

On the Role of Kerato-Epithelin in the Pathogenesis of 5q31-Linked Corneal Dystrophies

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PURPOSE. Recently, the authors identified a gene, *BIGH3*, in which different mutations cause a group of hereditary corneal dystrophies: lattice type I and IIIA (CDLI and CDLIIIA), granular Groenouw type I (CDGGI), Avellino (CDA), and Reis-Bücklers' (CDRB). All these disorders are characterized by the progressive accumulation of corneal deposits with different structural organization. Experiments were conducted to determine the role of kerato-epithelin (KE), the product of *BIGH3*, in the pathogenesis of the diseases.

METHODS. KE-15 and KE-2, two rabbit antisera raised against peptides from the 69–364 and 426–682 amino acid regions of KE respectively, were used for immunohistology of the corneas obtained after keratoplasty in six CDLI patients, three CDGGI patients, and one CDA patient.

RESULTS. The nonamyloid deposits observed in CDGGI stained intensively with KE-15 and KE-2, whereas the amyloid deposits in all analyzed CDLI corneas reacted to KE-2 but not to KE-15. In the CDA cornea, where amyloid and nonamyloid inclusions were present, positive staining with both antisera was observed.

CONCLUSIONS. Pathologic amyloid and nonamyloid deposits observed in CDLI, CDGGI, and CDA-affected corneas are caused by KE accumulation. Different staining patterns of amyloid and nonamyloid deposits observed with antibodies against the amino and carboxyl termini of KE suggest that two mechanisms of KE misfolding are implicated in the pathogenesis of 5q31-linked corneal dystrophies. (*Invest Ophthalmol Vis Sci.* 1999;40:2213–2219)

Corneal dystrophies are inherited disorders characterized by progressive accumulation of deposits in the cornea followed by visual impairment. Recent progress in the elucidation of the molecular nature of this heterogeneous group of conditions comprises the identification of a common genetic basis for Groenouw type I (CDGGI), Reis-Bücklers' (CDRB), lattice type I and IIIA (CDLI, CDLIIIA), and Avellino (CDA) corneal dystrophies.^{1–5} It has recently been shown that specific but different mutations in the same gene, *BIGH3*, are responsible for each particular type of dystrophy.^{6–9} Having a common genetic basis, these hereditary corneal dystrophies linked to chromosome 5q could now be referred to as five forms of the same entity sharing common features including an autosomal dominant mode of inheritance, complete penetrance, age of onset between the first and fourth decades of life, and visual loss during the third through sixth

decades, often requiring corneal transplantation. At the same time, each form can be distinguished clinically and by laboratory examination. First, they can be differentiated by the location of deposits: in the epithelium and Bowman's layer for CDRB and in the subepithelial layer and the stroma for CDGGI, CDLI, CDLIIIA, and CDA. Second, they differ by the structure of the deposits: In CDLI and CDLIIIA, the deposits consist of amyloid fibers, whereas in CDGGI and CDRB the accumulated material is of nonamyloid nature. CDA is characterized by both amyloid and nonamyloid deposits in the same cornea.^{10–15}

To investigate the role of KE, the protein encoded by *BIGH3*, in the pathogenesis of corneal dystrophies, we generated antibodies against two nonoverlapping parts of the protein. Here, we report the results of immunostaining of corneal sections from patients diagnosed with CDLI, CDGGI, and CDA.

METHODS

Patients and Corneas

A panel of human corneas obtained after keratoplasty at the Jules Gonin Eye Hospital, University of Lausanne, (Switzerland); Bascom Palmer Eye Institute, University of Miami, Florida; and Keio University Hospital, Tokyo, was used for this study. Diagnosis of CDLI, CDGGI, or CDA was made clinically and confirmed histopathologically in all cases. Molecular screening was performed by direct sequencing of *BIGH3* exons 4 and 12 as described by Korvatska et al.⁷ Three normal and one CDGGI cornea were used for crude protein extract preparation and for establishing cultures of corneal fibroblasts.

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Ten other corneas, six CDLI, three CDGGI and one CDA, routinely fixed and embedded in paraffin, were used for immunohistology.

Cell Cultures

Nontransformed cultures of fibroblast-like cells were established from biopsy of normal human skin and from normal and CDGGI corneas. All cell cultures were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum in a 5% CO₂ atmosphere at 37°C.

