Journal of Cellular Biochemistry 87:208–220 (2002)

The Roles Of Types XII And XIV Collagen In Fibrillogenesis And Matrix Assembly In The Developing Cornea

Blanche B. Young,[1] Guiyun Zhang,[1] Manuel Koch,[2] and David E. Birk[1]*

[1]Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania
[2]Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts

Abstract Corneal transparency depends on the architecture of the stromal extracellular matrix, including fibril diameter, packing, and lamellar organization. The roles of collagen types XII and XIV in regulation of corneal fibrillogenesis and development were examined. The temporal and spatial expression patterns were analyzed using semi-quantitative RT-PCR, in situ hybridization, Western analysis, and immunohistochemistry. Expression of types XII and XIV collagens in cornea development demonstrated that type XII collagen mRNA levels are constant throughout development (10D-adult) while type XIV mRNA is highest in early embryonic stages (10D–14D), decreasing significantly by hatching. The spatial expression patterns of types XII and XIV collagens demonstrated a homogeneous signal in the stroma for type XIV collagen, while type XII collagen shows segregation to the sub-epithelial and sub-endothelial stroma during embryonic stages. The type XII collagen in the anterior stroma was an epithelial product during development while fibroblasts contributed in the adult. Type XIV collagen expression was highest early in development and was absent by hatching. Both types XII and type XIV collagen have different isoforms generated by alternative splicing that may alter specific interactions important in fibrillogenesis, fibril–fibril interactions, and higher order matrix assembly. Analysis of these splice variants demonstrated that the long XII mRNA levels were constant throughout development, while the short XII NC3 mRNA levels peaked early (12D) followed by a decrease. Both type XIV collagen NC1 splice variants are highest during early stages (12D–14D) decreasing by 17D of development. These data suggest type XII collagen may have a role in development of stromal architecture and maintenance of fibril organization, while type XIV collagen may have a role in regulation of fibrillogenesis. J. Cell. Biochem. 87: 208–220, 2002.

Key words: corneal stroma; corneal development; fibril formation; matrix assembly; extracellular matrix; collagen types XII and XIV

The mature cornea is a strong and transparent tissue. The cornea is composed of four parallel extracellular matrices, the epithelial basement membrane, Bowman's layer, the stroma, and Descemet's layer. An integration of these layers during development is important for the acquisition of structural integrity as well as transparency. The epithelial basement membrane is a continuous sheet of specialized extracellular matrix that serves as the substrate for epithelial attachment [Linsenmayer et al., 1998]. Bowman's layer is at the interface between the epithelial basement membrane and the stroma proper. It is composed of small diameter collagen fibrils arranged as an interwoven meshwork. The stroma is the major corneal matrix composed of small diameter, striated collagen fibrils arranged in orthogonal lamellae. The fibrils have homogenous small diameters and a constant center-to-center spacing, and are arranged into orthogonal lamellae [Hay and Revel, 1969; Hay, 1980; Birk and

Grant sponsor: National Institutes of Health; Grant number: NEI EY05129.

Manuel Koch's present address is Institute for Biochemistry II, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, 50931 Cologne, Germany.

*Correspondence to: David E. Birk, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, JAH 543, Philadelphia, PA 19107. E-mail: david.birk@mail.tju.edu

Received 20 June 2002; Accepted 22 July 2002

DOI 10.1002/jcb.10290

Trelstad, 1984]. The formation and maintenance of small diameter fibrils, followed by the assembly of fibrils into tissue-specific matrices is essential for optical function [Cox et al., 1970]. Descemet's layer is a specialized acellular extracellular matrix between the stroma and the endothelium. These matrices are organized and integrated together at different levels during the development of a functional cornea.

The major structural element of Bowman's layer and the stroma is the heterotypic type I/V collagen fibril. The assembly and growth of collagen fibrils, the grouping of fibrils into lamellae, and the organization of lamellae within the stroma all contribute to tissue structure and function. We propose that macromolecules associated with the fibrils regulate the events that lead to corneal function. These macromolecules include the family of fibril-associated collagens with interrupted triple helical domains (FACIT collagens). The members of this family include collagen types IX, XII, XIV, XVI, XIX, and XX [Shaw and Olsen, 1991; Pan et al., 1992; Yamakoshi and Nagai, 1992; Yoshioka et al., 1992; Myers et al., 1994; Ricard-Blum et al., 2000; Koch et al., 2001]. Type XII and XIV collagens are expressed in a wide variety of tissues including the developing avian cornea [Nishiyama et al., 1994; Walchi et al., 1994; Koch et al., 1995; Gordon et al., 1996].

Types XII and XIV collagen are structurally similar homotrimers, composed of two collagenous domains (COL1–COL2) and three non-collagenous domains (NC1–NC3) [Shaw and Olsen, 1991; Ricard-Blum et al., 2000]. Both types XII and XIV collagen are found to associate with type I collagen fibrils [Keene et al., 1991; Young et al., 2000; Schuppan et al., 2001]. These multidomain collagens can interact with more than one extracellular component simultaneously allowing integration of developing matrices. For example, type XII collagen has been shown to bind to proteoglycans, decorin and fibromodulin [Font et al., 1996]. Type XIV collagen has been shown to bind with the proteoglycan decorin [Font et al., 1993] and heparin [Brown et al., 1993; Giry-Lozinguez et al., 1998]. The two carboxy terminal domains, NC1 and COL1, of both type XII and XIV collagen are believed to interact with the type I collagen fibrils [Keene et al., 1991; Schuppan et al., 2001]. While the large globular NC3 domain on the amino terminus projects into the matrix and is hypothesized to interact with other matrix components and may bridge adjacent fibrils [Shaw and Olsen, 1991; Ricard-Blum et al., 2000].

The NC1 and NC3 domains have different isoforms generated by alternative splicing. Both collagens XII and XIV have splice variants in these domains [Trueb and Trueb, 1992; Walchi et al., 1993; Imhof and Trueb, 1998; Kania et al., 1999]. Alternative isoforms can affect either interactions with the type I collagen fibril (NC1 splice variants) or interactions with other matrix components (NC3 splice variants) [Imhof and Trueb, 2001]. There is speculation that these different type XII and XIV isoforms have different roles in regulating developing tissues [Koch et al., 1995; Wessel et al., 1997; Kania et al., 1999; Young et al., 2000].

Our hypothesis is that type XII and XIV collagens are important regulatory macromolecules in corneal development. Specifically, type XII collagen functions in the integration of the developing corneal extracellular matrices providing stability in the mature stroma and type XIV collagen functions in the regulation of fibril growth in the stroma.

MATERIALS AND METHODS

Tissue Harvest

White leghorn chicken embryos (Spafas, CT, USA) were incubated in a humidified atmosphere and staged according to Hamburger and Hamilton [1951]. Corneas were dissected from staged embryos (8–19 day) as well as hatchling and adult (2 month old) chickens.

Immunofluorescence Microscopy

Immunolocalization of types XII and XIV collagen was done as described previously [Young et al., 2000]. Briefly, corneas were fixed with 4% paraformaldehyde in PBS, pH 7.3 for 30 min on ice. The tissues were incubated with 7% sucrose–PBS, frozen in OCT (Tissue Tek, Miles Laboratories, Naperville, IL) and sections (6 μm) were cut and picked up onto poly-L-lysine coated slides. Sections were treated with sodium borohydride (50 mg/100 ml PBS) and non-specific binding sites were blocked by incubation in 5% normal goat serum (NGS). Sections were then incubated in primary and secondary antibodies described below. Negative controls were slides incubated with buffer without primary antibodies. Slides were mounted in

Vectashield mounting media (Vector Labs, Burlingame, CA) with 10 μg/ml Hoechst 33258 (Pierce, Rockford, IL). Images were captured using an Optronics Digital camera using set integration times and identical conditions to facilitate comparisons between samples. These experiments were performed on sections from at least five different chickens from each stage.

