Hereditary Lattice Corneal Dystrophy Is Associated with Corneal Amyloid Deposits Enclosing C-Terminal Fragments of Keratoepithelin

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PURPOSE. To investigate the molecular basis of hereditary lattice corneal dystrophy (LCD) type IIIA associated with corneal amyloid deposits afflicting several members of a four-generation family.

METHODS. Histologic, immunohistochemical and biochemical studies were performed on corneal tissue samples obtained after perforating keratoplasty. DNA was extracted from peripheral blood leukocytes. All exons of the keratoepithelin-encoding TGFBI gene were amplified and sequenced. The presence of a mutation was confirmed by digestion of the isolated PCR product with the restriction enzyme AluNI.

RESULTS. The cornea of the index patient (II-1) contained large patchy deposits of amyloid, which were immunoreactive for the C terminus of keratoepithelin. Western blot analysis of the polypeptide chains extracted from the amyloid deposits of paraffin-embedded tissue revealed that these represented mainly fragments of the full-length protein. The smallest fragments were 6.5 and 6.9 kDa. DNA analyses of the TGFBI gene revealed a heterozygous T→C transition at the second position of codon 540 in exon 12, indicating that replacement of phenylalanine by serine (Phe540Ser) leads to dominant disease. The mutation creates a new restriction site for the enzyme AluNI. Five of the examined family members carried this mutation. Three of them (aged ≥41 years) had the disease, two family members (aged <20 years) do not yet show any clinical symptoms. An additional inconsecutive single-nucleotide polymorphism (T1667C) was found at the third position of the same codon (Phe540Phe) in three unaffected family members.

CONCLUSIONS. This is the first report of a single-nucleotide mutation at codon 540 of TGFBI leading to LCD, and the first to demonstrate that the amyloid deposits in LCD contain proto-lytic fragments of keratoepithelin. (Invest Ophthalmol Vis Sci. 2005;46:1133–1139) DOI:10.1167/iovs.04-1319

The human transforming growth factor β-induced (TGFBI; OMIM 601692; Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) gene located on chromosome 5 at q31 encodes keratoepithelin, a 68-kDa protein that is expressed on the surface of corneal epithelial cells.1 Mutations in the TGFBI gene have been linked with five distinct corneal dystrophies (CDs): granular Groenouw type 1, Reis-Bückler CD, lattice (LCD) types I and IIIA, and the Avellino CD. Most forms of CD are inherited in an autosomal dominant manner and lead to visual impairment by interfering with corneal transparency. LCD encompasses a group of diseases that is characterized by the deposition of amyloid in the cornea. LCD I is the classic form of the disease. Onset of symptoms (epithelial erosions and decreased visual function) is at the end of the first decade, with a slow progression. Substantial discomfort and visual impairment occur late in the fifth decade. A net of fine linear opacities within the corneal stroma, as a result of the accumulation of amyloid, is characteristic of LCD I. In LCD IIIA, the opacities are markedly thicker, but both LCD I and IIIA start in the center and at the surface of the corneal stroma. In time, the opacities spread to the deeper corneal layers and to the periphery.

Studies of the molecular basis of the corneal dystrophies have revealed that LCD I and - IIIA are caused by single point mutations in exons 4, 11, 12, and 14 of the TGFBI gene.2–7 Herein, we describe a family that was found to have amyloid deposits within the cornea caused by a novel mutation in the TGFBI gene, leading to marked loss of visual acuity in the late fourth and early fifth decades of life.

MATERIALS AND METHODS

Patients

All patients studied were examined with DNA sequencing, as described later in this section. In tissue from the index patient, histologic, immunohistochemical, biochemical, and molecular biological studies were performed. Full informed consent was obtained from all family members for molecular biological analyses, in keeping with the guidelines of the Declaration of Helsinki.

