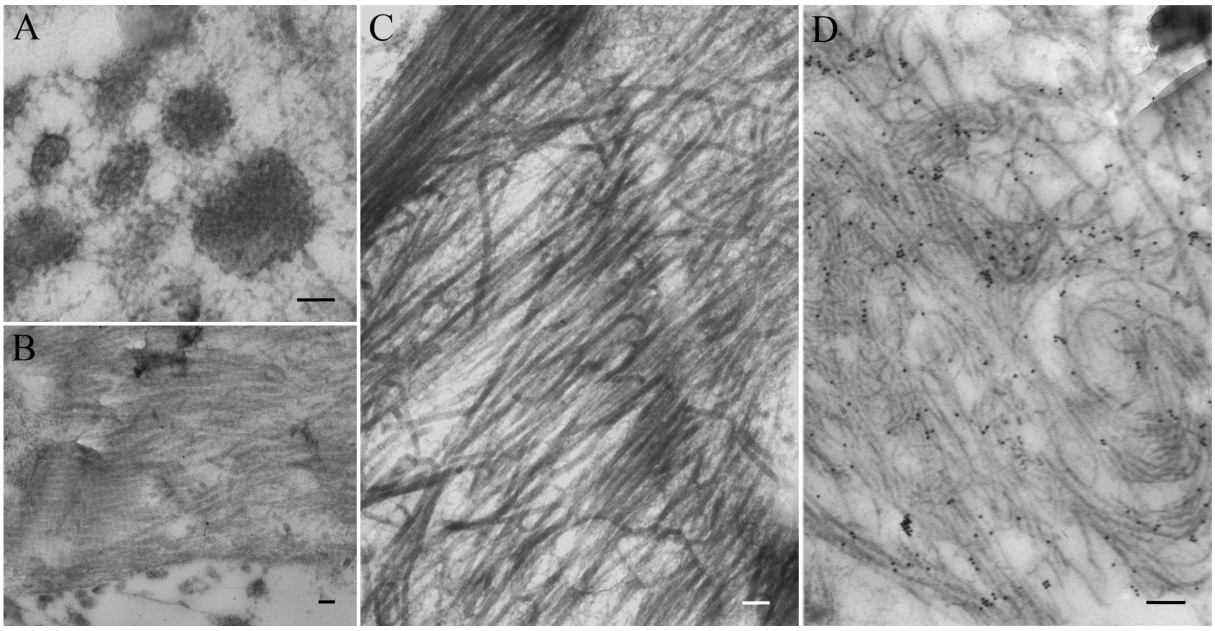


Figure 5-16 Suprastructures of matrices deposited by tenocytes. Control condition allowing for LOX-derived cross-link formation (A-C) or in the presence of LOX-inhibitor ßAPN (D). Small gold particles in micrograph D indicate the localization of collagen I. Bars: 200 nm.

5.10.2 Addition of ßAPN changed the fibril diameter distribution in tenocytes.

Analysis of fibril diameter distribution contributes further morphological details of the collagen fibrils formed by tenocytes in culture. The diameter of collagen fibrils was measured on images from ultrathin sections of 17-day-old embryonic chicken tendon (Fig. 5-17 A) or from cultures of tenocytes from 14-day-old chicken embryos (B, C).

Fibrils formed by tenocytes under control conditions showed a similar diameter distribution (B: 54.60 ± 8.62 nm, n = 604) comparing with the fibrils in metatarsal tendon tissue (A: 56.35 ± 11.33 nm, n = 701). In the presence of ßAPN the fibrils became thinner and had a broader distribution (C: 45.78 ± 14.12 nm, n = 517).

The results suggest that the arrangement of parallel fibrils and fibril bundles have a relationship to LOX-derived cross-links. Cross-links of collagens also contribute to fibril diameter control in tendon, for maintaining the thickness and arrangement of tendon fibrils.