Antibodies

Two different monoclonal antibodies against type XII collagen were used. One, (C19), detected all forms of type XII collagen while another, (SE14), detected only the long isoform of type XII collagen [Koch et al., 1995]. Both anti-type XII collagen antibodies were used at 5 μg/ml followed by secondary goat anti-mouse DTAF conjugated antibody used at a 1:300 dilution. For type XIV collagen, an affinity purified rabbit polyclonal antibody was used [Castagnola et al., 1992]. This antibody detected all forms of the protein. Rabbit anti-chicken primary antibody was used at a 1:500 dilution followed by secondary goat anti-rabbit IgG DTAF conjugated antibody used at 1:300 dilution.

Western Analysis

Western blots were done as previously described [Young et al., 2000]. Briefly, corneas were extracted according to Castagnola et al. [1992]. The extract was centrifuged, and the supernatant was collected. Total protein was measured using the BCA Protein Assay (Pierce) and protein samples ranging from 10 to 20 μg were electrophoresed under reducing conditions on 5% acrylamide gels. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed [Laemmli, 1970] and the gels were transferred to nitrocellulose [Towbin et al., 1979]. The blot was blocked in a 5% non-fat dry milk/PBS solution, washed in PBS buffer and incubated for 2 hours at room temperature with either anti-type XIV collagen antibody at 1:2,000 dilution or anti-type XII collagen antibody C19 or SE14 at 5 μg/ml. For type XIV detection, a goat anti-rabbit peroxidase secondary antibody was used, for type XII detection, a goat anti-mouse peroxidase secondary antibody was used. For detection of the peroxidase the ECL detection system (Amersham Life Science, England) was used. Western blots were performed in quadruplicate on at least three different cornea extractions. Relative density was measured from films with the BioRad Gel Doc 2000 system and Quantity One (Biorad, Richmond, CA).

Preparation of RNA and cDNA

The central corneas were isolated with a 2 mm dermal punch beginning at day 12, while whole corneas from earlier time points were dissected. Total RNA was isolated by homogenization in Trizol reagent (Gibco BRL, Gaithersburg, MD) and stored at −80°C. cDNAs were obtained by reverse transcription of 800 ng of total RNA with random primers using the Superscript II preamplification system (Gibco BRL). The final volume of the reverse transcription reaction was 20 μl.

PCR Analysis of Type XII and XIV Collagen mRNA

Each PCR reaction contained the following: 1.25 mM of each dNTP (Gibco BRL), 1 μM of each primer, PCR buffer with 1.5 mM $MgCl_2$, and 2.5 U of Taq polymerase (Perkin Elmer, Branchburg, NJ). For most experiments, all PCR reactants except the DNA template were first combined in a master mixture and then distributed in 24 μl aliquots into 0.2 ml microamp tubes. DNA samples were added (1 μl per reaction) and PCR was performed in a Perkin Elmer 9600 thermal cycler. For semi-quantitative analysis, each reaction was prepared with a combination of target primers and the internal control 18S primer competimer mix (Ambion Austin, TX). The following program was used for semi-quantitative analysis of either type XII collagen or type XIV collagen: 94°C for 30 sec followed by 26 cycles (type XII collagen) or 28 cycles (type XIV collagen) of 94°C for 15 sec, 56°C for 25 sec, and 72°C for 1.5 min followed by 72°C for 10 min, and stored at 4°C until used. The cDNAs from corneas at multiple developmental time points were isolated and combined in a multiplex PCR. For type XII collagen analysis the first set of primers targeted the NC3 domain of type XII collagen, while the second set of primers targeted the 18S ribosomal RNA. Because the type XII collagen products were close in size, separate reactions to detect short XII and long XII were prepared. For type XIV collagen analysis, the first set of primers targeted splice variants that generate the NC1 domain and the second set of primers targeted the 18S control. All PCR products were

resolved on 1.4% agarose gels and visualized with ethidium bromide at 0.5 μg/ml. Images were captured with the BioRad Gel Doc 2000 system and analyzed using the Quantity One software package.

These PCR products were resolved on 1.4% agarose gels and visualized by ethidium bromide (0.5 μg/ml). The analysis of type XII collagen produced two bands per lane, one representing either the short or long type XII (226 or 299 bp) and one band representing the 18S band (489 bp). The analysis of type XIV collagen produced three bands per lane, two representing the two NC1 domain splice variants of type XIV (223 or 316 bp) and a third band representing the 18S band (489 bp). This protocol was standardized by determining the linear range of the cycle number for the target products to be 28 cycles for type XII collagen and 26 cycles for type XIV collagen. In addition, the correct ratio (2:8) of the 18S primers to competimers was determined according to manufacturers' instructions.

Primers

For analysis of type XII collagen NC3 domain splice variants primers were used to detect both long and short forms as previously published [Gordon et al., 1996]. The forward primer was used for both long and short XII collagen (ATGAGGACAGCGCTGTGCTCG). Two reverse primers were utilized, TACCTCATCATATGAAGCTATT) used to detect the long XII variant collagen, and AGACCAATCTGTACTTTGTCA) used to detect the short XII variant collagen. The amplified short XII product is 226 bp while the long XII product is 299 bp. The primers for type XIV collagen NC1 splice variants were, CCTGTGCTGGCTATGGATG (forward), and TGTTCCAGAGTCTTGATGCT (reverse). Amplification will detect two splice variants, the long XIV variant (316 bp) and the short XIV variant (223 bp) in the target cDNA. The primers for the 18S control and competimers were from a kit (Ambion). The 18S primers generated a 489 bp product.

In Situ Hybridization

A cDNA fragment within the NC3 domain of type XII collagen was amplified by PCR using cDNA template synthesized from chicken embryo cornea RNA. The forward primer sequence is 5′-GCAGAACCAAACCTCTCACT while the reverse primer is 5′-TTCTTGGTGTTCCTCTCTCC. T7 phage RNA polymerase promoter adaptor (Ambion, Austin, TX) was ligated to the PCR product. Using gene-specific reverse primer and T7 promoter adaptor forward primer (from Lig'nScribe No-cloning Promoter Addition Kit, Ambion), or gene-specific forward primer and T7 promoter adaptor reverse primer, templates for in vitro transcription with T7 promoter at either 5′ or 3′ end were generated by PCR amplification from ligation reaction. Antisense or sense RNA probes labeled with Dig-11-UTP (Roche, Indianapolis, IN) were yielded by in vitro transcription (Ambion).

