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## Keratoepithelin in secondary corneal amyloidosis

Received: 14 March 2005  
Revised: 10 September 2005  
Accepted: 18 September 2005  
Published online: 6 December 2005  
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**Abstract** *Background:* Amyloid is found in several corneal dystrophies, including distinct lattice corneal dystrophies (LCD) and Avellino corneal dystrophy. Recently, point mutations in the transforming growth factor-beta-induced gene (TGFB1) encoding for keratoepithelin (KE) have been demonstrated in these corneal disease entities. We intended to investigate if KE was also a component of the rarely seen secondary corneal amyloid deposits. *Methods:* Immunohistochemical staining with a polyclonal antibody against KE was performed on formalin-fixed paraffin-embedded tissue of five corneal buttons with secondary amyloid obtained after keratoplasty. Secondary amyloidosis was due to Fuchs' endothelial dystrophy (FED) with bullous keratopathy and/or recurrent erosions in all cases. The diagnosis had been established by light microscopy using Congo red staining. Two cases of LCD type I served as positive controls

and three corneas with FED and one with keratoconus without amyloid served as negative controls. *Results:* All corneas with secondary amyloidosis as well as LCD type I revealed positive staining in the respective amyloid deposits. KE was localized in the subepithelial pannus and in the anterior stroma in the corneas with secondary amyloidosis. In the specimens with LCD type I it was distributed in the amyloid deposits located in the anterior and mid-stroma. Staining for KE showed a granular appearance in all cases. The intensity of staining was variable among the specimens. *Conclusions:* KE is found not only in primary amyloid deposits of hereditary corneal dystrophies, but also in secondary amyloidosis of the cornea of diverse etiologies.

**Keywords** Amyloid · Keratoepithelin · Cornea · Immunohistochemistry

### Introduction

Amyloid deposits are found in many corneal dystrophies, including lattice dystrophy (LCD) type I, IA, II, IIIA, IIIB, IV, V, VI and VII and granular dystrophy type II, also called Avellino dystrophy. Recently, point mutations in the transforming growth factor-beta-induced gene (TGFB1) encoding for the protein keratoepithelin (KE) have been found to be associated with these corneal disease entities [11]. To date, several point mutations in the TGFB1 gene on

the long arm of chromosome 5 (5q31) are believed to cause different corneal dystrophies with amyloid and non-amyloid deposits [13, 17, 18, 32].

Until today, the precise function of KE is not yet known. Several authors ascribe it a role in growth and differentiation and in cell adhesion and cell-to-cell communication [16, 24]. In some studies KE had also a negative influence on the ability of cells in tumor formation [25]. KE is synthesized by epithelial cells as well as by mesenchymal cells [16, 24, 25]. In the human cornea, KE is synthesized

in the normal epithelium, in the endothelium of Fuch's dystrophic corneas and in endothelial and stromal cells in healing corneal wounds [6, 22]. KE plays a role in the formation of extracellular matrix during normal development and healing of the cornea [22]. KE is, among other things, associated with collagen type VI in the corneal stroma. It is supposed that this complex has an anchoring function between stroma and Descemet's membrane [6]. Consequently, KE is supposed to have an important function with regard to corneal integrity and corneal wound healing processes.

Furthermore, corneal amyloidosis exists as a systemic form in LCD type II (Meretoja syndrome), where a mutation has been found in the gelsolin gene on chromosome 9 [3, 5, 26]. In gelatinous drop-like dystrophy with a mutation in MIS1 gene on chromosome 1 (gastrointestinal tumor associated gene; carcinoma associated gene [31] and as such, a systemic disorder), amyloid is present as central prominent subepithelial corneal nodules.

