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Biochemical studies on human corneal proteoglycans – a comparison of normal and keratoconic eyes

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Abstract. Human corneas from normal (healthy) donors and patients with keratoconus were either metabolically labelled under organ culture conditions or investigated without preincubation. The sulfated proteoglycans were isolated from a 4 *M* guanidinium chloride/2% Triton X 100 extract. Two predominant proteoglycans were obtained from normal cornea after digestion of total sulfated proteoglycans with chondroitin ABC-lyase or endo-β-galactosidase. One had an overall mass of 150 kDa, two dermatan sulfate chains ($M_r \approx 50$ kDa) with an iduronic acid content of 24%–28% and, after chondroitin ABC-lyase digestion, a core protein of 48 kDa. The other proteoglycan had an overall mass of 110 kDa, one keratan sulfate chain of ≈60 kDa and, following endo-β-galactosidase (keratanase) digestion, a core protein of 46 kDa. Each proteoglycan population was further fractionated into two subpopulations by chromatography on concanavalin A-Sepharose. The dermatan sulfate- and keratan sulfate-containing proteoglycans isolated from keratoconic and healthy cornea had comparable M_r values and core proteins with identical molecular weights, but the ratio of dermatan sulfate/keratan sulfate proteoglycan was increased in keratoconic cornea and the keratan sulfate chains of two keratan sulfate proteoglycans from keratoconic cornea were considerably shorter (M_r 44 and 33 kDa) than those from normal corneas.

Introduction

Proteoglycans are complex glycoconjugates that consist of a protein backbone to which one or more glycosaminoglycan chains and *N*- and *O*-linked oligosaccharides are covalently attached. After cellular synthesis, the proteoglycans can have a large variety of fates and functions, depending on the cell type and localization of the macromolecule (for review see [13, 30]).

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Corneal stroma proteoglycans [2] have been isolated from the ox [3, 11], rabbit [9, 10], embryonic chicken [19, 20] and rhesus monkey [12, 22] and have been partially characterized with respect to their macromolecular and biochemical properties, but comparable data on human corneal proteoglycans are missing. Earlier studies [1, 4] have revealed that chondroitin sulfate/dermatan sulfate (CS/DS) and keratan sulfate (KS) are the predominant glycosaminoglycan constituents in human corneal stroma, but the structure of the native proteoglycans was not reported.

In mammalian cornea, two major proteoglycan populations of small molecular size were identified. One proteoglycan with hybrid CS/DS chains that were *O*-glycosidically linked to a protein core had a molecular weight of 100–150 kDa and one CS/DS side chain. The other proteoglycan contained two KS side chains and an overall mass of 100 kDa [12]. In embryonic chicken cornea, both a proteoglycan with an overall mass of 150 kDa that has a single DS chain attached to a protein core of 45 kDa and a proteoglycan with three KS chains have been found [20].

The hydrodynamic size of the corneal proteoglycans enables them to fit well within the space between the regular lattice of defined domains of collagen fibrils [29]. An interaction of proteoglycans with specific domains of collagen maintains and stabilizes the orderly packing of collagen fibrils and guarantees optical transparency and mechanical properties.

Keratoconus is an ocular disease of unknown etiology that is characterized by a progressive alteration of the corneal shape accompanied by a thinning of the corneal tissue. In the development of keratoconus, decreased resilience and low mechanical strength of corneal tissue seem to be important pathogenetic factors [8], but the basic molecular defect is unknown. Comparative studies on collagen types I, III, IV and V in normal and keratoconic cornea revealed neither differences in amino acid composition and the type and number of cross-links [25] nor changes in the distribution and spe-

cific immunostaining of the various collagen types [16, 21, 33, 35]. Buddecke and Wollensak [4] found an increased hexosamine content in keratoconic cornea but no changes in the ratio of CS/KS as compared with normal cornea. Comparative studies by Wollensak et al. [31] revealed higher viscoelasticity for keratoconic than for normal corneas. A markedly increased amount of protein, uronic acid, neutral hexoses and *N*-acetylgalactosamine in keratoconus extracts as compared with controls has been reported by Critchfield et al. [5] and Yue et al. [34], but it remains to be determined whether these abnormalities are a primary factor associated with the pathogenesis of keratoconus.