Cryosections were prepared as described in the section of IF microscopy. After fixation in 4% paraformaldehyde and rinsing in PBS, the sections were treated with 0.2 N HCl to inactivate endogenous alkaline phosphatase activity and digested with 0.1% pepsin (Dako, Carpinteria, CA). Then the sections were acetylated in 0.25% acetic anhydride in 0.1 M triethylnolamine. Following prehybridization of 1 hr with hybridization buffer (Dako) after equilibrating in 5 × SSC, hybridization was carried out at 55°C overnight with probe at concentration of 150 ng/ml. Digest excess probe from slides by incubating with Rnase A/T1 (Ambion) followed by washing consequently in 2 × SSC, TNE buffer (10 mM Tris pH 7.5, 500 mM NaCl, 1 mM EDTA), 50% formamide DI in 2 × SSC and 0.08 × SSC. Before detection of digoxigenin (Dig) by anti-Dig antibody conjugated with HRP, the sections were blocked with 20% rabbit Ig fraction in blocking buffer (100 mM Tris pH 7.5, 150 mM NaCl, 1% purified casein). The signal was amplified by consequent incubations with biotinyl-tyramide, anti-biotin-HRP (Dako), biotinyl-tyramide and anti-biotin-alkaline phosphatase, with washing in TBST (50 mM Tris pH 7.6, 300 mM NaCl, 0.1% Tween-20) between steps. The signal was detected by incubation with fast red (Sigma, St Louis, MO) in dark. Then the sections were counterstained with hematoxylin and the nucleus staining was enhanced by 0.08% ammonium hydroxide. After rinsing with water followed by drying, the slides were mounted with permount (Fisher, Houston, TX).

RESULTS

Type XII Collagen in Developing Cornea

Type XII collagen was neutral salt extractable from the cornea from day 10 of develop-

ment until hatching. Western blot analyses demonstrated relatively high expression during this developmental period (Fig. 1A,B). In contrast, very little type XII collagen was observed on Western blots of neutral salt extracted adult corneas (data not shown). This observation was inconsistent with the immunochemical localization studies that demonstrated type XII collagen expression throughout the adult stroma (Fig. 2, and see results below). To address this inconsistency, we directly extracted the 17D and adult corneas with SDS sample buffer. This more stringent approach still only extracted relatively small amounts of type XII collagen from the adult cornea compared to the 17D cornea (Fig. 1A). The inability to extract type XII collagen from the mature stroma suggests that with maturation this collagen becomes firmly integrated into the corneal stroma, possibly by covalent crosslinking.

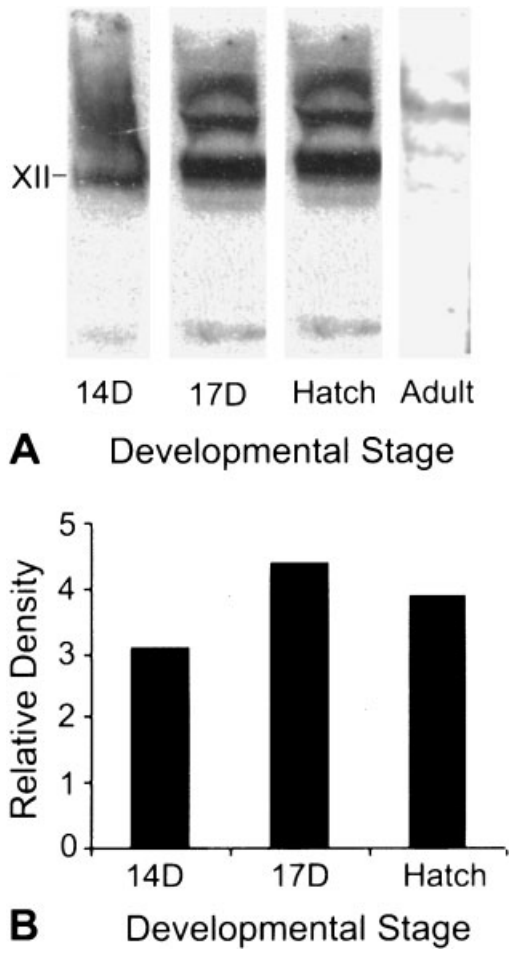

Fig. 1. Type XII collagen content is constant during corneal development. **(A)** Western blot analysis of type XII collagen neutral salt extracted from 14 days of development to hatching (14D, 17D, Hatch). Expression is maintained at a relatively high level during this period. In the adult cornea, type XII collagen is not extractable using neural salt buffers (data not shown). This Western blot (adult) shows the type XII collagen extracted from adult cornea utilizing SDS loading buffer and even in SDS containing buffers only a small amount is extractable. This is inconsistent with the immunofluorescence data for adult stroma (Fig. 2). Integration within the mature stroma may be a cause for low extraction. **(B)** The graph presents the quantitative densitometric analyses of the Western Blot from gel (A).

The spatial expression pattern of type XII collagen during corneal development was analyzed by immunofluorescence microscopy (Fig. 2A,B). Spatial expression of type XII collagen was not homogeneous during development. The expression of type XII collagen was spatially restricted to interfacial matrix regions during development. Specifically, Bowman's layer and the anterior stroma, as well as Descemet's layer and the adjacent posterior stroma reacted prominently with antibodies against type XII collagen. In contrast, the bulk of the stroma proper was negative between day 14 and hatching. At hatching, there was an obvious infusion of type XII collagen reactivity into the anterior stroma. In the mature adult cornea (2 months), the type XII collagen signal was detected throughout the stroma proper. The negative control experiments showed no reactivity.

The two major isoforms of type XII collagen were examined utilizing an antibody that recognized only the long type XII collagen isoform (Fig. 2C). Comparable with the data presented above for localization of both forms, the long NC3 (XII) isoform was present at all developmental stages studied and showed spatial segregation. Specifically, the long type XII collagen isoform was expressed in anterior and posterior regions, but not in most of the stroma proper, during early stages of corneal development (14D–19D). At hatching, the long isoform begins infiltrating the anterior stroma. In the adult, the mature stroma is strongly reactive for the long type XII collagen isoform. In contrast to developmental stages, this reactivity is present throughout the corneal stroma.

Type XII collagen mRNA expression in19 day embryo and adult chicken corneas was characterized by in situ hybridization (Fig. 3). In the developing cornea (19 day embryo), the fibroblasts in the anterior stroma are all negative, while the basal epithelial cells show strong collagen XII mRNA expression. This indicates that the type XII collagen localized in the interfacial matrix regions such as Bowman's layer