Reverse Transcription–Polymerase Chain Reaction Analysis

mRNA was isolated from these cell lines using oligo (dT) magnetic beads according to the protocol provided by the manufacturer (Dynal, Oslo, Norway). Reverse transcription–polymerase chain reaction (RT-PCR) was performed using a gene amplification kit (Perkin-Elmer Gene-Amp; Roche Molecular Systems, Nutley, NJ), a reverse primer BIG2120R (5'-TTC CTC CTG TAG TGC TTC AA-3'), and one of the following forward primers: BIG11F (5'-CGG TCG CTAGCT CGC TCG GT-3'), BIG20F (5'-GCT CGC TCG GTG CGC GTC GT-3'), BIG541F (5'-TTG AGC TGC TCA ATG CCC TC-3'), BIG2-1F (5'-CTG GAG GGC ACG ACA CTG GA-3') and BIG1321F (5'-CTC CAA TTG ATG CCC ATA CA-3'). Amplified products were subcloned in a vector (p(T)GEM; Promega, Madison, WI) and sequenced using a kit (AutoRead, Pharmacia Biotech, Piscataway, NJ).

Preparation of Total Protein from Conditioned Medium

Conditioned media from growing fibroblasts were periodically harvested and stored at –20°C. Total protein was then precipitated by addition of solid ammonium sulfate to 30%, 50%, and 75% saturation. Aliquots obtained from each fraction were used for immunoblot analysis.

Preparation of Crude Protein Extracts from Corneal Tissue

Two normal corneas were frozen in liquid nitrogen, cut into small slices, and homogenized in a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 5% 2-mercaptoethanol, and 50 mM Tris-HCl (pH 6.8). Samples were subjected to reduced sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis.

Antisera Production

Two portions of the *BIGH3* coding region, an N-terminal part from amino acid 69-364 for pKE-15 and a C-terminal part from amino acid 426-682 for pKE-2, were cloned in the pQE32 vector (Qiagen, Hilden, Germany) and expressed in *Escherichia coli*. After isopropyl this d-galactoside induction, pKE-15 and pKE-2 peptides, both containing a 6-histidine tag, were isolated from bacterial lysates under denaturing conditions (6 M guanidine HCl) using an Ni-NTA agarose column (Qiagen). Polyclonal antisera against pKE-15 and pKE-2 were produced by Eurogentec (Ougrée, Belgium) using two rabbits for each sample. Preimmune sera used for control staining were from the same rabbits.

Western Blot Analysis

Protein preparations were subjected to 10% to 12% SDS-PAGE under reducing conditions. The separated proteins were trans-

ferred to cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany) using a mini transblot apparatus (Bio-Rad, Milan, Italy). Membranes were blocked overnight in Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5] and 0.5 M NaCl) containing 0.05% Tween-20 and 5% (wt/vol) skim milk. Diluted antisera KE-15 (1:2000) and KE-2 (1:2000) were applied for 1 hour at room temperature. Peroxidase-conjugated secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit Ig diluted 1:60,000; Bio-Rad, Hercules, CA) was incubated for 1 hour at room temperature. Immunostaining was visualized using the enhanced chemiluminescence system (ECL; Amersham Life Science, Buckinghamshire, UK).

Immunohistology

Routinely processed corneal samples fixed in buffered formalin and embedded in paraffin were immunostained using the peroxidase-anti-peroxidase (PAP) technique of Sternberger et al.¹⁶ Five-micrometer-thick tissue sections were mounted on aminopropyl-methoxysilane-coated glass slides, deparaffinized in xylol, taken through to absolute alcohol, blocked for endogenous peroxidase with 0.1% hydrogen peroxidase in methanol for 45 minutes, and rehydrated through graded alcohols. The sections incubated with KE-15 and KE-2 were first digested in 0.05% Pronase (Dako, Glostrup, Denmark) for 15 minutes at room temperature. To reduce nonspecific primary antibody binding, they were incubated for 10 minutes with normal goat serum (Pel-Freez Biologicals, Rogers, AR) diluted 1:30 in TBS. The primary antibodies, KE-15 (1:10), KE-2 (1:10), and anti-P component (1:40; Dako); secondary antibodies, goat anti-rabbit immunoglobulins (1:100) and PAP complex (1:400; Sternberger Monoclonals, Baltimore, MD), were diluted in TBS containing 2 mg/ml skim milk. All incubations were for 30 minutes at room temperature, followed by washing in TBS.