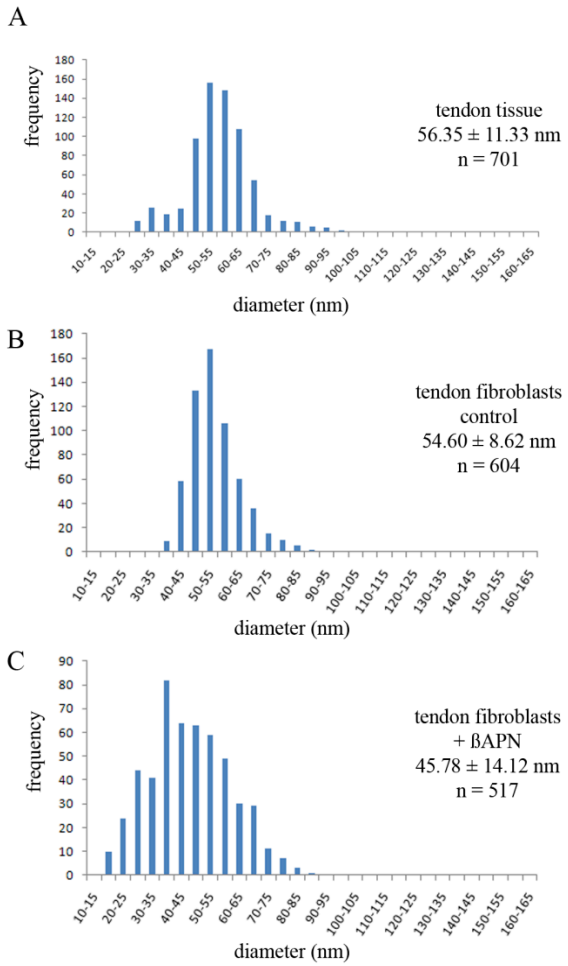


Figure 5-17. Analysis of fibril diameter distribution: The diameter of collagen fibrils were measured on ultrathin sections of authentic tissue (A) or of 3D matrices from chick cultures of tenocytes from metatarsal tendons by electron microscopy (B, C).

5.10.3 Lysyl oxidase activity is detectable and varied during the process of tenocytes proliferation.

The activity of lysyl oxidase in tendon fibroblast was also measured during the cell growth by a tritium-release assay (see 4.9). The measurement of activity was tracked for 6 days. After 24h cultivation, LOX-activity was detectable and was discontinuous over a 6-day period. Similarly to keratocytes, it reached a maximum at day 3. Thereafter, LOX activity decreased until cultures were discontinued at day 6 (Fig. 5-18). The changes of LOX-activity in tenocytes suggested that LOX had distinct effects on collagen fibril organization as time progressed in tendon fibroblast proliferation. These effects are also different from those in corneal cultures.

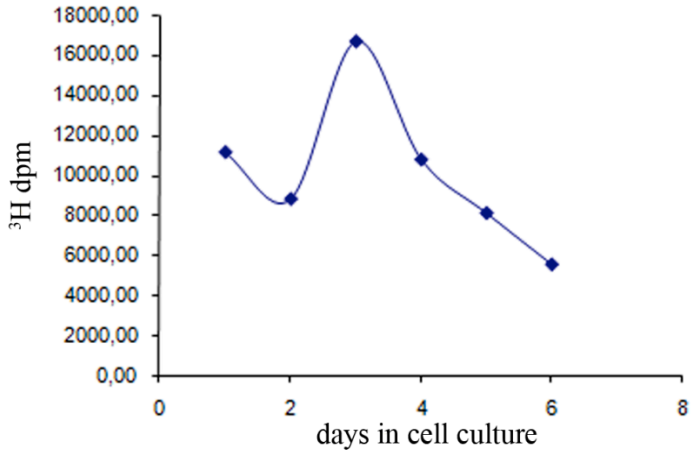


Figure 5-18. The activity of lysyl-oxidase in tendon fibroblast was measured per a tritium-release assay using L-[4, 5-^{3}H]lysine labeled chicken aortas as elastin substrate.