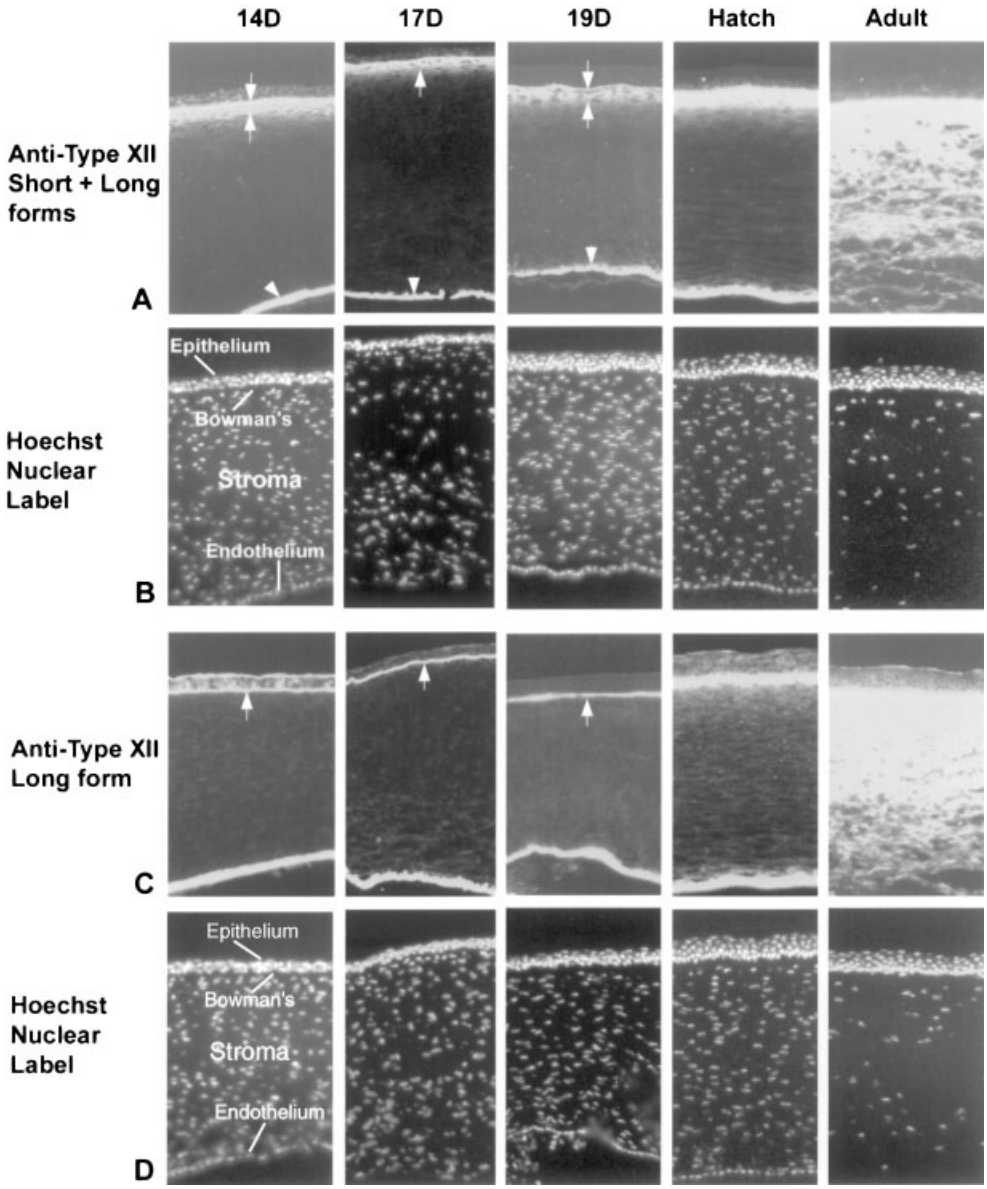

Fig. 2. Type XII collagen is localized to the interfacial matrix regions of developing cornea. Immunofluorescence microscopy of day 10–19 embryos, hatchling, and adult chicken corneas were reacted with antibodies against either type XII collagen or the long type XII collagen isoform. **(A)** In developing cornea, between day 14 and 19 the type XII collagen is sequestered in the sub-epithelial, Bowman's layer and anterior most stroma (arrow) and endothelial regions (arrowhead) of the cornea. In contrast, by hatching and beyond the type XII is clearly in the stroma proper (S). There are multiple splice variants of type XII collagen. **(C)** An antibody that recognized the long splice variant isoform, that produces the NC3 domain, was also utilized in these studies. The long XII collagen isoform is also localized to the interfacial matrices of the developing cornea. By immunofluorescence observation, the majority of type XII in the Bowman's layer (arrow) is long XII collagen, while the signal that appears in the anterior stroma (arrow in 17D panel A) is probably the short XII isoform. Furthermore, the majority of the type XII collagen in the adult stroma appears to be the long isoform. **(B)** and **(D)** For tissue orientation, Hoechst stain of fibroblast nuclei is presented below the immunostained sections. In all cases, the negative controls (absence of primary antibodies) were negative. Tissues were fixed in 4% paraformaldehyde and cryosectioned at 6 mm. Sections were reacted with mouse monoclonal antibodies conjugated to DTAF. Bar = 100 μm.

and the anterior stroma is derived from basal epithelial cells and not fibroblasts in the anterior stroma. However, collagen type XII in the posterior stroma is probably derived from both the endothelium and fibroblasts in the posterior stroma. In the adult cornea, the mRNA expression changes. Collagen type XII mRNA continues to be expressed by basal epithelial cells,

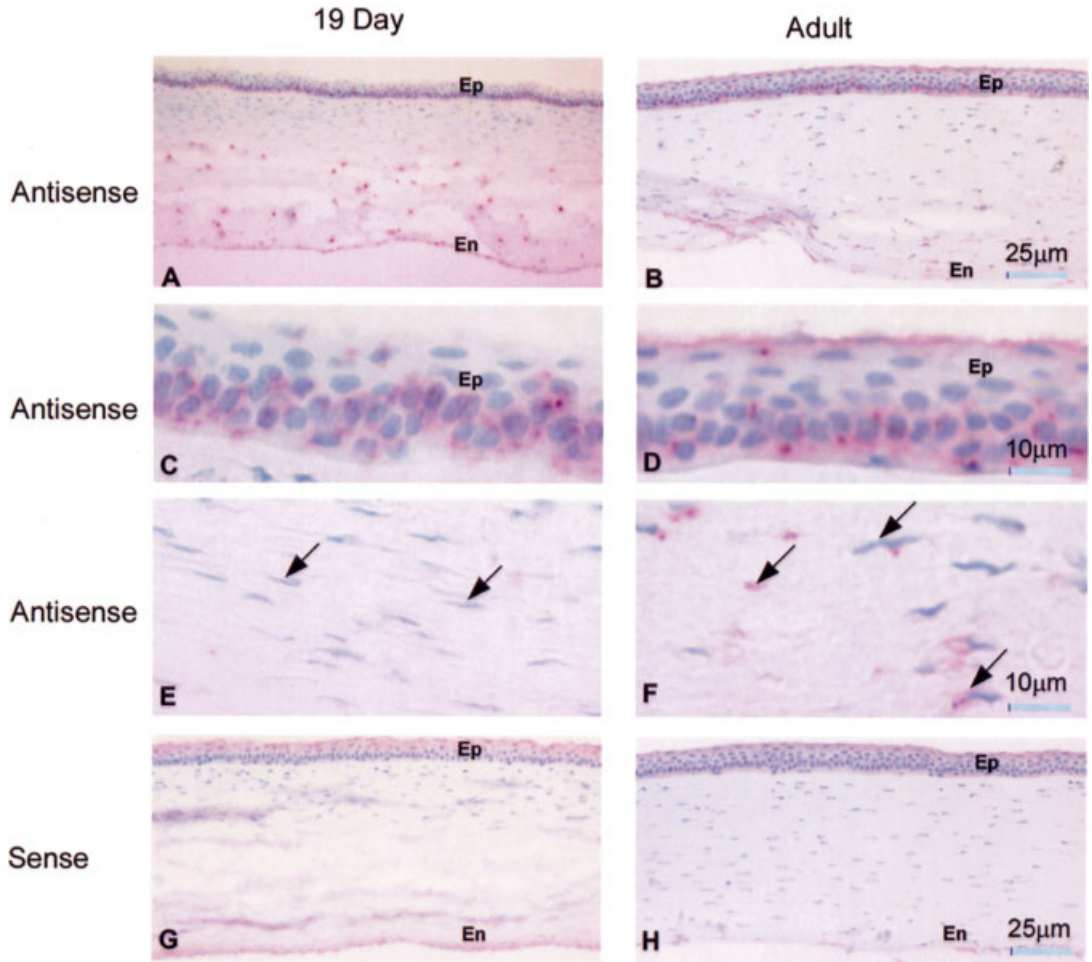

Fig. 3. Type XII collagen mRNA expression patterns change during corneal development. Type XII collagen mRNA expression in 19 day embryos and adult chicken corneas was detected by in situ hybridization with RNA probes that recognized both the long and short isoforms. **(A)** In the 19 day embryonic cornea, mRNA expression was detected in basal epithelial cells, some posterior keratocytes, and endothelium cells. The basal epithelia cells **(C)** showed strong reactivity, while fibroblasts in the anterior stroma were all negative (arrows) **(E)**. **(B)** In the adult cornea, type XII collagen mRNA was expressed in fibroblasts throughout the whole cornea stroma, basal epithelial cells, **(D)** and endothelial cells. **(F)** shows the positive fibroblasts in the anterior stroma (arrows). Sections incubated with the sense probe **(G,H)** were all negative. Ep, epithelium; En, endothelium. Bars = 25 μm (A,B,G,H) and 10 μm (C,D,E,F).

endothelial cells and fibroblasts in the posterior stroma, but in addition the fibroblasts in the anterior stroma demonstrate reactivity. This indicates that the collagen type XII localized throughout the adult stroma is derived from fibroblasts with contributions from the epithelial and endothelial cells in the anterior and posterior interfacial matrices, respectively.