The present paper describes the macromolecular and biochemical properties of native proteoglycans isolated from metabolically and chemically labelled normal and keratoconic human cornea. Analysis of the overall mass as well as the relative molecular mass (M_r) of the protein core and the glycosaminoglycan side chains of proteochondroitin sulfate (CS-PG)/dermatan sulfate (DS-PG) and proteokeratan sulfate (KS-PG) revealed differences between normal and keratoconic cornea in the ratio of DS-PG/KS-PG[1] and in the chain length of KS chains.

Materials and methods

Chemicals

Sodium [^{35}S]-sulfate (carrier-free, 0.9–1.5 TBq/mg sulfur), [6-^{3}H]-glucosamine (spec. act., 0.37–1.11 TBq/mmol) and L-[4,5-^{3}H]-leucine (spec. act., 4.4–7 TBq/mmol) were obtained from Amersham Buchler (Braunschweig); cell culture media were purchased from Seromed (Berlin) and fetal calf serum was supplied by Boehringer (Mannheim). Chondroitin ABC-lyase (5 units/vial) and endo-β-galactosidase (keratan sulfate 1,4-β-D-galactanohydrolase (spec. act. 5 units/mg) were preparations of Seikagaku Kogyo (Tokyo) and Boehringer (Mannheim). All other chemicals were of analytic grade or the best grade available and were purchased from Merck (Darmstadt), Serva (Heidelberg) or Sigma (Munich).

Cornea

Keratoconus buttons (diameter, 7 mm) were obtained from 16 patients (32–54 years old) at the time of penetrating keratoplasty. The buttons were immediately placed in sterile corneal storage medium and stored at 4°–8° C. Normal corneas were obtained from ten age-matched patients within 24 h of death and from two eyes with melanoma and one with neuroblastoma. Buttons with a diameter of 7.0 mm were excised from the central area and stored in the same manner as the keratoconic specimens.

Tissue culture

Normal and keratoconic cornea explants were equilibrated in Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37° C. For labelling experiments, explants were incubated in 2 ml of the same medium containing 10% fetal calf serum in the presence of 10 MBq/ml [^{35}S]-sulfate, 7.5 MBq/ml [^{3h}]-glucosamine or 7.5 MBq/ml [^{3h}]-leucine for 24 h at 37° C under a gas phase comprising 95% air/5% CO_2. Then, explants were washed three times with phosphate-buffered solution (PBS) and the epithelium and endothelium were scraped off using a scalpel blade and a fine forceps. After a brief blotting the wet weight was determined and the tissue was stored at −70° C until further analysis.

Extraction of corneal stroma proteoglycans

Frozen corneas were thawed and extracted in 2 ml 4 *M* guanidinium chloride and 2% Triton X-100 buffered with 50 m*M* sodium acetate (pH 5.8) and containing fresh protease inhibitors according to Oegema et al. [24], supplemented with 1 m*M* phenylmethylsulfonylfluoride. Tissue was rotary-shaken in this solution for 48 h at 6° C. The extraction was repeated once, extracts were pooled and the residual material was dialyzed against deionized water, lyophilized and digested with papain [15] to solubilize unextracted radioactivity.

