OPHTHALMIC MOLECULAR GENETICS

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Immunohistochemical and Molecular Genetic Evidence for Type IV Collagen α5 Chain Abnormality in the Anterior Lenticonus Associated With Alport Syndrome

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Objective: To present evidence for a type IV collagen α5 chain (α5[IV]) abnormality in the anterior lens capsule of a patient with anterior lenticonus associated with Alport syndrome.

Methods: The anterior lens capsule obtained from a 54-year-old man with anterior lenticonus associated with Alport syndrome was examined ultrastructurally and stained immunohistochemically for the α chains of type IV collagen, α1(IV) to α6(IV). A search was also made for a mutation in the COL4A5 complementary DNA encoding the α5(IV) chain by reverse transcription–polymerase chain reaction of illegitimate transcripts.

Results: The anterior lens capsule of the patient was much thinner than that of normal subjects and lacked the α3(IV) to α6(IV) chains immunohistochemically, while control specimens stained positively for all of the α(IV) chains. The patient had a C-to-T transition at nucleotide 5231 causing a nonsense mutation, R1677X, in the COL4A5 complementary DNA.

Conclusion: Our findings demonstrated that normal anterior lens capsules express all of the α(IV) chains and that a patient with anterior lenticonus associated with Alport syndrome had a mutation in the COL4A5 gene resulting in the lack of immunoreactivity to α3(IV) to α6(IV) chains in the anterior lens capsule.

Clinical Relevance: This study showed abnormal composition of α(IV) chains in the anterior lens capsule of a patient with anterior lenticonus caused by a nonsense mutation in the COL4A5 gene. Further investigation of the phenotype-genotype relationship will provide a better understanding of the molecular pathogenesis of anterior lenticonus.

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CASE REPORT

Alport syndrome (AS) is a hereditary multisystem disorder characterized by progressive nephritis, sensorineural deafness, and ocular abnormalities, including anterior lenticonus. Anterior lenticonus is a pathognomonic finding in AS. The disease arises primarily from a genetically induced disorder of the α chains of type IV collagen (α[IV]). Six types of α(IV) chains have been reported, namely, the α1(IV) to α6(IV) chains, and a compound helix of 3 α(IV) chains makes 1 molecule of type IV collagen.¹

Two types of AS are known: an autosomal recessive type² that has mutations of the genes encoding the α3(IV) and α4(IV) chains (COL4A3 and COL4A4 genes, respectively) on chromosome 2, and an X-linked type³,⁴ that has mutations of the COL4A5 gene encoding the α5(IV) chain on the X chromosome. We present ultrastructural, immunohistochemical, and genetic analyses of a case of AS associated with bilateral anterior lenticonus.

Methods

A 44-year-old man was first seen on April 25, 1988, complaining of decreased vision in his left eye. The best-corrected visual acuity was 20/25 OD and 20/100 OS. Anterior lenticonus and an anterior polar cataract were noted bilaterally, and yellowish flecks surrounded both maculas. Au-dimetry revealed a perceptive hearing loss. He had been on hemodialysis since age 27 because of chronic glomerulonephritis. His family history was negative for renal diseases, early-onset cataract, and hearing loss.

He underwent cataract surgery on both eyes, and the anterior lens capsule of his right eye was preserved for histological study. The lens capsules from 4 age-matched patients with age-related cataracts served as controls. The patient with AS and the control subjects had no history of other systemic diseases or medication use that might affect collagen expression. An informed consent for the research use of the lens capsule, the molecular genetic analysis, and the publication of the results was obtained from each subject.

The authors have no relevant financial interest in this article.
ELECTRON MICROSCOPY

The central portion of the anterior capsule was used for the ultrastructural study. The specimens were immersed in a phosphate buffer (pH, 7.2-7.4) containing 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide in the same buffer. The specimens were dehydrated in graded ethanol and embedded in epon using standard procedures. Ultrathin sections for electron microscopy were stained with uranyl acetate and lead citrate and were observed by transmission electron microscopy (H-7100; Hitachi, Tokyo, Japan).

