Immunolocalization of Type XII Collagen at the Corneoscleral Angle of the Embryonic Avian Eye

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A monoclonal antibody specific for the chicken a1(XII) collagen chain was used to immunolocalize type XII collagen in the corneosclera of 17–19-day-old chicken embryos. These immunofluorescence studies localized type XII collagen around the scleral cartilages and ossicles. There was also a striking positive staining in the scleral spur and stroma of the corneolimbus beneath the external scleral sulcus. By immunofluorescence the corneal stroma proper was found to be relatively poor in type XII collagen. Immunoblot analysis confirmed the relative absence of a1(XII) in the corneal stroma and demonstrated the presence of a 240-kD polypeptide representative of a1(XII) in extracts of the corneoscleral angle. It was suggested that type XII collagen in the corneoscleral angle reinforces the collagenous matrix at specific sites which require high tensile strength.

Determining the composition of the extracellular matrix at the corneoscleral angle is fundamental to our understanding of this anatomically and physiologically complex area. This area represents an important transition between the transparent cornea and the dense fibrous scleral tissue, anchors the ciliary body and iris, and is the site of the main pathway of aqueous outflow.

Type XII collagen is a member of the group of nonfibrillar collagens which are associated with fibrillar collagen. The nonfibrillar collagens, such as type IX collagen, found in cartilage, and type XII, found in dense type I collagen-containing connective tissue such as tendons, are distinctly different in molecular structure from the well-known fibrillar collagens (types I, II, III, V, and XI). The nonfibrillar collagens contain triple-helical domains interspersed with nontriple-helical regions. As a rule, they do not form supramolecular aggregates alone but may associate with the surface of fibrillar collagens. The molecular structure and location of types IX and XII at the surface of collagen fibrils supports the assertion that these collagens may link the classic collagen fibrils to the surrounding matrix. Such an association may anchor fibrillar collagen to the surrounding matrix and be crucial for the generation and maintenance of the three-dimensional macromolecular pattern in different matrices.

Type XII collagen was identified initially by isolating and analyzing the sequence of the cDNA pMG377 from an embryonic chick tendon cDNA library. Subsequently, we generated a monoclonal antibody specific for type XII collagen. Taking advantage of the known sequence of the a1(XII) chain, we generated a monoclonal antibody against a synthetic peptide derived from the conceptual translation product of pMG377. Using this antibody, we then identified the a1(XII) polypeptide. Type XII collagen was localized immunohistochemically in dense connective tissues such as ligaments, tendons, perichondria, and periosteum. The immunodistribution of type XII collagen is consistent with the role for type XII collagen in reinforcing type I collagen fibrils in matrices requiring increased tensile strength.

I used this monoclonal antibody to immunolocalize type XII collagen in the avian eye. Although type XII collagen appears to be relatively absent from the cornea, type XII collagen was found at discrete locations in the corneoscleral angle. The scleral spur, a fibrous wedge which affords attachment to the ciliary muscle and the ciliary trabeculum is rich in this type of collagen. It is also abundant in the stroma of the corneolimbus subjacent to the external scleral sulcus, tempting us to speculate that type XII may play a role in the maintenance of the differential curvature between the sclera and cornea.
Materials and Methods

Antibodies

We developed a monoclonal antibody against a synthetic peptide whose sequence was derived from the nucleotide sequence of a cDNA encoding α1(XII) collagen. The antibody 75d7 has been used to identify the α1(XII) chain on immunoblots of tendon extracts and immunolocalizations of type XII collagen in various dense connective tissues. A monoclonal antibody to type I collagen was generously provided by Dr. Thomas Linsenmayer (Tufts University, Boston, MA), and a monoclonal antibody to chick type III collagen was generously provided by Dr. Richard Mayne (University of Alabama, Birmingham, AL).

