Tenascin-C expression in normal, inflamed, and scarred human corneas

H Maseruka, R E Bonshek, A B Tullo

Abstract
Aims/background—In adult tissues the expression of tenascin-cytotactin (TN-C), an extracellular matrix glycoprotein, is limited to tumours and regions of continuous renewal. It is also transiently expressed in cutaneous and corneal wound healing. There are limited data regarding its expression in inflammation and scarring of the adult human cornea. In this study, TN-C expression patterns in normal, inflamed, and scarred human corneas have been examined.

Methods—Penetrating keratoplasty specimens were selected from cases of herpes simplex keratitis, herpes zoster ophthalmicus, rheumatoid arthritis ulceration, bacterial keratitis, rosacea keratitis, interstitial keratitis, and previous surgery so as to encompass varying degrees of active and chronic inflammation and scarring. TN-C in these and in normal corneas was immunodetected using TN2, a monoclonal antibody to human TN-C.

Results—There was no TN2 immunopositivity in normal corneas except at the corneoscleral interface. In pathological corneas, TN2 immunopositivity was localised in and around regions of active inflammation, fibrosis, and neovascularisation. TN2 positivity was less in acute inflammation than in active chronic inflammation. Mature, avascular scar tissue and epithelial downgrowth were TN2 negative.

Conclusion—These results indicate that in the adult human cornea, TN-C expression is induced in regions of inflammation, fibrosis, and neovascularisation, but that expression is absent in mature, avascular scar tissue. This suggests a role for this glycoprotein in inflammation, healing, and extracellular matrix reorganisation of the cornea.

Tenascin is a large extracellular matrix glycoprotein (~190–320 kD) which has been described variously as cytotactin, glioma-myotendinous associated extracellular matrix (GMEM) protein, myotendinous antigen, hexabranchion, and J1-200/220. Currently, this glycoprotein is referred to as tenascin-cytotactin (TN-C).

Several in vitro studies have shown that TN-C mediates a number of cellular activities. For example, it stimulates and inhibits the proliferation of cells. It also interacts with fibronectin (FN), and supports adhesion of a variety of cell types including fibroblasts, glia, neurons, and endothelial cells. Paradoxically, antiadhesive properties have also been described, providing evidence for multifunctionality of this glycoprotein.

TN-C shows a time and tissue specific expression in the developing embryo. It is, for example, present at the epithelial mesenchyme interfaces of skin, oral mucosa, lung, genitourinary tract, gastrointestinal tract, kidney, breast, and in the developing brain and cornea.

In adult tissues TN-C expression is limited to tumours, regions of continuous renewal, and wound healing (reviewed by Sakakura and Kusano).

Although TN-C is expressed in developing human fetal cornea, in normal adult human cornea very low expression has been demonstrated in corneal epithelial cells only, with a greater degree of expression at the limbal region. In rabbits, however, TN-C has been detected in the entire normal corneal epithelium, but it is absent in the stroma. As seen in cutaneous wound healing, TN-C is transiently expressed in corneal wound healing. However, to date there are limited data regarding the expression of TN-C in both normal and pathological adult human corneas. This, together with a body of evidence supporting a role for this glycoprotein in influencing several cellular activities, prompted us to examine TN-C expression in inflamed, vascularised, and scarred human corneas. Comparison of patterns of TN-C localisation in pathological and normal corneas was aimed at determining whether a relation exists between the progression of events in inflammation, healing, and scarring, and TN-C expression in the human cornea.

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Table 1 Source of reagents and antibodies employed

<table>
<thead>
<tr>
<th>Reagents/antibodies</th>
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<tr>
<td>Mouse anti-human tenascin-C (clone TN2, IgG1, kappa)</td>
<td>Dako, UK</td>
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<td>Mouse anti-human desmin (clone D33, IgG1, kappa)</td>
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<tr>
<td>Rabbit anti-mouse IgG (RAM)</td>
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<td>Avidin-biotin/horseradish peroxidase complex (ABC/HRP)</td>
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<td>Industrial methylated spirit (IMS)</td>
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<td>3-Aminopropyltriethoxysilane (APES)</td>
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<td>Trypsin tablets</td>
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<td>Normal rabbit serum (NRS)</td>
<td>Sigma Chemical Company, USA</td>
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<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
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Materials and methods

Thirty five corneal specimens obtained from patients aged from 3 to 97 years, who had undergone penetrating keratoplasty, were selected to encompass varying degrees of acute and chronic inflammation, and scarring. These included the following cases—bacterial keratitis (three); herpes simplex keratitis (HSK) (10); rosacea keratitis (three); herpes zoster ophthalmicus (HZO) (five); interstitial keratitis (IK) (seven); rheumatoid arthritis (RA) ulceration (five); and previous surgery (two). Ten globes enucleated for choroidal melanoma but without anterior segment pathology provided normal corneas. All tissues were fixed in 10% (v/v) phosphate buffered formalin, pH 7.4, before being dehydrated and embedded in paraffin wax.