Anion-exchange chromatography

Tissue extracts were dialyzed against a solution containing 6 *M* urea and 0.5% Triton X-100 buffered with sodium acetate (pH 6.8) and containing protease inhibitors (see above) for 24 h. The extract was diluted to a conductivity of 75 µS and applied to a 1-ml DEAE-cellulose (DE 52 Whatman) column equilibrated with 50 m*M* TRIS(trishydroxymethyl aminomethane) HCl buffer (pH 7.0) containing 0.1% Triton X-100 and proteinase inhibitors (buffer A). The column was then washed with 10 ml buffer A and stepwise eluted with 50 mM TRIS-buffered 0.1M sodium chloride and 0.7 *M* sodium chloride. The total sulfated proteoglycans were eluted with 0.7 *M* sodium chloride. This fraction was reduced to a small volume and divided into two aliquots: one was degraded with chondroitin ABC-lyase according to Saito et al. [27] and the other, with endo-β-galactosidase (keratan sulfate 1,4-β-D-galactanohydrolase; EC 3.2.1.103). The digests were subjected to sodium chloride gradient chromatography on diethylaminoethanol (DEAE)-cellulose (0–0.5 *M* NaCl). A single radioactivity peak eluted from the column; it was reduced to a small volume and subjected to gel chromatography on Sepharose CL-4B columns (0.8 × 200 cm) equilibrated and eluted with 4 *M* guanidinium chloride buffered with 20 m*M* TRIS/HCl (pH 7.0) containing 0.1% Triton and proteinase inhibitors.

Chemical labelling of proteoglycans

t-Butoxycarbonyl-L-[^{35}S]-methionine *N*-hydroxysuccinimidyl ester was used as a [^{35}S]-labelling reagent (Amersham, code SJ 440). Coupling of the labelling reagent was carried out according to the instructions of the manufacturer. In all, 11.2 MBq (300 µCi) of the dried labelling reagent was cooled on ice and about 30 µg proteoglycan was added in 0.1 *M* borate buffer (pH 8.5). After 30 min, the reaction was terminated with 100 µl of 0.2 *M* glycine in 0.1 *M* borate buffer (pH 8.5). The labelled proteoglycans were then purified by rechromatography on DEAE-cellulose.

Analysis of [^{35}S]-labelled CS/DS-PG and KS-PG

The M_r of the [^{35}S]-labelled proteoglycans was calculated from the elution profile obtained from gel-filtration experiments. A Sepharose 2B column equilibrated and eluted with 0.5 *M* sodium acetate buffer and calibrated with reference proteoglycans was used as previously described [28]. The CS/DS side chains were released from the metabolically labelled [^{35}S]-DS-PG by β-elimination in 0.15 *M* NaOH for 4 h at 37° C, neutralized and chromatographed on a Sephacryl S-300 column calibrated with dextran sulfate and CS standards [28]. CS/DS side chains from non-labelled DS-PG

[1] DS-PG, Hybrid proteoglycan containing chondroitin sulfate and dermatan sulfate (due to presence of iduronic acid residues in its glycosaminoglycan chains), it is referred to as dermatan sulfate proteoglycan; KS-PG, Keratan sulfate containing proteoglycan

were released and then labelled with tritium by β-elimination in the presence of [^{3H}]-$NaBH_4$. KS side chains of [^{35}S]-KS-PG were obtained by exhaustive degradation with papain. Unlabelled KS chains were oxidized by sodium periodate and then chemically labelled by reduction with [^{3}H]-$NaBH_4$.

HPLC analysis

DS-PG samples were exhaustively digested with 60 mU chondroitin ABC-lyase in a total volume of 100 µl TRIS buffer (pH 7.4) at 37° C for 6 h. After thermal denaturation of the enzyme, the digest was lyophilized and the disaccharide mixture was solubilized with 100 µl methanol and separated by high-performance liquid chromatography according to Hollmann et al. [14].

Con-A-Sepharose chromatography

A concanavalin A (Con-A)-Sepharose suspension (Pharmacia, Freiburg) was packed into a 1-ml column and washed with 10 column volumes of starting buffer (0.02 *M* TRIS-HCl, 0.5 *M* NaCl; pH 7.4). In all, 50,000 cpm of DS-PG or KS-PG were applied to the column and proteoglycans not bound to Con-A-Sepharose were eluted with 3 column volumes of starting buffer. Bound material was eluted by 1 *M* α-D-methylmannoside in starting buffer.

Other methods

The glucuronic acid/iduronic acid ratio of DS-PG was determined by digestion of samples with chondroitin ABC- and AC-lyase in parallel followed by chromatography on Sephadex G 50. Deglycosylated DS-PG and KS-PG was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [18], using a gel gradient from 5% to 15%. Radioactivity was measured with a liquid scintillation spectrometer (Packard model 4430) using Instagel. Total hexosamine content was assayed by a standard method (see [27]).