RESULTS

Immunoreactivity of Antisera

Polyclonal antibodies, KE-15 and KE-2, were raised in rabbits against the amino- and carboxyl-terminal parts of KE, respectively. The sensitivity and specificity of the antibodies were tested by immunoblot analysis. Each antibody recognized the polypeptide against which it was raised (data not shown). Because KE is one of the major proteins in adult cornea,¹⁷ we used Western blot analysis of corneal crude protein extracts with both antisera to check their reactivity with native KE. Figure 1 shows that both KE-15 and KE-2 recognize a single band of 68 kDa corresponding to the predicted molecular weight of secreted KE.

Expression of *BIGH3* at Both Transcriptional and Translational Levels in Cultured Fibroblasts from Normal and CDGGI Corneas

Because *BIGH3* is implicated in corneal dystrophies, we were interested in determining whether corneal fibroblasts express *BIGH3* mRNA and KE and whether the expression pattern differs between fibroblasts from normal and CDGGI corneas. We thus established fibroblasts cell cultures from skin and from normal and CDGGI corneas. Because skin fibroblasts are known to express *BIGH3* mRNA at high levels,¹⁸ we used them as a positive control. RT-PCR revealed *BIGH3* transcript in all analyzed cell lines (Fig. 2). Additional northern blot analysis of

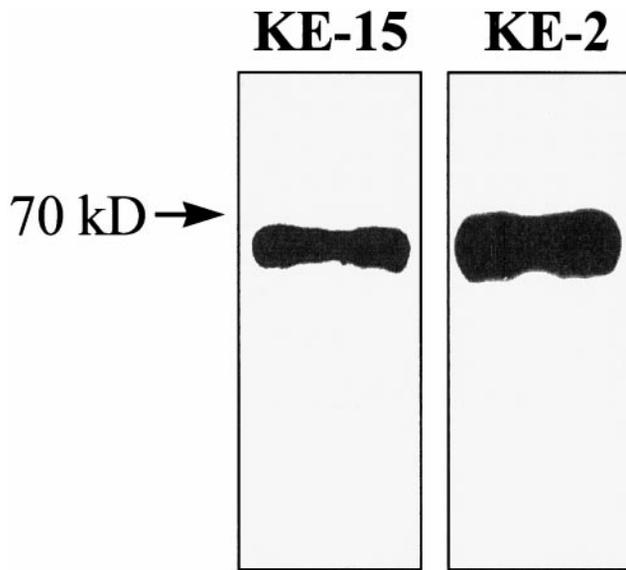


FIGURE 1. Immunoblot analysis of crude protein extract from normal cornea. The same membrane was stained with KE-15 and KE-2.

mRNA from normal and CDGGI fibroblasts revealed no appreciable difference in *BIGH3* transcription level or truncated transcript (data not shown). Because KE is a secreted protein, we also analyzed total protein harvested from conditioned media of normal and CDGGI fibroblast cultures. In all cases, we

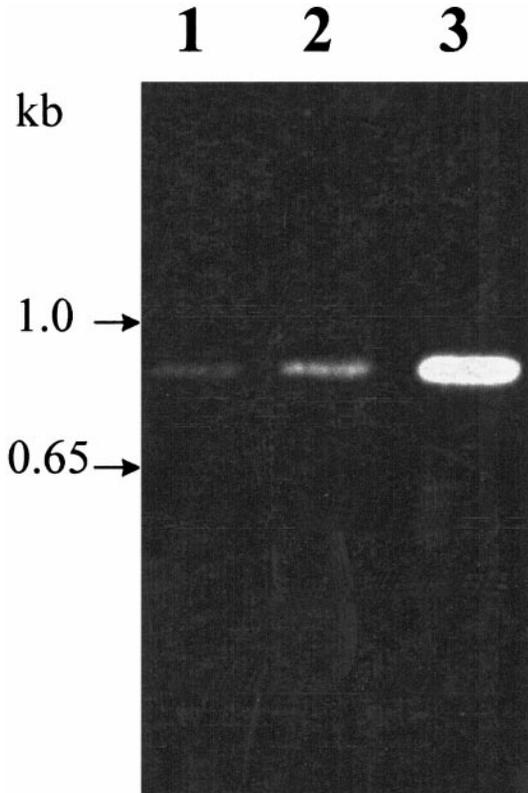


FIGURE 2. RT-PCR of mRNAs isolated from fibroblast-like cell cultures initiated from: normal cornea (lane 1), CDGGI-affected cornea (lane 2), and normal skin (lane 3). An 80-bp fragment of *BIGH3* coding sequence was amplified with primers BIG1321F and BIG2120R.

