Lateral Growth Limitation of Corneal Fibrils and Their Lamellar Stacking Depend on Covalent Collagen Cross-linking by Transglutaminase-2 and Lysyl Oxidases, Respectively

Received for publication, June 24, 2013, and in revised form, November 11, 2013. Published, JBC Papers in Press, November 21, 2013, DOI 10.1074/jbc.M113.496364

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Background: Mechanisms of growth limitation and lamellar stacking of collagen fibrils in cornea remain elusive.

Results: Covalent collagen cross-links are formed by catalysis involving both lysyl oxidases and tissue transglutaminase-2.

Conclusion: Aldehyde-derived and isopeptide cross-linking of collagen determine lamellar stacking and lateral fibril growth, respectively.

Significance: Two types of covalent collagen cross-linking are indispensable for correct corneal morphogenesis.

Corneal stroma contains an extracellular matrix of orthogonal lamellae formed by parallel and equidistant fibrils with a homogeneous diameter of ~35 nm. This is indispensable for corneal transparency and mechanical functions. However, the mechanisms controlling corneal fibrillogenesis are incompletely understood and the conditions required for lamellar stacking are essentially unknown. Under appropriate conditions, chick embryo corneal fibroblasts can produce an extracellular matrix resembling primary corneal stroma during embryonic development. Among other requirements, cross-links between fibrillar collagens, introduced by tissue transglutaminase-2, are necessary for the assembly of uniform, small diameter fibrils but not their lamellar stacking. By contrast, the subsequent lamellar organization into plywood-like stacks depends on lysyl aldehyde-derived cross-links introduced by lysyl oxidase activity, which, in turn, only weakly influences fibril diameters. These cross-links are introduced at early stages of fibrillogenesis. The enzymes are likely to be important for a correct matrix deposition also during repair of the cornea.

The suprastructure of the corneal stroma is well adapted to its specific functions as a robust and transparent tissue. Vulnerable structures within the eye are protected against environmental insults. At the same time, the cornea is an important optical element in that it diffracts light but does not impede its passage into the interior of the eye. These properties result from an exquisite fibrillar organization in the extracellular matrix (1–3). The human tissue, similarly to that of other vertebrate species, contains a stack of lamellae formed by parallel and evenly spaced collagen fibrils with small and uniform diameters. Within the lamellae, individual fibrils are packed into almost hexagonal arrays having a thickness of ~1 μm, corresponding to ~20 collagen fibrils and their intervening spaces. The orientation of the fibrils in neighboring lamellae is almost orthogonal.

During development of the chick embryo, the corneal stroma arises from epithelial cells depositing an extracellular matrix called the primary stroma, which contains relatively few fibrils that already are arranged in patterns reminiscent of future lamellae. Neural crest-derived cells that thereafter differentiate into fibroblast-like keratocytes invade this primordial tissue model. These cells generate the secondary, mature stroma by apposition of further fibrils into the pattern already established in the primary stroma and by incorporation of hyaluronan and proteoglycans, causing swelling of the tissue by colloid osmotic water binding (4–6).

The requirements for the formation of the uniformly thin corneal collagen fibrils have been investigated extensively by a variety of approaches, including reconstitution of fibrils from soluble molecular components in vitro matrix formation by keratocytes (7) and corneal epithelial cells (8) in culture and by histological and electron microscope analysis of corneas from transgenic and knock-out mice. The aggregated evidence suggests that several molecular species are indispensably involved in the appropriate fibrillogenesis in the cornea. Depending on the developmental stages, the fibrils not only contain several collagen types, including fibrillar types I, III, V, and XXIV (9) as well as fibril-associated collagens XII and XIV. In the primary stroma of the developing avian cornea, collagens II and IX are also found. In addition, several small proteoglycans with core proteins containing leucine-rich repeats (SLRPs),2 particularly lumican, fibromodulin, decorin, and biglycan, keratocan, and mimecan are also essential components of corneal fibrils (for review, see Ref. 10). For example, lumican-deficient mice have structurally abnormal corneal fibrils and, as a result, opaque corneas (11–13). The same is true for decorin- or biglycan-null mice.

The abbreviations used are: SLRP, small proteoglycans containing leucine-rich repeat; βAPN, β-aminopropionitrilite; TG, transglutaminase.

* This work was supported in part by Deutsche Forschungsgemeinschaft Grant SFB 492 and Projects A2 and B3.
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mice and, to a greater extent, mice with a compound deficiency of decorin and biglycan, suggesting redundant functions of the SLRPs in corneal fibrillogenesis (14, 15).

