

Keratan sulphate levels in mucopolysaccharidoses and mucolipidoses

S. TOMATSU^{1*}, K. OKAMURA², H. MAEDA², T. TAKETANI³, S. V. CASTRILLON¹,
M. A. GUTIERREZ^{1,7}, T. NISHIOKA¹, A. A. FACHEL¹, K. O. ORII^{4,5}, J. H. GRUBB⁴,
A. COOPER⁶, M. THORNLEY⁶, E. WRAITH⁶, L. A. BARRERA⁷, L. S. LAYBAUER⁸,
R. GIUGLIANI⁸, I. V. SCHWARTZ⁸, G. SCHULZE FRENKING⁹, M. BECK⁹, S. G. KIRCHER¹⁰,
E. PASCHKE¹¹, S. YAMAGUCHI³, K. ULLRICH¹², M. HASKINS¹³, K. ISOGAI⁵, Y. SUZUKI⁵,
T. ORII⁵, N. KONDO⁵, M. CREER¹⁴, T. OKUYAMA¹⁵, A. TANAKA¹⁶ and A. NOGUCHI³

¹Department of Pediatrics, Cardinal Glennon Children's Hospital, Saint Louis University, USA; ²Seikagaku Corporation, Japan; ³Department of Pediatrics, Shimane Medical University, Japan; ⁴Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, USA;

⁵Department of Pediatrics, Gifu University School of Medicine, Japan;

⁶Willink Biochemical Genetics Unit, Royal Manchester Children's Hospital, UK;

⁷Institute of Inborn Errors of Metabolism, Javeriana University, Colombia; ⁸Medical Genetics Service, Federal University of Rio Grande do Sul, Brazil; ⁹Department of Pediatrics, University of Mainz, Germany; ¹⁰Institute of Medical Chemistry, University of Vienna, Austria; ¹¹Laboratory of Metabolic Diseases, Department of Pediatrics, University of Graz, Austria; ¹²Department of Pediatrics, University of Hamburg, Germany; ¹³School of Veterinary Medicine, University of Pennsylvania, USA; ¹⁴Department of Pathology, Saint Louis University School of Medicine, USA; ¹⁵National Center for Child Health and Development, Tokyo, Japan; ¹⁶Osaka City University Graduate School of Medicine, Japan

*Correspondence: Department of Pediatrics, Saint Louis University, Pediatric Research Institute, 3662 Park Ave., St. Louis, MO 63110-2586, USA.
E-mail: tomatsus@slu.edu

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Summary: The mucopolysaccharidoses (MPS) is characterized by accumulation of glycosaminoglycans (GAGs), and mucolipidosis (ML) by accumulation of GAGs and sphingolipids. Each type of MPS accumulates specific GAGs. The lysosomal enzymes *N*-acetylgalactosamine-6-sulphate sulphatase and β -galactosidase involve the stepwise degradation of keratan sulphate (KS). Deficiency of these enzymes results in elevation of KS levels in the body fluids and in tissues, leading to MPS IV disease. In this study, we evaluated blood and urine KS levels in types of MPS and ML other than MPS IV. Eighty-five plasma samples came from MPS I ($n = 18$), MPS II ($n = 28$), MPS III ($n = 20$), MPS VI ($n = 3$), MPS VII ($n = 5$) and ML ($n = 11$) patients while 127 urine samples came from MPS I

($n = 34$), MPS II ($n = 34$), MPS III ($n = 32$), MPS VI ($n = 7$), MPS VII ($n = 9$) and ML ($n = 11$) patients. KS levels were determined using the ELISA method. Plasma KS levels varied with age in both control and patient populations. In all age groups, the mean values of plasma KS in MPS and ML patients were significantly higher than those in the age-matched controls. Plasma KS values in four newborn patients were above the mean + 2SD of the age-matched controls (mean, 41 ng/ml). Overall, 85.9% of individual values in non-type IV MPS and ML patients were above the mean + 2SD of the age-matched controls. For urine KS levels, 24.4% of individual values in patients were above the mean + 2SD of the age-matched controls. In conclusion, KS in blood is elevated in each type of non-type IV MPS examined, in contrast to the conventional understanding. This finding suggests that measurement of KS level provides a new diagnostic biomarker in a wide variety of mucopolysaccharidoses and mucopolysaccharidosis in addition to MPS IV.