IMMUNOHISTOCHEMISTRY

The specimen of the patient with AS was snap-frozen in a dry ice and acetone bath, transferred without thawing, and stored at −70°C for several months until use in the immunohistochemical experiments. The anterior capsules from 2 of the 4 control subjects were processed and stored on the same occasion. The specimens from 2 other control subjects were obtained 2 years later and processed in the same way.

Cryostat sections (3 µm) of the peripheral portion of the anterior capsule were cut at a constant temperature (−25°C) and fixed for 10 minutes in acetone. The sections were placed in an acid buffer made by mixing an equal volume of 0.1M potassium chloride and 0.1M hydrochloric acid (pH, 1.5) for 10 minutes and rinsed 3 times with phosphate-buffered saline (PBS). The sections were then immersed in 10% normal goat serum in PBS for 10 minutes and rinsed 3 times with PBS.

Serial sections were stained by an indirect method using primary antibodies against the α1(IV) to α6(IV) chains prepared as reported previously. Briefly, rat monoclonal antibodies were raised against synthetic peptides of nonconsensus amino acid sequences close to the carboxyl terminus of the noncollagenous (NC) domain of each α(IV) chain by a method using rat medial iliac lymph nodes. The antibodies were diluted 1:10 with PBS containing 1 mg/mL of bovine serum albumin. After a 60-minute incubation with the primary antibodies at room temperature, the sections were rinsed 3 times with PBS. Then the secondary antibody, fluorescein isothiocyanate–conjugated goat F(ab′)2 anti-rat IgG (Organon Teknika-Cappel, Durham, NC) was added to the sections. The secondary antibody was diluted 1:50 with PBS containing 5% normal human serum prepared in our laboratory. After a 30-minute incubation, the sections were rinsed 3 times with PBS and mounted on coverglass with 20% glycerin (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The sections were viewed with a reflecting microscope (BH2-RFCA; Olympus Optical Co, Tokyo).

Immunostaining of the specimens from the patient and control subjects was performed simultaneously using the same solutions.

MUTATION ANALYSIS OF THE COL4A5 COMPLEMENTARY DNA

The complementary DNA (cDNA) sequence encoding the noncollagenous (NC1) domain of the α5(IV) chain was determined by reverse transcription–polymerase chain reaction products using illegitimate COL4A5 transcripts as described by Inoue et al. Polyadenylated RNAs were extracted from peripheral leukocytes of each subject and reverse transcribed. The cDNA region encoding the NC1 domain of the α5(IV) chain was amplified by polymerase chain reaction with 2 sets of oligonucleotide-primer pairs. Direct sequencing of the polymerase chain reaction products was performed on an automated DNA sequencer.

RESULTS

ULTRASTRUCTURE

The thickness of the anterior capsule of the patient with AS was 3 µm in the central portion and noticeably thinner than that of the controls (20 µm) (Figure 1). Dehiscences were noted up to two thirds of the depth from the lens epithelium (Figure 1A, arrows). In contrast, such lesions were not noted in the control capsule (Figure 1B), suggesting that the observed dehiscences were not artifactual. The lens epithelial cells from the patient with AS were slightly flatter and the nuclei were pyknotic, but the numbers were not reduced compared with the controls.
IMMUNOHISTOCHEMICAL STAINING

Figure 2B demonstrates the α1(IV) to α6(IV) chain-like immunoreactivities present throughout the anterior lens capsules in controls. No difference in location of the immunoreactivity between different α chains was observed. In contrast, the lens capsule of the patient with AS was negative for the α3(IV) to α6(IV) chain-like immunoreactivities (Figure 2A). Even undiluted primary antibodies of α3(IV) to α6(IV) chains gave the same results, indicating the absence of immunoreactivity to these chains in the anterior capsule of the patient (data not shown).

The staining patterns of α1(IV) to α6(IV) chains in the lens epithelial cells were identical with the remaining parts of the anterior capsule, although the positive staining might be partly artifactual because of the dissociation of the epithelium during the tissue processing (Figure 2A, arrows).

MUTATION ANALYSIS OF THE COL4A5 cDNA

The patient had a C-to-T transition at nucleotide 5231 resulting in a nonsense mutation, R1677X, in the COL4A5 cDNA (Figure 3). Genomic DNA sequencing of the...
COL4A5 gene verified the sequence alteration (data not shown).