Whereas the molecular requirements for the generation of individual, small diameter fibrils in cornea have begun to emerge, the mechanisms controlling formation of lamellae or their stacking are an essentially uncharted territory. Keratocytes in culture can deposit a matrix, in which primordia of corneal lamellae are established (6, 7), and which is reminiscent of the primary or early secondary corneal stroma (2). However, the role of the macromolecular components remains to be identified. The regular arrangement of glycosaminoglycans between corneal fibrils suggested indirectly that anti-parallel association of glycosaminoglycans results in an even spacing (16). This would indicate that the essential information for lamellar stack formation already is available in the appropriate mixture of macromolecular constituents of the corneal stroma. By implication, the role of the cells consists of the biosynthesis of the macromolecules required but not necessarily in the building of the corneal architecture. However, keratocytes may well accelerate the deposition of the lamellae by forming compartments conducive to lamellar stacking. Here, we have studied further the role of keratocytes from 17-day-old chick embryos in forming lamellar primordia resembling those of primary corneal stroma. Surprisingly, we found that early formation of collagen cross-links is essential for both the limitation of fibril diameters and their organization into lamellae.

EXPERIMENTAL PROCEDURES

Three-dimensional Cell Culture— Fibroblasts were isolated from corneas of 17-day-old chicken embryos. After dissection of central regions of the corneas, tissues were washed in Krebs buffer. Subsequently, epithelia and endothelia were removed from tissue fragments by digestion in Krebs buffer containing 0.25% (w/v) trypsin and 2 mM EDTA for 15 min. After washing, tissues were minced and matrix-free fibroblasts were obtained by incubation with 1 mg/ml collagenase B from Clostridium histolyticum (0.191 units/mg, lyophilized, Roche Applied Science) in DMEM supplemented with 1% (w/v), each, of penicillin and streptomycin. Therefore, the cells were initially matrix-free. Cells were kept overnight at 37 °C and 5% CO2 in a humidified atmosphere incubator, washed three times with DMEM (10% FCS), and resuspended in DMEM (10% FCS), and distributed in 24-well plates (Nunc, Roskilde, Denmark) at 2 × 105 cells per well. Cells were cultured overnight and then supplemented with 0.14 mM L(+)-ascorbic acid, 1 mM sodium pyruvate, and 1 mM L-cysteine. In some experiments, cross-link inhibitors were added to give final concentrations of 0.2 mM β-aminopropionitrile (βAPN) and/or 5 μM transglutaminase (TG) inhibitor (Boc-DON-Gln-Ille-Val-OMe, ZEDIRA). After 14 days of cultivation, the cells formed three-dimensional cell-matrix constructs that were used for further experiments.

Collagen Purification—17-Day-old embryonic chicken corneas were washed three times in PBS and were extracted twice in 15 volumes of 0.5 mM acetic acid by stirring overnight at 4 °C. The tissue fragments were removed by centrifugation, and the combined extracts were supplemented slowly with solid NaCl to give final concentrations of 25% (w/v), precipitating a mixture of corneal collagens and non-collagenous macromolecules. The precipitates were recovered by centrifugation, dissolved in 50 mM Tris-HCl containing 2 mM NaCl and 200 mM NaCl, pH 7.4, dialyzed against the same buffer, and passed over a DEAE-cellulose column (3.5 × 21 cm, 200 ml, DE52; Whatman, Ltd.) equilibrated in the same buffer. A mixture of corneal collagens, without proteoglycans and other acidic components, were recovered from the breakthrough fraction. Both corneal collagens obtained by DEAE chromatography and crude corneal protein (before chromatography) mixtures were then dialyzed extensively against 100 mM Tris-HCl, pH 7.4, containing 400 mM NaCl (storage buffer). Proteins were precipitated by adding solid sodium chloride to a final concentration of 4.5 M. After centrifugation, pellets were redissolved in storage buffer and clarified by centrifugation. These materials were used for in vitro fibrillogenesis as described previously (17).

In Vitro Fibrillogenesis—The crude mixture of corneal collagens in storage buffer was degassed, and in vitro fibrillogenesis was carried out in a microcuvette (Multicell, light path, 1 cm, Beckman, Palo Alto, CA). Fibril formation was initiated by diluting the collagen mixtures with an equal volume of distilled water. In some experiments, tissue transglutaminase, CaCl2, or factor XIII and thrombin were added to the mixtures directly after dilution. The reconstitution products were examined by transmission electron microscopy and immunoelectron microscopy with antibodies to collagen I essentially as described (18).