The mucopolysaccharidoses (MPSs) are a group of lysosomal storage diseases (LSDs) caused by deficiency of the lysosomal enzymes needed to degrade glycosaminoglycans (GAGs) such as dermatan sulphate (DS), heparan sulphate (HS), keratan sulphate (KS), chondroitin sulphate (CS) and hyaluronan (Neufeld and Muenzer 2001). In the MPSs, the undegraded or partially degraded GAGs are stored in lysosomes and/or secreted into the bloodstream and excreted in urine. Mucopolysaccharidosis II or III (ML) is a disorder of the lysosomal enzyme phosphorylation and localization caused by deficiency of *N*-acetylglucosaminyl-1-phosphotransferase, leading to accumulation of GAGs and sphingolipids in cells (Kornfeld and Sly 2001). In ML, a number of biochemical defects have been observed including multiple lysosomal enzyme deficiencies in cultured fibroblasts and their presence in the cell culture medium at abnormally high levels (Tondeur *et al* 1971). ML II (so-called I-cell disease) has characteristics of both MPS and sphingolipidosis, storing GAGs and sphingolipids in the cells; however, no mucopolysacchariduria (excessive urinary excretion of GAGs) is observed (Leroy *et al* 1967). In spite of the fact that both MPS and ML involve abnormal metabolism of GAGs, neither total GAG nor a specific GAG level in blood has been measured owing to lack of an appropriate method.

The incidence of MPS and ML is estimated as 1 in 20 000–50 000 live births (Meikle *et al* 1999). In general, MPS and ML are asymptomatic in newborns, with subsequent onset of clinical signs that include abnormal development of bones, short stature and coarse hair during infancy or childhood. In some patients, mental retardation progresses gradually over years. MPSs are theoretically amenable to exogenously supplied enzymes. Clinical trials of enzyme replacement therapy on MPS I, II and VI are in progress (Kakkis *et al* 2001; Wraith *et al* 2004). Successful treatment of these disorders depends on early diagnosis and a protocol for enzyme replacement therapy that quantifies its clinical effectiveness. Identification of disease markers is of paramount importance in diagnosis and treatment.

There are established procedures for measuring total urinary GAGs using dye-spectrometric methods such as dimethylmethylene blue (de Jong *et al* 1992; Whitley

et al 2002) and alcian blue (Karlsson et al 2000). Thin-layer chromatography has been utilized for identification of each specific GAG. These methods are not applicable to blood or tissue extracts without prior protease, nuclease or hyaluronidase digestion. HPLC is a sensitive and accurate method to measure each specific GAG (Kinoshita and Sugahara 1999; Toyoda et al 1998; Yamada et al 2000). Tandem mass spectrometry is sensitive and accurate but its application is costly (Chai et al 1998; Oguma et al 2001a,b). The lysosome-associated membrane proteins were also measured as potential markers for the screening of LSD patients using the ELISA method (Chang et al 2000; Hua et al 1998; Meikle et al 1997). The newly developed ELISA assay of KS in blood and urine has been evaluated and it clearly separated MPS IVA patients from the controls, indicating a better solution compared with the dimethylene blue method (Tomatsu et al 2004). The KS concentrations correlated with clinical severity, especially with the progression of bone dysplasia (Tomatsu et al 2004).

It is generally accepted that each type of MPS is characterized by accumulation of specific GAGs (Neufeld and Muenzer 2001). MPS IV patients have deficiency of lysosomal enzymes involving the stepwise degradation of keratan sulphate (KS), leading to accumulation of KS in the body fluid. However, accumulation of KS in other types of MPS has not been considered or found, since the enzyme deficient in those types of MPS does not involve KS catabolism and no sensitive method has been available to detect KS in blood.

In this study we have evaluated KS levels in blood and urine for other types of MPS and ML patients in addition to MPS IV patients. We report, surprisingly, that all mucopolysaccharidoses and mucopolidoses are associated with elevation of KS levels.

MATERIALS AND METHODS

Materials

Eighty-five blood (plasma) samples came from MPS and ML patients except MPS IV ranging between 0 and 55 years of age (MPS I, $n = 18$; MPS II, $n = 28$; MPS III, $n = 20$; MPS VI, $n = 3$; MPS VII, $n = 5$; ML, $n = 11$). Sixty-two blood samples from MPS IV patients including 49 type A and 3 type B (2–66 years old) and 59 samples from other LSD patients (0–55 years old) were collected for comparison. Forty-five MPS IVA patients have been described previously (Tomatsu et al 2004). Four hundred and fifty control samples were collected (0–80 years old).