Histology

Corneal tissue specimens were fixed in formalin and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin. The presence of amyloid was demonstrated by the appearance of green birefringence from Congo red staining under polarized light.9


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Immunohistochemistry

Immunostaining was performed with monoclonal antibodies directed against AA amyloid (1:400; Dako, Hamburg, Germany) and with polyclonal antibodies directed against the amyloid P-component (1:1600), fibrinogen (1:1000), lactoferrin (1:900), lysozyme (1:5000), transthyretin (1:5000), a-light chain (1:10,000), k-light chain (1:10,000; all Dako), apolipoprotein Al (apoAl; 1:1500), and keratoepithelin (KE2 and KE15). The antibodies directed against keratoepithelin had been raised in rabbits by using the C (amino acids 426-682) and N (amino acids 69-364) terminal portions of keratoepithelin, as described elsewhere. Before they were immunostained, the specimens were treated with 10 mM EDTA (two times for 10 minutes each, 450-W microwave oven; amyllo P component, gelsolin, and transthyretin). Immunoreactions were visualized with the avidin-biotin complex method, with the use of an ABC alkaline phosphatase kit (Vectastain; Biogene-Alexis GmbH, Grünberg, Germany) or a diaminoenzidine (DAK) Detection kit (VIW: Ventana, Illkirch, France). Neufuchsin and 3,3-diaminobenzidine-tetrahydrochloride, respectively, served as chromogens. The specimens were counterstained with hematoxylin. The specificity of immunostaining was verified with specimens containing known classes of amyloid (AA amyloid, apoAl, transthyretin, a-light chain), by using positive controls recommended by the manufacturers (remaining antibodies) and by omitting the primary antibodies. Immunostaining with anti-KE2 and anti-KE15 antibodies was performed as described previously, with some modifications. Briefly, after digestion with 1 U Pronase (15 minutes at room temperature; Sigma-Aldrich), the sections were blocked with normal goat serum (1:2000 dilution of goat anti-rabbit-AP). The substrate (BCIP/NBT solution 1:2000) and anti-KE15 (1:100) as primary antibodies (room temperature, 1 hour). Blocking was performed in Tris-buffered saline containing 3% bovine serum albumin and 0.05% Tween-20. The membranes were then incubated for 1 hour with the secondary antibody (1:2000 dilution of goat anti-rabbit-AP). The substrate (BCIP/NBT (Pierce, Rockford, IL) was left on the membrane until distinct bands had developed.

SDS-PAGE and Western Blot Analysis

Amyloid fibril proteins were extracted from formalin-fixed and paraffin-embedded specimens and resolved by SDS polyacrylamide gel electrophoresis, as described elsewhere, using specimens from the index patient (II-1). As negative controls, we used amyloid fibril proteins, denatured tissue specimens, obtained from a patient with immunoglobulin-associated AL amyloidosis and from a patient with secondary AA amyloidosis. Proteins were visualized by Coomassie blue staining or by Western blotting onto polyvinylidene difluoride (PVDF) membranes. Immunostaining of the transferred proteins was performed with anti-KE2 (dilution 1:2000) and anti-KE15 (1:100) as primary antibodies (room temperature, 1 hour). Blocking was performed in Tris-buffered saline containing 3% bovine serum albumin and 0.05% Tween-20. The membranes were then incubated for 1 hour with the secondary antibody (1:2000 dilution of goat anti-rabbit-AP). The substrate (BCIP/NBT (Pierce, Rockford, IL) was left on the membrane until distinct bands had developed.

