Tenascin-cytotactin (TN-C) variants in pseudophakic/aphakic bullous keratopathy corneas

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LABORATORY STUDY

Abstract

Purpose **To examine pseudophakic/aphakic bullous keratopathy (PBK/ABK) human corneas for patterns of expression of tenascin-cytotactin (TN-C) variants known to mediate specific cellular functions, *viz.* anti-adhesion (high molecular mass (M_r)) and adhesion (low/intermediate M_r).**

Methods **PBK/ABK corneas were selected to encompass only those with bullae and without inflammation, scarring or neovascularisation. Serial sections from these and normal corneas were labelled with antibodies BC-4 (recognising all TN-C variants) and BC-2 (specific for the high M_r TN-C variant). Bound antibody was revealed with an avidin–biotin peroxidase technique. In a given pair of corneal sections, positivity with BC-4 but not BC-2 indicates localisation of low/intermediate M_r TN-C variants and absence of the high M_r TN-C variant. BC-2 identifies the high M_r variant.**

Results **There was no immunostaining with either BC-2 or BC-4 in normal corneas except at the corneoscleral interface, where both BC-2 and BC-4 were immunolocalised. In PBK/ABK corneas, BC-2 staining was seen in 5 of 13 corneas and was restricted mainly to epithelial basement membrane (BM) overlying bullae. BC-2 did not label the stroma. BC-4 immunostaining was present in all PBK/ABK corneas and was localised in epithelial BM, both epithelial and stromal borders of bullae, pannus, endothelial BM and in oedematous stromal regions.**

Conclusions **TN-C variants are differentially expressed in PBK/ABK corneas. The high M_r variant is restricted mainly to epithelial BM overlying bullae, while low/intermediate M_r variants occur in epithelial BM, both epithelial and stromal borders of bullae, and in pannus. Given the *in vitro* functions of TN-C, a role for promoting epithelial dehiscence and re-attachment to the substratum in PBK/ABK corneas by high and low/intermediate M_r variants respectively is likely.**

Key words Adhesion, Anti-adhesion, Bullous keratopathy, Extracellular matrix, Matricellular, Tenascin

Pseudophakic and aphakic bullous keratopathy (PBK and ABK) are corneal complications of intraocular surgery that are leading indications for penetrating keratoplasty.[1–3] Clinically, pathologically and histologically, PBK and ABK are similar to each other, and are only categorised separately on the basis of the presence or absence of an intraocular lens (IOL).[4–7] Both conditions are epitomised by corneal opacity and chronic oedema as a result of fluid retention in the corneal structures. In the corneal epithelial layer, retention of fluid may cause 'blisters' or bullae, which are not only painful on rupturing but may also interfere with vision.[6,7]

Although the cause of PBK and ABK is endothelial cell dysfunction[8,9] and a persistent decrease in endothelial cell numbers[10] following cataract surgery, PBK and ABK corneas show structural and compositional changes in glycosaminoglycans and extracellular matrix (ECM) proteins.[11,12] This is especially evident at the epithelial–mesenchymal interface where the epithelial basement membrane (BM) lacks 'adhesive' ECM proteins such as fibronectin, laminin and collagen type IV.[12] It has been suggested that lack of these adhesive ECM proteins in epithelial BM of PBK/ABK corneas reduces epithelial cell adhesion to the substratum and thus enhances bulla formation.[12,13] A common histological observation in PBK/ABK is loss of contact of corneal epithelial cells with each other and with underlying sub-epithelial tissues, and widespread loss of adhesion to the substratum in regions of bullae. It is therefore possible that in the epithelial–mesenchymal interface of PBK/ABK there is lack of adhesive ECM, or/and presence of 'anti-adhesive' matricellular proteins, such as tenascin-cytotactin (TN-C), thrombospondin-1 (TSP-1) and SPARC (secreted protein acidic and rich in