One hundred and twenty-seven urine samples came from MPS and ML patients except MPS IV ranging between 0 and 40 years of age (MPS I, $n = 34$; MPS II, $n = 34$; MPS III, $n = 32$; MPS VI, $n = 7$; MPS VII, $n = 9$; ML, $n = 11$). Eighty-five urine samples from MPS IV patients (1–66 years old) and 20 from other LSD patients (0–35 years old) were collected. Fifty-nine MPS IVA patients have been described previously (Tomatsu et al 2004). Four hundred and fifty control samples were also collected (0–64 years old). Overall, we collected 206 and 450 plasma samples from LSD patients and normal controls, respectively, and 232 and 450 urine samples from LSD patients and normal controls, respectively (Table 1).

Table 1A Age-dependent plasma KS (ng/ml) by ELISA method

	Mean KS (ng/ml)	SD	<i>p</i> -value*	Max.	Min.	<i>N</i>	Mean age (y)
<i>Control</i>							
Newborn ^a	41	18.6		77	2	100	0
0 < age ≤ 5	119	65.2		279	18	100	1.53
5 < age ≤ 10	239	70.2		323	80	50	6.8
10 < age ≤ 15 y	197	61		328	130	50	12.3
15 < age ≤ 20 y	158	50.5		352	22	50	26.4
Over 20 y	151	48.2		250	15	100	42.6
<i>MPS and ML patients except IVA</i>							
Newborn ^a	104	27.3	<0.0001	145	87	4	0
0 < age ≤ 5 y ^b	315	186	<0.0001	916	71	38	1.9
5 < age ≤ 10 y	634	309	<0.0001	1167	191	20	6.4
10 < age ≤ 15 y	642	320	<0.0001	1034	227	6	11.6
15 < age ≤ 20 y	724	431	<0.0001	1363	213	6	17.7
Over 20 y	613	327	<0.0001	1102	147	11	31.6
<i>Type of MPS and ML</i>							
I	254	118		517	87	18	5.5
II	742	327		1363	206	28	12.9
III	411	175		871	173	20	5.9
VI	523	113		625	401	3	4.9
VII	219	128		427	89	5	12.1
ML	340	331		1034	71	11	6.6
<i>MPS IV</i>							
IVA	569	345		1525	101	59	14.2
IVB	226	123		362	123	3	11.3
Other LSDs	130	91		435	17	59	17.1

y, year; max., maximum; min., minimum

^a Within 1 month after birth

^b Over 1 month

**p* value was calculated in comparison with the age-matched controls and patients

The urine and blood samples from the patients were collected at the following institutes: Department of Pediatrics, Shimane Medical University, Japan; Department of Pediatrics, Gifu University School of Medicine, Japan; Willink Biochemical Genetics Unit, Royal Manchester Children's Hospital, UK; Institute of Inborn Errors of Metabolism, Javeriana University, Colombia; Medical Genetics Service, Federal University of Rio Grande do Sul, Brazil; Department of Pediatrics, University of Mainz, Germany; Institute of Medical Chemistry, University of Vienna, Austria; Laboratory of Metabolic Diseases, Department of Pediatrics, University of Graz, Austria; Department of Pediatrics, University of Hamburg, Germany. The samples were sent to the Department of Pediatrics, Saint Louis University, for further analysis.

All the patients showed enzyme activity below 5% normal activity and one specimen of urine and/or blood was taken from each patient. Written informed consent was obtained from each patient at entry to the study at each institute. The study protocol was approved by the institutional review board at Saint Louis University.

Table 1B Age-dependent urine KS (mg/gCre) by ELISA method

	Mean KS (mg/gCre)	SD	p-value*	Max.	Min.	N	Mean age
<i>Control</i>							
0 < age ≤ 5 y	0.23	0.16		0.97	0	200	1.1
5 < age ≤ 10 y	0.21	0.13		0.49	0.07	50	6
10 < age ≤ 15 y	0.29	0.14		0.62	0.05	50	11.8
15 < age ≤ 20 y	0.14	0.14		0.2	0.07	50	16.2
Over 20 y	0.13	0.13		0.42	0.03	100	38.5
<i>MPS and ML patients except IVA</i>							
0 < age ≤ 5 y	0.51	0.39	<0.0001	2.2	0.055	69	2
5 < age ≤ 10 y	0.4	0.27	<0.0001	1.05	0.06	32	6.3
10 < age ≤ 15 y	0.6	0.55	<0.001	1.86	0.22	8	11.8
15 < age ≤ 20 y	0.32	0.24	<0.0001	0.78	0.075	6	18
Over 20 y	0.28	0.23	<0.0001	0.88	0.075	12	27.7
<i>Type of MPS and ML</i>							
I	0.48	0.4		1.9	0.06	34	4.1
II	0.49	0.33		1.86	0.17	34	10.1
III	0.42	0.28		1.05	0.06	32	5.1
VI	0.56	0.73		2.19	0.09	7	5
VII	0.3	0.26		0.93	0.08	9	14.2
ML	0.53	0.36		1.23	0.13	11	5.9
<i>MPS IV</i>							
IVA	9.1	9.75		46.3	0.11	78	12.7
IVB	2.6	2.55		7.02	0.5	7	16.9
Other LSDs	0.63	0.79		3.05	0.01	20	11

