Human Corneal Keratan Sulfates*  

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The keratan sulfate-containing proteoglycans were isolated from fourteen pooled human corneas (thirteen from 61- to 86-year-olds, plus one from a 12-year-old). These proteoglycans were subjected to digestion with the enzyme keratanase II, and the released oligosaccharides, which included nonreducing termini and repeat region oligosaccharides but not linkage regions, were reduced with alkaline borohydride and identified on two separate ion-exchange columns. Both of the latter had been calibrated with samples, most of which had been derived from bovine corneal keratan sulfate (Tai, G.-H., Huckerby, T. N., and Nieduszynski, I. A. (1996) J. Biol. Chem. 271, 23535–23546) and all of which had been fully characterized by NMR spectroscopic analysis.

The capping structures identified in human corneal keratan sulfates occurred in the relative proportions: NeuAcα(2-6) - NeuAcα(2-3) - GalNAc(S)β(1-3). The other groups of capping structures which had been identified in bovine corneal keratan sulfate, i.e. NeuGcα(2-3), NeuGcα(2-6), GlcNAc(S)β(1-3) were absent, although the possibility of the presence of some Galo(1-3)-structures could not be excluded. In addition, the human sample showed significantly higher levels of α(1-3)-fucosylated repeat region structures than did the bovine sample, and it is not clear whether this reflects a species or age dependence as the bovine corneas were from young animals, whereas the human corneas were predominantly from an older group. The charge densities and keratan sulfate chain sizes of the human and bovine keratan sulfate-containing proteoglycans were seen to be similar.

The corneal stroma is a transparent tissue predominantly comprised of regularly arranged collagen fibrils as well as small proteoglycans and matrix proteins. The proteoglycans, which are associated with the collagen fibrils (1), include the keratan sulfate (KS) and the chondroitin/dermatan sulfate (CS/DS) families. Electron microscopic studies have demonstrated that several proteoglycan binding sites lie within the D period of the collagen fibrils in the bovine cornea (2) with the KSPGs at the a and c band steps and the CS/DSFGs at the dle gap zone. Scott (3) has proposed a model for the structure of the corneal stroma in which duplexed glycosaminoglycan chains (both double-stranded CS/DS and KS) bridge collagen fibrils and maintain the precise interfibrillar distances required for transparency of the tissue.

Molecular biology studies of bovine corneal KS have shown the existence of several discrete proteoglycans. These have been named lumican (4), keratocan (5), and osteoglycin (6).

Keratan sulfate was first isolated from bovine corneal stroma by Meyer et al. (7), who subsequently classified KS types (8). Corneal KS with an N-linkage between N-acetylgalactosamine and asparagine was called KS-I, and skeletal KS with an O-linkage from N-acetylgalactosamine to serine or threonine was designated KS-II. Extensive structural studies of corneal KS showed the linkage region to be of the biantennary complex type in both bovine (9) and monkey (10) corneas. Other studies examined the distribution of sulfation along the KS chain in porcine KS (11) and characterized oligosaccharides from the repeat region in bovine corneal KS (12). Recently, a considerable diversity of capping structures such as α(2-6)- and α(2-3)-linked N-acetylenuraminic acids, α(2-6)- and α(2-3)-linked N-glycolylenuraminic acids, α(1-3)-linked galactose, as well as β(1-3)-linked and 6-sulfated N-acetylgalactosamine and N-acetylgalactosamine have been shown to be present on bovine corneal KS chains (13), but neither their functions nor their KSPG distributions are currently understood.

Despite the many structural studies there has been little investigation of human corneal keratan sulfate. Thus, this investigation seeks to extend our previous work on bovine cornea (13), and it applies keratanase II fingerprinting methodology (14, 15) to the elucidation of the capping and repeat region structures in the entire population of human corneal KSPGs.

EXPERIMENTAL PROCEDURES

Materials—All materials used in this study were described previously (13) except that papain (EC 3.4.22.2) was purchased from Sigma (Poole, Dorset, UK), a Superox 6 column (10 × 300-mm) from Pharmacia Biotech Inc. (Uppsala, Sweden), a Bio-Gel TSK-30 XL column (300 × 7.8-mm) from Bio-Rad Laboratories Ltd. (Watford, Herts., UK), an analytical Spherisorb SS SAX column (4.6 × 250-mm) from Phase Separation Ltd. (Deeside, Clwyd, UK), and an IonPac AS4A SC column (4-mm) from Dionex (Camberley, Surrey, UK).