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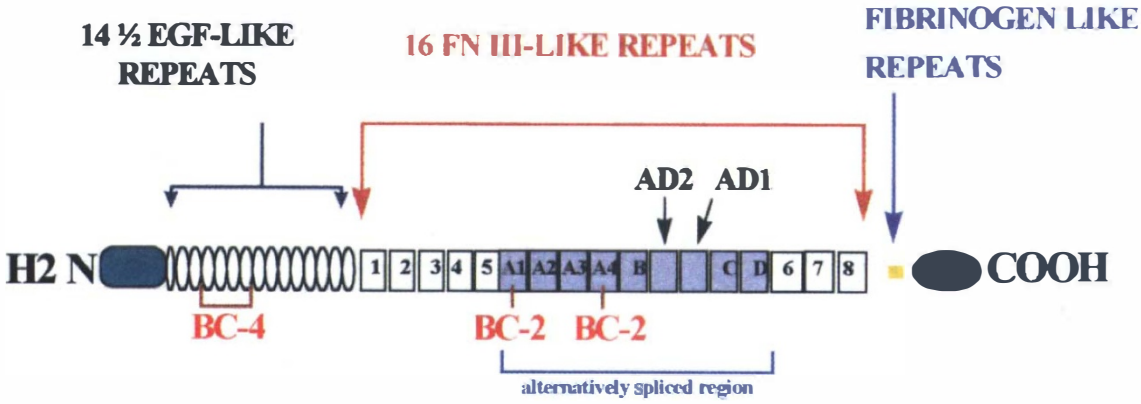


Fig. 1. *One of the six monomers of human tenascin-cytotactin (TN-C), its modular domains and location of epitopes for antibodies BC-2 and BC-4. After Siri* et al.[20] *and Sriramarao and Bourdon and Mighell* et al.[26,28]

cysteine).[14–17] The term 'matricellular', coined by Bornstein in 1995,[18] refers to a group of modular ECM proteins whose functions are achieved by binding to other ECM proteins as well as cell surface receptors. *In vitro* studies have demonstrated that TSP-1, SPARC and TN-C inhibit the adhesion of cells to normally adhesive substrata.[14–17]

Although there are no data on the expression of TSP-1 and SPARC, the expression of TN-C in PBK/ABK human corneas has been previously reported.[13,19] TN-C is a multifunctional glycoprotein whose pleiotropic nature is due, in part, to alternatively spliced mRNA-generated variants with relative molecular masses (M_r) ranging from ~190 to ~320.[20–27] Patterns of alternative splicing of human TN-C mRNA, known to occur in the region covering nine TN-C fibronectin (TNCfn) type III repeats, TNCfnA1 to TNCfnD (Fig. 1), have been revealed by cDNA and polymerase chain reaction (PCR) analytical studies.[26,28] At least 13 TN-C variants may exist, in which splicing of mRNA has resulted in either deletion of all the nine repeats, TNCfnA1 to TNCfnD (low M_r variants), a few of these repeats (intermediate M_r variants), or none (high M_r variants).[26,28] It is not known whether all the identified TN-C variants are expressed *in vivo* in humans. However, using variant-specific antibodies, TN-C variants with relative molecular masses ~190 and ~280 have been reported in the ECM of cultured human fetal lung fibroblasts.[22] In long-term cultures of human bone marrow, and in tissue sections of native bone, two TN-C variants of relative molecular mass ~220 and ~280 are expressed.[27] Whilst specific biological functions of individual TN-C variants are yet to be elucidated, *in vitro* studies and tissue distribution studies suggest that the high M_r variant mediates cell anti-adhesion and migration while low/intermediate M_r variants may mediate cell adhesion, proliferation and differentiation.[21,24,25,29–31]

Table 1. *Pseudophakic/aphakic bullous keratopathy (PBK/ABK) patients' clinical data and immunolocalisation of tenascin-cytotactin (TN-C) variants with antibodies BC-2 and BC-4*