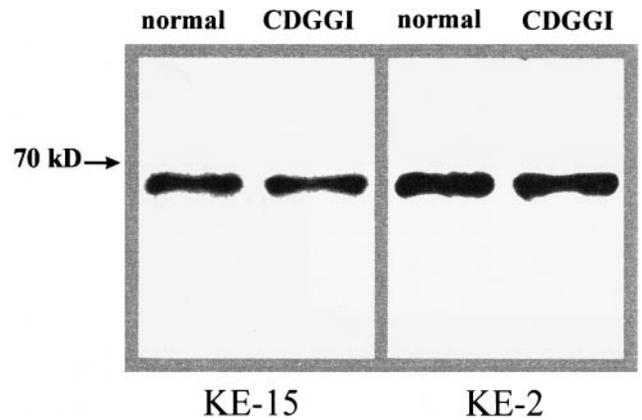


FIGURE 3. Western blot analysis of proteins precipitated from conditioned media of normal and CDGGI corneal fibroblasts stained with KE-15 and KE-2.

observed a single 68-kDa band corresponding to the native KE in Western blot analysis of protein preparations stained with KE-15 and KE-2 (Fig. 3).

Immunohistology of Affected Corneas

Corneal Dystrophy Lattice Type I. Corneas from six unrelated CDLI patients were used for immunohistology. Mutation screening revealed the presence of the R124C mutation in all cases. Histologic examination of corneal sections showed fusiform deposits concentrating in the anterior two thirds of the stroma, as visualized by hematoxylin staining (Fig. 4A). The amyloid nature of the deposits was confirmed by Congo red staining and by immunostaining using monospecific antisera to amyloid P (pentagonal) component (Figs. 4B, 4D), two methods used for histopathologic diagnosis of amyloid regardless of disease type.¹⁹ We observed the appearance of subepithelial layers of even thickness in all samples. In four of six cases they contained amyloid.

Immunostaining of corneal samples revealed a positive reaction with antibodies to the carboxyl terminal of KE in all examined cases (Fig. 4F) in which both the amyloid subepithelial layer and intrastromal deposits showed intense staining with KE-2. However, antibodies to the amino terminal of the protein reacted differently: We observed no staining of intrastromal amyloid deposits with KE-15 in all six samples (Fig. 4E). In four of the six cases mentioned earlier, in which the subepithelial layer was positive for amyloid, KE-15 did not recognize it (Fig. 4E). In the two other CDLI corneas, the nonamyloid subepithelial layer was weakly positive for KE-15 and strongly reactive for KE-2.

Granular Corneal Dystrophy Groenouw Type I. The presence of the R555W mutation in *BIGH3* was confirmed by direct sequencing in all three CDGGI patients whose corneas were analyzed in this study. Corneal changes consisted of accumulation of eosinophilic granular deposits, mostly involving Bowman's layer and the superficial and middle central stroma (Fig. 5A). The posterior stroma was clear in all cases. All CDGGI corneas contained nonamyloid deposits, which was confirmed by a negative Congo red stain (not shown) and by the absence of reactivity with antisera to amyloid P component (Fig. 5B). We observed a partial destruction of Bowman's layer by subepithelial accumulation of granular material, and in some

