A comparison of proteoglycan arrangement in normal and keratoconus human corneas

NIGEL J. FULLWOOD,* KEITH M. MEEK,* NAGEENA S. MALIK* and STEPHEN J. TUFTs
*The Open University, Oxford Research Unit, Foxcombe Hall, Boars Hill, Oxford OX1 5HR, U.K. and †Moorfields Eye Hospital, City Road, London EC1V 2PD, U.K.

Keratoconus is an eye disease characterized by a slow progressive thinning and cone shaped protrusion of the central region of the cornea, resulting in loss of vision due to irregular astigmatism and scarring [1]. It usually begins in the early teens, is more prevalent in women, and most estimates of its frequency are within the range 4-230 per 100,000 individuals [2]. At the present time there is no definite explanation as to the origin of this disorder although it is known that patients with atopic diseases have a higher incidence of keratoconus [2]. It has also been suggested that the condition may result from ocular trauma such as contact lens wear or eye rubbing [2], although the evidence for this is not as strong. There appear to be no differences in collagen type between normal and keratoconus specimens [3], although there is evidence to suggest that there is some alteration in collagen cross-linking [4]. An altered relative biosynthesis of collagen, proteoglycan, and glycoprotein has also been reported in keratoconus corneas [5].

Our investigations have centred on the nature and distribution of the proteoglycans relative to the collagen fibrils within the corneal stroma. The proteoglycans within keratoconus and normal human corneas were stained with Cuprolinic Blue using the "critical electrolyte" method [6]. Normal corneas showed clearly stained proteoglycan filaments, the majority of which were oriented crosswise to the collagen fibrils (Fig. 1a). Keratoconus corneas also exhibited this pattern of staining over most of their stroma; however, in some regions keratoconus corneas had fewer filaments than normal and those present were orientated parallel to the collagen fibrils (Fig. 1b).

The results from the Cuprolinic Blue staining clearly demonstrate an abnormal arrangement of proteoglycans in keratoconus corneas. This arrangement may be due to stresses within the stroma causing slipping between adjacent collagen fibrils and thus displacing the proteoglycans into a parallel arrangement with respect to collagen fibrils; this explanation obviously supports the theory that keratoconus results from ocular trauma. An alternative possibility is that the abnormal arrangement is due to a difference in the nature of the proteoglycans; this is supported by a recent report [7] which states that in keratoconus corneas the keratan sulphate proteoglycan core has fewer keratan sulphate chains attached. This difference in proteoglycan structure could explain the lower levels of staining and abnormal proteoglycan arrangement which we have observed. Corneas were also investigated by X-ray diffraction using the SERC synchrotron source at Daresbury U.K. X-Ray diffraction has the advantage over electron microscopy of allowing examination of tissues in their natural state without the need for fixation, dehydration, and embedding. The first-order diffraction ring in the low-angle X-ray pattern allows the mean centre-to-centre spacing of the fibrils to be calculated. Wide-angle X-ray diffraction allows the mean centre-to-centre spacing of the molecules within the fibrils to be calculated. Normal and keratoconus corneas were weighed before and after exposure to the X-ray beam, and again after drying to allow their state of hydration to be calculated. Preliminary results from the low-angle patterns of a limited number of specimens gives a mean intersubmolar spacing of 56 ± 4 nm for keratoconus corneas and 61 ± 5 nm for normal corneas at physiological hydration (H = 3.2). Results from the wide-angle patterns indicate that the mean intermolecular spacing is 1.58 ± 0.04 nm for keratoconus corneas and 1.61 ± 0.07 nm for normal corneas at H = 3.2.

We wish to thank Rosalind Harrison and Paul Brittain for providing samples for this project. Supported by grants from the National Keratoconus Foundation, the Discovery Fund for Eye Research, the M.R.C., the N.I.H., the Wellcome Trust, and the Nuffield Foundation.


---

Fig. 1. Normal human (a) and keratoconus corneas (b) were stained for proteoglycans with Cuprolinic Blue in a solution of 0.1 M-MgCl₂, the collagen fibrils were counterstained with tryanyl acetate and phosphophtungstic acid.

In the normal cornea (a) the proteoglycan filaments (arrows) are small and mostly orientated crosswise to the collagen fibrils. The keratoconus cornea (b) has much larger proteoglycan filaments (arrows) oriented parallel to the collagen filaments (scale bars = 100 nm).

Vol. 18
Modulation of extracellular matrix proteins and the influence of fucoidan on cell proliferation of smooth muscle cells

PETER VISCHER
Institut für Arterioskleroseforschung, Domagkstrasse 3, D-4400 Münster, F.R.G.

Cellular glycoconjugates serve as specific information-bearing components involved in several biological functions such as cell adhesion, cell growth or cell differentiation. One group of molecules which perform such functions are proteoglycans, owing to their versatility and capacity for multiple interactions with other molecules. Recent studies have indicated that heparin or heparin-like molecules are potent inhibitors of smooth muscle cell proliferation in vivo and in vitro [1, 2]. During our investigations on the biological role and cellular organization of thrombospondin in arterial wall cells, we observed, in addition to heparin and heparan sulphate, a strong influence of fucoidan, a sulphated fuco-polysaccharide of the brown marine algae Fucus vesiculosus, on this extracellular matrix protein. This prompted us to investigate in more detail the influence of this sulphated polysaccharide on several biological functions of smooth muscle cells. Porcine aortic smooth muscle cells were grown by the explant method and cultured as described previously [3]. For cell proliferation studies, cells were sparsely plated in Dulbecco’s modified Eagle’s medium (DMEM) + 10% (v/v) fetal calf serum (FCS) and placed after 24 h in DMEM + 0.5% (v/v) FCS for 72 h. Cell growth was initiated by replacing this medium with DMEM + 10% (v/v) FCS containing 1 μCi [3H]thymidine/ml in the presence or absence of fucoidan (Sigma, Munich, F.R.G.) or other polysaccharides. Inhibition of cell proliferation was calculated on the basis of [3H]thymidine incorporation into DNA. For the analysis of the influence of fucoidan on cellular and secreted proteins, confluent smooth muscle cells were pretreated with fucoidan (100 μg/ml) for 24 h and metabolically labelled with [35S]methionine. Cellular and secreted proteins were precipitated with trichloroacetic acid (10%, w/v) and separated by SDS/PAGE (7.5%, w/v) under reducing conditions. Immunoprecipitation of radioactively labelled proteins was performed as previously described, using a polyclonal antibody raised against porcine platelet thrombospondin [4] and a polyclonal antibody of commercial source for immunoprecipitation of fibronectin.

Fucoidan is capable of inhibiting smooth muscle cell proliferation with 50% inhibition at a concentration of 5–10 μg/ml. In contrast to fucoidan, heparan sulphate from calf aortic tissue or dextran sulphate (5000 kDa) needed a five times higher concentration to achieve a similar antiproliferative effect. Heparin of commercial source was even less active (150 μg/ml). Sulphation of the native fucoidan molecule was found to be necessary for the expression of antiproliferative activity. The effect of fucoidan on smooth muscle cell growth was connected with substantial altera-

![Fig. 1. Influence of fucoidan on secreted thrombospondin and fibronectin](image-url)

Confluent smooth muscle cells were cultured in the presence or absence of fucoidan (100 μg/ml) and pulse labelled for 4 h with [35S]methionine. Radioactively labelled thrombospondin and fibronectin was immunoprecipitated from the medium fraction and analysed by SDS/PAGE and fluorography.