Two splice variants in the NC3 domain of chicken type XII collagen have been described [Trueb and Trueb, 1992; Koch et al., 1995]. It has been suggested that these splice variants may change the interactions between adjacent fibrils or affect the binding affinity between type XII collagen and other matrix components [Koch et al., 1995; Wessel et al., 1997]. Semi-quantitative RT-PCR was used to determine the relative amounts of each type XII collagen mRNA splice variant for different stages in developing chicken cornea.

Total RNA from day 10, 12, 14, 17, 19 embryos in addition to hatchling and adult chicken corneas was used. Equal amounts of total RNA was used for generation of cDNA and subsequent PCR analyses. The multiplex PCR reactions amplified target mRNAs in addition in the 18S rRNA control. The linear range of amplification for 18S was adjusted to the target type XII collagen mRNAs. The results demonstrate that both alternatively spliced type XII collagen NC3

transcripts were present throughout corneal development (Fig. 4). Long type XII collagen variant mRNA was present from day 10 until adult. Expression of the long NC3 variant gradually increased with development and maturation (Fig. 4B). In contrast, the short type XII collagen variant mRNA appeared to peak at day 14 (14D) and decreased significantly with development (Fig. 4C). There is a reciprocal expression pattern seen during corneal development for the two NC3 splice variants. The short form decreases while expression of the long form increased with corneal development (Fig. 4B vs. C)

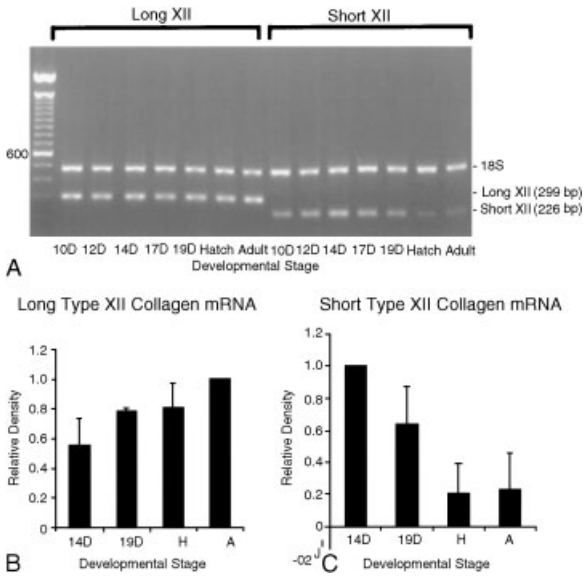

Fig. 4. Expression of type XII collagen mRNA splice variants in corneal development. Two amino terminus splice variants, generating the different forms of the NC3 domain, were analyzed in developing cornea. **(A)**This ethidium bromide agarose gel of type XII collagen message expression in the embryonic chicken cornea demonstrates long XII is present throughout corneal development into the adult while short XII is present until day 19 of development. **(B)** This graph presents the quantitative densitometric analyses of type XII collagen splice variants. The long type XII collagen message was maintained throughout cornea development from embryonic stages to adult. **(C)** This graph presents the quantitative densitometric analyses of short type XII collagen splice variant message that decreased during cornea development. Both graphs (B) and (C) represent the averages from three separate semi-quantitative PCR experiments from at least three pooled samples of cDNA. The values were normalized to 1.0 for the developmental stage that was highest in each graph. A semi-quantitative PCR approach was used to determine relative amounts of the two type XII collagen splice variants in developing cornea. This multiplex PCR utilized two primer pairs to amplify the target type XII splice variants and an internal 18S rRNA control. Two type XII collagen splice variants producing the NC3 domain have been described [Koch et al., 1995]. We refer to these as long XII (299 bp) and short XII (226 bp) collagen. This PCR analyses was optimized for both the correct cycle number for amplification in the linear range and the correct ratio of the internal control primers.

Type XIV Collagen in Developing Cornea

The expression of type XIV collagen in developing cornea was analyzed by immunofluorescence microscopy with an antibody directed against type XIV collagen (Fig. 5). Type XIV collagen was present early in the developing cornea until day 17 of development. Beginning at day 19 of corneal development, there was a dramatic decrease in reactivity. During the development period (10D–17D) type XIV collagen signal was homogeneously distributed throughout the Bowman's layer and the corneal stroma. The hatching and adult were not reactive with the type XIV collagen antibodies. These changes in type XIV collagen reactivity correlate with specific stages of fibrillogenesis. The controls without primary antibodies were negative at all stages.

To demonstrate that the absence of signal in the stroma of the mature corneas was not due to masking of the epitope, type XIV collagen in the developing cornea was further analyzed using Western blot analyses (Fig. 6). Type XIV collagen was present at relatively high levels during day 12–17 of corneal development with an apparent peak at day 14 of development. Type XIV collagen was present in low amounts by hatching (Fig. 6B).

Expression of two NC1 type XIV collagen mRNA splice variants was examined using semi-quantitative RT-PCR. The results of the semi-quantitative analysis of type XIV collagen in developing cornea demonstrated both the long and short NC1 splice variants were present from day 10 to 17. There was a significant decrease in expression of both variants at day 19. No expression could be measured in hatching and adult corneas (Fig. 7A). The two NC1 splice variants showed a coordinate expression pattern (Fig. 7B,C). Both variants had peak expression at 14 days of development. There was decreased expression at 17 and 19 days followed by no expression in the mature cornea (hatchling and adult).

DISCUSSION

The present study characterizes the expression of types XII and XIV collagen and their splice variants in relation to different stages of