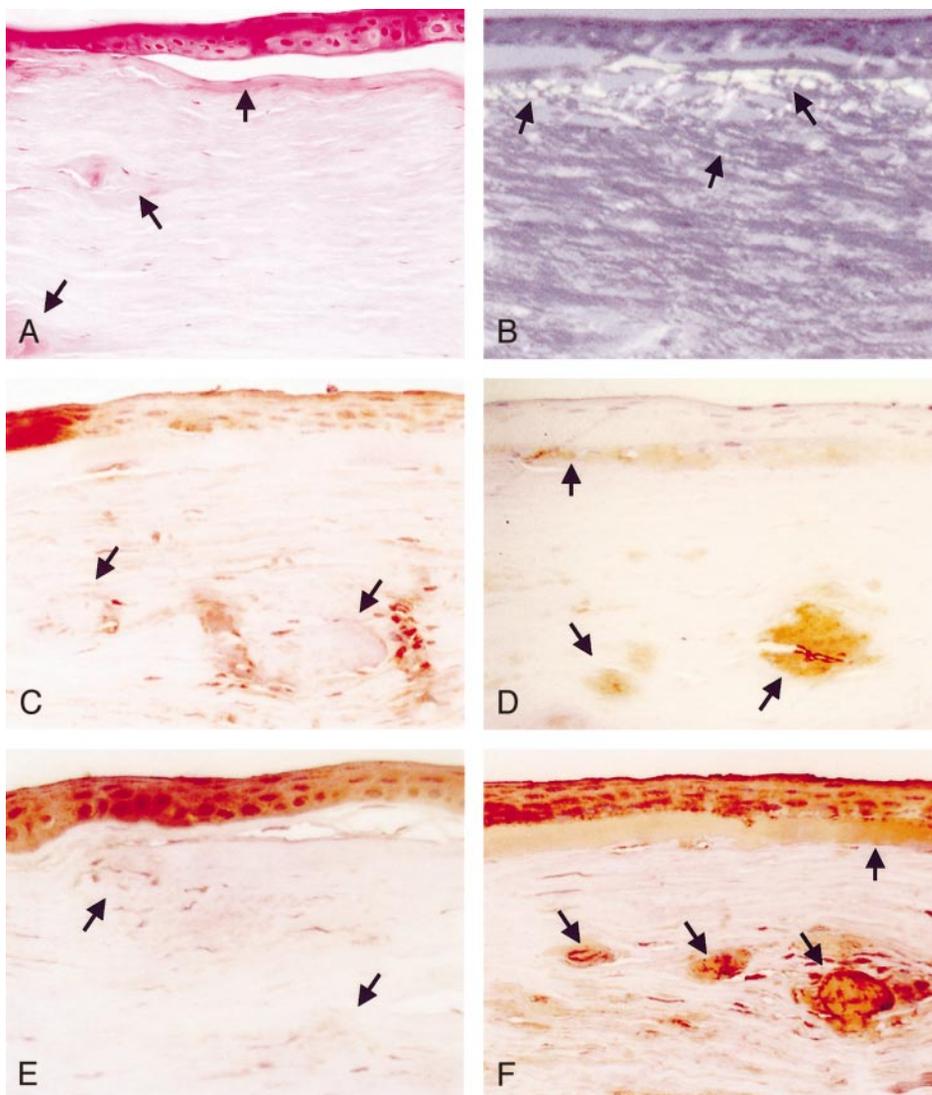


FIGURE 4. Tissue sections of the cornea from a CDLI patient stained with (A) hematoxylin and eosin, (B) Congo red under polarized light, (C) pre-immune serum, (D) antibody against amyloid P, (E) KE-15, and (F) KE-2. Subepithelial layer and stromal deposits are indicated by *arrows*. Magnification, $\times 100$.

cases, the deposits were spread between the epithelium and Bowman's layer. Immunostaining showed that all observed deposits reacted with KE-15 and KE-2 (Figs. 5D, 5E).

Avellino Corneal Dystrophy. A corneal button from a CDA patient whose R124H mutation was documented by direct sequencing was reviewed histologically. The corneal stroma was of normal thickness. Masses of deposited material were accumulated in the upper two thirds of the stroma. This material was strongly and homogeneously positive for KE-2 (Fig. 6F). Fusiform amyloid deposits were located mainly in the middle third of the stroma, confirmed by positive staining with Congo red and antisera to amyloid P (Figs. 6A, 6B, 6D). Staining with KE-15 was observed through the upper two thirds of the central corneal stroma (Fig. 6E). It was difficult to assign precisely the staining patterns of granular and amyloid deposits because nonamyloid inclusions were tightly colocalized with the amyloid material.