Immunofluorescence

The animal studies conformed to the ARVO Resolution on the Use of Animals in Research. The eyes were removed from 7-, 10-, 17-, and 19-day-old chick embryos and 2-day-old hatchlings. The anterior portions were harvested by excising 5 mm posterior to the anterior margin of the scleral cartilage. The anterior segments were then either fixed in 3% paraformaldehyde for 30 min followed by Tris-buffered saline washes and then mounted in OCT embedding medium (Miles Inc., Elkhart, IN) for cryostat sectioning or directly mounted in OCT without fixation. Seven-micron cryostat sections were cut and prepared for immunofluorescence microscopy. Routinely, sections were digested with testicular hyaluronidase (3.3 mg/ml phosphate buffer, pH 6.0) for 30 min at 30°C. Incubations in primary antibody, hybridoma supernatant, or ascites fluid diluted 1:1000 (~3.5 μg/ml), were done for 1 hr at room temperature. After washing in phosphate-buffered saline (PBS), secondary antibody incubations, using fluorescein-labeled goat anti-mouse immunoglobulin (IgG) (Boehringer Mannheim, Indianapolis, IN) were done for 1 hr at room temperature. Additional attempts to unmask potential type XII collagen in the corneal stroma included treatment in 0.5 M acetic acid, 20 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and heat denaturing.

Extraction of type XII collagen

The 17- and 19-day-old chick anterior segments were harvested from 36 chicks, and the collagen was extracted from both central corneal buttons (removed with a 3-mm diameter trephine) and corneoscleral angles (dissected free of attached corneal tissue and scleral ossicles). Collagen was extracted for 24 hr at 4°C after homogenization in 50 mM Tris-HCl, pH 8.0, containing 1 M NaCl and protease inhibitors (1 mM phenylmethyl-sulfonyl fluoride (PMSF), 1 mM p-aminobenzamidine, 10 mM N-ethylmaleimide, and 10 mM EDTA). We previously showed that type XII collagen is found in the 1 M NaCl solubilized fraction. These extracts were precipitated with ammonium sulfate, at 40% saturation, and the precipitate was centrifuged (12,000 × g for 20 min). The pellet was washed three times with 75% ethanol containing 1.0% potassium acetate and solubilized in 0.5 ml sample buffer containing 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol.

Immunoblotting

The SDS polyacrylamide slab-gel electrophoresis was done according to Laemmli, and the samples were reduced with 1% mercaptoethanol. After electrophoresis, the gel was soaked in transfer buffer (10 mM 3-[cyclohexyl amino]-l propanesulfonic acid, with 10% methanol, pH 11.0) for 5 min. The gel was sandwiched between a sheet of Immobilon membrane (polyvinylidene difluoride; Millipore Corp., Bedford, MA) and blotting paper and was electroblotted for 18–20 hr at 60 V in the transfer buffer.

The membranes were treated with 5% nonfat dry milk in PBS, then incubated for 1 hr in 75d7 antibody (1:1000 dilution). After rinsing in PBS (three times for 5 min each), the membranes were incubated for 1 hr in peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim) in PBS containing 5% normal goat serum. After additional washes, the membranes were treated with 3,3'-diaminobenzidine 0.5 mg/ml in PBS containing H2O2 (0.03%).

Tissue Culture and Biosynthetic Labeling

Epithelia from 19-day-old chick corneas were harvested by forceps dissection after a 30-min treatment in EDTA (0.8 mg/ml). Epithelial sheets were placed on Millipore filter discs and cultured at the air–media interface as reported previously. Fibroblasts from these corneas and from leg tendons were isolated by digestion with collagenase (sigma type I, 1.5 mg/ml) in Hank's buffered saline for 30 min. The cells were recovered after passage through 50-μm nylon mesh and plated at density of 5 × 106 cells/cm2 in HAM's F12 medium supplemented with 10% fetal calf serum (Gibco, Grand Island, NY) and fresh ascorbic acid, pyruvate, and glutamine. For metabolic labeling, after a 12-hr incubation, the culture medium was changed to medium containing 35 μCi of 35S-methionine. After an additional 18-hr incubation, the medium proteins were precipitated with ammonium sulfate at 30% saturation. The ammonium sulfate precipitate was centrifuged (12,000 × g, for 30 min), and the pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.6, containing 0.2 mM NaCl, 0.5% Triton X-100, and 1 mM EDTA. After centrifugation, the supernatant was...
dialyzed against the same buffer used for immunoprecipitation.