The ultrastructural changes of the anterior lens capsule in our patient with AS were in agreement with those described in previous reports. Streiten et al first reported that the anterior lens capsule in AS was markedly thin and showed numerous dehiscences extending up to two thirds of the depth from the lens epithelium. Later, Kato and Junk et al reported similar lesions, with Junk and colleagues concluding that ultrastructural changes of the anterior lens capsule should be included in the differential diagnosis of AS.

Cheong et al described 2 male patients with AS; one showed α3(IV) to α5(IV) chain-like immunoreactivities in the anterior lens capsule, while the other did not, reflecting a genetic heterogeneity in AS. Takei et al described a female patient whose anterior lens capsule showed normal immunoreactivity to the α2(IV) chain and decreased immunoreactivity to the α5(IV) chain. Therefore, the anterior lens capsule from patients with AS may show abnormal immunostaining of the α3(IV) to α5(IV) chains, depending on individual genetic defect. However, neither study identified the possible gene mutations responsible for the abnormal α(IV) chains. In addition, there has been no examination of the α6(IV) chain in the anterior lens capsule of patients with AS or of normal subjects.

As demonstrated, the normal anterior lens capsule expressed the α1(IV) to α6(IV) chains, but only the α1(IV) and α2(IV) chains were expressed in our patient with AS. Because α1(IV)/α1(IV)/α2(IV), α3(IV)/α4(IV)/α5(IV), and α5(IV)/α5(IV)/α6(IV) triple helical molecules are the only known components of the type IV collagen network, our results indicate that the normal anterior lens capsule exhibits not only α1(IV)/α1(IV)/α2(IV) and α3(IV)/α4(IV)/α5(IV) molecules but also the α5(IV)/α5(IV)/α6(IV) molecule. Our patient lacked the α3(IV)/α4(IV)/α5(IV) molecule and the α5(IV)/α5(IV)/α6(IV) molecule. The most plausible interpretation of our results is that this patient had a defective α5(IV) chain.

Recently, several studies have identified a close relationship between the clinical severity of nephritis (phenotype), the immunohistochemical findings of the α(IV) chains in the glomerular basement membrane, and the genotype of the α5(IV) chain. Inoue et al showed that mutations in the NC1 domain of the α5(IV) chain were associated with an absence of immunostaining of the α3(IV) to α5(IV) chains in the glomerular basement membrane and with severe renal dysfunction. Similarly, our patient had renal dysfunction and was negative for the α3(IV) to α5(IV) (and α6(IV)) chain-like immunoreactivities in the anterior lens capsule. We therefore searched for a mutation in the cDNA sequence corresponding to the NC1 domain of the α5(IV) chain and found the nonsense mutation R1677X. This is one of the mutations reported by Inoue at al. The R1677X nonsense mutation is thought to produce a truncated NC1 domain of the α5(IV) chain lacking 9 amino acids, including 2 cysteine residues involved in intermolecular cross-linkages, leading to a malformation of collagen network made by the α3(IV)/α4(IV)/α5(IV) and α5(IV)/α5(IV)/α6(IV) triple helical molecules. We suggest that the mechanisms causing the defective glomerular basement membrane also caused fragility in the anterior lens capsule in our patient with AS.

Pajari et al reported that the ocular manifestations in AS develop with age but independently of the genotype. However, they identified the gene mutations in only a few of their patients; therefore, the correlation between the genotype and the ocular phenotype in AS needs further investigation. Systematic examinations, in-

Figure 3. The COL4A5 complementary DNA sequences of the patient with AS (A) and of a normal subject (B). Arrows point to the C-to-T transition corresponding to a nonsense mutation, R1677X, changing a codon for arginine (Arg) to a stop codon (Stop) at the 1677 amino acid residue of COL4A5 in the patient.
cluding electron microscopy, immunohistochemistry, and molecular genetics, in multiple patients with AS with anterior lenticonus will provide a better understanding of the physiological role played by the α(IV) chains in the anterior lens capsule and in the pathogenesis of anterior lenticonus.

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