Preparation of Human Corneal Proteoglycans—Fourteen human eyes (thirteen from 61- to 86-year-olds, plus one from a 12-year-old) were obtained from the Manchester Eye Bank and came from individuals with no history of ocular disease. The corneas had been removed within 48 h after death and stored in organ culture for periods ranging from 2 to 3 weeks. The organ culture medium (16) consisted of Eagle’s minimal essential medium with Earles salts and HEPES buffer, 2% heat-inactivated fetal calf serum, 24 mM sodium bicarbonate, 2 mM l-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.2 units/ml neomycin. The corneas were rinsed several times with phosphate-buffered saline to remove culture medium, and the corneas (with endothelium) were excised immediately, as described previously (13).

The pooled human corneas (2 g wet weight) were extracted three times each with 10 ml of 4 M guanidine-HCl containing 0.1 M 6-aminoheptonic acid, 0.01 M EDTA, 0.005 M benzamidine-HCl, and 0.05
M sodium acetate, pH 5.8, at 4 °C for 24 h. These three extracts were combined and dialysed thoroughly against 7 M urea, 50 mM Tris-HCl, pH 7.0. The dialysate was applied to a Q-Sepharose column (15 × 50-mm) and washed with sufficient 0.15 M NaCl, 7 M urea, 50 mM Tris-HCl, pH 7.0, to remove the unbound material. The bound material was eluted with a linear gradient of 0.15 M to 2 M NaCl containing 7 M urea and 50 mM Tris-HCl, pH 7.0, over 30 min at a flow rate of 1 ml/min. The eluate was monitored (Fig. 1) by absorbance at 280 nm and assayed for KSPGs by direct enzyme-linked immunosorbent assay (13) using an anti-KS monoclonal antibody, 5D4. The KSPG-containing material was recovered by dialysis against water (yield, 11 mg). In a parallel experiment, 3 g of bovine corneas (wet weight, from 4 eyes) were extracted in the same way, and 24 mg of bovine proteoglycans were isolated. These preparations of proteoglycans were directly used for keratanase II digestion and oligosaccharide fingerprinting.

A proportion of the human proteoglycans (~1.1 mg) and bovine proteoglycans (~1.2 mg) were further purified by gel permeation chromatography on a Superose 6 column (10 × 300-mm) (see Fig. 2 for human proteoglycan data). The KSPG-containing material (positive to 5D4) was recovered and used for KS chain-sizing experiments.

KS-sizing Experiment—Aliquots of the purified bovine and human proteoglycans were treated with chondroitin ABC lyase as described above. After precipitation with 3 vol of ethanol to remove CS/DS-oligosaccharides, the KSPGs were recovered and subjected to papain digestion. 100 mg of KSPGs were dissolved in 100 mM NaH2PO4, pH 7.0, containing 0.2 M NaCl, 50 mM EDTA, and 10 mM cysteine hydrochloride, and incubated with 20 milliunits papain at 65 °C for 24 h. The digests were dialysed using Spectra/Por membrane 1 (molecular mass cut-off: 6–8000 kDa protein) to remove small peptides and chromatographed on a Bio-Gel TSK-30 XL column (300 × 7.8-mm). The column was eluted with 0.15 M NaCl and monitored by absorbance at 208 nm (Fig. 3).

Characterization of Oligosaccharides Derived from Keratanase II Digestion—Crude preparations of human and bovine proteoglycans (~10 mg) were dissolved in 1 ml of 10 mM sodium acetate, pH 6.5, and incubated with 20 milliunits keratanase II. After 30 h of incubation at 37 °C the digests were heated at 100 °C for 5 min followed by centrifugation at 100,000 × g for 2 h to remove precipitate. The supernatant was chromatographed on a Bio-Gel P30 column (100 × 10-mm) to separate the oligosaccharides derived by keratanase II digestion from the intact CS/DS/PGs and the core proteins of KSPGs, which eluted in the void volume (data not shown). After reduction with NaBH4 the oligosaccharides were chromatographed on an analytical Spherisorb S5 SAX column (4.6 × 250-mm), eluted first with 2 mM LiClO4, pH 5.0, for 10 min, followed by a linear gradient from 2 to 250 mM LiClO4 within 60 min and finally from 250 to 500 mM LiClO4 within 10 min, at a flow rate of 1 ml/min and monitored by absorbance at 206 nm. Partial profiles are shown in Fig. 4. Fractions corresponding to

FIG. 1. Q-Sepharose ion-exchange chromatography of 4 M guanidine-HCl extract of human corneal stroma. Human corneal extract was loaded onto a Q-Sepharose column (15 × 50-mm) and eluted with 0.15 M NaCl, 7 M urea, 50 mM Tris-HCl, pH 7.0 (data not collected). The gradient started at fraction 20, and the eluate was monitored by absorbance at 280 nm. Fractions (2 ml) were assayed using 5D4 and pooled as shown by the horizontal bar.