**Immunoprecipitation**

For immunoprecipitation of labeled type XII collagen, purified 75d7 IgG and protein-G Sepharose (Pharmacia, Piscataway, NJ) were used. Two milliliters of ammonium sulfate precipitated fibroblast proteins in 25 mM Tris HC1, pH 8.0, with 0.5% Triton X-100 and 0.1 M NaCl were preincubated with 50 µl of protein-G Sepharose. After centrifugation in a table-top centrifuge, the supernatant was brought to a final concentration of 30 µg/ml of IgG from 75d7. After mixing for 30 min at room temperature, 50 µl of protein-G Sepharose was added. Then there was an additional incubation of 30 min, and the protein-G Sepharose–antibody–antigen complexes were centrifuged. Supernatants were discarded, and the pellets were washed repeatedly in Tris HC1 buffer containing 0.5 M NaCl and 0.5% Triton X-100. For gel electrophoretic analysis of the immunoprecipitated proteins, sample buffer containing 1% SDS and 1% mercaptoethanol was added to the pellets, and the samples were heated to 95°C for 1 min.

**Results**

Immunofluorescence analysis of cryostat sectioned anterior segments with antibody 75d7, against type XII collagen showed immunostaining of the dense connective tissues surrounding the skeletal tissues in the avian eye. The type XII staining was clear surrounding the scleral ossicles and the scleral cartilages (Fig. 1D). This result was consistent with our earlier results demonstrating the immunolocalization of type XII in perichondria and periossea. However, abundant staining was also seen in the fibrous scleral...
spur and the corneoscleral limbus connective tissue beneath the external scleral sulcus (Figs. 1B, 2).

Using monoclonal antibodies to the fibrillar collagens types I and III, we showed that the type XII staining does not strictly colocalize with either of these fibrillar collagens. Type I collagen, as expected, was very plentiful in the corneal stroma and found throughout scleral connective tissue including the scleral ossicles (Fig. 1C). Type III was found in the loose connective tissue of the sclera and lid but mostly excluded from the corneal stroma (Fig. 1D).

From our previous studies, we had speculated that type XII collagen may be present on the surface of type I fibrils in dense connective tissues (excluding bone) and may link the type I-containing fibrils to the surrounding matrix. The exclusion of type XII immunostaining from the cornea was therefore an unexpected result. Attempts to unmask potential type XII staining in the stroma failed. Unmasking procedures, including heat denaturing, detergent (Triton X-100, 1%), EDTA, and acid (0.05M acetic acid) treatments, did not significantly increase the immunostaining for type XII collagen (data not shown).

In addition to 17- and 19-day-old embryonic chick eyes, 7- and 10-day-old embryonic eyes and those of 2-day-old hatchlings were examined. The early chick eye (7-day-old) showed no significant staining of the cornea or of the corneoscleral angle (Fig. 3A). However, 10-day-old eyes had staining at the corneoscleral angle. The staining seen here was more diffuse than that seen in older eyes, however, the stroma beneath
the external corneoscleral sulcus and the region that will later become the scleral spur were positive for type XII collagen (Fig. 3B). The staining pattern of the 2-day-old hatchling was very similar to that described for the 17-19-day-old embryonic eyes (data not shown). Because the latter eyes had a pattern seen in the hatched chicken, we concentrated our efforts on examining this late embryonic developmental stage.