DISCUSSION

We identified four missense mutations in the *BIGH3* gene that were specific to four forms of corneal dystrophies: R124C in

CDLI, R124H in CDA, R555W in CDGGI, and R555Q in CDRB.⁶ Independently, several groups confirmed these findings.^{20,21} Recently, Yamamoto et al.⁸ identified another mutation in the *BIGH3*, P501T, associated with CDLI. Mutation screening of families of different origin showed that the alterations predominantly found in the patients affected with CDGGI, CDLI, and CDA occurred at two arginine codons, R124 and R555; that these amino acids represent two mutation hot spots; and that mutated R124 and R555 were associated with different types of deposition, amyloid for R124 and nonamyloid for R555.⁷ However, the exact role of the protein encoded by *BIGH3* in the pathogenesis of the 5q-linked corneal dystrophy was unclear. We thus decided to establish whether the protein itself constitutes the deposits or whether KE is only part of the metabolic pathway that leads to aggregation of other proteins.

Immunostaining of corneal sections shows that KE is specifically accumulated in the pathologic deposits of amyloid and nonamyloid nature in all analyzed forms of 5q31-linked dystrophy. Corroborating genetic evidence, our immunohistology data provide the proof of the direct role of KE in the development of these diseases. In the case of CDGGI, both KE-15 and KE-2 stained the granular inclusions positively. It points out

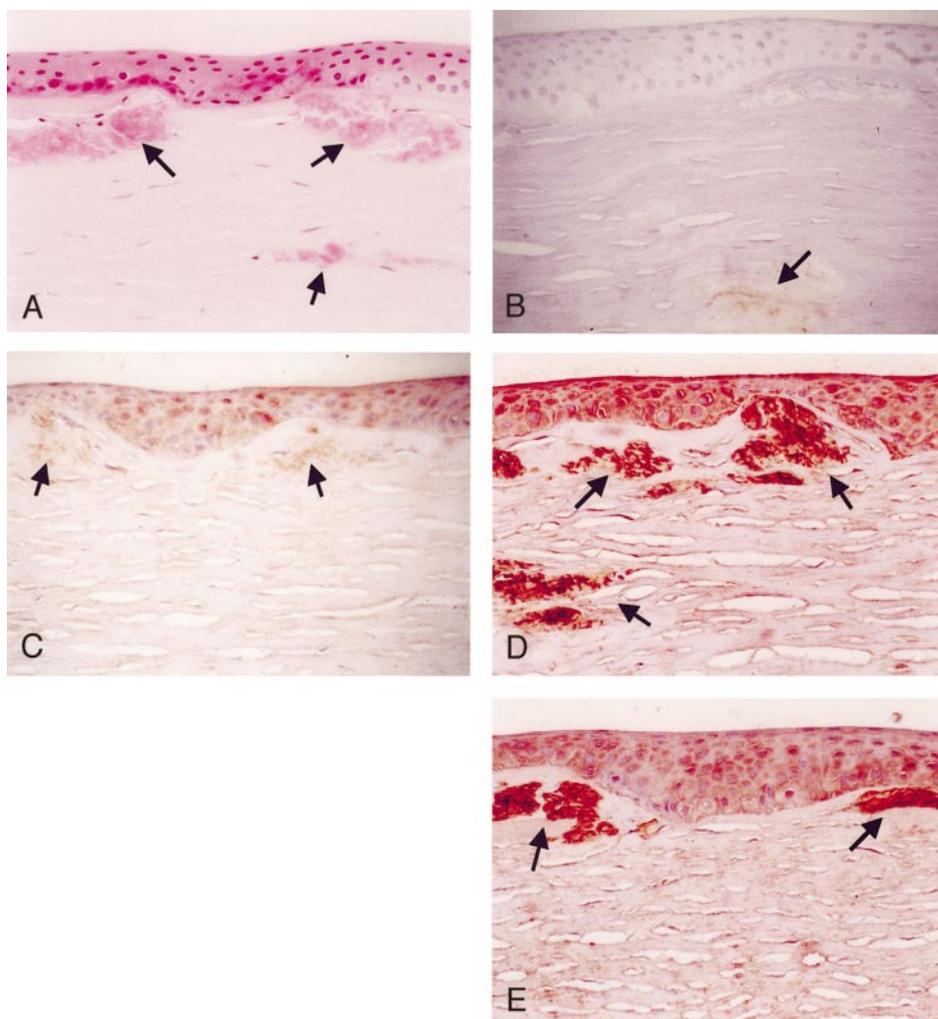


FIGURE 5. Tissue sections of the cornea from a CDGGI patient stained with (A) hematoxylin and eosin, (B) antibody against amyloid P, (C) preimmune serum, (D) KE-15, and (E) KE-2. Subepithelial and stromal deposits are indicated by *arrows*. Magnification $\times 100$.

that the R555W mutation results in the misfolding of whole protein followed by aggregation in deposits. In a recent report Klintworth et al.¹⁷ demonstrated by biochemical methods that CDGGI corneas contain an excess of full-length KE. Thus, KE is the major, if not the sole, component of the nonamyloid deposits in CDGGI.