Results

Isolation and quantitation of proteoglycans

Radiolabelled or unlabelled corneal stromata were extracted using buffered 4 *M* guanidium chloride containing Triton-X 100 and were then dialyzed in the presence of protease inhibitors. In all, 90%–92% of the bound radioactivity was recovered in the dialyzed extracts of normal and keratoconic cornea. The extracted macromolecules were applied to a DEAE-cellulose column, which was eluted sequentially with starting buffer and with a NaCl gradient. The proteoglycans were released from the column at an NaCl concentration of 0.3–0.6 *M*.

Each of the dermatan sulfate- and keratan sulfate-containing proteoglycans (DS-PG, KS-PG) eluted as a single peak on rechromatography on DEAE columns after digestion with either chondroitin ABC-lyase or β-endogalactosidase (keratanase) (Fig. 1). DS proteoglycan, which was identified by its susceptibility to chondroitin ABC-lyase, was released at an NaCl concentration slightly higher than that required for release of the KS-PG, which was sensitive to endogalactosidase.

No differences were observed in the elution profile of DS-PG and KS-PG from normal and keratoconic

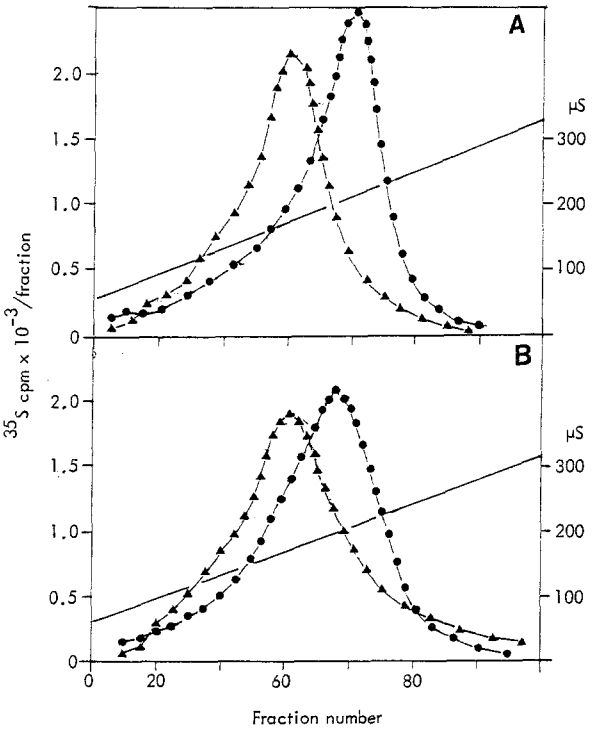

Fig. 1. Separation of DS-PG and KS-PG from **A** normal and **B** keratoconic cornea by DEAE-cellulose anionic exchange chromatogratography. ●, DS-PG; ▲, KS-PG

Table 1. Tissue concentration and specific relative radioactivity of proteoglycans from normal and keratoconic cornea. Total proteoglycans were extracted with 4 *M* guanidinium chloride, resolved into dermatan sulfate (DS)- and keratan sulfate (KS)-containing proteoglycans and monitored for hexosamine content and radioactivity. The sum of DS- and KS-containing proteoglycans was set to 100%

Cornea	PG type	DS-PG/KS-PG ratio (%)[a]	Relative specific activity[b] (cpm/mol HexN)	
			[^{35}S]	[^{3}H]
Normal	DS-PG	47.7±5.4	2.5	1
(n=4)	KS-PG	52.3±5.4	0.74	1
Keratoconic	DS-PG	56.8±5.9	3.0	1
(n=5)	KS-PG	43.2±5.9	0.90	1

[a] Mean values and standard deviation calculated on the basis of hexosamine content
[b] Double labelling with [^{35}S]-sulfate and [^{3}H]-glucosamine

corneas. However, when the relative amounts of DS-PG and KS-PG were determined on the basis of the hexosamine content, a significantly lower KS-PG value and a correspondingly higher DS-PG proportion was found in keratoconic corneas (Table 1).