Isolation and Amplification of Genomic DNA

Genomic DNA was extracted from peripheral blood leukocytes with a kit (NucleoSpin Blood L Kit; Macherey & Nagel, Düren, Germany). Exons 1 to 17 of the TGFBI gene were amplified by PCR with 1 µg of genomic DNA of individual II-1, and the primers are listed in Table 1. Initial denaturation and activation of Taq polymerase at 95°C for 5 minutes was followed by 35 cycles (30 for exons 4 and 11) with denaturation (95°C, 30 seconds), annealing for 30 seconds (temperatures listed in Table 1), an extension at 72°C for 60 seconds, and a final extension at 72°C for 15 minutes. The coding region for the classic mutation Asp187 of the gelsolin gene was amplified with the following primers: 5'-ACT GGT GTA CTG TG TGT CTA TA-3' and 5'-TCT CAA TCA CTT CCC TGG AG-3'. The PCR profile for gelsolin was as follows: 94°C, 3 minutes; 35 cycles (94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute); and an extension of 7°C, 10 minutes. The PCR products were isolated (NucleoSpin Extract; Macherey & Nagel) and sequenced with dye-termination chemistry (Prism Big Dye Terminator ver.1.1, Ready Reaction kit; Applied Biosystems, Inc. [ABI], Darmstadt, Germany) by a capillary sequencer (Primer model 310; ABI).

Restriction Enzyme Digestion with AlwNI

Restriction enzyme digestion analysis was performed to confirm the mutation found. This mutation creates a new restriction site for AlwNI (CAGNNN/CTG). One microgram of the purified DNA fragment obtained by the PCR with the primer for exon 12 (Table 1) was incubated with 5 U AlwNI at 37°C for 2 hours. The digestion was stopped by heat inactivation (65°C, 30 minutes) according to the manufacturer’s instructions. The samples were separated on a 9% polyacrylamide gel and visualized with a silver staining procedure.

Homology Modeling

The model was generated using MODELLER based on a sequence alignment of keratoepithelin domain 4 (Swiss Prot: Q15582, residues 502-635; http://www.expasy.org; provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland) to the known Drosophila melanogaster fascin 1 domain four-template structure according to a procedure by Clout and Hohenester.
RESULTS

Family

The family tree is shown in Figure 1.

The index patient (II-1) was referred to the Department of Ophthalmology of the Klinikum Kassel at the age of 42 years with a visual acuity loss to 0.5 in the right eye and 0.1 in the left eye. The patient refused medical treatment and did not return to the department until 11 years later. Visual acuity had further deteriorated and was found to be 1/20 in the right eye and 1/35 in the left eye (Fig. 2). Another three years later, at age 56 years, she underwent perforating keratoplasty on her left eye and another year later on her right eye. This improved visual acuity to 0.4 in the left eye and to 0.25 in the right. The corneal specimens obtained at surgery were submitted for histologic examination in this study.

The index patient’s mother (I-1) had also had LCD. She underwent three keratoplasties: at age 46 (left eye), 53 (right eye), and 56 (left eye) years. No blood or tissue samples were available from her.

The patient’s brother (II-2) underwent two keratoplasties: at age 46 (left eye) and 49 (right eye) years. Peripheral blood samples were available from the patient, his wife, and his three daughters.

The patient’s daughter (III-1) is a 41-year-old woman. She presented at age 40 with LCD (Fig. 2). Her visual acuity was 0.8 in both eyes. One year later, her visual acuity declined to 0.2 in her right eye and 0.1 in her left eye. Peripheral blood samples were available from the patient and her daughter.

Histology and Immunohistochemistry

The biopsy specimens obtained from the cornea of the index patient II-1 showed large patchy deposits of homogeneous cosinophilic material in the subepithelial layer and deeper corneal stroma, which showed green birefringence in polarized light after Congo red staining, characteristic of amyloid (Fig. 3). The amyloid deposits were immunoreactive for amyloid P component, but not for amyloid A, apolipoprotein A1, fibrinogen, gelsolin, \( \lambda \) - and \( \kappa \)-light chains, lysozyme, lactoferrin, or transthyretin.