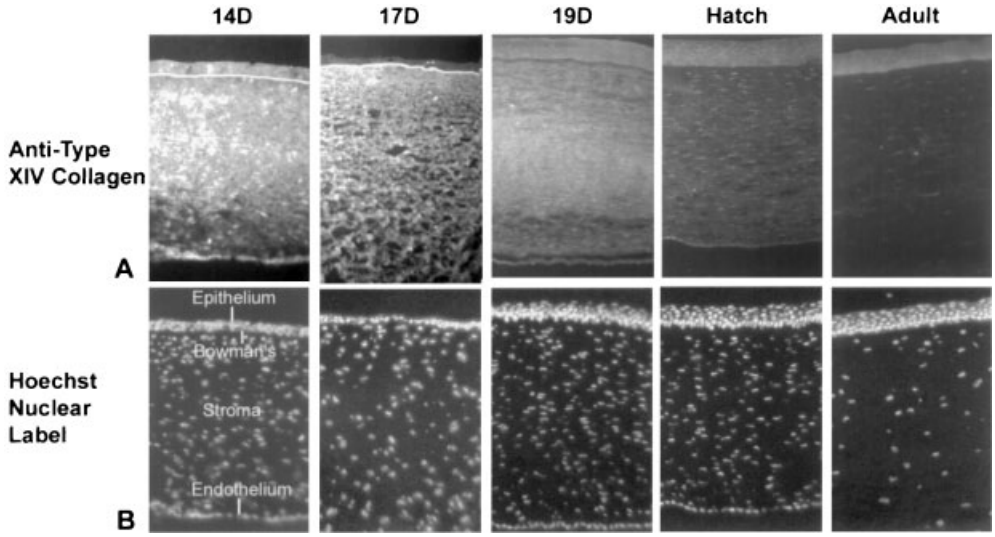

Fig. 5. Type XIV collagen reactivity is localized in the developing stroma of the cornea. This reactivity is high early (14D) and disappears with maturation (19D) in the developing cornea. Immunofluorescence microscopy of developing chicken corneas from day 10 until adult were reacted with antibodies against type XIV collagen. **(A)** Reactivity with antibodies against type XIV collagen is high in day 14–17 embryonic corneal stromas (S). In contrast, reactivity is very low in day 19 corneas and beyond. This indicates type XIV expression is low in the relatively mature cornea matrix. Also, note the high signal of type XIV collagen in the Bowman's layer (arrows) at day 14 (14D) and day 17 (17D). **(B)** For tissue orientation, Hoechst stain of fibroblast nuclei is presented below the immunostained sections. In all cases, the negative controls (absence of primary antibodies) were negative. Tissues were fixed in 4% paraformaldehyde and cryosectioned at 6 μm. Sections were reacted with rabbit polyclonal antibodies against type XIV collagen, followed by secondary antibodies conjugated to DTAF. Bar = 100 μm.

corneal development. Type XII collagen was restricted to interfacial regions throughout corneal development suggesting role in the integration of the multiple corneal layers. The absence of type XII collagen from the stroma proper during periods characterized by rapid fibrillogenesis indicates that type XII collagen is not directly involved in the regulation of fibrillogenesis during development of the corneal stroma. In contrast, type XIV collagen expression is homogeneous throughout the developing stroma and decreases dramatically in the mature stroma. This expression pattern suggests that type XIV collagen has a regulatory function in corneal fibrillogenesis. Previous studies of type XIV collagen expression during tendon development indicated a potential role in regulating the transition to linear growth during fibrillogenesis [Young et al., 2000]. The type XIV collagen expression was reduced and linear growth increased during this period of down regulation. In the corneal stroma immature fibrils undergo significant linear growth during the period where type XIV collagen expression is down regulated [Birk et al., 1996]. During normal corneal development there is no lateral fibril growth, however, in tendon lateral growth occurs when type XIV collagen is absent. The comparison of the two tissues suggests that type XIV collagen does not play a significant role in regulation of lateral growth. In fact the proteoglycans decorin, lumican, and fibromodulin have been shown to alter normal lateral growth [Iozzo and Murdoch, 1996; Svensson et al., 1999; Chakravarti et al., 2000; Ezura et al., 2000].

The characterization of type XII collagen in developing tissues has been studied by different groups to determine its functional significance [Nishiyama et al., 1994; Walchi et al., 1994; Koch et al., 1995; Gordon et al., 1996; Sasaki et al., 1996; Wessel et al., 1997; El Shabrawi et al., 1998; Anderson et al., 2000]. Our data demonstrate the localization of both forms of type XII collagen is consistent with a role in structural integration of the developing matrices. Type XII collagen is found in the interfacial regions, Bowman's layer and Descemet's layer, on either side of the stroma. These are regions that show increased stability compared to adjacent regions [Linsenmayer et al., 1986; Fitch et al., 1988]. Others have demonstrated that type XII collagen localizes to dense connective tissues subjected to stress, again supporting a structural function for type XII collagen [Walchi et al., 1994; Koch et al., 1995; Akutsu et al., 1999]. Interestingly, type XII collagen

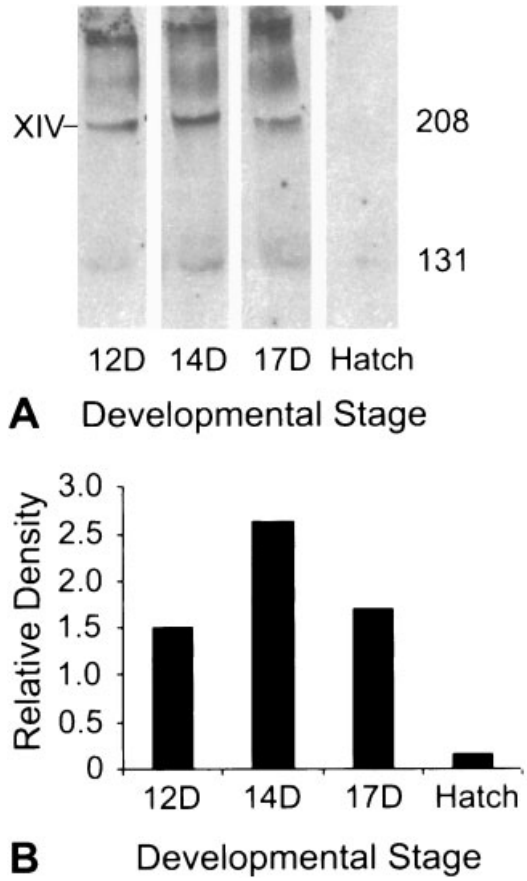

Fig. 6. Western analyses of type XIV collagen in cornea development demonstrated high levels during early stages of development and a significant decrease by hatching. (**A**) This is a representative Western blot demonstrating a significant decrease in type XIV collagen reactivity between 17D and hatching in corneal development. (**B**) The graph presents quantitative densitometry of the data from gel (A). At embryonic day 14 (14D) the α1(XIV) chain is approximately 17-fold more abundant than in hatchling cornea (H). These analyses were repeated four times on pooled protein samples with consistent results. A general trend of peak type XIV collagen expression between 14D and 17D followed by a dramatic decrease at hatching was observed.