6. DISCUSSION

The size and organization of collagen fibrils within the ECM are crucial parameters for tissue structure and function. Corneal fibrils are heterotypic fibrils containing collagen I co-assembled with collagen V. Tendon fibrils contain predominantly of collagen type I, and to a lesser degree collagens III and V. Although both tissues contain similar collagenous components, the fibrillar organizations are vastly different. Mature cornea consists of orthogonally stacked lamellae formed by uniformly thin collagen fibrils evenly spaced in parallel. In contrast, tendons contain fibrils with heterogeneous diameters arranged into parallel fibrous bundles.

The aim at the beginning of my work was to obtain further insight into the molecular control of fibril formation in *in-vitro* experiments by finding differentially expressed matrix proteins in tendon and corneal fibroblast cultures. The proteins produced in these cultures were to be analyzed by 2D-electrophoresis, followed by identification of differential protein spots by mass spectrometry. But I got no exciting results using these biochemical methods. Thereafter, a change of the culture conditions led to a delectable finding. In our lab the routine fibroblast cells are cultured with supplement of ßAPN, in order to reduce the collagen cross-linking. In my 3D cell cultures I was not satisfied with the dense of matrix deposition using the routine method. A remove of ßAPN may increase the matrix deposition in fibroblast cell cultures. Also, a fortuitous discovery came out when this new culture condition was employed.

The essential role of covalent collagen cross-links in tissue-specific fibril formation became evident with the seminal observations that fibrils in primary cultures of chicken embryonic keratocytes had a typical lamellar organization that was disrupted in the presence of ßAPN. Furthermore, the introduction of γ-glutaminyl-ε-lysyl isopeptide bonds by tissue transglutaminase was required for the tight restriction of fibril diameters. This was lost in the presence of transglutaminase inhibitors. Therefore, in addition to lysyl oxidase, another important enzyme introducing collagen cross-links, i.e. tissue transglutaminase, has been identified in my work as an essential instrument of corneal matrix organization.

The same culture conditions were applied in tendon fibroblasts. Although parallel organization of fibrils and few discrete bundles of fibrils were found in the absence of ßAPN, the importance of cross-links was less prominent.

In this study, the original materials, collagens and cells, were from 17-day-old chicken embryos. Thus, all discussions and conclusions apply to this avian species and it remains to be investigated whether or not they can be extended to other organisms. The issue of corneal matrix organisation has been addressed predominantly by three complementary approaches:
(i) suprastructural analysis of electron micrographs derived from ultrathin sections of authentic tissues and matrices deposited in cell cultures;
(ii) immunofluorescence analysis of ECM components of interest on cell cultures;
(iii) *in-vitro* fibrillogenesis of purified collagens or mixtures thereof and analysis of the reconstituted fibrils by electron microscopy.

These strategies were implemented for cornea and metatarsal tendon tissues, for matrices deposited in 3D keratocytes and tenocytes, and for keratocytes proliferated directly on µ-slides. Small tissue pieces or cell matrices were embedded and cut into ultrathin sections for examination by electron microscopy or immuno-gold electron microscopy. After negative staining the fibril suprastructures were visualised in more details. In addition, compositional analysis is possible at early time points directly on the ECM deposited by the cells on µ-slides. In the 30 µl volume, keratocytes grow to confluence within two days. Moreover, collagen mixtures extracted and purified from authentic embryonic cornea were employed for *in-vitro* reconstitution. The *in-vitro* data complemented the results from cell culture system.