Pt	Age/sex	Diagnosis	Clinical details	CE-PK	BC-2 stain	BC-4 stain						
1	57 ♀	PBK	CE + IOL 1989→ IOL exchange 1989 →PK+ IOL exchange 1994	66	-	-	BM	AS	-	PS	DM	-
2	80 ♀	PBK	Comp. CE 1983→ Cornea-IOL touch→PK + IOL removal 1996	36	-	-	BM	AS	*MS*	PS	DM	*Endo*
3	64 ♂	PBK	Comp. CE 1994→ IOL displaced into AC→PK + IOL exchange 1995	10	-	-	BM	AS	*MS*	PS	DM	*Endo*
4	89 ♀	PBK	CE + IOL 1985→ PK + IOL removed 1995	124	-	*SE*	BM	AS	MS	PS	-	-
5	81 ♀	ABK	CE + Trab 1986→ PK + 2° IOL 1995	108	-	SE	BM	-	-	-	-	-
6	81 ♀	ABK	Trab 1972, CE 1982→ Vitreous cornea touch→ PK + Anterior vitrectomy 1995	156	-	SE	BM	AS	-	PS	-	*Endo*
7	72 ♀	PBK	CE + Iris clip IOL 1980→ IOL cornea touch→PK + IOL exchange 1997	170	-	SE	BM	AS	*MS*	PS	DM	
8	54 ♀	ABK	Congenital glaucoma surgery→ CE 1984→ Cyclocryotherapy for IOL control 1989→ PK 1997	156	*SE*, BM	*SE*	BM	AS	-	PS	-	*Endo*
9	69 ♀	PBK	Comp. CE + IOL 1986→ Trab 1995→ PK + IOL exchange 1997	132	-	SE	BM	*AS*	-	*PS*	-	*Endo*
10	53 ♂	PBK	CE + Vitreous loss 1992→ Secondary ACIOL→ PK 1996	54	*SE*, BM	-	BM	AS	-	-	-	*Endo*
11	86 ♀	PBK	CE + iris clip IOL 1983→ IOL loop-corneal touch→ PK + IOL removal 1996	155	BM	SE	BM	AS	-	*PS*	-	-
12	80 ♂	PBK	CE + IOL 1982→ 1st PK for PBK 1988→ 2° IOL 1992→ Repeat PK 1996	168	BM	-	BM	AS	-	PS	DM	-
13	68 ♀	ABK	CE + IOL 1981→ IOL exchange 1993→ IOL removed 3/12 later→ PK + IOL 1996	180	BM	*SE*	BM	AS	-	PS	-	-

Pt, patient; Comp., complicated; Trab, trabeculectomy; CE, cataract extraction; PK, penetrating keratoplasty; CE-PK, period of time from CE to PK (in months); IOL, intraocular lens; ACIOL, anterior chamber IOL; 2°, secondary; SE, sub-epithelial layer; BM, basement membrane; AS, anterior stroma; MS, mid stroma; PS, posterior stroma; DM, Descemet's membrane; Endo, endothelium.
Stain localisation is shown in bold print (strongly positive), italics (moderately positive) or as – (negative).

Given these functional differences, especially with regard to adhesion and counter-adhesion, we postulate that these molecules may be involved in the pathogenesis of corneal bullous disease. We have thus examined the patterns of expression of high and low/intermediate M_r TN-C variants, with special reference to the epithelial–mesenchymal interface, in PBK/ABK and in normal human corneas.

Materials and methods

Tissues

Thirteen PBK/ABK corneas obtained at penetrating keratoplasty (Table 1) were chosen to include only those without neovascularisation, scarring or inflammation, since TN-C is known to be induced in these conditions.[32,33] Normal corneas were obtained from globes enucleated for choroidal melanoma but without anterior segment pathology. Specimens were immediately fixed in buffered formalin, processed and embedded in paraffin wax.