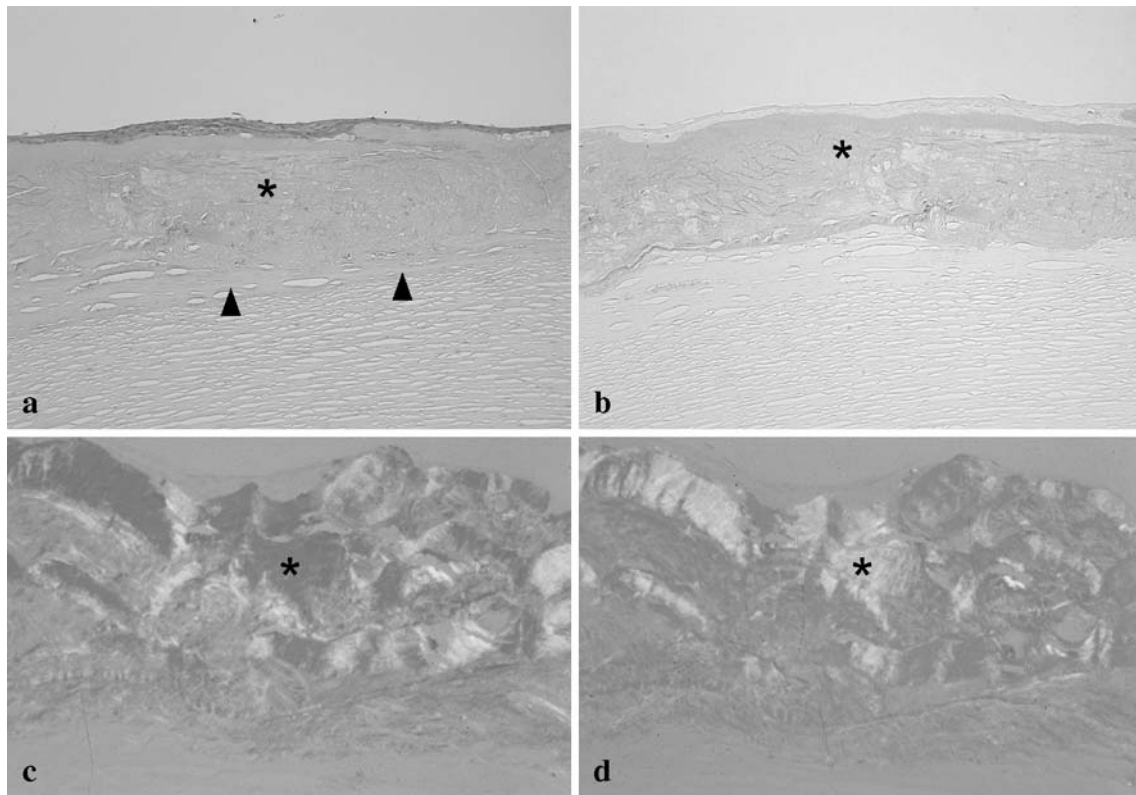
In addition to these primary amyloid deposits, a variety of corneal and ocular diseases such as trachoma, lepra, sarcoidosis, interstitial keratitis, phlyctenular keratitis, uveitis, chronic post-traumatic inflammation, glaucoma, keratoconus and retinopathy of prematurity [7] can develop

secondary amyloid deposits. In the present study, we intended to investigate if keratoepithelin could also be demonstrated in secondary corneal amyloid deposits.

## Materials and methods

Five different corneal buttons obtained after penetrating keratoplasty for visual rehabilitation were diagnosed as secondary amyloidosis on sections stained with hematoxylin-eosin, periodic-acid Schiff, and Congo red (Fig. 1a–d). Keratoplasty had been performed because of bullous keratopathy due to endothelial decompensation of various causes (for further details, see Table 1). Immunohistochemistry was performed on paraffin sections using a polyclonal antibody (pAb) directed against TGFBI (Schorderet and Munier) in a dilution of 1:1400. The TGFBI antibody was produced in rabbits to recombinant protein, including the full-length complementary DNA sequence (210–683) of human TGFBI [27]. Aminoethylcarbazol was used as a chromogen to detect the immunoreaction.

Two corneal buttons with LCD type I were used as positive controls, and omitting the primary antibody as well as using a non-specific Ab served as negative



**Fig. 1 a:** Cornea with flattened epithelium, degenerative subepithelial corneal pannus with amyloid (*asterisks*) and intact Bowman's layer (*arrowheads*) (specimen 4, H&E,  $\times 200$ ). **b:** Positive labeling of subepithelial amyloid deposits (*asterisks*), using Congo red

staining (specimen 4, Congo red,  $\times 200$ ). **c and d:** Characteristic dichroism of amyloid deposits within the subepithelial pannus (*asterisks*) (specimen 4, Congo red,  $\times 400$ )

**Table 1** Synopsis of the corneal specimens used for immunohistochemical staining with pAb against KE. (KE keratoepithelin, LCD type I lattice corneal dystrophy type I, FED Fuchs' endothelial dystrophy)

