Decreased GlcNAc 6-O-Sulfotransferase Activity in the Cornea with Macular Corneal Dystrophy

Nobuko Hasegawa,1 Takayoshi Torii,2 Takuji Kato,1 Hiroaki Miyajima,5 Atsushi Furuhata,4 Kiyoo Nakayasu,1 Atsushi Kanai,1 and Osami Habuchi2

PURPOSE. Macular corneal dystrophy (MCD) is an autosomal recessive inherited disorder that is accompanied by corneal opacity. Explants from MCD-affected corneas have been reported to synthesize low-sulfated KS, suggesting that sulfate groups attached to KS may play critical roles in maintaining corneal transparency. To clear the biosynthetic defect in the MCD cornea, sulfotransferase activities were determined that are presumably involved in the biosynthesis of KS: galactose-6-sulfotransferase (Gal6ST) activity and N-acetylglucosamine 6-O-sulfotransferase (GlcNAc6ST) activity.

METHODS. Gal6ST and GlcNAc6ST activities, which were contained in the corneal extracts from corneas affected by MCD and keratoconus and from normal control corneas, were determined by measuring the transfer of 35SO4 from [35S]3-phosphoadenosine 5′-phosphosulfate into the Gal residue of partially desulfated KS and the nonreducing terminal GlcNAc residue of GlcNAcβ1-3Galβ1-4GlcNAc (oligo A), respectively.

RESULTS. The level of Gal6ST activity in corneal extracts from eyes with MCD, which was measured by using partially desulfated KS as an acceptor, was nearly equal to that in eyes with keratoconus and normal control eyes. In contrast, GlcNAc6ST activity in the extracts from MCD-affected corneas, which was measured by using oligo A as an acceptor, was much lower than in those in corneas with keratoconus and in normal control corneas.

CONCLUSIONS. The decrease in GlcNAc6ST activity in the cornea with MCD may result in the occurrence of low- or nonsulfated KS and thereby cause corneal opacity. (Invest Ophthalmol Vis Sci. 2000;41:3670–3677)

Macular corneal dystrophy (MCD) is an autosomal recessive inherited disorder that causes bilateral corneal opacity. This disorder begins in the first decade of life, manifesting as a fine, superficial, central stromal haze that spreads to the periphery and develops into multiple nodular opacities. Histologically, the disease is characterized by the accumulations of glycosaminoglycan within the keratocyte, the surrounding stroma, the subepithelial area, Bowman’s layer, Descemet’s membrane, and the endothelium.1,2 The immunohistochemical evaluations of the corneal tissue and its accumulations, together with the measurement of the level of serum keratan sulfate (KS) with a sensitive enzyme-linked immunosorbent assay (ELISA) using an anti-KS monoclonal antibody (5D4)3 has allowed us to subdivide patients with MCD into two types.4–11 In MCD type I, KS is absent from both serum and corneal tissue; in MCD type IA, although KS is absent from serum and corneal stroma, accumulations within the keratocytes react with the 5D4 antibody; in MCD type II, the serum KS level is often normal, and the corneal accumulations react with 5D4 antibody. In addition to these types, MCD types that could not be classified into these types have been reported.8 Because the 5D4 antibody recognizes the sulfate residue on the linear poly-N-acetyllactosamine sequence of KS,5,12,13 the storage materials in corneas with MCD type I have been thought to be nonsulfated or low-sulfated forms of KS proteoglycans (KSPG). The nonsulfated form of KSPG has also been demonstrated in the nasal cartilage of patients with MCD type I.5 Corneal keratocytes of patients with MCD type I are reported to synthesize nearly normal amounts of the fully glycosylated core proteins of KSPG but fail to sulfate poly-N-acetyllactosamine backbone structures.14–17 Thus MCD has been hypothesized to have some defects in the sulfotransferase activities involved in biosynthesis of KS.