In normal and keratoconic corneas the ratio of [^{35}S]-sulfate/[^{3}H]-glucosamine radioactivity was significantly higher in DS-PG than in KS-PG. However, this observation must be related to the fact that the cornea buttons

were incubated without an intact scleral rim and underwent considerable swelling under these conditions. Disturbance of the environment of corneal keratocytes is known to depress their ability to synthesize KS-PG [22]. Other minor proteoglycans in the stromal extract included proteoheparan sulfate. In [^{35}S]-labelled total stromal proteoglycans, 5%–7% of the radioactivity could be released as low-molecular-weight fragments after treatment with microbial heparitinase (Seikagaku).

M_r of DS-PG and KS-PG and their glycosaminoglycan side chains

When metabolically labelled DS-PG and KS-PG were subjected to gel chromatography on 4 B CL columns, a higher hydrodynamic volume was found for DS-PG than for KS-PG, but no significant differences in the elution profiles of proteoglycans from normal and keratoconic corneas were detectable.

This experiment was repeated with chemically labelled proteoglycans (see Materials and methods) isolated from corneas that were not preincubated under organ culture conditions. Figure 2 shows that both DS-PG and KS-PG isolated from normal cornea eluted as one peak each, but the broad elution profile suggests molecular polydispersity, which is expecially pronounced for KS-PG isolated from keratoconic cornea. Gel-filtration experiments on calibrated Sepharose CL 2B columns [28] revealed an M_r of $\approx$150 kDa for DS-PG and that of $\approx$110 kDa for KS-PG.

The M_r of DS and KS side chains was determined in corneas labelled metabolically with [^{35}S]-sulfate as well as in those labelled chemically (see Materials and methods). Table 2 shows that metabolic labelling leads to shorter glycosaminoglycan chains as compared with chemically labelled glycosaminoglycans. Especially the length of the KS chains was dramatically reduced under organ culture conditions, whereas only minor reductions in the length of the CS/DS chains was observed. A further noteworthy finding after chemical labelling of proteoglycans was a reduction in the molecular weight of the KS side chains in keratoconic cornea as compared with normal cornea (Table 2).

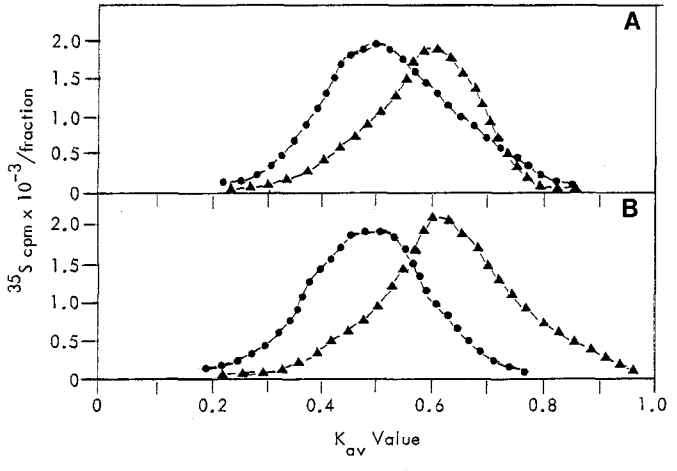

Fig. 2. Elution profile of individual [^{35}S]-labelled proteoglycans from **A** normal and **B** keratoconic cornea on Sepharose 4B-CL. DS-PG and KS-PG were isolated from corneal tissue as described in Materials and methods and then chemically labelled using [^{35}S]-*t*-butoxycarbonyl-L-methionine-*N*-hydroxysuccinimidyl ester (Amersham S J 440) as a labelling reagent. ●, DS-PG; ▲, KS-PG

Table 2. Relative molecular mass (M_r) of dermatan sulfate and keratan sulfate chains in normal and keratoconic cornea as determined by metabolic and chemical labelling of the glycosaminoglycans (see Materials and methods). M_r values were determined by gel filtration on calibrated Sepharose S 300 columns