Immunodetection of TN-C variants

Following pretreatment of sections with trypsin (7 min, 37 °C), a standard avidin–biotin peroxidase (ABC) technique,[34] and monoclonal antibodies, BC-4 and BC-2 (isotype IgG1), to known epitopes in the human TN-C molecule,[20] were employed in detecting TN-C variants. As shown in Fig. 1, the epitope recognised by BC-4 lies in a conserved epidermal growth factor (EGF)-like domain present in all TN-C variants. Thus BC-4 detects all TN-C variants. BC-2, however, whose antigenic epitopes are located in the alternatively spliced fibronectin type III repeats TNCfnA1 and TNCfnA4, which exhibit a high degree of homology, can detect only TN-C molecules with either one or both of these domains intact (i.e. the high M_r variant). Therefore, positivity with BC-4 and negativity with BC-2 indicates the presence of low/intermediate M_r variants, but not high M_r variants. BC-2 positivity identifies high M_r variants.

To enable comparison of immunostaining patterns for the two antibodies in a given cornea a pair of microtome serial sections (6 μm) was taken from each cornea, one of which was incubated (4 °C, overnight) with BC-4 and the other with BC-2. Bound antibody was detected by incubating sections (30 min, room temperature) with biotinylated rabbit anti-mouse IgG1 immunoglobins (Dako, UK) and was revealed by incubating sections (30 min, room temperature) with avidin–biotin peroxidase (Dako, UK) followed by reacting with a substrate composed of 3,3'-diaminobenzidine (DAB) tetrachloride and 1% hydrogen peroxide (H_2O_2) to give a brown reaction product. Sections were viewed under a light microscope. In a given pair of corneal sections, positivity with BC-4 but not BC-2 indicates localisation of low/intermediate TN-C variants and absence of high M_r TN-C variants. BC-2 positivity identifies the high M_r variants. Negative immunohistochemical controls included substitution of the TN-C antibodies with an irrelevant antibody, anti-desmin (Dako, clone D33, IgG1), or normal rabbit serum. There was no staining seen in negative controls. BC-2 and BC-4 positive immunoreaction control was provided by sclera.[32,33] The immunolocalisation of each antibody given in Table 1 was based on the intensity of the brown DAB coloration and is shown either in bold print (strongly positive), in italic print (moderately positive) or by a minus sign (negative).