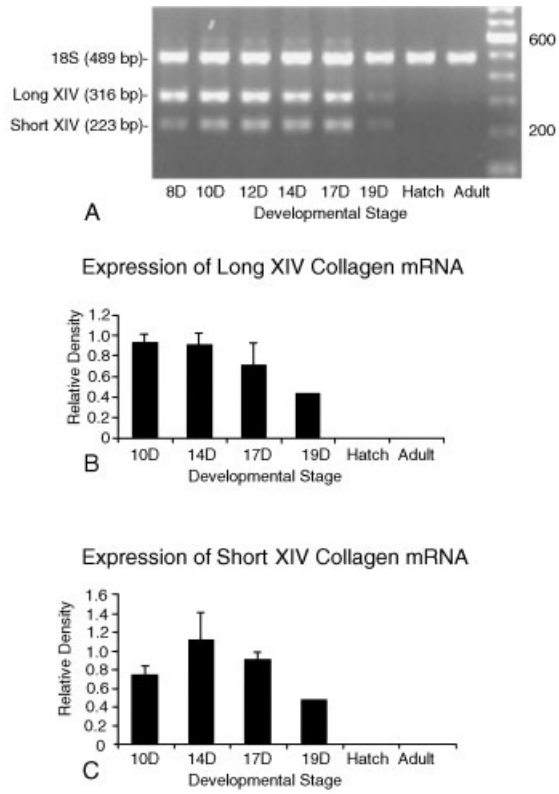

Fig. 7. Type XIV collagen mRNA expression decreases dramatically between day 17 (17D) and day 19 (19D) during corneal development. (**A**) This ethidium bromide agarose gel demonstrates that both the long and short type XIV collagen NC1 splice variants are present from embryonic day 8 (8D) until day 17 (17D) during the period of rapid fibril growth. The long XIV splice variant message is consistently higher relative to the short splice variant message at all stages. By hatching, type XIV collagen is no longer detectable. (**B**) This quantitative densitometric graph demonstrates the expression of long XIV collagen message is high during early stages of fibril growth in cornea. (**C**) This quantitative densitometric graph demonstrates the expression of short XIV collagen message peaks at day 14 and decreases during fibril growth in cornea. Graphs (B) and (C) represent the averages of four separate semi-quantitative PCR experiments on multiple samples of pooled cDNA. Again, the levels of both type XIV splice variants are present during stages of early development and rapid fibrillogenesis (10D–17D) when fibrils are growing primarily in length. A semi-quantitative PCR approach was used to determine relative amounts of the two type XIV collagen splice variants in developing cornea. This multiplex PCR utilized two primer pairs to amplify the target type XIV splice variants and an internal 18S rRNA control. Two type XIV collagen splice variants in the NC1 domain have been described [Walchi et al., 1993]. We termed these the long XIV and short XIV variants. The −93 bp deletion has a region between bases 5,747 and 5,839 spliced out. The primer pairs designed would recognize both splice variants and yield PCR products of 316 bp for the long XIV variant and 223 bp for the short XIV variant. This PCR analyses was optimized for both the correct cycle number for amplification in the linear range and the correct ratio of the internal control primer.

begins infusing the anterior stroma at hatching and is found throughout the mature stroma of the adult. This change implies a more structural role stabilizing the layers of the cornea.

Our data indicate that the long NC3 isoform is the predominant form present in the adult stroma. The observed isoform switching is presumably involved in the change from developmental regulation of matrix assembly and integration to stabilization of lamellae in the adult. The switch to predominantly the long isoform also may partially explain the unextractability of type XII collagen in adult

corneas. Analysis of the type XII splice variants in separated epithelium and stroma layers demonstrated a contribution by both tissue layers [Gordon et al., 1996]. The contribution of two NC3 isoforms from two adjacent sources may be required for tissue integration. Our present data demonstrated a strong localization to Bowman's layer from day 14 to day 17. This further supports a role for type XII collagen in the integration of the epithelial and endothelial matrices with the stroma during development.

The temporal expression of type XII collagen remains constant in the developing Bowman's layer indicating a maintenance role for this FACIT collagen. Studies in human corneas demonstrated the rigidity of the anterior stroma preserves corneal curvature [Bron, 2001; Muller et al., 2001]. At day 19, the type XII collagen begins infusing the anterior stroma again, suggesting a role in maintaining the structural integrity of the anterior stroma that is important in corneal curvature and function. There are two major isoforms of type XII collagen that differ in their NC3 domain. Since the NC3 domain projects into the surrounding matrix away from the collagen I/V fibril, different isoforms can affect matrix–collagen interactions or collagen–collagen interactions. Our analysis of the type XII NC3 splice variants indicated the presence of the long type XII splice variant throughout development while the short type XII variant was highest during earlier stages and decreased in the mature stages. Nishiyama reported that the NC3 domain of type XII caused gel contraction suggesting a macromolecular bridge between the fibrils [Nishiyama et al., 1994]. Both our morphological data and molecular analyses indicated long type XII collagen is predominant during development and in the adult stroma. Since this is the isoform with the larger NC3 domain, that contains more type III FN repeats, it suggests that this isoform would function in holding together and integrating the developing matrices. Other groups have investigated type XII in developing rabbit, bovine and human cornea [Sasaki et al., 1996; Wessel et al., 1997; Anderson et al., 2000]. The data presented by others in the rabbit cornea demonstrated the long XII isoform is predominant in the newborn rabbit throughout the stroma. This group further suggests that the long isoform is anchoring the epithelium to the stroma promoting condensation or compaction. The presence of the long isoform in human corneas at the interfacial matrices and stroma supports a role in integration of matrices. [Wessel et al., 1997].

Both type XII and XIV collagens have multiple splice variants. We analyzed both splice variants that affected the amino terminus of type XII collagen and the carboxyl terminus of type XIV collagen. We propose that these splice variants generate different isoforms that will affect interactions with the developing fibrils or interactions between the fibrils and the matrix. The type XII collagen splice variants affect the NC3 domain that projects into the matrix. These alternative isoforms may ultimately change interactions between fibrils or between fibrils and matrix components. Our data demonstrated long XII collagen is present throughout the stages of the developing cornea. We hypothesize this larger isoform is responsible for structural maintenance of Bowman's layer in the developing tissue. The presence of the long isoform in the mature stroma proper suggests a role in organizing the fibrils. It has been suggested that type XII collagen may have a role in maintenance of fibril spacing in the stroma [Wessel et al., 1997]. The analyses of the type XIV collagen splice variants demonstrated that both variants are present in early corneal development until day 19. The long splice variant is consistently higher relative to the short variant. As we stated in our earlier report, the long NC1 type XIV splice variant introduces a neutral region into a highly charged area. This change in charge may affect the interaction with the developing fibril in a growing tissue [Young et al., 2000]. We hypothesize that long type XIV may aid in stabilizing the fibril intermediates guiding the growth process of the developing fibrils.

In summary, we have demonstrated that two FACIT collagens, types XII and XIV, have unique localization patterns in the developing cornea. These data suggest different roles for these collagens and their different isoforms in fibrillogenesis and matrix assembly in the developing cornea.

ACKNOWLEDGMENTS

Supported by a grant from the, NIH-NEI EY05129. We thank Jessie Feng for expert technical assistance. Helpful discussions with Dr. Jeffrey Marchant and Dr. Marion Gordon are gratefully acknowledged.

REFERENCES

Akutsu N, Milbury CM, Burgeson RE, Nishiyama T. 1999. Effect of type XII or XIV collagen NC-3 domain on the human dermal fibroblast migration into reconstituted collagen gel. Exp Dermatol 8:17–21.

Anderson S, SundarRaj S, Fite D, Wessel H, SundarRaj N. 2000. Developmentally regulated appearance of spliced variants of type XII collagen in the cornea. Invest Ophthalmol Vis Sci 41:55–63.

Birk DE, Trelstad RL. 1984. Extracellular compartments in matrix morphogenesis: Collagen fibril, bundle, and lamellar formation by corneal fibroblasts. J Cell Biol 99:2024–2033.

Birk DE, Hahn RA, Linsenmayer CY, Zycband ZI. 1996. Characterization of fibril segments from chicken embryo cornea, dermis, and tendon. Matrix Biol 15:111–118.

Bron AJ. 2001. The architecture of the corneal stroma. Br J Ophthalmol 85:379–381.

Brown JC, Mann K, Wiedemann H, Timpl R. 1993. Structure and binding properties of collagen type XIV isolated from human placenta. J Cell Biol 120:557–567.

Castagnola P, Tavella S, Gerecke DR, Dublet B, Gordon MK, Seyer J, Cancedda R, van der Rest M, Olsen BR. 1992. Tissue-specific expression of type XIV collagen—a member of the FACIT class of collagens. Eur J Cell Biol 59:340–347.

Chakravarti S, Petroll M, Hassell J, Jester J, Lass JH, Paul J, Birk DE. 2000. Corneal opacity in lumican-deficient mice due to collagen fibril structure and packing defects in the posterior stroma. Invest Ophthal Vis Sci.