In the case of CDLI, a hereditary, localized amyloidosis,¹¹ all pathologic deposits found in the corneal stroma and subepithelial layer were positive for KE. This demonstrates the direct implication of KE in the formation of amyloid deposits and suggests that KE is the amyloidogenic protein.

Amyloid diseases are characterized by extracellular deposition of abnormally folded protein precursors that form fibrils of characteristic structure.²² Today, more than 20 different proteins are known to be amyloidogenic. In addition to the major protein constituting the fibril, approximately 10% of the deposit consists of components ubiquitously found in all amyloidoses. Although immunostaining of corneal sections does not allow the quantification of KE in deposits, it is rather unlikely that protein(s) other than KE constitutes the amyloid fibrils in CDLI. Studies of hereditary amyloidoses show that, as a rule, the involved genes encode the amyloidogenic protein-precursors. In addition, several amyloidogenic proteins (amyloid beta, prion protein, and immunoglobulin light chains) can exhibit multiple aggregation states (amyloid and nonamy-

loid).²³ In the case of KE, different mutations in the protein sequence may predispose it to different misfolded states.

The immunohistology of CDLI corneas revealed different staining patterns with the antibodies to amino- and carboxyl-terminal parts of KE. Unlike nonamyloid inclusions in CDGGI, amyloid deposits did not react with KE-15, an antibody to the amino-terminal polypeptide comprising R124. At the same time, KE-15 showed strong specific binding to the entire protein on western blot analysis and to nonamyloid inclusions on CDGGI sections. Thus, KE-15 was able to recognize KE in the denatured form and histochemically as a constituent of nonamyloid aggregates. Moreover, in those CDLI sections where the subepithelial layer consisted of nonamyloid material, we observed weak reactivity with KE-15, but never in the amyloid-containing layer. It seems that in CDLI there was an inverse relationship between the presence of amyloid and staining with KE-15. Our data on CDLI were recently confirmed by Takacs et al.²⁴ who also reported negative staining of amyloid deposits with monoclonal antibodies raised against a 21-amino-acid peptide of the amino terminus.

Two hypotheses can be proposed to explain the differential staining of amyloid inclusions. First, amyloid deposits in CDLI may consist of truncated KE, which lacks, at least, its amino-terminal region. In fact, in many amyloidoses, proteolysis precedes or accompanies amyloid conversion whereby the

