Amyloid and Non-amyloid Forms of 5q31-linked Corneal Dystrophy Resulting from Kerato-epithelin Mutations at Arg-124 Are Associated with Abnormal Turnover of the Protein*

(Received for publication, December 20, 1999, and in revised form, January 20, 2000)

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Mutations in kerato-epithelin are responsible for a group of hereditary cornea-specific deposition diseases, 5q31-linked corneal dystrophies. These conditions are characterized by progressive accumulation of protein deposits of different ultrastructure. Herein, we studied the corneas with mutations at kerato-epithelin residue Arg-124 resulting in amyloid (R124C), non-amyloid (R124L), and a mixed pattern of deposition (R124H). We found that aggregated kerato-epithelin comprised all types of pathological deposits. Each mutation was associated with characteristic changes of protein turnover in corneal tissue. Amyloidogenesis in R124C corneas was accompanied by the accumulation of N-terminal kerato-epithelin fragments, whereby species of 44 kDa were the major constituents of amyloid fibrils. R124H corneas with prevailing non-amyloid inclusions showed accumulation of a new 66-kDa species altogether with the full-size 68-kDa form. Finally, in R124L cornea with non amyloid deposits, we found only the accumulation of the 68-kDa form. Two-dimensional gels revealed mutation-specific changes in the processing of the full-size protein in all affected corneas. It appears that substitutions at the same residue (Arg-124) result in cornea-specific deposition of kerato-epithelin via distinct aggregation pathways each involving altered turnover of the protein in corneal tissue.

5q31-linked corneal dystrophies are autosomal dominant disorders characterized by age-dependent progressive accumulation of protein deposits in the cornea followed by visual impairment. The pathological inclusions display two different types of ultrastructure: amyloid fibrils in lattice type I dystrophy (CDLI) and non-amyloid amorphous aggregates in Groenouw type I (CDGGI) and Reis-Bücklers (CDRB). The fourth, Avellino corneal dystrophy (CDA), is a mixed form, which is characterized by amyloid and non-amyloid lesions in the same cornea. Mutations in the BIGH3 gene encoding for kerato-epithelin (KE)3 were found to be responsible for this entire group of conditions (1). BIGH3 is expressed in many tissues, including cornea, and is up-regulated by transforming growth factor-β (2). The function of KE is not yet understood, although it is thought to be an extracellular matrix protein mediating cell adhesion (3).

Population analysis revealed two hot spots of mutations in KE, Arg-124 and Arg-555 (4). There is a strong correlation between each particular mutation and the affected phenotype. Changes of Arg-555, R555W and R555Q, result in the conditions of non-amyloid nature, CDGGI and CDRB, respectively. Changes of Arg-124 result in a more complex pattern of deposition: R124C causes CDLI, a localized amyloidosis; R124H causes a mixed form, CDA. The most recently described mutation, R124L, results in non-amyloid deposition (5).

Recently, by using immunohistology we demonstrated that KE is specifically co-localized with pathologic inclusions in all analyzed forms of the disease (6). This supports the idea that the mutated protein aggregates in pathologic deposits (similarly to other deposition diseases). However, the immunohistology does not allow us to conclude whether KE is a major component of the deposits. In addition, using antibodies against two non-overlapping parts of the protein we observed different staining patterns of amyloid (R124C) and non-amyloid (R555W) deposits. To get more insight into the mechanism of amyloid and non-amyloid conversion of KE we analyzed pathological corneas with different mutations at the single site Arg-124.

EXPERIMENTAL PROCEDURES

Materials—Corneal buttons were obtained after keratoplasty of seven patients with different forms of 5q31-linked corneal dystrophies. Two control corneas were obtained from the corneal bank of Jules Gonin Hospital. Each cornea was divided into two parts: one was used for histology, and the other was reserved for protein analysis. The latter ones were immersed in tissue-freezing medium (Jung, Germany) and stored at –80 °C before the assays. Three patients (P1–P3) were unrelated heterozygous with R124C mutation of the BIGH3 gene (6); P4–P6 were heterozygous individuals with the R124H mutation (cases 2, 5, and 6, respectively (7)); and P7 was a heterozygous patient with the R124L mutation (5). None of these patients had systemic diseases known to be associated with amyloidosis. Clinical diagnosis was confirmed histopathologically in all cases. The presence of amyloid deposits was tested by Congo Red staining under polarized light. Mutations in BIGH3 were determined by direct sequencing of exon 4 (4).