Isolation of Fragments of Collagen Fibrils from Cornea—Human corneas were obtained from voluntary donors at autopsy and in agreement with local ethics regulations concerning recovery of human tissues. The corneas were stored in the tissue bank of the clinic of ophthalmology of the University Hospital of Münster. Pieces of corneas rejected for transplantation were homogenized in PBS, and fragments of collagen fibrils were isolated as described (19, 20). Briefly, homogenates were subjected to centrifugation at low speed, and fragments of tissue suprastructures in the low speed supernatants were directly analyzed by transmission electron microscopy at appropriate dilutions.

Transmission Electron Microscopy and Immunogold Electron Microscopy—Multilayer corneal fibroblast-matrix constructs were cultured for 14 days, gently detached from the culture dishes, and subjected to fixation at 4 °C overnight in 100 mM cacodylate buffer, containing 2% (v/v) formaldehyde and 0.25% (v/v) glutaraldehyde, pH 7.4. After washing in PBS, specimens were dehydrated in an ascending ethanol series from 30 to 70%, incubated overnight at 4 °C in LR White (Agar Scientific, Stansted, UK) and 70% ethanol (2:1) followed by several steps of pure embedding medium. Finally, LR White was polymerized under UV light according to the manufacturer’s instructions. Ultrathin sections were cut on an Ultramicrotome at random orientation of the specimen and collected on nickel grids coated with Formvar/carbon. Grids were floated on drops of 100 mM glycine in PBS for 30 s. Alternatively, 20 μl of reconstitution products from in vitro fibrillogenesis or fibrils isolated from homogenates of human cornea were absorbed for 10 min to Formvar/carbon-coated grids, washed with PBS, and treated for 30 min with 2% (w/v) skim milk in PBS. The ultrathin sec-
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Fibrils extracted from homogenized human cornea comprised a very rare population of strongly cross-striated fibrils with variable diameters (40–80 nm), in addition to the abundant and characteristic fibrils with small diameters described above (Fig. 1B). The large fibrils were not derived from scleral tissue because precautions were taken to use only central portions of the corneas. Interestingly, such fibrils were labeled with monoclonal antibodies to collagen III by immunogold electron microscopy (Fig. 1B), whereas the major population of small diameter fibrils was systematically devoid of such labeling even after demasking of the epitopes by treatment of the fibrils with acetic acid (26). The specificity of the commercial collagen III antibodies was suitable for this investigation, as revealed by immunoblotting on collagens I (negative) and III (positive) purified from human skin (data not shown). Keene and colleagues (27) have described previously a small population collagen III-positive fibrils in human corneas, but a preferential labeling of strongly banded, large diameter fibrils was not apparent. However, Bruns et al. (28) investigated tissue sections of embryonic and adult chick cornea and have observed large banded fibrils at very low abundance and in conjunction with microfibrillar material. Although this was not studied at the time, it is tempting to speculate that the large banded collagen fibrils in fact were similar to the collagen III containing fibrils described here. Such fibrils, and their aligned microfibrillar material, may serve to prevent mechanical stress-induced delamination of the lamellar stacks in cornea.

Next, we prepared suspensions of keratocytes obtained from corneas of 17-day-old chick embryos by enzymatic digestion of the tissue with collagenase. The cells were plated at high density and were cultured for 14 days in medium containing fetal calf serum. Ultrathin sections of the cultures containing the newly synthesized matrix were analyzed by transmission electron microscopy after negative staining. As shown in Fig. 2A, the organization of the fibrils was highly ordered and resembled that of the primary corneal stroma during development. Bundles of parallel, thin fibrils were deposited in orthogonal arrays. The fibrils contained collagen I, the quantitatively major constituent of corneal collagen fibrils, as revealed by immunogold electron microscopy (data not shown). Fibril diameters (n = 758) had a uniform Gaussian distribution centered at 45 nm (σ = 12 nm, Fig. 3B). Our observations are consistent with those reported previously on cultures of keratocytes emigrating from fragments of human corneal stroma (7). However, the experimental protocol employed here excludes the possibility that authentic tissue fragments influence or control the organization of fibrils deposited de novo. In our experiments, the cells were devoid of extracellular matrix at the time of initiation of...
the cultures. However, the cells still generated a de novo matrix with corneal characteristics.