Reagents and antibodies employed in the study are shown in Table 1. A mouse anti-human TN-C (clone TN2, isotype IgG1, kappa) obtained from Dako, UK, was employed in this study. TN2 has been employed in a number of studies investigating tissue distribution of TN-C. The corneal stroma was separated from epithelium in all cases by subepithelial staining (Fig 2). At higher magnification (Fig 3) focal TN2 staining was seen in occasional keratocytes/fibroblasts in acutely inflamed tissue. It was apparent that much of the intense stromal TN2 immunoreaction around the inflamed areas was associated with chronic inflammatory cell infiltrate, although the infiltrating cells were themselves negative. An intermittent line of staining was also seen at the junction between Descemet's membrane and the posterior stroma (Fig 3).

Cases of HSK, rosacea keratitis, and HZO showed heterogeneity of stromal staining for TN2. However, in all cases there was stromal TN2 immunoreactivity associated with active chronic inflammation (Fig 4). As in the chronic inflammation around the area of acute inflammatory activity shown in Figures 2 and 3, the inflammatory cells were TN2 negative, and there was focal positivity of the stroma/
Descemet's membrane interface. There was no
difference in the patterns of TN2 staining seen
with respect to any of these three aetiologies.

IK corneas were negative except for occa-
sional vessels where there was weak TN2
immunopositivity in vascular endothelial cells
(not shown). In the majority of RA corneas
there were few infiltrating inflammatory cells
and there was focal faint TN2 immunostaining
associated with these cells. One RA cornea had
perforated and contained a strip of intact
epithelium which spanned the perforation.
This cornea showed strong TN2 positivity in
stroma adjacent to the perforation, especially
in early subepithelial fibrous scar tissue, and in
basement membrane-like material which lay
beneath epithelium spanning the defect and
which was devoid of substratum (Fig 5).

In corneas with previous surgery there was
TN2 immunostaining in regions of active scar
tissue, associated with some chronic inflamma-
tory cells, where stromal and lymphocyte cyto-
plasmic membrane staining was seen, and
some vascular endothelial cells, where there
was intracytoplasmic staining (Fig 6). How-
ever, where scars were uncomplicated, no TN2
immunopositivity was seen (Fig 7). In one case
with an epithelial downgrowth, but with no
scarring, TN2 immunoreaction was negative
(Fig 8). In some pathological corneas, without
any specificity for any disease type, occasional
epithelial cells showed granular epithelial
cytoplasmic TN2 immunostaining (Figs 6
and 7).

Discussion

Tenascin was not detected in normal human
corneal structures except at the corneoscleral
interface. This observation is consistent with
the findings of Uusitalo who employed the
same antibody (clone TN2) which recognises
all TN-C variants. However, Tervo et al, have
reported immunodetectable TN-C in normal
human corneal epithelium, but in agreement
with our observation, these authors noted a
greater localisation of TN-C at the corneoscle-
ral margin. It is unfortunate that their
description of corneal epithelial staining is
found only in the abstract of their paper and is
not mentioned in either the results or discus-
sion, nor do the photomicrographs illustrate
this corneal epithelial staining. These authors
employed a different monoclonal antibody
(100EB2), which recognises an epitope within
a conserved FN type III repeat region of the
TN-C molecule. Thus differences in epitope
specificity may be involved, or there may be
differences due to masking of epitopes because
of fixation, as they used an immunofluores-
cence method with fresh tissue. We have
recently used BC4, a monoclonal antibody
described by Siri et al, with results identical
to those of TN2 (unpublished observations).

Species differences in TN-C expression exist,
as TN-C has also been immunodetected in the
entire epithelium of normal rabbit corneas. In
pathological corneas and those with previ-
ous surgery, localisation of TN-C was observed
around active ulcers and foci of acute inflam-
mation, within chronically inflamed tissue and
granulation tissue, and in vascularised regions.
Old/mature avascular scar tissue was TN-C
negative.

The absence of TN-C in normal corneas, its
expression in inflammation and neovasculari-
sation, and its disappearance in quiescent
fibrosis, as seen in mature scar tissue, mirrors
the situation described in cutaneous and
experimental corneal wound healing. In
cryptogenic fibrosing alveolitis (CFA), TN-C
expression has also been reported in areas of
inflammation and active disease but not in end
stage scarred tissue. Of interest, the heteroge-
nity of TN-C expression which is a feature
seen in our HSK, rosacea, and HZO corneas
was also observed in CFA. Moreover, there
was no difference in the patterns of staining
which could be ascribed to a viral aetiology.
Morphologically, there are no histological features which allow an aetiological distinction between chronic HSK, rosacea, or HZO; thus, the TN-C immunoreactivity observed presumably reflects factors associated with the chronic inflammatory response, fibrosis, and neovascularisation common to these conditions. Data from in vitro and tissue distribution studies have implicated TN-C in signalling cells to alter the expression of genes involved in extracellular matrix (ECM) synthesis and remodeling processes as seen in wound healing.\textsuperscript{23} TN-C is produced by cultured fibroblasts,\textsuperscript{24} and an upregulation of synthesis of proteolytic enzymes, such as collagenase, stromelysin, and the 92 kDa gelatinase is commonly observed in fibroblasts plated on a mixed substrate of fibronectin and TN-C.\textsuperscript{25} TN-C expressed around ulcers and acute inflammation, and in chronically inflamed and fibroblastic areas of these corneas, possibly modulates ECM remodelling during the healing and repair processes which are a component of the chronic inflammatory response. This is supported by the absence of TN-C in quiescent, burnt out scars.