Two rabbit polyclonal antisera against human KE (Swiss-Prot Q15582) were described previously (6). Antiserum anti-KE 69–364 was directed to KE residues from 69 to 364 amino acids; antiserum anti-KE 426–682 was directed to KE residues from 426 to 682.

* This work was supported by Grants 31-052940.97 (to D. F. S.) and 32-58439.99 (Marie Heim-Vögtlin Beiträge, to E. K.) from the Swiss National Science Foundation and from the Foundation of the 450th Anniversary of the University of Lausanne (to E. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: KE, kerato-epithelin; PAGE, polyacrylamide gel electrophoresis.
Preparation of Protein Extracts from Cornea—The tissues were ground in liquid N$_2$ and then homogenized with a Teflon potter in 8 mM urea; 10% v/v glycerol, 2% w/v SDS; 5% v/v 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin, 1 µM leupeptin in 50 mM Tris-HCl, pH 6.8. Samples were centrifuged for 5 min at 16,000 × g, and the pellets were discarded. Protein content of the supernatants were determined by using the bicinchoninic acid assay (BCA, Pierce). To eliminate the interference of 2-mercaptoethanol with the BCA assay, proteins were first precipitated in 6% (w/v) trichloroacetic acid, 0.0125% (w/v) sodium deoxycholate, and 1 mM urea. The samples were centrifuged for 10 min at 16,000 × g, and the pellets were washed twice in ice-cold acetone, dried, and dissolved in 1% (w/v) SDS, 0.1 M NaOH.

One- and Two-dimensional Gel Analysis—SDS-PAGE was carried out on 100 × 70 × 1 mm gels containing 10% (w/v) acrylamide and piperazine diacrylamine as a cross-linker (Bio-Rad). High resolution two-dimensional PAGE was prepared according to the method described by Henry et al. (8, 9). Molecular weights and isoelectric points were calculated using two-dimensional PAGE standards as reference (Bio-Rad). Silver staining was done as described (10). For Western blotting, proteins were transferred from SDS-PAGE and two-dimensional PAGE to Immobilon-P membrane (Millipore) by semi-dry electrophoblotting according to Laurie`re (11). The anti-KE 69–364 and anti-KE 426–682 antibodies were diluted 5000- and 10,000-fold, respectively, in blocking buffer (Tris-buffered saline containing 5% (w/v) skim milk and 0.05% (w/v) Tween-20) followed by alkaline phosphatase-linked secondary antibodies (Bio-Rad) diluted 4000-fold in blocking buffer. The immunodetected KE species were visualized by use of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad). The blots were scanned with a laser densitometer (ImageQuant v3.3 software, Molecular Dynamics) for band quantification. Polypeptides of interest were subjected to N-terminal sequencing. Theoretical molecular mass and isoelectric point were calculated by use of the online software ComputeP/Mw (http://www.expasy.ch/).

RESULTS

Analysis of KE Content in Normal Cornea—To characterize the KE content in normal tissue, corneal protein extracts were subjected to one- and two-dimensional gels followed by Western blotting or silver staining. One-dimensional PAGE following immunostaining with both anti-KE 69–364 and anti-KE 426–682 antibodies revealed a major band migrating at 68 kDa, which corresponds to full-size secreted protein (Fig. 1, A and B). Apart from this major form, we observed several minor species that showed differential staining by the antibodies. Anti-KE 426–682 recognized fragments of 64, 57, 47, and 29 kDa. In contrast, anti-KE 69–364 stained much more weakly 64-, 57-, and 47-kDa bands and did not detect the 29-kDa fragment. This differential staining suggests that all the observed minor KE species were N-terminal deletions.

Fig. 1 demonstrates that all of the KE species seen on the one-dimensional gel are resolved on a two-dimensional gel as a series of spots with different pI migrating at the same molecular weight. They may represent different posttranslational modifications of KE. The major 68-kDa KE fraction constituted 8 spots with a pI range within 6.3–6.8. The minor KE species were resolved as follows: the 64-kDa fragment was formed by 8 spots within pI 5.3–5.8; the 57-kDa fragment was formed by 7 spots within pI 5.6–6.3; the 47-kDa fragment was formed by 4 spots within pI 5.4–5.9, and the 29-kDa fragment was formed by 5 spots within pI 6.6–6.9. N-terminal sequencing of the 68-kDa fraction yielded the expected processed KE starting at position 24 of the protein sequence. Sequencing of the 64- and 47-kDa species confirmed these fragments had N-terminal deletions that start at positions 105 and 273, respectively. Sequencing data revealed no heterogeneity of the N termini. Although the 57- and 29-kDa species were not sequenced, their pI and molecular weights suggest N termini starting near residues 160 and 420, respectively. These sequencing data, together with immunostaining, demonstrate that species resulting from normal KE turnover in the cornea are C-terminal fragments of the protein (Fig. 1C).