Cornea	M_r ($\times 10^{-3}$) of glycosaminoglycan chains:			
	Metabolic labelling ($n=2$)		Chemical labelling ($n=2$)	
	CS/DS	KS	CS/DS	KS
Normal	34 38	~6	44 49	58 60
Keratonic	32 36	~6	52 38	33 44

Analysis of CS/DS side chains

Complete degradation of CS/DS chains by chondroitin ABC-lyase yielded a similar pattern for normal and keratoconic glycosaminoglycans (Fig. 3, Table 3). About 40% of the disaccharides are non-sulfated. The CS/DS ratio was determined by gel chromatography on Sephadex G 50. A DS content of 24%–28% was calculated from the radioactivity of [^{3}H]-glucosamine-labelled disaccharides (Table 4). The higher value (50% DS) calculated from [^{35}S]-sulfate labelling could be due to a differ-

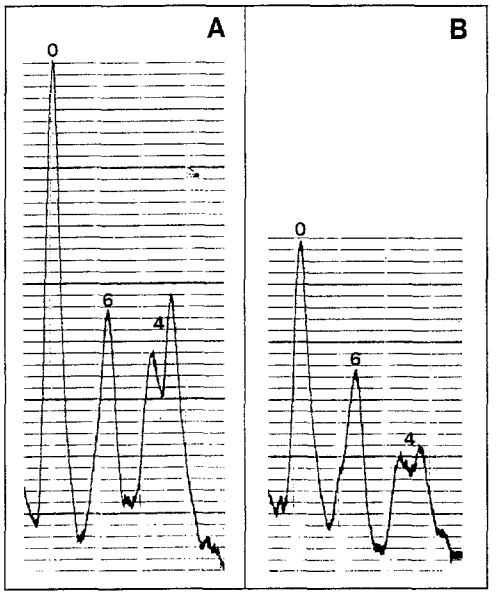

Fig. 3. Separation of unsaturated disaccharides of DS-PG from **A** normal and **B** keratoconic cornea. DS-PG was digested by chondroitin ABC-lyase, and the resulting 0-, 4- and 6-sulfated unsaturated disaccharides were subjected to HPLC (see Materials and methods)

Table 3. Separation of unsaturated 0-, 6- and 4-sulfated disaccharides on high-performance liquid chromatography (HPLC). DS/CS proteoglycans were isolated from normal and keratoconic cornea, submitted to exhaustive digestion with chondroitin sulfate ABC-lyase and applied to an HPLC column

Cornea	Percentage of sulfate ester group in position		
	0	6	4
Normal (22)	39.6	22.4	40.0
Kerato-conic (18)	38.9	28.7	32.4

Table 4. DS/CS ratio in DS/CS proteoglycans from normal and keratoconic cornea. [^{35}S]-sulfate- and [^{3}H]-GlcN-labelled DS/CS proteoglycans were digested with chondroitin sulfate lyase ABC or AC and the released di- and oligosaccharides were separated from the non-digested material by gel filtration on Sephadex G 50

Cornea	Percentage of radioactivity found in V_t after:				% DS calculated from	
	ABC degradation		AC degradation			
	[^{35}S]	[^{3}H]	[^{35}S]	[^{3}H]	[^{35}S]	[^{3H}]
Normal ($n=4$)	84±4	84±2	40±2	44±3	51	24
Kerato-conic ($n=5$)	73±5	80±1	32±4	50±5	59	28

ent degree of sulfation of the CS and DS disaccharide units. Therefore, the value found for the [^{3}H]-glucosamine-labelled disaccharides is considered to be valid.

M_r of core proteins

For determination of the M_r of core proteins, corneas were incubated with [^{35}S]-sulfate and [^{3}H]-leucine and were then processed for DS-PG and KS-PG as described. The native proteoglycan and the core proteins obtained by exhaustive digestion with chondroitin ABC-lyase or β-endogalactosidase were subjected to electrophoresis on a 4%–20% polyacrylamide gradient slab gel and were detected by subsequent fluorography (Fig. 4A, B). The core proteins of DS-PG formed a single main band at 48 kDa (Fig. 4A) and those of KS-PG formed double bands at 46 and 44 kDa. All core proteins still contained oligosaccharide units that were not attacked by chondroitin ABC-lyase or β-endogalactosidase. There were no differences between normal and keratoconic material.