KS, the major glycosaminoglycan of corneal stroma, is composed of the repeating disaccharide unit of Galβ1-4GlcNAc (poly-N-acetyllactosamine) with sulfate groups at position 6 of each sugar. The highly anionic nature of the sulfate moiety of this molecule confers a water-holding ability that contributes to maintaining corneal transparency.18 For example, lower sulfation has been suggested in scarred cornea,19 wound cornea,20 and keratoconus-affected cornea,21–25 and increasing sulfation of KSPG occurs as the cornea acquires transparency during development.24–27 Various sulfotransferases that are
likely to be involved in the biosynthesis of KS and chondroitin sulfate have been purified\(^{28,29}\) and cloned.\(^{30-35}\) These sulfotransferases show strict specificities in the type of glycosaminoglycans and the sulfation site on each sugar residue. For the biosynthesis of KS, at least two types of sulfotransferases are required: one catalyzes sulfation of position 6 of the Gal residue, and another catalyzes sulfation of position 6 of the GlcNAc residue. As the sulfotransferases that are capable of transferring sulfate to position 6 of the Gal residues of KS, we have cloned two enzymes: chondroitin 6-sulfotransferase (C6ST)\(^{30,36}\) and KS Gal-6-sulfotransferase (KSGal6ST).\(^{31}\) Human KSGal6ST was mapped to chromosome 11p11. Both enzymes also showed an ability to sulfate N-acetylatedlysaminoglycans.

Northern blot analysis,\(^{31,36}\) although expression of C6ST message was much lower in the 12-day-old chick embryo corneas than in cultured chondrocytes; therefore, it is possible that both KSGal6ST and C6ST may be involved in the biosynthesis of KS in the cornea. Sulfotransferases that are involved in the formation of (6-sulfogluco)N-acetylD-glucosamine have been cloned.\(^{32-34}\) GlcNAC 6-O-sulfotransferase (GlcNAC 6-O-ST) cloned by us, catalyzes the sulfation of position 6 of nonreducing terminal GlcNAc residue and has been mapped to chromosome 7q31.\(^{35}\) However, at present it is not clear whether either or both of these GlcNAC 6-O-STs participate in the sulfation of KS in the cornea.

To clear the mechanism by which low-sulfated KS is synthesized in MCD-affected cornea, we measured the activity of the two sulfotransferases contained in the cornea: one catalyzes the transfer of sulfate to position 6 of Gal residue (Gal6ST) and another catalyzes the transfer of sulfate to position 6 of nonreducing terminal GlcNAc residue (GlcNAC6ST). As reported previously for serum sulfotransferases,\(^{39}\) we measured Gal6ST activity and GlcNAC6ST activity using partially desulfated KS and a trisaccharide, GlcNAc\(^1\)-Gal\(^1\)-4GlcNAc (oligo A), respectively, as acceptors. As controls, we used keratoconus-affected corneas, in which sulfated KS was reported to be synthesized,\(^{16,17,40}\) and normal corneas. As a result, we found that the level of Gal6ST activity in corneas with MCD was nearly the same as that of keratoconus-affected normal control corneas, but the level of GlcNAC6ST activity in corneas with MCD was much lower than in the presence of keratoconus and in normal control. From these observations, it is possible that the reduced GlcNAC6ST activity may result in the formation of the low-sulfated KS accumulated in MCD-affected corneas.

**MATERIALS AND METHODS**

**Tissues**

Corneas from patients with MCD (\(n = 2\)) and keratoconus (\(n = 3\)) were obtained during penetrating keratoplasty. Patients with MCD were a 39-year-old man and a 42-year-old woman. Both patients had no detectable KS in the serum (<3 ng/ml; 152 ± 48 ng/ml in the normal control subjects). Patients with keratoconus were a 33-year-old, 24-year-old, and 26-year-old men. Three donors of eyes at autopsy were aged 62, 69, and 72 years; the peripheral corneas of these eyes were used as normal control corneas. All human tissues were supplied by the Juntendo Hospital, Tokyo, Japan, and the experiments followed the tenets of the Declaration of Helsinki for human experimentation. Corneal buttons (7.0 mm) removed during keratoplasty were immediately dissected. One fourth of the corneas were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde before they were frozen in optimal cutting temperature (OCT; Tissue Tek II; Baxter Scientific, Columbia, MD), and the remaining three fourths of the corneas were processed for preparation of the extracts within 12 hours after removal.