To determine whether antigen immunolocalized by 75d7 corresponded to the α1(XII) chain found in tendon, I repeated the extraction scheme previously shown to yield abundant type XII collagen from chick tendons. Similar to the tendon, the type XII collagen immunoreactivity was abundant in the 1 M NaCl extract from the corneolimbus. In addition, consistent with the immunofluorescence results, the extract of central corneal buttons had relatively little α1(XII) immunoreactivity (Fig. 4). The weak immunoreaction product seen in central button extracts most likely represents a very low expression of α1(XII) which is not detectable by immunofluorescence microscopy.

The immunoblots of the 1 M NaCl extracted limbus material demonstrated a single immunoreactive polypeptide migrating around 240 kDa compared with globular molecular weight standards (Fig. 4); the tendon extracts had immunoreactive polypeptides at 220, and 240 and a complex around 300 kDa (Fig. 4).

Immunoprecipitation of type XII collagen synthesized by fibroblasts in culture also demonstrated the discrepancy in molecular weight between corneoscleral angle α1(XII) and tendon α1(XII). A metabolically labeled polypeptide of 240 kD was immunoprecipitated from culture medium of fibroblast harvested from the corneoscleral angle; two polypeptides of 220 and 260 kD were immunoprecipitated from tendon fibroblast cultures (Fig. 5). The heterogeneity of the polypeptides from tendon is currently under examination. Possible posttranslational processing and nonreducible crosslinking to other matrix components are currently being considered.

I next examined the extracts and medium of cultures of isolated cells from different regions of the chick eye for α1(XII) chains. Fibroblasts harvested from the central cornea, peripheral cornea, scleral angle as well, and corneal epithelia were cultured for 48 hr. Cell layers and media were extracted and immunoblotted with 75d7 (Fig. 6). There were α1(XII) chains detected in all the extracts. These data demonstrate that cells derived from tissues that contain little type XII collagen in vivo are capable of synthesizing and secreting detectable quantities α1(XII) chains in vitro. This observation raises the intriguing question...
Fig. 6. Epithelial sheets and isolated fibroblasts derived from central and peripheral cornea and sclera were placed in culture for 48 hr. Cells were extracted with 1 M NaCl. Extracts were then dialysed and concentrated. Samples were electrophoresed on 6% SDS-PAGE. Western blots were reacted with monoclonal 75d7. Lanes 1-4 are coomassie stained gel. Lanes 4-8 are immunoblots. Extracts from central corneal fibroblasts are shown in lanes 1 and 5, peripheral corneal fibroblasts shown in lanes 2 and 6, scleral fibroblasts shown in lanes 3 and 7, and corneal epithelia shown in lanes 4 and 8.

of what factors may control the production and deposition of type XII collagen in the tissues of the eye.

Discussion

Type XII collagen has been proposed to be a member of a distinct class of extracellular matrix proteins found in association with the classic quarter-staggered collagen fibrils. It was first identified by the isolation of a cDNA encoding for a polypeptide with collagenous (triple-helical) domains interspersed with noncollagenous domains; these afford the molecule both rod-like and flexible qualities. The structural and molecular similarity of type XII to type IX collagen is striking and has prompted the speculation that type XII may serve to link type I collagen-containing fibrils to the surrounding matrix, much like the postulated role of type IX collagen in cartilage. Although the carboxyl end of α1(XII) is similar to type IX collagen, α1(XII) chain has a large NC3 domain at the amino end not found in type IX chains. If the proposed association to type I collagen fibrils is valid, the large NC3 domain would extend laterally into the surrounding matrix. This proposed heteromolecular association would provide firm anchorage for the collagen fibrils and may enable the linearly packed collagen fibrils greater tensile strength as would be required in tendons and ligaments, perichondria, and periostea. The immunodistribution of type XII collagen in the avian eye described is consistent with this postulated function. The presence of type XII in the sclera surrounding the ossicles and scleral cartilage was as expected from our previous work. However, the abundance of the type XII in the scleral spur or roll and corneoscleral stroma beneath the external scleral sulcus is remarkable, as is the relative absence of α1(XII) from the corneal stroma.