Immunostaining of the cornea with the polyclonal antisera directed against keratoepithelin showed a strong positive reaction of the intrastromal amyloid deposits and of those in the subepithelial layer with KE2, which was raised against the C terminus of the protein (residues 426-682; Fig. 3). By contrast, the polyclonal antibody KE15, which was generated against the N terminus of the protein (residues 69-364), showed no immunostaining within these amyloid deposits. However, both...
Amyloid fibril proteins were extracted from formalin-fixed and paraffin-embedded biopsy specimens of the cornea, which were shown histologically to enclose amyloid deposits, and were resolved by denaturing gel electrophoresis. The amount of amyloid proteins extracted was low and could not be visualized by Coomassie blue or silver staining (data not shown). However, using Western blot analysis with the antibodies KE15 and KE2, we were able to detect a protein band at 68 kDa, which represents mature, full-length keratoepithelin (Fig. 4). In addition, the amyloid-specific antibody KE2 detected a range of further bands, which shows that keratoepithelin or fragments of the full-length protein are smeared across most of the lane, a property that is commonly encountered when trying to separate tissue-purified amyloids on an SDS-gel. This effect is caused most likely by cross-linking of the amyloid proteins after formalin fixation, as this smear was not observed after immunoblot analysis with the antibody directed against the N-terminal portion of keratoepithelin. In addition, KE2 but not KE15 detects in the low molecular range several distinct bands corresponding to masses of 6.5, 6.9, 14, 17, and 21 kDa, representing peptide fragments arising from the C terminus of keratoepithelin (Fig. 4). These data demonstrate that amyloid deposits derived from keratoepithelin do not contain mainly the full-length protein, but rather fragments thereof. Native, unfixed corneal tissue was not available from any of the family members. The specificity of immunoblot analysis was further tested by using AL and AA amyloid proteins extracted from formalin-fixed tissue obtained from a patient with primary AL amyloidosis (Fig. 4, lane 5)\textsuperscript{15} and from a patient with chronic rheumatoid arthritis (Fig. 4, lane 6). Several amyloid proteins were found after Coomassie blue staining, some of which had been identified by amino acid sequencing.\textsuperscript{13} Similar to corneal amyloid (Fig. 4, lane 2), a protein smear related to cross-linking of the amyloid proteins after formalin fixation was found (Fig. 4, lanes 5, 6). However, neither the AA nor the AL amyloid proteins immunoreacted with the two antibodies directed against keratoepithelin (Fig. 4, lanes 3, 4).

**DNA Analyses**

Analysis of the exons 1 through 17 of the keratoepithelin encoding \textit{TGFBI} gene of patient II-1 revealed a point mutation in exon 12. A heterozygous T→C transition at the second position of codon 540 was found by double-strand sequencing, leading to the replacement of the normal phenylalanine by a serine residue (Phe540Ser; Fig. 5). It is of note that this mutation occurs within the same part of keratoepithelin that was found by our antibody. Moreover, the same mutation was found in the index patient’s daughter (III-1), the patient’s brother (II-2), the patient’s niece (III-4), and one granddaughter (IV-1; Fig. 1).

Another base change, a T→C transition at the third position of the same codon 540 was found in individuals II-3, III-2, III-3, and III-4, but has no effect on the amino acid encoded (Phe540Phe). This single nucleotide polymorphism is a well-documented base change in this exon.\textsuperscript{16} The sequence of individual III-4 revealed the Phe540Phe polymorphism in conjunction with the Phe540Ser mutation.

The mutation at the second position of codon 540 introduces a restriction site for the endonuclease \textit{Alu}NI. The wild-type sequence of exon 12 (ACA GTC TTT GCT, codon 538-541) changes into ACA GTC T\_ CT GCT, containing the recognition sequence for \textit{Alu}NI (CAG NNN \_ CTG). Accordingly, three distinct bands were found after the incubation of the DNA with \textit{Alu}NI in all affected family members investigated: the full-length fragment of exon 12 of the nonmutated allele, and two shorter fragments yielded by the digestion of the mutated allele (Fig. 6). Proband II-3 served as a negative control and showed a single band only (Fig. 6, lane 3).