The culture media were then supplemented with βAPN, a natural drug inhibiting the activity of all isoforms of lysyl oxidase, the enzymes catalyzing post-translational oxidative deamination of (hydroxy)lysyl residues to yield (hydroxy)lysyl aldehydes. In the absence of βAPN, the aldehydes spontaneously react and mature into several bivalent, trivalent, and oligomeric molecular species that covalently cross-link corneal fibrillar components, including collagens (29). Strikingly, the culture regimen with βAPN completely abrogated the formation of lamellae. Only residual assemblies sometimes remained of parallel and approximately equidistant fibrils alternating with non-fibrillar material as typically seen within corneal lamellae. By contrast, abolition of lysyl oxidase activity with βAPN had no major influence on the diameter of most fibrils (Fig. 2B). However, as revealed by the occurrence of clusters of gold particles in immunogold electron microscopy, the fibrils were identified as collagen I-containing when sectioned in oblique or longitudinal orientation (Fig. 2B, inset, arrows). Sections not exposed to antibodies to collagen I, were essentially devoid of gold particles and clusters were never observed (data not shown). 62% of the fibrils had diameters with a distribution centered at 44 nm (σ = 10 nm), which closely corresponds to the values observed in control cultures without βAPN. However, several minor populations of thicker fibrils also occurred, which resulted in a tailing of the distribution toward higher values (Fig. 3C).

We observed the formation of divalent and tetravalent but not trivalent cross-linked species labeled with 14C-labeled proline during early phases of our cultures. A fluorogram of a SDS-PAGE gel of collagen metabolically labeled during the first 24 h is shown in Fig. 4A, lane 1. By contrast, trivalent species appeared at the expense of dimer and tetramer species on a fluorogram of newly synthesized collagens labeled for 6 days (Fig. 4B, lane 1).

As shown in Fig. 4, A and B, lane 3, addition of βAPN to the culture medium does not entirely suppress the formation of cross-linked collagenous polypeptides at any time during culture. However, it is well known that βAPN, at the concentrations employed, inactivates all lysyl oxidase isoenzymes and, hence, eliminates all cross-links of the aldehyde variety (30).
Therefore, the persistence of cross-linked collagen polypeptides in cultures with βAPN necessitates alternative enzyme activities leading to collagen cross-linking. Candidates are transglutaminases catalyzing the formation of isopeptide bonds.

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JOURNAL OF BIOLOGICAL CHEMISTRY

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FIGURE 2. Suprastructures of matrices deposited by keratocytes. A, control condition allowing for normal lysyl oxidase- and TG-derived cross-link formation; B, in the presence of lysyl oxidase inhibitor βAPN (inset in B); C, in the presence of a TG inhibitor (Boc-DON-Gln-Ile-Val-OMe) (inset at higher magnification highlights the clear banding pattern of the fibrils); or D and E, with both inhibitors. Arrows in B (inset) and E emphasize gold particles labeling collagen I; arrowheads in B point toward residual parallel fibrils in the presence of βAPN. K, keratocytes. Bars, 500 nm (B, inset, 50 nm; C, inset, 200 nm)

FIGURE 3. Analysis of fibril diameter distribution by electron microscopy. Diameters of collagen fibrils were measured on ultra-thin sections of authentic embryonic chick tissue shown in Fig. 1A or of three-dimensional matrices from chick cultures of keratocytes from cornea (Fig. 2, A–E). The bars represent the number of fibrils falling into distinct 5-nm intervals of diameters. The solid lines are the Gaussian distributions calculated from the experimentally determined fibril diameters. A, tissue control (μ₁ = 35 nm, σ₁ = 6 nm, f₁ = 0.86; μ₂ = 52 nm, σ₂ = 8 nm, f₂ = 0.14; Q = 0.63). B, control without inhibitors (μ₁ = 35 nm, σ₁ = 8 nm, f₁ = 0.17; μ₂ = 48 nm, σ₂ = 12 nm, f₂ = 0.83; Q = 0.05). C, cultures with βAPN (μ₁ = 44 nm, σ₁ = 10 nm, f₁ = 0.62; μ₂ = 68 nm, σ₂ = 8 nm, f₂ = 0.22; μ₃ = 91 nm, σ₃ = 6 nm, f₃ = 0.08; μ₄ = 112 nm, σ₄ = 8 nm, f₄ = 0.04; μ₅ = 147 nm, σ₅ = 8 nm, f₅ = 0.02; Q = 0.17). D, cultures with transglutaminase inhibitor (μ₁ = 107 nm, σ₁ = 13 nm, f₁ = 0.87; μ₂ = 134 nm, σ₂ = 8 nm, f₂ = 0.13; Q = 0.05). E, cultures with both inhibitors (μ₁ = 25 nm, σ₁ = 4 nm, f₁ = 0.14; μ₂ = 53 nm, σ₂ = 10 nm, f₂ = 0.16; μ₃ = 75 nm, σ₃ = 8 nm, f₃ = 0.16; μ₄ = 96 nm, σ₄ = 8 nm, f₄ = 0.08; μ₅ = 120 nm, σ₅ = 8 nm, f₅ = 0.03; μ₆ = 160 nm, σ₆ = 8 nm, f₆ = 0.01; Q = 0.08).
between appropriately spaced side chains of glutamine and lysyl residues. Tissue transglutaminase (transglutaminase-2, TG-2) is present in cornea (31, 32) and is up-regulated after injury, particularly in the corneal epithelium but also in the stroma (32) and, possibly, also during corneal development because similarities are frequently observed in wound healing and development (10). Therefore, we treated our cultures with a synthetic inhibitor specifically and irreversibly inactivating TG-2. The lamellar organization of the collagen fibrils essentially remained normal under these conditions (Fig. 2C). However, the fibrils were much thicker and had an obvious \( \pi \)-periodic banding pattern (Fig. 2C, inset). Diameters fell into two Gaussian distributions centered at 107 nm (\( \sigma = 13 \) nm, 87% of the fibrils) and 134 nm (\( \sigma = 8 \) nm, 13%; Fig. 3D). Therefore, the average fibril width was more than doubled in comparison with control cultures without TG-2 inhibitor, and the diameter distributions overlapped only slightly (compare Fig. 3, B and C, with 3D). Metabolically labeled collagens were then isolated from the cultures with the TG2 inhibitor and were analyzed by SDS-PAGE followed by fluorography. As shown in Fig. 5B, lane 2, the formation of trimeric species at late time points was almost unaffected by the inhibitor, whereas tetramers were reduced in quantity to about one-half.