FIG. 2. Superose 6 chromatography of corneal KSPG-containing fractions. Aliquots (1 mg) of the crude human corneal proteoglycans obtained as in Fig. 1 were applied to a Superose 6 HR column (10 × 300-mm) and eluted in 0.15 M NaCl, 7 M urea, 0.05 M Tris-HCl, pH 7.0, at a flow rate of 0.3 ml/min. Proteoglycans were pooled as shown by the horizontal bar.

FIG. 3. Bio-Gel TSK-30 XL chromatography of peptide-KS derived from bovine and human proteoglycans by papain digestion. The column (7.8 × 300-mm) was eluted with 0.15 M NaCl at a flow rate of 0.5 ml/min and monitored by absorbance at 208 nm. The chromatogram of free bovine corneal KS chains released by the enzy-
individual peaks, except peaks 7, 8, and 9, which were pooled, were recovered and re-chromatographed using a calibrated IonPac AS4A SC column (4-mm) as described previously (14). The chromatograms on IonPac AS4A SC of peaks 3, 4, 7, 8, 9, and 18 are shown in Fig. 5.

RESULTS AND DISCUSSION

KS Proteoglycans—The entire population of KSPGs from the human corneal stroma (plus endothelium) was prepared after dissociative extraction and initially, one stage of chromatography. This step (Fig. 1) involved ion-exchange chromatography to separate the proteoglycans from protein contaminants. The KS-containing proteoglycans from this human preparation elute at a similar salt concentration to those from the bovine preparation (13) indicating similar charge densities. The material recovered (Fig. 1, pooling bar) contained nearly all of the KS and presumably (see below) substantial amounts of decorin, although the lack of response with the antibody LF95 (data not shown) indicates that this antibody does not recognize human decorin. A small peak of 5D4-positive material is observed prior to the start of the salt gradient, which was possibly due to column overloading. The gel permeation chromatography step (Fig. 2) permitted further purification on the basis of size. The material recovered (pooling bar) contained all of the KSPGs and decorin but the procedure had removed protein contaminants such as the tyrosine-rich acidic matrix protein, TRAMP (17).

KS Chain Size—The gel permeation chromatographic behavior of the peptido-KS chains released from the entire population of human KSPGs was compared with that from the bovine KSPGs (Fig. 3), and they were seen to be of the same size. In the case of the bovine preparation the chromatogram of free corneal KS chains released by the enzyme peptide N-glycosidase F is shown. These chains were estimated (13) to have a weight average $M_w$ of 14,000 and a number average $M_n$ of about 10,000. It would, therefore, seem that these sizes also pertain to the human KS chains, although, as discussed before (13), the $M_w$ and $M_n$ values may be slight underestimates because the column was calibrated (18) with linear rather than biantennary oligosaccharides.

Keratanase II Fingerprinting—The nonreducing terminal and repeat region oligosaccharides derived from the entire population of human KSPGs after keratanase II digestion followed by borohydride reduction were examined on calibrated ion-exchange chromatography columns. The results (Fig. 4) of the chromatography on an analytical Spherisorb S5 SAX column for human and bovine samples are compared and the peaks numbered 1–21. Clearly, there are several resolution problems, particularly in peaks 7, 8, and 9. (In addition, only minor amounts of fucose-containing peaks had been observed in the bovine KSPG preparation and hence the SAX column had not been calibrated for such peaks.) Consequently, every peak from the SAX column was recovered, and these samples were individually re-chromatographed on a fully calibrated IonPac AS4A SC column. Those corresponding to peaks 3, 4, 18, and the composite of peaks 7, 8, and 9 are shown in Fig. 5. Each and every oligosaccharide fragment has been fully characterized previously by NMR spectroscopic studies and was used in calibrating the elution positions on both ion-exchange columns. Detailed structural analyses for fractions 2 and 21 have previ-
Oligosaccharides isolated from human and bovine corneal keratan sulfates