Analysis of KE Content in the R124C Cornea—Prior immunohistology of corneal sections revealed unusual accumulation of KE, which was co-localized with the pathological deposits in all R124C corneas used in this study (6). Analysis of corneal material from these patients heterozygous for R124C detected a 3-fold increase of KE content compared with normal cornea. Staining with both antibodies, however, showed that the amount of full-size protein (68-kDa band) did not significantly differ from the control. Instead, we observed massive accumulation of unusual KE fragments reacting exclusively with anti-KE 69–364 (Fig. 2A). The 44-kDa fragment was the major species (as much as double the amount compared with 68-kDa KE in normal cornea, see histogram 2C). We also observed several minor fragments migrating at 39, 26, and 17 kDa of which the 26-kDa band was the most abundant. All of these fragments were consistently missing from normal corneas. At the same time, in R124C corneas we observed all of the fragments characteristic for KE proteolysis in normal cornea (Fig. 2B). The only difference was a significant, 6-fold reduction in the amount of the 64-kDa frag-
ment seen with anti-KE 426–682.

Analysis of cultured primary skin fibroblasts established from patients with R124C mutation revealed no production of the disease-specific KE fragments found in affected corneas (data not shown). This suggests that other, tissue-specific, factors may be involved in the pathogenesis.

Two-dimensional gels of R124C corneal proteins revealed drastic changes in the pattern of pl isomers forming the major 68-kDa KE fragment (Fig. 3). This fraction was resolved as a train of 10 spots (compared with 8 spots in normal corneas) within pl range 5.8–6.8 (compared with pl 6.3–6.8 in controls). Although the substitution of arginine to cysteine may have explained this shift, we detected no changes in the pattern of pl isomers or number of spots for KE fragments of 64, 57, 47, and 29 kDa. Because the 64-kDa fraction still contains Arg-124 it is rather unlikely that R124C per se affects the electrophoretic behavior of the 68-kDa KE. However, we cannot exclude the possibility that the 64-kDa species in the R124C cornea consist exclusively of the nonmutated KE allele.

N-terminal sequencing of 3 spots from the 68-kDa fraction migrating within pl 5.8–6.2 and absent in normal cornea showed the N terminus at residue 26 (in contrast to 24 in the control). Interestingly, sequencing of the 44-kDa fragment also showed it to start at residue 26. In both cases sequencing revealed uniformity of the N termini. In addition, the 44-kDa fraction appears to be heterogeneous by molecular weight: the species are resolved as two closely migrating trains of 10 isoforms each (Fig. 3). The similar heterogeneity was observed for 26-kDa KE fraction consisting of 4 upper and 6 lower spots. Sequencing of the 26-kDa species yielded an amino terminus at residue 163. The C termini for both the 44- and 26-kDa fragments were calculated to be within the region surrounding residue 420 (Fig. 2D). It appears that the 26-kDa species are the products of further proteolysis of 44-kDa fragment. The other two species of 39 and 17 kDa characteristic for R124C cornea are likely to originate from the N-terminal region of the protein, according to their exclusive staining with anti-KE 69–364.

Analysis of KE Content in the R124H Cornea—Prior histological analysis revealed a predominantly non-amyloid type of deposition in all R124H corneas used in our study (12). The proportion between non-amyloid and amyloid deposits was roughly estimated as 10:1 by Congo Red staining of corneal sections. Immunohistology of R124H corneas showed specific, positive staining of non-amyloid deposits with both anti-KE antibodies (data not shown). Western blot analysis of R124H corneal extracts showed a characteristic pattern of KE species that was different from normal and R124C corneas (Fig. 4, A and B). We observed an accumulation of a 68-kDa fragment, which was about 2 times higher than in normal cornea (Fig. 4C). In addition, a new 66-kDa fragment accumulated in amounts comparable to those of 68-kDa KE in normal cornea. This species was consistently missing from all other studied pathological samples, as well as from normal controls. Although the 66-kDa KE reacted with both anti-KE 69–364 and anti-KE 426–682 its reactivity compared with the 68-kDa KE was somewhat lower with anti-KE 426–682. This suggests that 66-kDa fragment may lack part of its epitopes from the C terminus.