6.1 Cross-link dependent tissue-specific fibril formation and matrix organization in chicken embryonic cornea and tendon

The corneal stroma is unique in having a homogeneous distribution of small diameter fibrils that are regularly packed within lamellae and this arrangement minimises light scattering and permits transparency. The formation of small-diameter fibrils is commonly thought to be controlled by the globular domains of type V collagens (Birk et al., 1990; Marchant et al., 1996; Birk, 2001), or by co-assembly of the collagenous cores of the fibrils with decorin, biglycan (Zhang et al., 2009; Rada et al., 1993) or

lumican (Chakravarti et al., 1998, 2000; Rada et al., 1993). However, the formation of these thin fibrils into orthogonal stacked lamellae has not been explained. Ruberti and co-workers have shown that human corneal fibroblasts in culture produced lamellae of fibrils with similarity to the developing mammalian cornea (Guo et al., 2007). In this study, primary cultures of corneal fibroblasts were generated after their emigration from pieces of stromal tissue and the cells were passaged after 2-weeks of cultivation. Nevertheless, some questions have not been answered, e.g. how the lamellae of fibrils build in fibroblast cell cultures; it is still unknown that which factors or mechanism involve in this process. In the present work, fibroblast cells were isolated from chicken corneas digested with bacterial collagenase. In this way, matrix deposition and cross-linking occurred *de novo* after cell proliferation. Therefore, a study of the influence of cross-link formation on matrix organization in cell cultures could be achieved. A correlation between cross-link formation and suprastructural organization in cornea, especially with respect to diameter control of fibrils and their organization into lamellae has not been established before.

A major observation of the present work was that the unique suprastructure of corneal fibrils indeed depended on cross-linking. Cross-link inhibitors were added at the beginning of collagen production in cell cultures and, therefore, could exert their influence already at very early stages of fibril formation. The total collagen amounts deposited in matrices of keratocytes were measured and it was shown that cross-link inhibitors did not appreciably reduce collagen production. The arrangement of collagen fibrils in 3D keratocyte cultures was very similar to that in the developing chicken corneal stroma. Typical stacks of orthogonal sheets of parallel collagen fibrils were formed by keratocyte cell cultures in the absence of the cross-link inhibitor ßAPN. Such lamellae were not apparent in cultures with ßAPN. Therefore, LOX-derived cross-link formation is essential for fibril arrangement into lamellae in chicken cornea. By contrast, the diameter control in individual fibrils not only depended on collagen V (Birk et al., 1990, 2001) and SLRPs (Zhang et al., 2009; Chakravarti et al., 1998, 2000; Rada et al., 1993) but also on cross-linking. However, the chemical nature of such cross-links was isopeptide bonds introduced by tissue transglutaminases, but not factor XIII. Thicker fibrils with clearly visible banding pattern were formed in the presence of TG-inhibitor whereas the orthogonal arrangement of fibrils remained intact. Thus, TG-derived isopeptide cross-linking

seems to be an additional important requirement for a stringent control of fibril diameters to generate homogeneously thin fibrils in chicken cornea. A loss of both lamellae organization and diameter control of fibrils appeared when the formation of both LOX- and TG-derived cross-links was prevented. Interestingly, many thin fibrils with diameters of 20-25 nm which are even thinner than the authentic corneal fibrils were observed. However, structurally disrupted and thick fibrils were also observed in addition to the typically thin corneal fibrils. These results imply some functional connections between the formations of both cross-links. The suprastructural regulation in corneal fibrils is a mechanism with complementarity of LOX- and TG-derived cross-links. We presume that the stabilization of aggregates formed early during fibrillogenesis is essential for a tissue-specific outcome, i.e. the typical matrix organization in chicken embryonic cornea.

Analogous experiments were performed with tenocytes which resulted in comparable phenomena. Tendon tissue-specific bundles of fibrils and fibrils arranged in parallel were found only in controls allowing LOX-derived cross-link formation, albeit in low numbers. Such bundles were not apparent in cultures with ßAPN. The distribution of fibril diameters also changed slightly in the presence of ßAPN towards thinner fibrils with a broader diameter distribution. Thus, we conclude that LOX-activity may control collagen organization into bundles and parallel fibrils also in embryonic tendon at the tissue-level. LOX may also contribute to fibril diameter control and the maintenance of the thickness and tensile strength of chicken tendon fibrils.