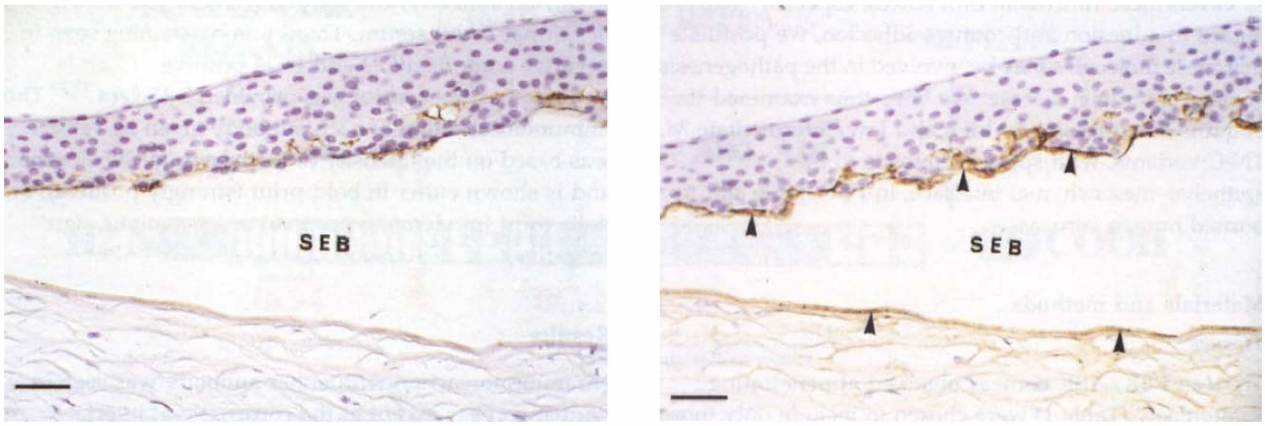
Results

No immunoreaction with either antibody was seen in normal corneas except at the corneoscleral interface, where both BC-2 and BC-4 were immunolocalised. In PBK/ABK corneas, BC-2 immunostaining was seen in 5 of 13 corneas while BC-4 staining was observed in all PBK/ABK corneas (Table 1). BC-2 immunoreaction was restricted to epithelial BM in cases where there were bullae (Fig. 2a). It did not label the stroma or BM where the epithelium was attached (Fig. 3a). BC-4 immunostaining, on the other hand, was localised in epithelial BM, both epithelial and stromal borders of bullae, pannus and endothelial BM (Figs. 2b, 3b, 4b). In 2 of 13 PBK/ABK corneas (Table 1), BC-4 immunoreaction was also localised within oedematous mid-stromal regions (Fig. 4b). None of the pathological corneas exhibited the posterior collagenous layer (PCL) that has been described in ABK/PBK corneas.[13]

As shown in Table 1, there is no correlation between the expression of TN-C and a patient's age or sex and no definite pattern was revealed by analysis of the clinical histories. In all corneas where there had been cornea–IOL touch after cataract extraction, BC-4 immunostaining was seen in mid-stroma. However, this mid-stromal BC-4 staining was not exclusively associated with cornea–IOL touch (see Table 1). Comparison of TN-C expression patterns with the degree of severity of bullous disease based on visual acuity (VA) was not undertaken in this study, given that several factors, including the state of the retina and optic nerve, and media opacification, are known to affect VA.

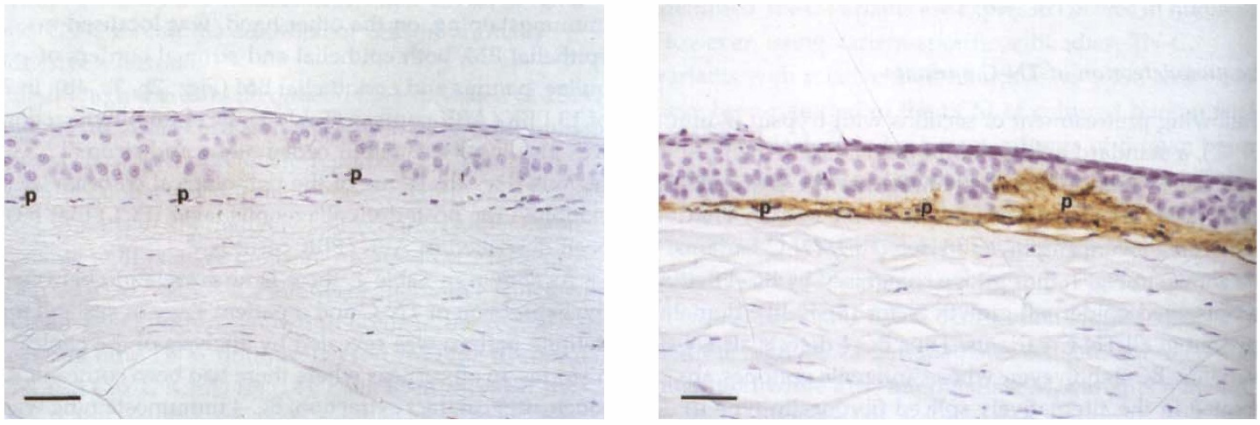
Discussion

In the normal adult cornea, immunodetectable TN-C glycoprotein is restricted to the limbus. This is further confirmation of our previous observations,[32] and is in agreement with observations reported by Tuori *et al.*[33] and Ljubimov *et al.*[13] The expression of TN-C in normal adult tissues is usually limited to regions of continuous renewal and repair, where TN-C may be involved in mediating cell growth and differentiation (reviewed by Mackie[35]). In PBK/ABK corneas, the high M_r TN-C variant was restricted to the epithelial BM overlying bullae (Fig. 2a) and was not immunodetected in either the stroma or the epithelial BM where the epithelium was attached to the substratum (Fig. 3a). While the specific biological functions of these variants are not fully understood, the high M_r variant has been shown, *in vitro*,