When both inhibitors were applied, fibrillogenesis was severely compromised. In general, fibrils were scarce and both the diameter control and the lamellar organization were lost (Fig. 2, D and E). The few fibrils, which still were formed, contained collagen I as revealed by immunogold labeling (highlighted by arrows in Fig. 2E). They also had irregular cross-sections that were similar to those of tendon or skin fibrils of decorin-deficient mice (33) because smaller fibrils appeared to fuse into larger and less organized fibril-like suprastructures. This event does not take place in corneal stroma under normal conditions because it would strongly impair tissue transparency. In addition, it prevented us from determining meaningful diameters of the thicker fibrils. However, there was an additional population of thin fibrils with a mean diameter of 25 nm (\( \sigma = 4 \) nm, Fig. 3E), which represented \( \sim 14\% \) of the fibrils. Such fibrils were not found in our cultures with or without addition of the single inhibitors.

Taken together, the results described above are consistent with the notion that the accurate deposition of collagen fibrils in cornea is specified, at least in part, by appropriate mixtures of macromolecules and their post-translational modifications, including intermolecular cross-linking by isopeptide bonds.
introduced by transglutaminase activity. To test this hypothesis, we generated crude extracts from chick embryo corneas comprising neutral salt-soluble collagens as well as acidic macromolecules normally eliminated by chromatography on DEAE-cellulose. These mixtures were subjected to reconstitution of fibrils in vitro (34) from buffers with or without Ca$^{2+}$. In some experiments, recombinant tissue transglutaminase-2 was also added. As shown in Fig. 5A, a heterogeneous population of fibrils as well as finely contoured aggregates without defined fibrillar features was formed when tissue transglutaminase-2 was not added. Therefore, a strict diameter control was not apparent under these conditions. By contrast, in the presence of tissue transglutaminase-2, thinner fibrils were formed, which massively accumulated non-fibrillar material on their surface (Fig. 5B). This effect was dose-dependent (Fig. 6). Fibrils were also reconstituted from collagen without material binding to DEAE-cellulose, i.e. without acidic components, including proteoglycans and glycosaminoglycans. As shown in Fig. 5C, average fibril diameters were also reduced in a dose-dependent manner when tissue transglutaminase-2 activated by Ca$^{2+}$ was added to the reconstitution mixtures. The blood-clotting factor XIII, which is known to occur in cornea (35), was ineffective although the enzyme was active (transfer of dansyl cadaverine to standard proteins; data not shown). Therefore, the restriction of lateral growth of fibrils during reconstitution in vitro is specified by the introduction of cross-links by tissue transglutaminase and not by factor XIII.

FIGURE 6. Fibril diameters inversely depend on the dose of transglutaminase activity added to the reconstitution mixtures. For details, see text. The bars represent the number of fibrils falling into distinct 25-nm intervals of diameters. The solid lines are the Gaussian distributions calculated from the experimentally determined fibril diameters. A, tissue control ($\mu = 540$ nm, $\sigma = 130$ nm, $f = 1.0$; $Q = 0.56$). B, transglutaminase, 2.5 $\mu$g/ml ($\mu = 390$ nm, $\sigma = 100$ nm, $f = 1.0$; $Q = 0.19$). C, transglutaminase, 5.0 $\mu$g/ml ($\mu = 290$ nm, $\sigma = 85$ nm, $f = 1.0$; $Q = 0.50$).