Two-dimensional gels of R124H corneas detected profound changes in the 68-kDa KE (Fig. 3). In contrast to the R124C cornea, this fraction appeared to be heterogeneous not only by charge, but also by molecular weight. It was resolved as two trains with close molecular weights each consisting of 12 isoforms within pl range 5.7–6.7 (compare with pl 6.3–6.8 in control). Interestingly, the 66-kDa species also migrated as two trains of 8–10 spots with close molecular masses. The distribution of other KE species, including a 64-kDa fraction still containing 124 residues, did not differ from normal cornea.

Analysis of KE Content in the R124L Cornea—Immunohis-
tology of this cornea showed strong positive staining of non-amyloid deposits with both anti-KE antibodies (data not shown). Western blot analysis revealed an ~2.5-fold excess of 68-kDa KE compared with normal cornea, whereas the pattern of other KE species did not significantly differ from normal control (Fig. 5). The 68-kDa fraction was resolved on two-dimensional gel as two trains of pI isomers with close molecular weight (Fig. 3). The upper train (8 spots within pI range 6.3–6.8) was similar to 68-kDa isomers seen in normal cornea. The lower train was formed by 10 (or 12) spots with a characteristic shift in charge toward both directions (pI 6.1–7.0), although the predicted effect of R124L would be only a loss of charge.

**DISCUSSION**

5q31-Linked corneal dystrophies are hereditary deposition diseases of amyloid and non-amyloid nature resulting from different mutations in KE. It is a protein of unknown function, which is secreted at a substantial level in the extracellular milieu of many tissues, including the cornea. Analysis of protein content shows that KE is one of the major corneal proteins. Apart from the major 68-kDa band corresponding to full-size KE compared with normal cornea, the pattern of other KE species did not significantly differ from normal control (Fig. 5). The 68-kDa fraction was resolved on two-dimensional gel as two trains of pI isomers with close molecular weight (Fig. 3). The upper train (8 spots within pI range 6.3–6.8) was similar to 68-kDa isomers seen in normal cornea. The lower train was formed by 10 (or 12) spots with a characteristic shift in charge toward both directions (pI 6.1–7.0), although the predicted effect of R124L would be only a loss of charge.

Recently, we showed specific co-localization of KE with all types of pathologic deposits in corneas with different forms of dystrophy (6). Here, we analyzed corneas with three different mutations of Arg-124 by immunohistochemical and biochemical approaches. All affected corneas demonstrated positive staining of the deposits with anti-KE antibodies. Western blot analysis of the unfixed parts of corneas revealed a significant, severalfold increase in the amount of KE compared with normal control. These data allow us to conclude that KE itself aggregates into amyloid deposits in R124C and non-amyloid deposits in R124H and R124L corneas. At the same time, we observed different staining patterns of amyloid (R124C) and non-amyloid (R124H and R124L) deposits with antibodies against two nonoverlapping parts of KE. Although non-amyloid, amorphous inclusions reacted with both antibodies, amyloid inclusions in R124C corneas did not react with antiserum to the amino-terminal part. To our surprise, we detected massive accumulation of unusual N-terminal fragments of 44 and 26 kDa in R124C corneas. The drastic changes of the protein structure when undergoing amyloid conversion could explain the absence of staining of amyloid deposits with antibodies specific to the N-terminal part of KE. Although non-amyloid, amorphous inclusions reacted with both antibodies, amyloid inclusions in R124C corneas did not react with antiserum to the amino-terminal part. To our surprise, we detected massive accumulation of unusual N-terminal fragments of 44 and 26 kDa in R124C corneas. The drastic changes of the protein structure when undergoing amyloid conversion could explain the absence of staining of amyloid deposits with antibodies specific to the N-terminal part of KE. Independently of us, the appearance of N-terminal fragments of similar molecular weights in CDLI corneas was observed by Takacs et al. (16), although the authors made no conclusion about the role of KE in the deposit formation.

The massive accumulation of unusual KE fragments and exclusive co-localization of KE having specific immunostaining properties with amyloid inclusions infer that the major 44-kDa fragment found in R124C corneas is the amyloidogenic unit that comprises the amyloid fibril. Thus, amyloidogenesis of KE in
R124C corneas is associated with abnormal proteolysis of the protein. This is consistent with other amyloid disorders where protein precursors generally undergo proteolysis during amyloid formation. Known examples are immunoglobulin, gelsolin, serum amyloid A, cystatin C, and amyloid β precursor protein (17, 18).