Furthermore, amino acid analysis of cell layers deposited in 14-days corneal and tendon fibroblasts was carried out (in cooperation with Prof. Jürgen Brinckmann, Institute of Virology and Cell Biology, University of Lübeck). The raw data showed different contents of each amino acid in both cell cultures, especially with respect to hydroxyproline, proline, glycine and tyrosine. These data may contribute towards an explanation of different extents and the nature of cross-link formation in keratocytes and tenocytes. However, these data cannot be interpreted strictly because the matrices could not easily be separated from the cells. Hence, the values obtained correspond to crude mixtures of proteins derived from both the matrices and the cells. Distinct cell numbers in the respective cultures also could influence the measurement. Trypsin digestion of cell layers to separate the matrices from cells may be a partial

solution to this problem which, however, will leave intact collagenous but not non-collagenous matrix components.

	Cornea	Tendon
Hyp	126,30	98,66
Asp	41,41	48,38
Thr	21,20	20,78
Ser	27,60	27,11
Glu	64,97	72,57
Pro	141,46	124,25
Gly	289,84	325,80
Ala	84,22	99,62
Val	21,77	17,33
Met	8,43	7,68
Ile	14,90	13,17
Leu	31,66	26,74
Tyr	8,00	4,80
Phe	16,21	14,35
His	11,42	7,18
Hyl	18,91	16,93
Lys	25,00	21,74
Arg	46,71	52,91
Hyp/Pro	0,893	0,794
Hyp/(Hyp+Pro)	0,472	0,443
Hyl/Lys	0,757	0,779
Hyl/(Hyl+Lys)	0,431	0,438
Hyl/Hyp	0,150	0,172
µg Protein inj.	1,50	5,68
µg Protein/sample	300,96	1136,63
µg Collagen	373,60	1130,12

Figure 6-1. Amino acid analysis of matrices deposited in 14-days corneal or tendon fibroblasts (residues per 1000, without cysteine) (In cooperation with Brinckmann, Uni. Lübeck).

6.2 Analysis of LOX- and TG-derived cross-links in chicken keratocytes

Different tissue types showed distinct patterns of cross-linking chemistry, though most of them were essentially based on the reactions of peptide-bound aldehydes created from specific lysine, hydroxylysine side chains during the assembly of collagen subunits into fibrils. Reports are found in the literature on the basic mechanisms, the principal pathways, and the interaction sites of collagen cross-linking analyzed directly by several techniques. These techniques included labeling of reducible cross-links with tritiated borohydride, isolating them for structural analysis after proteolysis or acid hydrolysis as tritiated peptides or amino acids, and similarly by monitoring 3-hydroxypyridinium cross-linking residues by their inherent

fluorescence (Eyre et al., 1984, 1987, 2005). New methods were also developed in recent years, such as peptide isolation by HPLC followed by sequence analysis or protein mass spectrometry (Eyre et al., 2008). The TG-derived cross-links base on the reaction of γ-glutaminyl-ε-lysyl isopeptide bonds. The isopeptide can be hydrolyzed by both acid and alkali hence isolation and identification involves extensive enzyme digestion. Its presence can be detected by antibodies to the cross-link and indirectly by amino acid analyses before and after fluorodinitrobenzaldehyde (FDNB) derivativization to distinguish free and bound ε-amino lysine residues (Fratzl ed., 2008).

In the present work, collagen-fluorography using ^{14}C-proline labeled keratocytes was carried out to indirectly visualize all the chains of collagens and collagen cross-linking products in cell culture. Detailed analysis of the chains visualized by collagen-fluorography showed that separate LOX-dependent as well as LOX- and TG-dependent cross-link products co-existed in keratocytes. However, formations of both cross-links in the ECM are not synchronous. Thus, their influences on fibril assembly are time-dependent. For further details of the structure of cross-links, e.g. of cross-links between staggered or unstaggered collagen molecules within the fibrils, mass spectrometry of cross-linked peptides will be necessary. Cross-link products containing lysyl- or glutamine-modified peptides can also analyzed in this manner.