Con-A-Sepharose binding

Con-A-Sepharose binds >50% of DS-PG and KS-PG (Table 5), which indicates the presence of *N*-glycosidically linked mannose-containing oligosaccharide units in these proteoglycans in addition to the glycosaminoglycan chains. Thus, DS-PG and KS-PG contain α-D-mannopyranosyl or sterically related residues [23], but

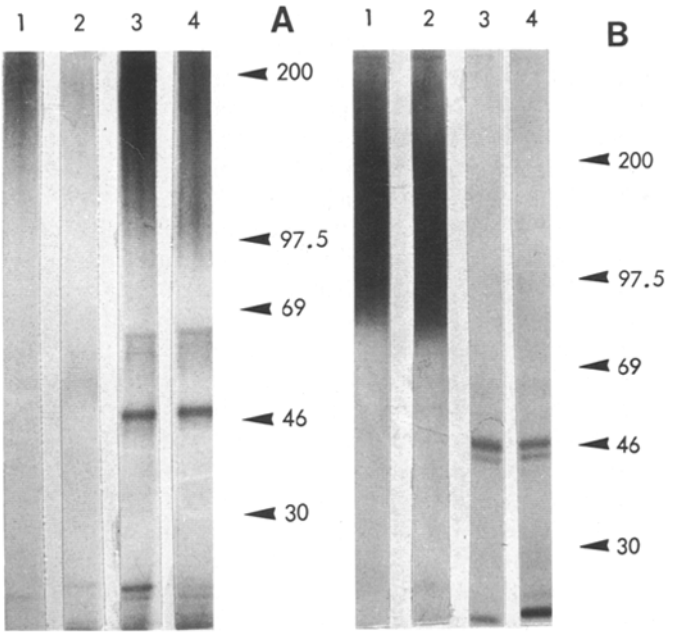

Fig. 4. A SDS electrophoresis of DS/CS-PG before and after degradation with chondroitin sulfate ABC-lyase. Keratoconic and native cornea were incubated under organ culture conditions in the presence of [^{35}S]-sulfate and [^{3}H]-leucine. *1*, Native DS/CS-PG from normal cornea; *2*, native DS/CS-PG from keratoconic cornea; *3*, DS/CS-PG from normal cornea after degradation by chondroitin sulfate ABC-lyase; *4*, DS/CS-PG from keratoconic cornea after degradation with chondroitin sulfate ABC-lyase. **B** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of keratan sulfate proteoglycan (KS-PG) before and after degradation with β-endogalactosidase (keratanase). Normal and keratoconic corneas were incubated in the presence of [^{35}S]-sulfate and [^{3}H]-leucine for 24 h. *1*, Native KS-PG from normal cornea; *2*, native KS-PG from keratoconic cornea; *3*, KS-PG from normal cornea after keratanase degradation; *4*, KS-PG from keratoconic cornea after keratanase degradation

Table 5. Binding of DS/CS-PG and KS-PG from normal and keratoconic cornea to Con-A-Sepharose. After application of the specified radioactivity and washing with equilibrating buffer, the proteoglycans bound to Con-A-Sepharose were eluted with the same buffer containing 1 *M* α-methylmannoside (α-Me mannoside)

Cornea	PG type	cpm applied to Con-A column ($\times 10^{-3}$)	cpm eluted by 1 *M* α-Me mannoside ($\times 10^{-3}$)	Bound to Con-A-Sepharose (%)
Normal	DS/CS-PG	112.0	58.3	52.0
	KS-PG	94.2	61.4	65.0
Kerato-conic	DS/CS-PG	98.1	49.1	50.0
	KS-PG	102.4	57.6	59.0

no significant differences between normal and keratoconic proteoglycans could be observed.