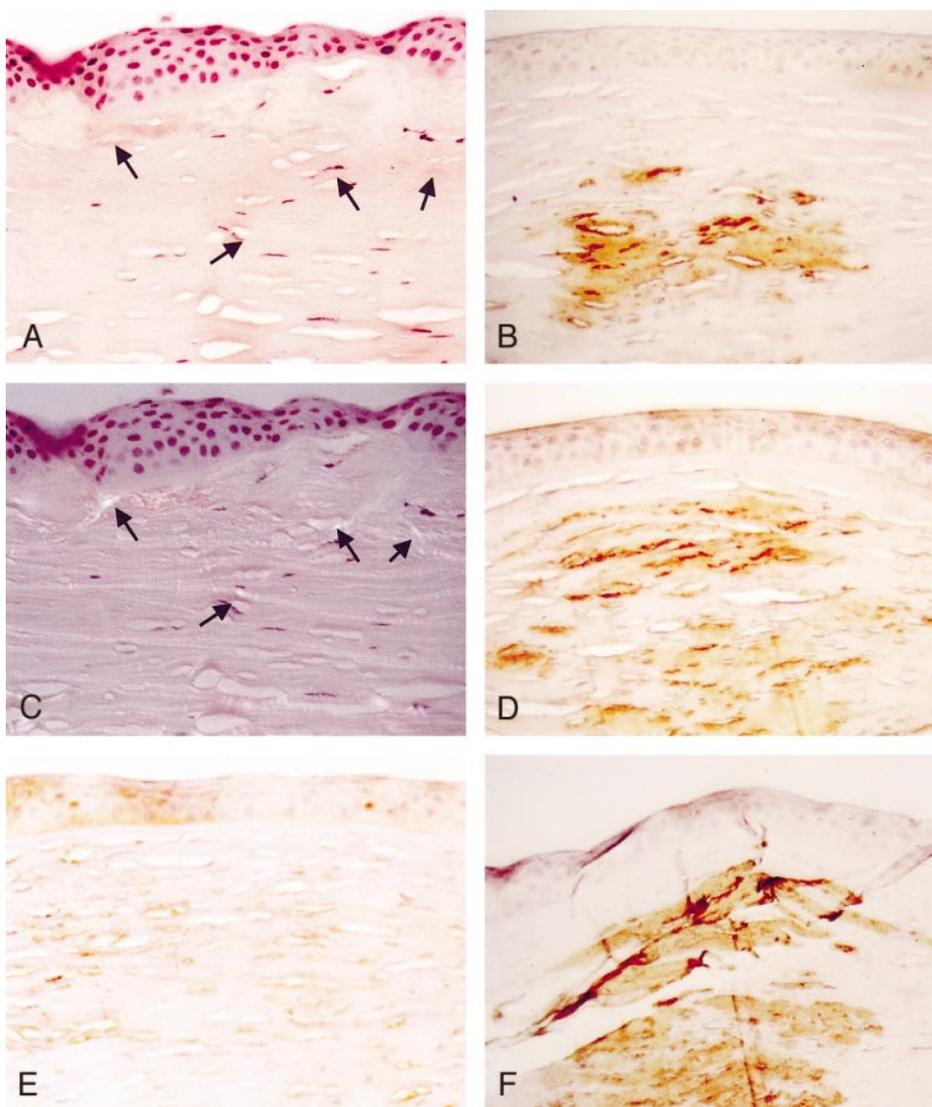


FIGURE 6. Tissue sections of the cornea from a CDA-affected patient stained with (A) Congo red; (B) Congo red under polarization optics (arrows mark amyloid inclusions), (C) pre-immune serum, (D) antibody against amyloid P, (E) KE-15, and (F) KE-2. Magnification $\times 100$.

deposited proteins have a lower molecular weight than their precursors.²² A recent biochemical study of total protein extracts of CDLI corneas showed the presence of KE in degraded form, which was absent in normal eyes.²⁴ This observation favoring the proteolysis hypothesis supports the amyloidogenic role of KE in CDLI, although further work is required to establish which part of the protein forms a fibril. Second, gross structural changes occurring during fibrillogenesis of KE may render a part of its epitopes inaccessible to KE-15. There is evidence that immunostaining properties of soluble and amyloid forms of the same protein may be different.^{25,26} The immunoreactivity of amyloid aggregates may also be changed by their association with other constituents of the deposits, such as glycosaminoglycans, as well as with intra- or extracellular structures.^{27,28} If the entire KE constitutes amyloid, the differential immunoreactivity of its amino and carboxyl termini could be explained by various degrees of their involvement in fibril formation. Therefore, the amino-terminal region, against which KE-15 was directed, would undergo the most profound structural changes.

What is the origin of deposited KE? mRNA of BIGH3 is expressed in a variety of organs and tissues^{29,30} As are the

majority of amyloidogenic proteins, KE is also present in blood serum.¹⁷ However, in the 5q31-linked dystrophies the cornea is known to be the only site where pathologic deposits are found,^{10-14,31} although more systematic histopathologic analysis of patients may be required. It is thus possible that KE of extracorneal origin migrates to the cornea to form deposits. Another possibility is the intracorneal source of deposited protein. Several lines of evidence indicate that both epithelium and stromal fibroblasts may be implicated in the generation of misfolded KE.^{11,14,31} The intensive synthesis of BIGH3 transcripts, as well as secretion of the protein, has been detected in the corneal epithelium of the adult eye.^{30,32} We report here that primary corneal fibroblasts established from normal and CDGGI-affected corneas expressed BIGH3 transcripts and that these cells secreted KE in the cultured medium. Whether the different forms of 5q31-linked dystrophies all have identical origins remains to be seen.

To our knowledge, KE is the first protein that causes a spectrum of inherited disorders of both amyloid (CDLI, CD-LIIIA) and nonamyloid (CDGGI, CDRB) types, as well as a mixed form (CDA). Studies of this group of corneal disorders may therefore provide direct insight into the mechanism of

conformational rearrangement as a function of the altered protein sequence.

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