Cox JL, Farrell RA, Hart RW, Langham ME. 1970. The transparency of the mammalian cornea. J Physiol 210:601–616.

El Shabrawi Y, Kublin CL, Cintron C. 1998. mRNA levels of alpha1(VI) collagen, alpha1(XII) collagen, and beta ig in rabbit cornea during normal development and healing. Invest Ophthalmol Vis Sci 39:36–44.

Ezura Y, Chakravarti S, Oldberg A, Chervoneva I, Birk DE. 2000. Differential expression of lumican and fibromodulin regulate collagen fibrillogenesis in developing mouse tendons. J Cell Biol 151:779–788.

Fitch JM, Birk DE, Mentzer A, Hasty KA, Mainardi C, Linsenmayer TF. 1988. Corneal collagen fibrils: dissection with specific collagenases and monoclonal antibodies. Invest Ophthalmol Vis Sci 29:1125–1136.

Font B, Aubert-Foucher E, Goldschmidt D, Eichenberger D, van der Rest M. 1993. Binding of collagen XIV with the dermatan sulfate side chain of decorin. J Biol Chem 268:25015–25018.

Font B, Eichenberger D, Rosenberg LM, van der Rest M. 1996. Characterization of the interactions of type XII collagen with two small proteoglycans from fetal bovine tendon, decorin, and fibromodulin. Matrix Biol 15:341–348.

Giry-Lozinguez C, Aubert-Foucher E, Penin F, Deleage G, Dublet B, van der Rest M. 1998. Identification and characterization of a heparin binding site within the NC1 domain of chicken collagen XIV. Matrix Biol 17:145–149.

Gordon MK, Foley JW, Linsenmayer TF, Fitch JM. 1996. Temporal expression of types XII and XIV collagen mRNA and protein during avain corneal development. Dev Dyn 206:49–58.

Hamburger V, Hamilton HL. 1951. A series of normal stages in development of the chick embryo. J Morphol 88:49–92.

Hay ED. 1980. Development of the vertebrate cornea. Int Rev Cytol 63:263–322.

Hay ED, Revel JP. 1969. Fine structure of the developing avian cornea. Monogr Dev Biol 1:1–14.

Imhof M, Trueb B. 1998. An alternative insert of three amino acids is incorporated into collagen XIV in a developmentally regulated fashion. FEBS Lett 438: 325–328.

Imhof M, Trueb B. 2001. Alternative splicing of the first F3 domain from chicken collagen XIV affects cell adhesion and heparin binding. J Biol Chem 276:9141–9148.

Iozzo RV, Murdoch A. 1996. Proteoglycans of the extracellular environment: Clues from the gene and protein side offer novel perspectives in molecular diversity and function. FASEB J 10:598–614.

Kania AM, Reichenberger E, Baur ST, Karimbux NY, Taylor RW, Olsen BR, Nishimura I. 1999. Structural variation of type XII collagen at its carboxyl-terminal NC1 domain generated by tissue-specific alternative splicing. J Biol Chem 274:22053–22059.

Keene DR, Lunstrum GP, Morris NP, Stoddard DW, Burgeson RE. 1991. Two type XII-like collagens localize to the surface of banded collagen fibrils. J Cell Biol 113:971–978.

Koch M, Bohrmann B, Matthison M, Hagios C, Trueb B, Chiquet M. 1995. Large and small splice variants of collagen XII: Differential expression and ligand binding. J Cell Biol 130:1–10.

Koch M, Foley JE, Hahn R, Zhou P, Burgeson RE, Gerecke DR, Gordon MK. 2001. Alpha 1(XX) collagen, a new member of the collagen subfamily, fibril-associated collagens with interrupted triple helices. J Biol Chem 276:23120–23126.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Linsenmayer TF, Gibney E, Fitch JM. 1986. Embryonic avian cornea contains layers of collagen with greater than average stability. J Cell Biol 103:1587–1593.

Linsenmayer TF, Fitch JM, Gordon MK, Cai CX, Igoe F, Marchant JK, Birk DE. 1998. Development and roles of collagenous matrices in the embryonic avian cornea. Prog Retin Eye Res 17:231–265.

Muller LJ, Pels E, Vrensen GF. 2001. The specific architecture of the anterior stroma accounts for maintenance of corneal curvature. Br J Ophthalmol 85:437–443.

Myers JC, Yang H, D'Ippolito JA, Presente A, Miller MK, Dion AS. 1994. The triple-helical region of human type XIX collagen consists of multiple collagenous subdomains and exhibits limited sequence homology to alpha 1(XVI). J Biol Chem 269:18549–18557.

Nishiyama T, McDonough AM, Bruns RR, Burgeson RE. 1994. Type XII and XIV collagens mediate interactions between banded collagen fibers in vitro and may moduate matrix deformity. J Biol Chem 269:28193–28199.

Pan TC, Zhang RZ, Mattei MG, Timpl R, Chu ML. 1992. Cloning and chromosomal location of human alpha 1(XVI) collagen. Proc Natl Acad Sci USA 89: 6565–6569.

Ricard-Blum S, Dublet B, van der Rest M. 2000. Unconventional collagens types VI, VII, VIII, IX, X, XIV, XVI, and XIX (Oxford University Press).

Sasaki T, Akutsu N, Nishiyama T, Burgeson RE, Nakajima H. 1996. Type XII collagen in human skin: Studies on its localization with monoclonal antibodies. Arch Dermatol Res 289:62–64.

Schuppan D, Cantaluppi MC, Becker J, Veit A, Bunte T, Troyer D, Schuppan F, Schmid M, Ackermann R, Hahn EG. 2001. Undulin, an extracellular matrix glycoprotein associated with collagen fibrils. J Biol Chem 265:8823–8832.

Shaw LM, Olsen BR. 1991. FACIT collagens: diverse molecular bridges in extracellular matrices. Trends Biochem Sci 16:191–194.

Svensson L, Aszodi A, Reinholt FP, Fassler R, Heinegard D, Oldberg A. 1999. Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon. J Biol Chem 274:9636–9647.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Trueb J, Trueb B. 1992. The two splice variants of collagen XII share a common 5′ end. Biochim Biophys Acta 1171: 97–98.

Walchi C, Trueb J, Kessler B, Winterhalter KH, rueb B. 1993. Complete primary structure of chicken collagen XIV. Eur J Biochem 212:483–490.

Walchi C, Koch M, Chiquet M, Odermatt BF, Trueb B. 1994. Tissue-specific expression of the fibril-associated collagens XII and XIV. J Cell Sci 107:669–681.

Wessel H, Anderson S, Fite D, Halvas E, Hempel J, SundarRaj N. 1997. Type XII collagen contributes to diversities in human corneal and limbal extracellular matrices. Invest Ophthalmol Vis Sci 38:2408–2422.

Yamakoshi H, Nagai Y. 1992. Molecular cloning and partial characterization of a novel collagen chain, alpha 1(XVI), consisting of repetitive collagenous domains and cysteine-containing non-collagenous segments. J Biochem Tokyo 112:856–863.

Yoshioka H, Zhang H, Ramirez F, Mattei MG, Moradi-Ameli M, van der Rest M, Gordon MK. 1992. Synteny between the loci for a novel FACIT-like collagen locus (D6S228E) and alpha 1 (IX) collagen (COL9A1) on 6q12-q14 in humans. Genomics 13:884–886.

Young BB, Gordon MK, Birk DE. 2000. Expression of type XIV collagen in developing chicken tendons: association with assembly and growth of collagen fibrils. Dev Dyn 217:430–439.