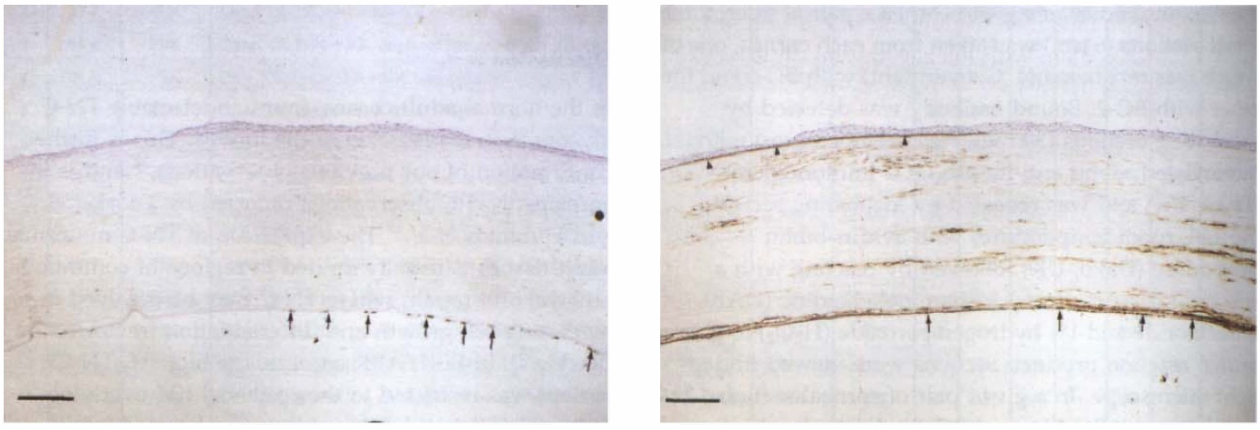
(a) (b)

Fig. 2. *Subepithelial bulla (SEB). (a) BC-2 immunopositivity is demonstrated by immunoreactivity in the epithelial basement membrane (BM) zone of the detached corneal epithelium, including areas where there is BM and epithelial reduplication. (b) BC-4 intensely labels these areas (arrowheads) and also labels the anterior surface of Bowman's layer. DAB with Maeyer's haematoxylin counterstain. Scale bar represents 30 μm.*



(a) (b)

Fig. 3. *Superficial and basal epithelial oedema, and early pannus (p) accumulation without bulla formation. (a) BC-2 immunoreaction is negative. (b) BC-4 immunopositivity is seen in epithelial BM, in and around pannus and along the anterior border of Bowman's layer. DAB with Maeyer's haematoxylin counterstain. Scale bar represents 30 μm.*



(a) (b)

Fig. 4. *Whole section of cornea. Endothelium is severely depleted; iris pigment (arrows) is present within remaining endothelial cells. (a) BC-2 immunoreaction is negative. (b) BC-4 is positive in the epithelial BM (arrowheads), in oedematous stromal regions and along both sides of Descemet's membrane. DAB with Maeyer's haematoxylin counterstain. Scale bar represents 300 μm.*

to inhibit the adhesion of several cell types including eosinophils, epithelial and endothelial cells to laminin, fibronectin and multi-extracellular matrix gels.[28,29,35–37] Interestingly, in these PBK/ABK corneas the high M_r TN-C variant was immunolocalised to the epithelial BM of bullae and was not seen in epithelium that was attached to the substratum. Therefore, the high M_r TN-C variant expressed in the bullae of PBK/ABK corneas may play a role in epithelial dehiscence. Moreover similar patterns of expression of the high M_r TN-C variant have been reported in epidermal–dermal separation of skin.[38]