Despite almost ubiquitous KE expression in the organism, patients affected with 5q31-linked dystrophy, even homozygous ones, do not manifest any sign of systemic abnormality, suggesting a tissue-specific character of pathologic KE deposition. Because cultured fibroblasts established from R124C patients do not express disease-specific KE fragments, it seems that amyloid conversion occurs at later stages of the protein life cycle and that it is mediated by tissue-specific factors.

A critical question is how a given mutation predisposes a protein to misfolding and aggregation. At present, even for the best studied amyloidosis there is no predictable pattern of mutations resulting in amyloid aggregation. However, many amyloid mutations appear to affect the structure of protein precursors by increasing β-sheet content. According to secondary structure predictions (19), R124C, but not R124H and R124L, would introduce additional β-sheet content. In addition, in all analyzed R124C corneas we observed a dramatic, 6-fold decrease of the content of a 64-kDa KE species compared with normal cornea. We propose that R124C abolishes the site of KE proteolysis located near residue 105, which may be required for normal turnover of the protein. We speculate that this event initiates further steps leading to the accumulation of abnormal proteolytic fragments of KE and fibril formation.

Our data also show that R124C influences the processing and post-translational modifications of full-size KE. We found that the N terminus of the 68-kDa form starting at residue 24 in normal cornea is changed for residue 26 in the R124C cornea. The same N terminus was found in the amyloidogenic 44-kDa fragment. Our sequencing data were consistent with those reported by other groups (16, 20). Moreover, the R124C cornea demonstrates a remarkable shift in charge and increase in the number of species constituting the 68-kDa KE. The charge heterogeneity already present in wild type KE may be attributed to posttranslational modifications. The observed loss of positive charge in the 68-kDa KE found in R124C cornea may be because of the arginine to cysteine substitution, but it can also reflect mutation-specific changes of post-translational modifications of KE. It has been proposed that post-translational modifications of precursor proteins may be implicated in amyloid formation (18).

Analysis of several R124H corneas showed that the pathologic KE accumulation is also accompanied by abnormal proteolysis of the protein. We observed a new unusual KE fragment migrating at 66 kDa, which was absent in others pathological corneas, as well as in normal controls. This specific 66-kDa KE form could be responsible for the deposit formation in R124H cornea though the involvement of the major 68-kDa form cannot be excluded because of its massive accumulation. The isoforms comprising this band showed the loss of positive charge. However, the observed value of pI shift as well as the number of isoforms were different from R124C cornea. Moreover, unlike in normal and R124C corneas, the species comprising the 68-kDa band manifested a heterogeneity by molecular weight. All this points to the effect of R124H mutation on KE processing and turnover in corneal tissue.

All R124H corneas used in our study demonstrated the predominance of a non-amyloid type of deposition. Although clinical form of the disease, CDA, corresponding to this mutation was described as having a mixed type of deposition, it is important to note that granular, non-amyloid inclusions are the earliest manifestation and that amyloid deposition occurs later in the course of the disorder (21). Moreover, not all patients with R124H develop a distinct deposition of amyloid (7). Several reported R124H homozygous patients exhibited exclusively non-amyloid deposition occurring very early in life. These data suggest that the primary pathological impact of R124H is the destabilization of KE that renders it prone to nonordered aggregation. In this respect, amyloid fibril formation can be viewed as a secondary event.

The R124L mutation is associated with a severe, early onset form of the disease, which is characterized by a non-amyloid type of deposition (5). In contrast to R124C and R124H corneas, we observed no accumulation of abnormal fragments. A seversfold increase of the 68-kDa form suggests that full-size protein constitutes non-amyloid inclusions in the R124L cornea. Two-dimensional gel resolution reveals a characteristic pattern of the 68-kDa species different from all other analyzed corneas. We conclude that the R124L mutation affects processing and stability of the major 68-kDa form but does not result in abnormal proteolysis of KE.

Replacements of Arg-124 lead to different types of KE aggregation. In each case, misfolding and aggregation of this protein was accompanied with characteristic changes of its processing and metabolism in corneal tissue. It invokes that different therapies may be required for treatment/prevention of each particular form of disease. In view of this unique pleiotropic effect of KE substitutions, 5q31-linked dystrophy represents an attractive model for protein misfolding/aggregation studies.

Acknowledgments—We thank Barbara Eilers for excellent technical assistance, Dr. Silvia Barcelo-Batllo for help with image quantification, and Dr. Susan Marqusee for critical reading of the manuscript.

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J. Biol. Chem. 2000, 275:11465-11469.
doi: 10.1074/jbc.275.15.11465

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