**Immunohistochemistry and Immunofluorescence Isolation and Cloning of Sulfotransferase Activity in MCD**

Cryostat sections (7 μm) were mounted on silane-coated slides, air-dried, and stained with the avidin-biotin immunofluorescence complex technique. Before staining, sections were blocked with a biotin blocking system (Dako, Carpinteria, CA) to inhibit nonspecific staining due to endogenous biotin and also with 5% normal goat serum and then were incubated with a monoclonal antibody that recognizes human KS (5D4; Seikagaku, Tokyo, Japan) at 1:400 dilution. For negative control specimens, normal mouse IgG1 or 5D4 antibody preincubated for 30 minutes with 1 mg/ml of shark KS (Seikagaku) was used in place of the primary antibody. After incubation with primary antibody, sections were incubated with biotinylated goat anti-mouse IgG antibody in PBS, rinsed in PBS for 5 minutes, and then incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Dako). Slides were mounted in antifade reagent (Anti-FluoroGuard; Bio-Rad, Hercules, CA) and photographed under an epifluorescence microscope (Carl Zeiss, Oberkochen, Germany). The measurement of serum KS levels was performed by inhibition enzyme-linked immunosorbent assay (ELISA), as has been described,\(^{4}\) with minor modifications.

**Preparation of the Extracts of Cornea**

Corneas were rinsed with PBS and homogenized with a glass homogenizer in 50 mM NaCl in buffer A (10 mM Tris-HCl [pH 7.2], 20 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM 2-mercaptoethanol, 20% glycerol, and 0.1% Triton X-100). The homogenate was centrifuged at 10,000g for 10 minutes, and the supernatant was used as the corneal extract.

**Assay of Sulfotransferases**

Gal6ST activity was determined by measuring the transfer of \(^{35}\)SO\(_4\) \(^{2-}\) from \([^{35}S]^{3'}\)-phosphoadenosine 5'-phosphosulfate (PAPS) to partially desulfated KS, because, as will be shown, \(^{35}\)SO\(_4\) \(^{2-}\) was incorporated to only position 6 of Gal residues when desulfated KS was used an acceptor. The reaction mixture contained, in a final volume of 50 μl, 2.5 micromoles imidazole-HCl (pH 6.8), 0.5 micromoles MnCl\(_2\), 0.1 micromoles 5'-AMP, 1 micromole NaF, 25 nanomoles (as glucosamine) partially desulfated KS, 50 picomoles \([^{35}S]PAPS\) (approximately 1 \(\times\) 10\(^9\) cpm), and the corneal extracts (2 μg as protein). After incubation at 20°C for the indicated time, the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 minute. The denatured proteins formed after heating were solubilized by digestion with 100 μg Pronase-P (Kaken
Seiyaku, Tokyo, Japan) for 2 hours at 37°C. 35S-labeled glycosaminoglycans were separated from 35SO4 and [35S]PAPS with the fast desalting column,28 and digested with chondroitinase ABC.41 To the chondroitinase ABC digests, two volumes of ethanol containing 1.3% potassium acetate was added, and the mixtures were centrifuged at 10,000g for 10 minutes. The radioactivity of the chondroitinase ABC-resistant glycosaminoglycans recovered in the precipitates was measured by liquid scintillation counting. Incorporation of 35SO4 into chondroitinase ABC-sensitive materials, which were presumably formed from endogenous acceptors, varied with individual cornea and fell within 4% of total 35S-glycosaminoglycans. GlcNac6ST activity was determined using GlcNacβ1-3Galβ1-4GlcNac (oligo A) as an acceptor. The reaction mixture and the incubation conditions were the same as those described earlier for Gal6ST, except that 25 nanomoles oligo A was added to the reaction mixture in place of the desulfated KS. 35S-labeled oligosaccharides were separated by gel chromatography (Superdex-30; Amersham Pharmacia Biotech). Incorporation of 35SO4 into the nonreducing terminal GlcNac residue was determined by measuring the radioactivity of (6-sulfo)2,5-anhydromannitol (AMan-ol) formed from the 35S-labeled oligo A after a reaction sequence of hydrazinolysis, deaminative cleavage, and NaBH4 reductions described later.