Low/intermediate M_r TN-C variants were localised in both epithelial and stromal borders of bullae, pannus, endothelial BM and in oedematous stromal regions (Figs. 2b, 3b, 4b). In PBK/ABK, detachment and re-attachment of the epithelium to the substratum are common phenomena. Considering that low/intermediate TN-C variants mediate, *inter alia*, cell adhesion,[25,28] their role in PBK/ABK may be to promote epithelial attachment and/or re-attachment to the substratum. Although a PCL was not seen in our cases, the presence of low/intermediate M_r TN-C variants we saw in the area of DM and the endothelium corresponds to the staining described by Ljubimov *et al.*[13] where BC-4 labelled the PCL in their cases. The lack of a PCL in our cases presumably reflects less severe pathology, as corneas with scarring, vascularisation or inflammation were excluded from the study, given the known association of TN-C with these processes.[32,33]

While the initiating factor in PBK/ABK is known to be endothelial dysfunction and/or loss of endothelial cells causing corneal oedema, a lack of adhesive ECM proteins such as fibronectin, laminin and collagen type IV in epithelial BM has been implicated in enhancing bulla formation.[11,12] In this study we have found the expression of the high M_r TN-C variant, an anti-adhesive glycoprotein, to be associated with bullae. The anti-adhesive activities of the high M_r TN-C variant are attributed to the alternatively spliced fibronectin type III repeats, TNCfnA1 to TNCfnD.[29] This anti-adhesive effect, as of other matricellular proteins, has been widely assumed to be mediated through an interaction of the matricellular protein with the substratum, thus blocking binding to cells. However, *in vitro* studies have suggested that TN-C mediates its anti-adhesive effects through interactions with cell surface receptors.[39,40] These interactions are as avid as those between 'adhesive' ECM proteins and their cell surface receptors, yet result in anti-adhesion presumably by generating transmembrane signals that inhibit tyrosine phosphorylation[41] of certain proteins such as $pp125^{FAK}$, which are known to be essential to focal contact assembly.[42] It is, therefore, possible that at the epithelial–mesenchymal interface of PBK/ABK corneas, anti-adhesive signals provided by the high M_r TN-C variant antagonise those provided by epithelial BM adhesive molecules such as fibronectin, laminin and glycosaminoglycans, resulting in epithelial dehiscence.

Several factors (reviewed by Chiquet-Eherismann *et al.*[43]) are known to induce the expression of TN-C. These include angiotensin II and reduced low density lipoprotein (LDL),[44] and cytokines such as tumour necrosis factor-alpha (TNF-α), basic fibroblast growth factor (bFGF), transforming growth factor-beta 1 (TGF-β1), interleukin-1 alpha (IL-1α), interleukin-4 (IL-4) and interleukin-6 (IL-6).[45,46] Interestingly, it has also been demonstrated *in vitro* that mechanical stress can induce fibroblasts to synthesise TN-C.[47] Considering that the PBK/ABK corneas in this study are neither inflamed nor vascularised, the repertoire and levels of the cytokines mentioned above are likely to be similar to those in the normal corneas which are TN-C negative. However, the accumulation of fluid within stroma, epithelial and sub-epithelial layers of PBK/ABK corneas which results in formation of the so-called fluid-filled lakes[6–11] would be expected to produce mechanical stress in the corneal tissue which may induce epithelial cells and keratocytes/fibroblasts in such areas to synthesise TN-C. This, as suggested by Ljubimov *et al.*,[13] may explain the stromal localisation of TN-C seen in many of these corneas.

Conclusions

The normal adult human cornea is devoid of TN-C glycoproteins. TN-C variants are, however, differentially expressed in PBK/ABK human corneas. Given the *in vitro* functions of these variants and the patterns of expression observed in this study, roles for enhancing bulla formation (high M_r) and promotion of corneal epithelial re-attachment to the substratum (low/intermediate M_r variants) are likely.

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