The scleral spur is a firm, fibrous wedge that serves as the anterior insertion site for the ciliary muscles and the site of attachment of the ciliary trabeculae. The collagen fibrils in the spur are variable in diameter and arranged in a circular formation. There may be a role for type XII collagen in maintaining the collagen fibrils in a circular tendon for the ciliary muscle and thereby limiting the diameter of the scleral ring. After contraction of the ciliary muscle, the scleral spur (or ring) is pulled posteriorly, thus affecting the width of the spaces in the ciliary trabecular meshwork. I suggest that type XII collagen may be integral in limiting the circumference of the scleral ring.

The corneoscleral stroma beneath the external scleral sulcus is also rich in type XII collagen. The external sulcus is formed by the corneal curvature merging with the greater radius of the curvature of the sclera. This transition zone of the limbus is the site of merging of collagenous tissue of the corneal stroma with the collagenous tissue of the sclera. Type XII collagen at this location may reinforce the type I-containing fibrils, affording a tendon-like attachment for the cornea to the fibrous sclera. A belt-like tendon at this location may thereby assist in maintaining the differential curvature between cornea and sclera.

The relative absence of type XII collagen in the corneal stroma was demonstrated by immunofluorescence supported by immunoblot analysis of salt extracts of central corneal buttons and corneoscleral angles. The low expression of type XII collagen in the cornea would be consistent with a role of type XII collagen in areas of requisite tensile strength as opposed to a role in the packing of collagen fibrils into higher organizational arrays such as that found in the cornea. We previously showed that type IX collagen is found in very early chick corneal stroma, and there may be an additional, as yet unknown, member(s) of this nonfibrillar collagen family similar to types IX and XII in the adult corneal stroma.

The expression of type XII collagen described here poses many fascinating questions pertaining to the control of matrix expression and assembly, such as, what inhibits α1(XII) expression in the cornea but enhances it in the sclera? It is interesting that the α1(XII) chains could be detected in primary cultures of corneal fibroblasts and corneal epithelia. I speculate that the corneal cells in vivo contain the message.
for the α1(XII) chain, but the translation and/or processing of the collagen may be blocked in the in vivo cornea. There is a precedent for cells containing collagen mRNA but not expressing the protein. Others\(^5\) showed that the α2(I) collagen gene contains a cartilage-specific promoter that renders the mRNA product incapable of translation of collagen chains in chondrocytes.

I also showed that the apparent molecular weight of α1(XII) chain is different from that found in tendon and that not all forms found in tendon are present in the corneoscleral junction. The identification of unique collagenous molecules or altered forms of collagens in the eye is not unprecedented. We reported that in the embryonic chick cornea the mRNA coding for α1(IX) collagen chains are 700 nucleotides shorter than that found in cartilage.\(^4\)\(^6\) Another study\(^16\) found that the corneal collagen gene uses a different splice site for transcription and that this site is in an intron. However, at this time, it is too early to speculate whether or not the α1(XII) collagen chains of different sizes than those found in tendon result from potential differential gene splicing or posttranslational modifications of the α1(XII) chains in the avian eye.

Although it is tempting to extrapolate from the avian eye to the mammalian eye, we must be cautious. In fact, the corneosclera of the avian eye differs markedly from that of mammals. The eye of the chicken is reinforced posteriorly by cartilage and more anteriorly by the scleral ossicles. With these specializations, we would expect perichondrial and periosteal staining for type XII collagen. However, it will be interesting to determine if the presence of type XII collagen in the scleral spur and corneoscleral junction is restricted to avians. Whether or not type XII collagen is present in the angle of human eye must await the generation of antibodies and/or other probes for human type XII collagen.

**Key words**: type XII collagen, corneoscleral angle

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**References**