**DISCUSSION**

The corneal stroma is a particularly exquisite example for the adaptation of the suprastructural organization of the extracellular matrix to functional tissue requirements. The significance of the uniformity and of the small diameters of corneal fibrils has been studied extensively, and the aggregated evidence suggests that the control of fibril shapes in the cornea is rather complex. In cartilage, the correct stoichiometry of collagens II and XI (8:1) is sufficient for the rigorous diameter control of prototypic small diameter fibrils (17) that are typically found in immature cartilage or in territorial zones of adult cartilaginous tissues (36). In the corneal stroma, important roles in controlling collagen fibrillogenesis have been assigned to quantitatively minor collagen V, a structurally close relative of collagen XI, and to several SLRPs, including lumican (11), decorin, and biglycan (37) acting in a partially redundant manner (10). Here, we have discovered two additional factors contributing to the control of fibril shape and the formation of lamella. The diameter control depended on isopeptide bond formation catalyzed by transglutaminases, specifically tissue transglutaminase-2, whereas collagen cross-links of the classical, aldehyde variety were necessary for the correct organization of the fibrillar corneal lamellae. Both findings are surprising because the event of covalent cross-linking of collagen molecules is usually thought to occur after rather than during fibrillogenesis. In addition, cross-linking is generally associated with the stabilization of fibrils against their unraveling after assembly. Early isopeptide cross-link formation results in a limited lateral growth of the future fibrils. Presumably, this process also requires the integration of several types of SLRPs with partially redundant roles at later stages of fibrillogenesis. In addition, further cross-linking of the isopeptide variety introduced at later stages may serve to stabilize fibrils after their assembly is completed.

Less is known about the origins of the regular lateral spacing of parallel collagen fibrils and the formation of fibrillar lamellae in the cornea. It has been suggested that dermatan and/or keratan sulfate chains of SLRPs on the surfaces of neighboring fibrils serve as spacers by interacting in an anti-parallel direction (16). Based on this notion, one may predict that crude corneal extracts can be reconstituted in vitro into arrays of parallel collagen fibrils with uniform spacing. Our reconstitution experiments, however, have not yet revealed any indication for the establishment of this type of higher-order suprastructural organization. This may well be due to inappropriate molecular mixtures in our crude extracts or to still insufficient control of the experimental conditions, although it was possible to reduce fibril diameters by providing transglutaminase activity to the reconstitution mixtures. Therefore, further details of the exact mechanism controlling the spacing of corneal fibrils still remain to be elucidated.

It has been shown previously (for review, see Ref. 6) that keratocytes in culture can elaborate, at least in principle, the lamellar organization of collagen fibrils resembling that of early developmental stages in cornea. In addition, the cells retain this capacity even if their authentic extracellular matrix is entirely eliminated. However, the mechanistic origins of the formation of lamellae, their size limitation, and their orthogonal stacking
remained obscure. Here, we observed that covalent collagen cross-linking is essential for lamellar stacking. In this context, the chemistry of cross-linking is oxidative deamination of (hydroxy)lysyl residues by lysyl oxidases, followed by spontaneous condensation reactions between the side chains containing the enzymatically introduced aldehyde groups. In this way, aldehyde-type collagen cross-links formed early during fibrillogenesis appear to lay the ground for the generation of lamellae. In an earlier study (38), the formation of orthogonal lamellae was observed even though lysyl oxidase activity was inhibited starting at day 8 of in ovo development, i.e. 3 days after the deposition of the primary corneal stroma. Therefore, we surmise that the aldehyde-type cross-linking triggering orthogonal lamellar stacking must occur at early stages of fibrillogenesis long before matrix deposition is complete. Supporting this notion, we found that dimeric and tetrameric collagen polypeptides are formed in the matrix deposited by keratocytes. Tetramers are generated by covalently connecting two dimers. Trimmers are also formed, but only at later stages of the cultures. It is tempting to speculate that the trimers have a cross-link chemistry that differs from that of the dimers and tetramers even if they both originate from oxidative deamination. It also remains to be established by future studies whether the trimers only serve to stabilize the fibrils or whether they are also required for lamellar stack formation.

The principle that early cross-linking of collagens is required for appropriate tissue morphogenesis may well apply in general. Indeed, our preliminary experiments with cultured chick embryo tendon fibroblasts analogous to the cultures described here indicate that bundle formation of collagenous fibrils also depends on cross-linking of the aldehyde-derived variety. We conclude that the important roles of cross-link formation not only include the structural stabilization of mature connective tissues but also to achieve appropriate tissue organization.