	Specimen	Age (years)	Sex	Diagnosis	Amyloid localization
Secondary corneal amyloidosis	1	36	Male	Endothelial decompensation with secondary amyloid deposits	Subepithelial
Secondary corneal amyloidosis	2	Unknown	Unknown	Endothelial insufficiency with secondary amyloid deposits, chronic keratitis	Subepithelial
Secondary corneal amyloidosis	3	69	Female	Endothelial decompensation with secondary amyloid deposits	Subepithelial
Secondary corneal amyloidosis	4	59	Female	FED with secondary amyloid deposits	Subepithelial, anterior stroma
Secondary corneal amyloidosis	5	85	Male	FED with secondary amyloid deposits	Subepithelial, anterior and mid stroma
Positive control	6	73	Male	Recurrence of LCD I in the corneal transplant after keratoplasty	Subepithelial, anterior and mid stroma
Positive control	7	57	Male	LCD I	Subepithelial, anterior and mid stroma
Negative control	8	75	Male	FED	Not present
Negative control	9	70	Male	FED	Not present
Negative control	10	66	Female	FED	Not present
Negative control	11	29	Male	Keratoconus	Not present

control. For further comparison with amyloid-negative corneas, three corneal buttons diagnosed as Fuchs' endothelial dystrophy (FED) without amyloid deposits and one keratoconus cornea were also examined using the same immunohistochemical protocol (see Table 1 for summary and further details). These two entities were chosen because they were easily available due to the frequency of penetrating keratoplasty in advanced stages of the disease. In addition, we could compare antibody reactions with the results of other authors concerning these two corneal diseases [6, 28, 29, 33].

The amount of staining was analyzed semiquantitatively (–: no staining, (+): very weak staining, +: weak

staining, ++: moderate staining, +++: very intense staining).

## Results

### Secondary corneal amyloidosis

All five corneal buttons with secondary amyloidosis showed a positive reaction with the pAb against TGFBI. The staining intensity varied among the specimens but always appeared granular. Immunoreactivity was localized to extracellular material and most prominent in amyloid deposits, but in

**Table 2** Semiquantitative analysis of the staining pattern of the different corneal specimens with pAb against KE (–: no staining; (+): very weak staining, +: weak staining; ++: moderate staining; +++: very intense staining)

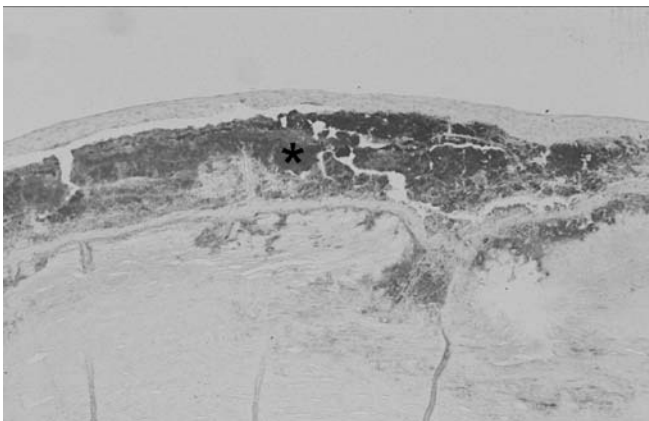
Specimen	Amyloid deposits	Epithelium	Bowman's layer	Descemet's membrane	Endothelium
1	+	–	–	–	–
2	+	–	–	+	–
3	++	–	–	(+)	–
4	++	–	++	++	–
5	+++	–	–	–	–
6	++	–	+	(+)	–
7	+++	–	+	(+)	–
8	Not present	–	(+)	+	–
9	Not present	–	(+)	(+)	–
10	Not present	–	(+)	+	–
11	Not present	–	–	(+)	–



**Fig. 2** Immunostaining with anti-KE reveals a positive and moderate (++) reaction of granular appearance within the pannus (*asterisk*), corresponding to the area of amyloid deposition. Note also granular and moderate (++) labeling of Bowman's layer (*arrowheads*) (specimen 4,  $\times 200$ )

most cases a patchy inhomogeneous granular staining pattern of Bowman's layer and Descemet's membrane was also observed. Neither the epithelium nor the endothelium of the five specimens revealed positive staining (see Table 2).

Case 4 showed moderate anti-KE immunoreactivity within the amyloid deposits in the subepithelial pannus and the anterior stroma as well as moderate staining of Bowman's layer and Descemet's membrane (Fig. 2). In specimen 5, there was intense labeling of the amyloid deposits located subepithelially and in the anterior and mid stroma (Fig. 3), while no other structures revealed any positive staining.



**Fig. 3** Similar granular and very intense (+++) immunolabeling within the subepithelial pannus in another specimen with secondary amyloid (*asterisk*) (specimen 5,  $\times 100$ )



**Fig. 4** Moderate (++) immunolabeling with the same antibody in the region of the amyloid deposits of a corneal button with LCD type I (*arrows*) (specimen 6, positive control,  $\times 400$ )

#### Lattice corneal dystrophy

The two cases with LCD type I serving as positive controls both showed moderate to intense anti-KE immunoreactivity in the respective amyloid deposits which were situated subepithelially and in the anterior to mid-stroma (Fig. 4). Labeling was granular as in the specimens with secondary amyloidosis. We could detect weak staining of Bowman's layer and very weak staining of Descemet's membrane in both cases.