6.3 Matrix components of keratocyte cultures possibly involved in cross-link formation

Collagen V, which constitutes 15-20% of the fibrillar collagens in cornea, is considered as an important factor specifying the thin fibrils with uniform diameter in cornea tissue (Birk et al., 1990; Marchant et al., 1996). Collagen VI is present as a network throughout chicken secondary stroma (Doane et al., 1992). Decorin and fibronectin are both ubiquitous components of ECM that were shown to interact with collagen molecules. Decorin has been shown to associate with collagen fibrils in many connective tissues including corneal stroma (Fleischmajer et al., 1991). It associates with collagen I and leads to a delayed initial assembly of collagen molecules resulting a decreased fibril diameter (Vogel and Trotter, 1987). In addition, fibronectin can bind to tissue transglutaminase (Radek et al., 1993) and also acts as a scaffold for active LOX (Fogelgren et al., 2005). Furthermore, fibronectin binds and

enhances the activity of BMP-1, which is required for LOX activation. Based on these arguments the corresponding corneal components were investigated in keratocyte cultures.

α-Chains of collagens were metabolically labeled with ^{14}C-proline in keratocyte cultures, digested with pepsin, and analysed by PAGE and fluorography. In the present work, different behaviors were demonstrated of collagen V at early and later time points of fibrillogenesis in corneal matrices which was also influenced by cross-link inhibitors. The results imply that the cross-link formation affects the proportion of collagen V in formed corneal fibrils in a time-dependent manner. In keratocytes, deposition of collagens changed during cell proliferation: the proportion of α2(I)- and α1(I)-chains changed slightly; LOX inhibitor decreased while TG-inhibitor increased the relative amount of collagen V; and simultaneous application of both inhibitors led to a slightly reduction of collagen V at day 6. Since collagen V is considered as an essential regulator in heterotypic collagen I/V fibrils, this change of collagen I/V proportions could change the pathway of fibrillogenesis and fibril organization because the corneal fibrils are heterotypic I/V fibrils.

In the present work, immunofluorescence staining of fibronectin and collagen VI in the ECM were nearly the same under four different conditions of cross-link formation. These results suggested that the synthesis and deposition of neither fibronectin nor collagen VI were altered by suppression of LOX- or TG-derived cross-link formation. On the other hand, the results showed that the deposition of decorin into ECM was delayed by either LOX- or TG-inhibitors. Thus, deposition of decorin was affected by both LOX- and TG-derived cross-link formations during the initial phase of cell proliferation and fibril formation. The correlative functions of LOX-derived cross-link formation and deposition of decorin into ECM may explain LOX-dependent differences in suprastructural organization of corneal fibrils, especially concerning the formation of lamellae.

6.4 *In-vitro* reconstitution of crude corneal collagens

The assembly of collagen molecules into fibrils is an entropy-driven process, similar to that occurring in other protein self assembly systems, such as microtubules, actin filaments and flagella (Kadler et al., 1987). The fibril-forming collagens in embryonic

chicken are synthesized as soluble procollagens, and then are converted into collagens by specific enzymatic cleavage of terminal propeptides by the procollagen metalloproteinases. Without these proteinases the synthesis of collagen fibrils would not occur. Collagens extracted in acetic acid from authentic tissue are triple helical monomers without N- and C-propeptides. Therefore, they are capable to reconstitute into fibrils under suitable conditions, e.g. appropriate buffer salts, pH, and temperature. *In-vitro* reconstitution of collagens into fibrils is a well-established strategy for a time-dependent observation of collagen fibril formation (Birk and Silver, 1984; McPherson et al., 1985; Birk et al., 1990; Hansen and Bruckner, 2003; Trelstad et al., 2006), sometimes modulated by the presence non-collagenous matrix components.