Discussion

In human corneal stroma, two predominant proteoglycan populations are observed, one containing dermatan sulfate (DS) and the other keratan sulfate (KS). Our data concerning DS-PG and KS-PG, their M_r, core pro-

teins and polysaccharide chains can be summarized as follows: DS-PG and KS-PG are small-type proteoglycans with a protein core bearing one (KS-PG) or two (DS-PG) glycosaminoglycan side chains. Thus, human corneal DS-PG and KS-PG appear to be similar to those isolated from the ox [2, 3, 11], rabbit [9, 10], monkey [12, 21] and embryonic chicken [19, 20].

The molecular weight was determined using chemically labelled CS-PG and KS-PG and the glycosaminoglycan chains derived therefrom, instead of metabolically labelled components. This method was chosen because metabolic labelling of cornea explants does not yield reliable data. Several investigators [6] have reported that the synthesis of KS by stromal fibroblasts (keratocytes) is lost with increasing duration of cultivation. Thus, the rate of KS-PG synthesis in explant cultures of embryonic chicken cornea drops by more than half of the value of fresh tissue within 20 h [9]. This is in accordance with our finding that the molecular weight of KS chains after metabolic labelling ($\approx$6 kDa) is considerably lower than that obtained after chemical labelling (48–60 kDa), which explains the lower specific radioactivity of KS-PG fractions relative to the DS-PG fraction (Table 1).

In addition, KS-PG synthesis is impaired in explant cultures by the considerable swelling of cornea buttons. The observed increase of 50%–80% in fluid content during in vitro incubation is assumed to be due to the absence of a scleral rim. Nakazawa et al. [22] reported that the scleral rim of cornea prevents the pronounced swelling of the corneal stroma.

Our data on the M_r of DS-PG and KS-PG and their glycosaminoglycan chains provide evidence for some differences in KS-PG and KS between normal and keratoconic corneas. Although the elution maxima of KS-PG on Sepharose 4B-CL do not differ significantly for healthy and keratoconic cornea, the tailing of KS-PG from keratoconic cornea towards lower hydrodynamic volumes (Fig. 2) would suggest a higher proportion of KS-PG molecules with a lower molecular weight. This supposition is confirmed by the molecular weight calculated for free KS chains. A reduction of about 40% in the length of KS chains relative to the length of KS chains from normal cornea was found in the two cases of keratoconus investigated after chemical labelling of the KS chains. Such a difference was not observed when the DS chains of the same corneas were compared.

Several studies have reported on altered biochemical parameters in keratoconic corneal tissue. Higher incorporation of [^{3}H]-proline into all cell layers [26], higher collagenase and gelatinase activity [17], increased content of type V collagen [32] and higher levels of protein, uronic acid and neutral hexosamine [5], as well as elevated amounts of corneal glycoconjugates [34] have been discussed as pathogenetic factors. As yet, there is no conclusive evidence as to whether any of these alterations might be associated with the pathogenesis of keratoconus or whether keratoconus is a heterogeneous disease, as has been suggested by Yue et al. [33].

The mechanical strength of the corneal collagen-fiber network depends not only on the formation of covalent cross-links between the collagen molecules but also on a precise interaction of DS-PG and KS-PG with collagen. This interaction stabilizes the collagen network and maintains the regular spacing of the collagen fibers. Taking this into consideration, it is conceivable that even minor deviations in the structure of proteoglycans could impair the stabilizing effect of proteoglycans on the collagen network and could cause an increased distensibility of corneal tissue [7, 31] when occurring over long intervals. This assumption is in agreement with the concept of a slowly progressive development of keratoconus, which requires months or years for its full expression.

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Note added in proof. While we found a reduction of about 40% in the length of KS chains in keratoconus, Funderburgh et al. concluded from solid phase immunoassay experiments that corneas with keratoconus contain a form of proteokeratan sulfate that containes fewer KS chains or in which the KS has a modified structure. (Funderburgh JL, Panjwany N, Conrad GW, Baum J (1989) Invest Ophthalmol Vis Sci 30:2278–2281)