Acknowledgments—We thank Gerburg Hölscher and Barbara Sched for excellent technical help.

REFERENCES
1. Komai, Y., and Ushiki, T. (1991) The three-dimensional organization of collagen fibrils in the human cornea and sclera. Invest. Ophthalmol. Vis. Sci. 32, 2244–2258
2. Birk, D. E., and Trebstad, R. L. (1984) Extracellular compartments in matrix morphogenesis: collagen fibril, bundle, and lamellar formation by corneal fibroblasts. J. Cell Biol. 99, 2024–2033
3. Hay, E. D., and Revel, J. P. (1969) Fine structure of the developing avian cornea. Monogr. Dev. Biol. 1, 1–144
4. Bard, J. B., and Higginson, K. (1977) Fibroblast-collagen interactions in the formation of the secondary stroma of the chick cornea. J. Cell Biol. 74, 816–827
5. Linsenmayer, T. F., Fitch, J. M., Gordon, M. K., Cai, C. X., Igge, F., Marchant, J. K., and Birk, D. E. (1998) Development and roles of collagenous matrices in the embryonic avian cornea. Prog. Retin. Eye Res. 17, 231–265
6. Rueter, J. W., and Zieske, J. D. (2008) Prelude to corneal tissue engineering—gaining control of collagen organization. Prog. Retin. Eye Res. 27, 549–577
7. Guo, X., Hutcheon, A. E., Melotti, S. A., Zieske, J. D., Trinkaus-Randall, V., and Rueter, J. W. (2007) Morphologic characterization of organized extracellular matrix deposition by ascorbic acid-stimulated human corneal fibroblasts. Invest. Ophthalmol. Vis. Sci. 48, 4050–4060
8. Bard, J. B., Hulmes, D. J., Purdom, I. F., and Ross, A. S. (1993) Chick corneal development in vitro: diverse effects of pH on collagen assembly. J. Cell Sci. 105, 1045–1055
9. Koch, M., Laub, F., Zhou, P., Hahn, R. A., Tanaka, S., Burgeson, R. E., Gerecke, D. R., Ramirez, F., and Gordon, M. K. (2003) Collagen XXIV, a vertebrate fibrillar collagen with structural features of invertebrate collagens: selective expression in developing cornea and bone. J. Biol. Chem. 278, 43236–43244
10. Hassell, J. R., and Birk, D. E. (2010) The molecular basis of corneal transparency. Exp. Eye Res. 91, 326–335
11. Chakravarti, S., Magnusson, T., Lass, J. H., Jepsen, K. J., LaMantia, C., and Carroll, H. (1998) Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. J. Cell Biol. 141, 1277–1286
12. Chakravarti, S., Paul, J., Roberts, L., Chervoneva, I., Oldberg, A., and Birk, D. E. (2003) Ocular and scleral alterations in gene-targeted lumican-fibromodulin double-null mice. Invest. Ophthalmol. Vis. Sci. 44, 2422–2432
13. Chen, S., Oldberg, A., Chakravarti, S., and Birk, D. E. (2010) Fibromodulin regulates collagen fibrillogenesis during peripheral corneal development. Dev. Dyn. 239, 844–854
14. Birk, D. E., and Bruckner, P. (2011) Collagens, suprastructures, and fibril assembly in The Extracellular Matrix: an Overview, pp. 77–115, Springer Verlag, Berlin Heidelberg, Germany
15. Zhang, G., Chen, S., Goldoni, S., Calder, B. W., Simpson, H. C., Owens, R. T., McQuillan, D. J., Young, M. F., Joozo, R. V., and Birk, D. E. (2009) Genetic evidence for the coordinated regulation of collagen fibrillogenesis in the cornea by decorin and biglycan. J. Biol. Chem. 284, 8888–8897
16. Scott, J. E. (1991) Proteoglycan: collagen interactions and corneal ultrastructure. Biochem. Soc. Trans. 19, 877–881
17. Blaschke, U. K., Ekenberry, E. F., Hulmes, D. J., Galla, H. J., and Bruckner, P. (2000) Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. J. Biol. Chem. 275, 10370–10378
18. Mendler, M., Eich-Bender, S. G., Vaughan, L., Winterhalter, K. H., and Bruckner, P. (1989) Cartilage contains mixed fibrils of collagen types II, IX, and XI. J. Cell Biol. 108, 191–197
19. Hansson, U., Hussain, M., Villone, D., Herrmann, M., Robenek, H., Peters, G., Sinha, B., and Bruckner, P. (2006) The anchorless adhesin Eap (extracellular adherence protein) from Staphylococcus aureus selectively recognizes extracellular matrix aggregates but binds promiscuously to monomeric matrix macromolecules. Matrix Biol. 25, 252–260