Analysis of 35S-Labeled Products

35S-labeled chondroitinase ABC-resistant glycosaminoglycans, which were formed from the partially desulfated KS after incubation with the corneal extracts and [35S]PAPS, were isolated as described earlier and subjected to the reaction sequence of N-deacetylation (70% hydrazine containing 1% hydrazine sulfate at 96°C for 24 hours), deaminative cleavage at pH 4, and reduction with NaBH4 as described previously.36,42 The degraded materials were separated by paper chromatography together with [3H](6-sulfo)Galβ1-4(6-sulfo)AMan-ol, [3H](6-sulfo)Galβ1-4AMan-ol, and [3H](6-sulfo)AMan-ol as the internal standards. The fractions that comigrated with [3H](6-sulfo)Galβ1-4AMan-ol and [3H](6-sulfo)AMan-ol were recovered from the paper and analyzed by high-performance liquid chromatography (HPLC; Partisil 10-SAX; Whatman, Clifton, NJ) as described later after purification with paper electrophoresis. 35S-labeled oligo A was degraded through the same reaction sequence of hydrazinolysis, deaminative cleavage, and NaBH4 reduction as described for the degradation of 35S-labeled glycosaminoglycans, except that the reaction products obtained after hydrazinolysis were purified with gel chromatography (Superdex 30; Amersham Pharmacia Biotech) and paper electrophoresis before the deamination reaction. Nonreducing terminal (6-sulfo)GlcNac, if present, should be converted to (6-sulfo)AMan-ol after the reaction sequence. The final degradation products were separated by paper chromatography together with [3H](6-sulfo)AMan-ol. The 35S-labeled materials that comigrated with [3H](6-sulfo)AMan-ol were further separated with HPLC, and the 35S-radioactivity of the peak fraction corresponding to (6-sulfo)AMan-ol was determined.

Gel Chromatography, Paper Electrophoresis, Paper Chromatography, and HPLC

The elution column (Hiload Superdex 30 16/60) was equilibrated at 1 ml/min with 0.2 M NH4HCO3. Fractions of 1 ml were collected, and the radioactivity was determined by liquid scintillation counting in 4 ml of a scintillation cocktail (Clearsol; Nakarai Tesque, Kyoto, Japan). Paper electrophoresis was performed in pyridine-acetic acid-water (1:10:400, by volume [pH 4]) at 30 V/cm for 40 minutes or 60 minutes with paper strips (2.5 × 57 cm; No. 3; Whatman; Clifton, NJ). For paper chromatography, samples were spotted on the same size paper strips (2.5 × 57 cm) and developed with 1-butanol-acetic acid-1 M NH4 (3:2:1, by volume). The dried paper strips after paper electrophoresis or paper chromatography were cut into strips (2.5 × 57 cm) and developed with 1-butanol-acetic acid-1 M NH4 (3:2:1, by volume). The dried paper strips after paper electrophoresis or paper chromatography were cut into strips (2.5 × 57 cm) and developed with 1-butanol-acetic acid-1 M NH4 (3:2:1, by volume).

RESULTS

Immunohistochemical Studies

Immunohistology of the cornea from the patients with MCD, those with keratoconus, and normal control subjects with the 5D4 anti-KS monoclonal antibody is shown in Figure 1. Stroma of normal and keratoconus-affected corneas were heavily and continuously stained (Figs. 1A, 1E). In contrast, stroma of the corneas with MCD was almost negative, although subepithelial accumulations and interlamellar linear structures were positively stained (Figs. 1F, 1G). Normal cornea stained with 5D4 antibody previously incubated with KS (Fig. 1C) or stained with normal mouse IgG1 (data not shown) was totally negative. Because the accumulations of the corneas of the patients with MCD were positive and continuous, the 5D4 antibody was not classified as having MCD type I. Stroma of the corneas of the patients with MCD were negative for 5D4, and KS level in the serum of these patients was below the detectable level (<3 ng/ml), suggesting that these patients could not be classified as having MCD type II. When the reactivity of the 5D4 anti-KS monoclonal antibody and the KS level in the serum was considered, these patients could not be classified as having any known type of MCD.