#### Fuchs' endothelial dystrophy

As in the corneas with secondary amyloid, granular labeling of Bowman's layer and Descemet's membrane was seen. No other corneal structures stained positively.

#### Keratoconus

Here, only in Descemet's membrane was very weak immunoreactivity present.

### Discussion

In our study of corneas with secondary amyloid in a subepithelial pannus we found KE in the region of the amyloid deposits themselves as well as to some degree also in the surrounding pannus. In comparison with the distinct labeling of the amyloid deposits of LCD type I, the staining of the secondary amyloid deposits in the subepithelial pannus is more diffusely distributed. In previous investigations, KE has been suggested to play a role in corneal

development, wound healing and scarring processes [22, 28, 29]. Hirano et al. immunohistochemically demonstrated KE in subepithelial newly synthesized connective tissue of corneas with FED [6]. The corneal buttons in our study were obtained after keratoplasty because of Fuchs' dystrophy, resulting in epithelial wound healing problems with subsequent scarring. It has been shown that KE is highly expressed under those conditions where healing is associated with increased synthesis of new ECM [28, 29].

Other authors have detected KE immunohistochemically also in primary amyloid and non-amyloid deposits of several corneal dystrophies. Korvatska et al. revealed a positive reaction in the amyloid deposits of LCD type I and Avellino corneal dystrophy and in non-amyloid deposits of granular corneal dystrophy Groenow type I using two polyclonal antibodies raised in rabbits against the amino- and carboxyl-terminal parts of KE [14]. Similar to our results, there was positive staining of subepithelial and stromal amyloid deposits in LCD type I. Streeten et al. studied corneas with LCD type I, granular corneal dystrophy, Avellino corneal dystrophy, Reis-Bücklers corneal dystrophy and Thiel-Behnke corneal dystrophy with the same antibody used in our study of secondary corneal amyloidosis. They found strong and diffuse immunostaining for KE in all lesions of these dystrophies [27].

In our study, in addition to the immunohistochemical demonstration of TGFBI in the region of the secondary amyloid deposition, Bowman's layer and Descemet's membrane also revealed a granular staining pattern. This finding parallels that of Streeten et al. and Akhtar et al., who also found TGFBI in these corneal structures in normal corneas but also found TGFBI-expression in the corneal stroma where we could not detect any significant staining. If KE acts as an anchoring component within the scope of the ECM it might act in such a function especially in an area of adjacent structures such as Bowman's layer between the epithelium and the stroma. This hypothesis is also supported by the work of Zhao et al., who found decreasing levels of stromal TGFBI with increasing degeneration of keratoconus corneas indicating a loss of adhesiveness between keratocytes and the surrounding ECM [33]. In addition, in keratoconus corneas, Takacs et al. found no expression of TGFBI in either Bowman's layer or Descemet's membrane at the site of gaps, tears, thinning and breaks, thus supporting the idea of a stabilizing, anchoring and adhesive role of TGFBI [28, 29]. In contrast to these findings, we saw only very little labeling of Descemet's membrane in the keratoconus corneas examined in our series. Most likely, this is due to the different antibody used by Takacs et al. that reacts with the N-terminal portion of TGFBI, since they also failed to demonstrate TGFBI in Bowman's layer and Descemet's membrane of normal corneas. Furthermore, the keratoconus sections in our series were taken from an area without obvious irregularities in Bowman's layer, probably repre-

senting fairly normal cornea with probably normal levels of TGFBI expression.

Normally, KE is synthesized in the corneal epithelium and is secreted into the subepithelial space [4, 24]. In our study, however, we were not able to demonstrate TGFBI immunohistochemically in the corneal epithelium. Similarly, Streeten et al. employing the same antibody also failed to detect any immunolabeling in the epithelium while KE expression was found to some degree in Bowman's layer, in the collagen lamellae, in the corneal stroma, and in Descemet's membrane [27]. With a different antibody (to amino acids 26–46 of TGFBI), Takacs et al. in normal and keratoconus corneas showed immunohistochemical staining of TGFBI in the basal layer of the corneal epithelium, in the stroma, and in the endothelium, whereas Bowman's layer and Descemet's membrane were not labeled [28, 29]. Zhao et al. detected levels of TGFBI mRNA in the stroma of normal corneas using in situ hybridization [33]. Akhtar et al. could demonstrate labeling for TGFBI in the subepithelial basement membrane, Bowman's layer, the stroma and in Descemet's membrane by immunoelectron microscopy, using a polyclonal rabbit anti-human TGFBI antibody [1]. Hirano et al. also could not show epithelial staining with their anti-TGFBI-antibody, but they could demonstrate TGFBI-mRNA synthesis in the epithelium of corneas with Fuchs' endothelial dystrophy by in situ hybridization [6]. Thus, these findings emphasize that the results of these studies are highly dependent upon the method as well as upon the respective antibody. We employed a polyclonal antibody to KE produced in rabbits against human KE, which was used also in other studies [27]. Hirano et al. were the only investigators using an antibody against  $\beta$ ig-h3 that was monospecific [6]. Using a semiquantitative grading of KE staining, it was not possible to see any correlation of the intensity of the immunoreaction with any of the specific features such as age of the patient, duration or etiology of the disease process or the location of the deposits. This might however, be possible if more specimens could be investigated.