20. Villone, D., Fritsch, A., Koch, M., Bruckner-Tuderman, L., Hansen, U., and Bruckner, P. (2008) Supramolecular interactions in the dermo-epidermal junction zone: anchoring fibril-collagen VII tightly binds to banded collagen fibrils. J. Biol. Chem. 283, 24506–24513
21. Bonner, W. M., and Laskey, R. A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 63, 83–88
22. Laskey, R. A., and Mills, A. D. (1975) Quantitative film detection of 3H and 14C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56, 335–341
23. Craig, A. S., and Parry, D. A. (1981) Collagen fibrils of the vertebrate corneal stroma. J. Ultrastruct. Res. 74, 232–239
24. Tsukahara, N., Tani, Y., Lee, E., Kikuchi, H., Endoh, K., Ichikawa, M., and Sugita, S. (2010) Microstructure characteristics of the cornea in birds and mammals. J. Vet. Med. Sci. 72, 1137–1143
25. Meek, K. M., and Fullwood, N. J. (2001) Corneal and scleral collagens—a microscopist’s perspective. Micron 32, 261–272
26. Linsenmayer, T. F., Fitch, J. M., Schmid, T. M., Zal, N. B., Gibney, E., Sanderson, R. D., and Mayne, R. (1983) Monoclonal antibodies against chicken type V collagen: production, specificity, and use for immunocytochemical localization in embryonic cornea and other organs. J. Cell Biol. 96, 124–132
27. Keene, D. R., Sakai, L. Y., Bächinger, H. P., and Burgeson, R. E. (1987) Type III collagen can be present on banded collagen fibrils regardless of fibril diameter. J. Cell Biol. 105, 2393–2402
28. Bruns, R. R., Press, W., and Gross, J. (1987) A large-scale, orthogonal network of microfibril bundles in the corneal stroma. Invest. Ophthalmol. Vis. Sci. 28, 1939–1946
29. Yamauchi, M., Chandler, G. S., Tanzawa, H., and Katz, E. P. (1996) Cross-linking and the molecular packing of corneal collagen. Biochem. Biophys. Res. Commun. 219, 311–315
30. Prockop, D. J., and Tuderman, L. (1982) Posttranslational enzymes in the biosynthesis of collagen: extracellular enzymes. Methods Enzymol. 82, 305–319
31. Raghunath, M., Cankay, R., Kubitscheck, U., Fauteck, J. D., Mayne, R., Aeschlimann, D., and Schlötzer-Schrehardt, U. (1999) Transglutaminase activity in the eye: cross-linking in epithelia and connective tissue structures. Invest. Ophthalmol. Vis. Sci. 40, 2780–2787
32. Zhang, W., Shiraishi, A., Suzuki, A., Zheng, X., Kodama, T., and Ohashi, Y. (2004) Expression and distribution of tissue transglutaminase in normal and injured rat cornea. Curr. Eye Res. 28, 37–45
33. Danielsen, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E., and Iozzo, R. V. (1997) Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J. Cell Biol. 136, 729–743
34. Gelman, R. A., Williams, B. R., and Piez, K. A. (1979) Collagen fibril formation. Evidence for a multistep process. J. Biol. Chem. 254, 180–186
35. Muszbek, L., Bereczky, Z., Bagoly, Z., Komáromi, I., and Katona, É. (2011) Factor XIII: a coagulation factor with multiple plasmatic and cellular functions. Physiol. Rev. 91, 931–972
36. Hunziker, E. B., Herrmann, W., Schenk, R. K., Mueller, M., and Moor, H. (1984) Cartilage ultrastructure after high pressure freezing, freeze substitution, and low temperature embedding. I. Chondrocyte ultrastructure—implications for the theories of mineralization and vascular invasion. J. Cell Biol. 98, 267–276
37. Zhang, G., Ezura, Y., Chervoneva, I., Robinson, P. S., Beason, D. P., Carine, E. T., Soslowsky, L. J., Iozzo, R. V., and Birk, D. E. (2006) Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development. J. Cell Biochem. 98, 1436–1449
38. Birk, D. E., Fitch, J. M., Babiarz, J. P., and Linsenmayer, T. F. (1988) Collagen type I and type V are present in the same fibril in the avian corneal stroma. J. Cell Biol. 106, 999–1008

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