In the present work, the structure and organization observed for fibrils reconstituted *in-vitro* in the presence of rhTG complemented and corroborated the results from the cell culture experiments in the presence of TG-inhibitor. In the presence of rhTG or factor XIII crude corneal collagens were differentially reconstituted into fibrils *in-vitro*. A sudden increase in turbidity at the initiation time of fibrillogenesis is consistent with the formation of specific early aggregates supported by TG activity at the beginning of fibril organization. Such small aggregates may serve as a nucleation site accelerating the formation of a large number of thin, tissue-specific corneal fibrils. Furthermore, analysis of diameter distribution showed that rhTG activity favored the formation of thin fibrils with crude corneal collagen mixtures. However, another transglutaminase, the factor XIII, does not have this capability. Interestingly, we also found that network-like structures composed of thin, banded, collagen I-containing fibrils only appeared in the reconstituted products in the presence of proteoglycans and TG activity. Here, fibrils reconstituted *in-vitro* from crude corneal collagens without proteoglycans exhibit obviously larger diameters in comparison to authentic corneal fibrils. In the presence of proteoglycans, fibrils became much thinner and similar to authentic 20-35 nm-fibrils. Therefore, we were able to show that two factors are essential for fibril diameter control in corneal stoma: co-polymerization of proteoglycans as well as the enzymatic activity of tissue transglutaminase (and not factor XIII).

In this experiment, the original materials of crude collagens should contain some cross-links, since initial cross-links were formed already in 17-day-old embryonic chicken cornea. We have tried to purify collagens from cell cultures of keratocytes in the presence of both LOX- and TG-inhibitors, in order to harvest more cross-link free material for reconstitution experiments. Because of the low yield of collagens in cell culture, as well as the loss during purification process, I failed to get enough materials for further experiments. To enlarge the cell culture system, to concentrate the purified collagen mixture, and to reconstitute fibrils directly on EM-grids in very small volumes, may represent possible strategies to achieve fibril reconstitution unaffected by a contribution of pre-existing cross-links.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

The data accumulated in this thesis show that the formation of cross-links is essential for the tissue-specific fibrillogenesis and matrix organization in chicken embryonic cornea and tendon. In cornea, the organization into lamellae was strongly effected by lysyl oxidase-derived cross-links whereas transglutaminase-derived cross-links have special effects on fibril diameter control. The inhibition of both LOX- and TG-derived cross-links leads to a loss of matrix organization (e.g. lamellae) and broad fibril diameter distribution with abnormal fibril morphology. Further, the deposition of collagen V and decorin into extracellular matrix was affected by alterations of cross-link formation. Parallel arrangement of fibrils and discrete bundles present in tendon fibroblast also depends LOX-derived cross-linking.

These observations and conclusions lead to the following prospections: (i) what is the mechanism of LOX-derived cross-link formation leading to an organization of fibrils into lamellae; (ii) do the early formed cross-linked small aggregates affect the further fibrils organization; (iii) which other factors are required for such tissue-specific matrix organizations; (iv) are there some transport canals, projections of the plasma membrane or some other special structures which may contribute to the orientation of corneal collagen fibrils into orthogonal arrangements.

Several methods are available helping to answer those questions, e.g. mass spectrometric analysis of cross-linked peptides which were deposited in fibroblast cultures; morphological studies of cross-linked small aggregates at early stage of fibrillogenesis on EM-level; employing purified LOX in *in-vitro* fibrillogenesis experiments; introduction of LOX siRNA into keratocyte cultures; analysis of differentially expressed matrix proteins under different cross-linking conditions using 2D electrophoresis. In addition, other components of interest, which may also involve in fibril formation and matrix organization in chicken cornea, should be studied.

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