Gal-6-O-Sulfotransferase Activity

When the extracts from keratoconus cornea and partially desulfated KS were used as enzyme and acceptor, respectively, the incorporation of 35SO4 into chondroitinase ABC-resistant materials proceeded linearly up to 30 hours (Fig. 2A), indicating that under the assay conditions, the sulfotransferase remained fully active. When the 35S-labeled glycosaminoglycans formed from partially desulfated KS were subjected to the reaction sequence of N-deacetylation, deaminative cleavage, and NaBH4 reduction, two radioactive peaks were observed on paper chromatogram: one migrated to the position of [3H](6-sulfo)Galβ1-4AMan-ol and the other to the position of a mixture of [3H](6-sulfo)Galβ1-4AMan-ol and [3H]Galβ1-4(6-sulfo)AMan-ol (Fig. 3A). The faster migrating fraction was thought to be composed of monosulfated disaccharide alditols, because the fraction comigrated with [3H](6-sulfo)Galβ1-4AMan-ol and [3H]Galβ1-4(6-sulfo)AMan-ol as a single peak on paper electrophoresis (data not shown). When the monosulfated disaccharide alditol fraction was applied to HPLC (Partisil SAX-10; Whatman) together with a mixture of [3H](6-sulfo) Galβ1-4AMan-ol and [3H]Galβ1-4(6-sulfo)AMan-ol, the 35S-ra-
dioactivity was coeluted with [3H](6-sulfo)Galβ1-4Man-ol, but no radioactive peak was detected at the position of [3H]Galβ1-4(6-sulfo)AMan-ol (Fig. 3B). The slower migrating peak in Figure 4A was not examined, but it is most probable that this peak consisted mainly of (6-sulfo)Galβ1-4(6-sulfo)GlcNAc unit contained in the partially desulfated KS. These results indicate that the sulfation occurred exclusively at Gal residue and that Gal 6-O sulfotransferase activity could be detected by measuring the transfer of 35SO4 to the partially desulfated KS. Table 1 shows Gal6ST activity measured by the rates of sulfation of the partially desulfated KS, when the corneal extracts from patients with keratoconus or MCD and normal control subjects were used. These results indicate that the level of Gal6ST activity contained in the cornea with MCD was nearly equal to the level contained in keratoconus-affected and normal cornea.

GlcNAc 6-O-Sulfotransferase Activity

Because GlcNAc6ST activity could not be detected when desulfated KS was used as the acceptor and the cloned GlcNAc6-O-sulfotransferase was found to catalyze the sulfation of nonreducing terminal GlcNAc residue,32,33 we tried to detect the activity by adopting an oligosaccharide, GlcNAcβ1-3Galβ1-4GlcNAc (oligo A), as the acceptor. The incorporation of 35SO4 into oligo A using the extract of keratoconus-affected cornea proceeded linearly up to 40 hours (Fig. 2B), indicating that under the assay conditions we used here the sulfotransferase remained fully active. To determine GlcNAc6ST activity, we degraded the 35S-labeled oligosaccharide products with the reaction sequence of N-deacetylation, deaminative cleavage, and NaBH4 reduction. If 35SO4 was transferred to nonreducing terminal GlcNAc residue of oligo A, 35S-labeled (6-sulfo)AMan-ol should be released after the reaction sequence. When the 35S-labeled oligo A formed after incubation with the extracts of cornea with keratoconus (Fig. 5A) or normal cornea (Fig. 4C) were degraded, a 35S-labeled peak was obtained that comigrated with [3H](6-sulfo)AMan-ol in paper chromatography. A major part of this fraction also migrated to the position of [3H](6-sulfo)AMan-ol in paper electrophoresis (data not shown). In the HPLC system used, the retention time of the material, which comigrated with [3H](6-sulfo)AMan-ol in both paper chromatography and paper electrophoresis was exactly the same as the retention time of [3H](6-sulfo)AMan-ol and was clearly distinct from that of [3H](3-sulfo)AMan-ol (Figs. 5A, 5C). These results indicate that the extracts of cornea with keratoconus and normal cornea catalyzed the transfer of sulfate to position 6 of nonreducing terminal GlcNAc residue of oligo A.

In contrast, when the 35S-labeled oligo A formed after incubation with the extracts of cornea with MCD were degraded, the proportion of the 35S-radioactivity found in the segments of [3H](6-sulfo)AMan-ol in paper chromatography was much lower than that observed in keratoconus-affected corneas.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933216/) Immuno(..., 605.0, 786.0, image)

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933216/) Time courses of incorporation of 35SO4 into partially desulfated KS (A) and oligo A (B). The incorporation of 35SO4 into partially desulfated KS and oligo A was determined using 2 μg (as protein) of extracts from keratoconus-affected corneas.