No mutations were found in the gelsolin gene, which is known to cause hereditary LCD II.\textsuperscript{17,18}

**DISCUSSION**

We describe here for the first time a family with hereditary LCD associated with corneal AKer amyloid deposits caused by a

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932933/)

**Figure 4.** Amyloid proteins were extracted from formalin-fixed specimens obtained from the cornea of the index patient (II-1). After SDS-PAGE and Western blot analysis with anti-KE15 (lane 1) and anti-KE2 (lane 2) antibody, a distinct band was found at 68 kDa that was compatible with intact keratoepithelin. Further bands were found after blot analysis with anti-KE2 (lane 2), probably representing the amyloid proteins. The anti-KE2 antibody did not stain amyloid proteins extracted from formalin-fixed tissue specimens containing AL (lane 3) or AA (lane 4) amyloid. Coomassie staining revealed the banding pattern of AL (lane 5) and AA (lane 6) amyloid proteins.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932933/)

**Figure 5.** Nucleotide sequence from exon 12 of the \textit{TGFBI} gene of the index patient II-1. The heterozygous T→C transition at position 1666 (codon 540, arrows) was confirmed by both sense (a) and antisense (b) primers. The transition of T to C results in the replacement of phenylalanine by serine.
dominant single-point mutation at the second position of codon 540 in exon 12 of the TGFBI gene, replacing phenylalanine by serine (Phe540Ser) in the adhesion molecule keratoepithelin.

Keratoepithelin causes six different LCDs (LCD I, -IA, -IIIA, -IV, -VI, and -VII), all of which are related to point mutations in the TGFBI gene. These mutations, including our novel mutation T1666C, are located in the exons 4, 11, 12, or 14. We believe that our family has hereditary LCD IIIA. This form of corneal dystrophy was first described by Stock et al. in an Italian family. LCD IIIA is characterized by thick, ropy lattice deposits consisting of an α-helical and a β-domain and that residue Phe540 is positioned within a β-strand. This strand runs through the center of the hydrophobic core of the β-domain (Fig. 7b). Considering the large size of phenylalanine and its ability to make very many hydrophobic contacts, replacement of such a burried residue by the much smaller side chain of polar serine readily suggests that the thermodynamic stability of the entire protein will be decreased dramatically. For comparison, when the fully buried Phe153 of the T4 lysozyme is replaced by the small hydrophobic residue Ala, it decreases the thermodynamic stability of the protein by ~5.5 kcal/mol, a value that is well within the range of values known to promote aggregation and amyloid formation. Indeed, amyloid structures are known to form by association of at least partly, if not entirely, unfolded polypeptide chains; and stable, globular proteins must be sufficiently unfolded to enable the polypeptide backbone to form intermolecular interactions. Moreover, unfolding also favors a proteolytic cleavage reaction.

Morand et al. demonstrated that the secretion of the recombinant proteins carrying the most common mutations (Arg124Cys and Arg555Trp) was unaffected in transfected HeLa and HCE cells, with the mutated proteins accumulating in the medium. They also showed that the accumulation of these proteins correlated with an increased apoptotic response of the affected cells. Whether the cornea lacks a general clearing mechanism in response to apoptosis, allowing for the progres-
sive accumulation and deposition of mutated keratoepithelin extracellularly, as suggested by Morand et al.,31 or the deposition of amyloid results from the secretion of the mutated proteins, remains unclear. As mutant forms of keratoepithelin do not form amyloid deposits in the skin of patients with CD,23 there seem to be specific components within the cornea that promote the accumulation of mutated keratoepithelin to form amyloid deposits. In this regard it was interesting to note that immunoblot analysis with site-specific antibodies detected proteolytic fragments derived from the C-terminal region of keratoepithelin in our index patient. After our amyloid protein extraction procedure, we also found mature keratoepithelin, which may originate from the surface epithelium. However, N-terminal cleavage products were not detected by immunoblot analysis. Further studies are needed to demonstrate whether proteolysis is a prerequisite and essential for the formation of amyloid, as in Alzheimer’s disease, or whether it occurs after amyloid has been formed and represents a frustrated attempt to clear pathologic corneal protein aggregates.32 This ultimately may improve our understanding of the pathologic course and pathogenesis of CDs and lead to the development of new treatment strategies, other than perforating keratoplasty.

References


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