Among the corneal dystrophies with amyloid deposits different point mutations on chromosomes 1, 5 and 9 result in the expression of altered proteins of amyloidogenic nature [11]. So far LCD type I, IA, II, IIIA, IIIB, IV, V, VI and VII [10, 11, 18, 19] and the Avellino corneal dystrophy have been analyzed with point mutations in the TGFBI gene.

The TGFBI gene was first identified in a human adenocarcinoma cell line of the lung after 3 days of treatment with TGF-beta [24]. The gene encodes for a protein named KE with a molecular mass of 68kDa, which consists of 683 amino acids. There is a high sequence homology between KE and the osteoblast specific factor 2 (OSF2) a protein which acts as an adhesion molecule in bone formation [30] and fascilin I, an adhesion molecule found in insects [30]. Therefore, KE is supposed to play a

role in cell adhesion, especially since it has been shown to be a component of the extracellular matrix (ECM) and to interact with other proteins of the ECM such as collagen type I, II, IV and V, fibronectin and laminin [9].

Amyloid is a congophil and hyaline material with characteristics of glycoproteins and has a specific microfibrillar structure. There are systemic or local amyloid deposits which give the respective tissue a waxy aspect. Systemic amyloidosis with amyloid deposits in many organs and tissues can be differentiated from local amyloidosis with amyloid material in one organ or tissue only. The various amyloid deposits consist of at least the following three components: a fibrillar protein [20, 23], the amyloid-p-component (a physiological serum protein in blood circulation), and heparan sulfate proteoglycans. As for the formation of amyloid, an incorrect synthesis or deficient or incomplete catabolism with subsequent aberrant protein folding has been suggested [20, 23].

For comparison, in corneal dystrophies where a point mutation results in a substitution of one amino acid, this substitution might also be responsible for the formation of amyloid.

Several authors explain amyloid development associated with corneal dystrophies with point mutations in the TGFBI gene by interfering with the protein folding or protein-to-protein interactions [2, 15].

Interesting conclusions were made by Ratnaswamy et al., who investigated amyloid due to gelsolin aggregation [21]. They examined the effects of a point mutation in the gelsolin gene (D187N) which leads to a familial form of amyloidosis. They showed that neither normal gelsolin of normal length nor the mutated gelsolin fragments were able to form amyloid fibrils at a pH of 7.5. Incubation at lower

pH-values (6.0–6.4), however, resulted in aggregation to amyloid fibrils [21]. In the case of monellin, a sweet-tasting plant protein, fibrous amyloid aggregates form in the heat-denatured state [12]. Based upon these results, it can be assumed that changes in pH or similar “external” factors are necessary for amyloid formation. In accordance with these conclusions, Kelly explains that the build-up of amyloid fibrils is the result of a combination of factors [8]. The primary structure of the polypeptide as well as the exact aqueous environment including pH, temperature, ionic strength etc. are determinants of the folding processes [8]. Since in corneal dystrophies resulting in amyloid deposition one point mutation in the TGFBI gene is obviously sufficient to initiate the disease process, it would also be reasonable to assume that in the case of secondary amyloid in our corneas KE is altered, e.g. by proteolysis in association with particular environmental factors in such a way that it is able to build fibrils which aggregate to form amyloid deposits.

Based upon the results of our study, KE can be immunohistochemically demonstrated within and around secondary corneal amyloidosis of different etiologies. However, point mutations in the gene encoding for this protein is probably not the only reason for amyloid formation under these circumstances. Future work is warranted to recognize the exact factors and changes in the KE-protein being responsible for the formation of secondary amyloid deposits.

If we could manage to recognize these factors, it would perhaps be possible to influence these factors to prevent the formation of amyloid and thereby a massive corneal opacity resulting in a drastic visual impairment for the patient.

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