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and normal corneas (Fig. 4B). When the fraction comigrating with [3H](6-sulfo)AMan-ol in paper chromatography was subjected to HPLC, no obvious peak of 35S-radioactivity was observed at the position of [3H](6-sulfo)AMan-ol (Fig. 5B). The same experiments using two other keratoconus-affected corneas, two other normal corneas, and one other MCD-affected cornea gave consistent results. GlcNAc6ST activity obtained from three corneas with keratoconus, two with MCD, and three normal corneas are shown in Table 1. These results suggest that the activity of GlcNAc6ST, which transfers sulfate to position 6 of nonreducing terminal GlcNAc, may be decreased or disappear in cornea with MCD. Alternatively, synthesis of putative inhibitors, which could selectively inhibit GlcNAc6ST activity, may be enhanced in corneas with MCD.

However, this possibility is unlikely, because the production of [35S](6-sulfo)AMan-ol catalyzed by the extract of keratoconus-affected cornea was not inhibited by the addition of the extract of MCD-affected cornea (data not shown). Taken together, it is most probable that in MCD the cornea has decreased activity of GlcNAc6ST, although the level of Gal6ST seems to be normal.

**DISCUSSION**

In this article, we determined the sulfotransferase activities contained in the extracts of corneas by using two acceptors: partially desulfated KS and a trisaccharide, GlcNAcβ1-3Galβ1-4GlcNAc (oligo A). When partially desulfated KS was used, only Gal6ST activity was detected in the corneal extracts from corneas with keratoconus or MCD and normal control corneas, because 35S-labeled (6-sulfo)Galβ1-4AMan-ol but not Galβ1-4(6-sulfo)AMan-ol was detected with HPLC in the monosulfated disaccharide alditol derivatives formed from 35S-labeled prod-

**FIGURE 3.** Separation of the disaccharide alditols derived from 35S-labeled partially desulfated KS with paper chromatography and HPLC. (A) Paper chromatographic separation of the degradation products formed from 35S-labeled partially desulfated KS after the reaction sequence of hydrazinolysis, deaminative cleavage, and NaBH₄ reduction. Partially desulfated KS was incubated with [35S]PAPS and the extract of keratoconus-affected cornea. Arrows: Migration position of 1, [3H](6-sulfo)Galβ1-4(6-sulfo)AMan-ol and 2, a mixture of [3H](6-sulfo)Galβ1-4AMan-ol and [3H]Galβ1-4(6-sulfo)AMan-ol. The peak fractions of monosulfated disaccharide alditols (indicated by a horizontal bar) were pooled and purified with paper electrophoresis for further analysis. (B) HPLC separation of the monosulfated disaccharide fractions from paper chromatography. The 35S-labeled monosulfated disaccharide fractions were subjected to HPLC together with 3H-labeled internal markers. Arrows: Elution position of 3, [3H](6-sulfo)Galβ1-4AMan-ol, and 4, [3H]Galβ1-4(6-sulfo)AMan-ol.

**FIGURE 4.** Paper chromatography of the 35S-labeled materials formed from 35S-labeled oligo A after the reaction sequence of hydrazinolysis, deaminative cleavage, and NaBH₄ reduction. 35S-labeled oligo A was prepared by incubation with 35 μg (as protein) of the extracts from control keratoconus-affected cornea (A), MCD-affected cornea (B), or normal cornea (C) and degraded with hydrazinolysis, deaminative cleavage, and NaBH₄ reduction. The degradation products were separated by paper chromatography together with [3H](6-sulfo)AMan-ol. (6-Sulfo)AMan-ol fractions (horizontal bars) were recovered for further analysis. (●), 35S-radioactivity; (○), 3H-radioactivity.
Table 1. Gal6ST Activity and GlcNAc6ST Activity of Control Cornea and MCD-Affected Cornea

<table>
<thead>
<tr>
<th>Extract</th>
<th>Gal6ST Activity*</th>
<th>n</th>
<th>GlcNAc6ST Activity†</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (keratoconus)</td>
<td>3.23 ± 1.16</td>
<td>3</td>
<td>0.28 ± 0.11</td>
<td>3</td>
</tr>
<tr>
<td>MCD</td>
<td>2.91 ± 0.90</td>
<td>2</td>
<td>ND†</td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td>2.44 ± 0.72</td>
<td>2</td>
<td>0.19 ± 0.05</td>
<td>3</td>
</tr>
</tbody>
</table>

Values indicate average ± SD for n = 3 or average ± range for n = 2. Data are expressed in picomoles per hour per milligram protein.