On the other hand, it has been suggested that TN-C acts as an immunomodulator by altering the adhesion of monocytes and B and T lymphocytes.\textsuperscript{27,28} Stromal TN2 positivity was strongest in areas of chronic inflammatory activity and the observation of occasional lymphocytes have cytoplasmic membrane staining. Elsewhere, there is cytoplasmic labelling of some vascular endothelial cells. There is also granular cytoplasmic staining of epithelial cells overlying this area (× 450, oil immersion).

Morphologically, there are no histological features which allow an aetiological distinction between chronic HSK, rosacea keratitis, and IK corneas, and in some active scar tissue may support suggestions that TN-C is expressed in activated vascular endothelial cells.\textsuperscript{29} TN-C expression has been reported in tissue surrounding hyperplastic capillaries around tumours.\textsuperscript{29,30} There is also evidence from in vitro studies that capillary endothelial cell morphology and behaviour are influenced by TN-C present in underlying medium via an integrin dependent process.\textsuperscript{31} Antibodies to TN-C have also been shown to inhibit angiogenesis in vitro.\textsuperscript{32} Our observed TN2 positivity around the periphery of areas of

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**Figure 4**  Herpes simplex keratitis cornea. There is stromal TN2 immunopositivity associated with chronic inflammation in the posterior stroma. Inflammatory cells are negative. Note that, as in Figure 3, there is also focal positivity at the interface between posterior stroma and Descement’s membrane (arrowheads). Endothelium is intact (× 370).

**Figure 5**  Rheumatoid perforation with an area of epithelium bridging a stromal defect. There is diffuse, extracellular, stromal TN2 immunopositivity around the defect. The small amount of subepithelial tissue remaining beneath the undermined epithelium is also strongly positive (small arrows). Iris pigment (IP) is present as discrete deposits in the posterior stroma below and adjacent to the perforation. Very few inflammatory cells are present (× 38).

**Figure 6**  Previous surgery. There is patchy chronic inflammation and vascularisation. TN2 immunopositivity is present in the stroma where there is an aggregate of chronic inflammatory cells. Here, occasional lymphocytes have cytoplasmic membrane staining. Elsewhere, there is cytoplasmic labelling of some vascular endothelial cells. There is also granular cytoplasmic staining of epithelial cells overlying this area (× 450, oil immersion).
acute inflammation and ulceration may, therefore, be consistent with a role for TN-C in promoting angiogenesis and vascular proliferation. The observed strong subepithelial TN2 staining in the rheumatoid arthritis (RA) cornea where the epithelium is detached (Fig 2) is of considerable interest. In this situation there appears to be increased TN-C expression by the epithelial cells, with immunolocalisation in the extracellular basement membrane (EBM) zone, which is thickened. A similar appearance is seen in a case of bacterial keratitis (Fig 2) where the epithelium has partially lifted from oedematous stroma. These findings, also reported in dermo-epidermal separation of human skin, indicate that TN-C expression is induced in situations where there is loss of attachment of epithelium to substratum. This may suggest that the epithelium is the source of TN-C in these cases. The granular cytoplasmic TN2 staining in the epithelium of several of the pathological corneas could also be interpreted in this way and may be consistent with the observations of Tervo et al. TN-C is known to bind to proteoglycans which are basement membrane components; thus, the TN2 positivity seen in the EBM zone and at the interface between Descemet's membrane and the posterior stoma may reflect this property.

Our findings, together with the body of data regarding the structure of TN-C and its expression in a variety of tissues during development, in wound healing events, and in pathological states, indicate a functional role for this molecule. The development of the 'tenascin gene knockout mouse', which develops normally despite an absent functional tenascin gene, presents a paradox, appearing to contradict this evidence. However, a number of gene deletions in mice have resulted in surprisingly mild or undetectable phenotypic alterations and, even in cases where a mutant mouse mimics a human disease, the mutant mouse does not always provide an exact model for the aetiology and pathogenetic mechanisms (reviewed by Smithies). Furthermore, other members of the tenascin family (reviewed by Chiquet-Ehrismann et al.) might compensate for the loss of one component.

In conclusion, the patterns of TN-C expression observed in this study indicate that in the human adult cornea, TN-C is induced in inflammatory states, especially where there appears to be fibroblastic activity, and that it is not expressed in mature avascular scar tissue. This pattern of expression suggests an important role for this glycoprotein in inflammation, healing, and in the extracellular matrix remodelling of the human cornea.

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