| Code | Structure | $^{\alpha}$ | $^{\beta}$ | Previous codes |
|------|-----------|-------------|------------|----------------|
| 1    | Galβ1-4(Fucα1-3)GlcNAc(S)-ol | + | + | F1 |
| 2    | Galβ1-4GlcNAc(S)-ol | + | + | R1 | 2 |
| 3a   | NeuAcc2-6Galβ1-4GlcNAc(S)-ol | + | + | 6 |
| 3b   | Galβ1-4GlcNAc(S)β1-3Galβ1-4(Fucα1-3)GlcNAc(S)-ol | + | + | F3 |
| 4a   | Galβ1-4Fucα1-3GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | F4 |
| 4b   | Galβ1-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | 7 |
| 5    | Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | R2 | 8 |
| 6    | Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | R3 | 9 |
| 7a   | NeuGcα2-6Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | – | – | 10i |
| 7b   | NeuGcα2-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | – | + | 10i |
| 8    | NeuAcc2-6Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | – | C1 | 11 |
| 9    | NeuAcc2-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | – | C2 | 12 |
| 10   | Galβ1-4GlcNAc(S)β1-3Galβ1-4Fucα1-3GlcNAc(S)-ol | + | + | R5 | 16 |
| 11   | Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | R3 | 17 |
| 12   | Galβ1-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | ? | + | Ref. 19 |
| 13   | Galβ1-4Fucα1-3GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | – | Ref. 20 |
| 14   | Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | Ref. 13 |
| 15   | Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | 18 |
| 16   | NeuGcα2-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | – | – | 19 |
| 17   | NeuAcc2-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | 19 |
| 18a  | NeuAcc2-6Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | C3 | 21 |
| 18b  | NeuAcc2-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | C4 |
| 18c  | GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | – | – | 20 |
| 19   | Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | R6 | 22 |
| 20   | NeuGcα2-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | Ref. 23 |
| 21   | NeuAcc2-3Galβ1-4GlcNAc(S)β1-3Galβ1-4GlcNAc(S)-ol | + | + | C5 |

$^{\alpha}$ Oligosaccharides present (+) or absent (−) in human corneal KS.

$^{\beta}$ Oligosaccharides present (+) or absent (−) in bovine corneal KS; ? presence uncertain.

Previously, we have examined the content of NeuAc β(1-3)-fucose (perhaps 1/10 chains) were observed. However, the bovine cornea was from young (15-month-old to 3-year-old) animals. By contrast, this study of human KS detects 2–4 times higher α(1-3)-fucose levels (see Figs. 4 and 5), but the human samples predominantly derive from older individuals (61- to 86-year-olds). Previous studies on bovine and human articular cartilage KS have shown that fucose levels increase with age, and thus it is probable that the fucose levels noted in this study relate more to age than species. The capping structures from human corneal KS do not contain NeuGcα(2-3)- or NeuGcα(2-6)-, which are generally absent from human adult tissues (23) nor was the GlcNAc(S)β(1-3)- caps found. Surprisingly, because humans do not normally have a functional α(1-3)-galactosyltransferase (22), a low level of Galα(1-3)- caps was detected and this was not believed to be a contaminant. When identified in humans the Galα(1-3)- structure is normally believed to be associated with autoimmune conditions. The relative abundance of the three major caps detected in the human corneal KS were NeuGcα(2-6)- > NeuGcα(2-3)- > GalNAc(S)β(1-3)- (although these data are not fully quantitative), and these relative proportions are similar to those observed in the bovine sample. It is interesting to note the differences here with the capping proportions in articular cartilage KS where for both bovine and human samples the content of NeuGcα(2-3)- > NeuGcα(2-6)-, and there are age-related changes in NeuGcα(2-6)- content. A further understanding of the significance of the diverse capping structures in corneal KS will require a closer description of how they are distributed between antennae and specific KSPGs.

In an as yet unreported part of these studies, we have made considerable but unsuccessful efforts to fractionate the bovine KSPGs on a preparative scale to permit characterization of the individual KS chains present. We believe that the best future chance of achieving this goal will depend upon antibody-based...
KSPG fractionation followed by high sensitivity keratanase II fingerprinting using a fluorescence-based method. Such a methodology should permit the full characterization of lumican and keratocan KS chains and may establish whether any relationship exists between specific chain capping and chain length, as the control of KS chain length in the cornea would seem to be a requirement for any precise collagen fibril spacer mechanism (3).

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