* Gal6ST activity was determined by measuring the incorporation of 35SO4 from [35S]PAPS into partially desulfated KS, because partially desulfated KS was sulfated exclusively at position 6 of the Gal residue. The reaction was continued for 5 hours.

† GlcNAc6ST activity was determined by measuring the incorporation of 35SO4 into [35S](6-sulfo)AMan-ol derived from 35S-labeled oligo A after the reaction sequence of hydrazinolysis, deaminative cleavage, and NaBH4 reduction. As indicated in the sulfation of glycoprotein oligosaccharide, GlcNAc 6-O-sulfotransferase should require the presence of nonreducing terminal GlcNAc residue in the acceptor. In contrast, when oligo A was used, activity of GlcNAc6ST, which transfers sulfate to nonreducing terminal GlcNAc residue, could be detected in the extract of keratoconus-affected and normal cornea, because 35S-labeled (6-sulfo)AMan-ol was obtained from 35S-labeled oligo A that was formed by the incubation of oligo A with the corneal extracts and [35S]PAPS. GlcNAc6ST activity was calculated from the total incorporation of 35SO4 into oligo A by multiplying the proportion of (6-sulfo)AMan-ol fraction in paper chromatography, paper electrophoresis, and HPLC. Recovery of radioactivity in each step was more than 80%. The reaction was continued for 40 hours. Five incubation mixtures were combined and analyzed. The limit of detection (50 cpm above background) was 0.006 picomoles/h/mg protein.

‡ Less than detectable limits.

FIGURE 5. Identification of [35S](6-sulfo)AMan-ol with HPLC. (6-sulfo)AMan-ol fractions (horizontal bars, Fig. 4) derived from 35S-labeled oligo A, which were formed after incubation with the extracts of keratoconus-affected control cornea (A), MCD-affected cornea (B), or normal cornea (C), were separated with HPLC. Arrows: Elution time of 1, [3H](3-sulfo)AMan-ol, and 2, [3H](6-sulfo)AMan-ol.
was not inhibited by the extracts of corneas with MCD. If the first possibility is the case, GlcNAc6ST involved in the biosynthesis of KS in the cornea should be different from GlcNAc6-O-sulfotransferase cloned previously by us, because human GlcNAc6-O-sulfotransferase is located on chromosome 7q31, whereas the MCD type 1 locus has been mapped to chromosome 16q22 by the previous linkage study. As observed in high endothelial venule-specific GlcNAc6-O-sulfotransferase, there may be an isofrom of GlcNAc6ST specifically expressed in the cornea, with a substrate specificity and amino acid sequence that may be similar to those of GlcNAc6-O-sulfotransferases cloned thus far.

We have previously determined GlcNAc6ST activity in the serum of normal control subjects and patients with MCD and have found no significant difference in the activity between control and MCD. It is thus likely that GlcNAc6ST present in the serum may be different from corneal GlcNAc6ST that was decreased in MCD. For elucidating the molecular basis of the manifestation of MCD, it is critically important to clear the molecular nature of GlcNAc6ST as well as the gene encoding GlcNAc6ST, which participates in the biosynthesis of KS in the cornea.

From the reactivity between 5D4 anti-KS monoclonal antibody and the KS level in the serum, patients with MCD in whom corneal sulfotransferase activities were determined in this report were found to be classified as neither type I nor type II. It is important to confirm the immunohistologic data with chemical analyses of the KS chain fine structure in this type of MCD.

References


**ERRATUM**


In the Note Added in Proof, the percentage of patients was incorrectly stated. The note should have read: In a follow-up test of around 20 patients suffering from occlusions of the posterior cerebral artery and associated large scotomata, about 20% were unable to perceive their visual field defects in any of the pattern of component perimetry. This test, however, employed circular stimuli of radius 30°.