*p value was calculated in comparison with the age-matched controls and patients

Methods

Sandwich ELISA assay: The KS standards for ELISA calibration and the anti-KS monoclonal antibody (5-D-4) (Tomatsu et al 2004) were obtained from Seikagaku (Japan). The ELISA procedure was performed as previously described (Cateron et al 1983). The absorbance was measured at 450 nm with microplate spectrophotometer reference to 650 nm. The KS concentration was read by applying the absorbances of each sample to the calibration curve.

For urine samples, creatinine was measured by mixing 10 µl of a 10-fold diluted urine sample with 50 µl saturated picric acid (Sigma, St Louis, MO, USA) and 50 µl 0.2 mol/L NaOH. Absorbance at 490 nm was read after 20 min and compared to a standard. We evaluated the cross-reactivity (%) with other KS-related GAG compounds including HS, DS and CS obtained from Seikagaku in an acid mucopolysaccharide (AMPS) kit (#400610). The KS assays all showed cross-reactivity of <0.1% against HS, DS and CS compared with the same concentration of KS. Repeated freezing at -20°C and thawing of the plasma did not affect the level of KS detected.

Keratanase II digestion: To confirm that the anti-KS monoclonal antibody did not recognize any other GAG except KS, the keratanase II treatment, which digests

KS specifically, was performed for the urine and plasma samples. The enzyme solution was prepared by dissolving keratanase II (Seikagaku, #100812) in 50 mmol/L Tris-HCl buffer (pH 7.0) at a concentration of 500 mU/ml. Keratanase II digestion was initiated by adding keratanase II to the plasma and urine samples to give a final concentration of 5 mU/ml. The reaction mixture was then incubated for 3 h at room temperature, followed by the ELISA.

HPLC assay: To prove elevation of KS in specimens of MPS and ML, HPLC assay was also used for measurement of plasma and urine KS concentration. Each 0.5 ml aliquot of the urine sample was chromatographed on a Q Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden) and desalted with a PD-10 column (Pharmacia). This fraction was concentrated to 0.2 ml with a centrifugal evaporator centrifuge EC-57C (Sakuma Seisakusyo Ltd, Tokyo, Japan). Each 50 μ l aliquot of the concentrated urine or plasma samples was pretreated with 1 mU of keratanase II in 200 μ l of 0.02 mol/L sodium acetate buffer (pH 6.0) at 37°C for 3 h, which produced saturated monosulphated disaccharide of KS (di-mono-KS) and saturated disulphated disaccharide of KS (di-di-KS). The di-mono-KS and di-di-KS were ultrafiltered using an Ultrafree C3GC system (molecular size cut-off 10 000; Japan Millipore Ltd, Tokyo, Japan). HPLC analysis of di-mono-KS and di-di-KS was performed as previously described (Caterson et al 1983; Shinmei et al 1992). The area of each peak corresponding to di-mono-KS and di-di-KS was calculated by the integrator and converted to an amount of KS against the areas of standard di-mono-KS and di-di-KS from bovine cornea KS digested with keratanase II (Yoshida et al 1989).

Data analysis: The data obtained were analysed to determine whether the levels of KS varied significantly with respect to age and each type of MPS and ML. Student's *t*-test or Welch's *t*-test was used to compare KS levels in the patient samples with the control samples with age dependency. Correlation in blood (or urine) KS concentrations assayed by ELISA and HPLC was evaluated using regression plot (Pearson's correlation coefficient). The mean value for KS levels in each type of MPS or ML was compared with that of the controls by one-way ANOVA, followed by Dunnet's post-test analysis. The KS levels were also each compared across type of MPS or ML by one-way ANOVA, followed by Bonferroni's post-test analysis.

All data analyses were performed with Statview statistical software (Abacus Concepts, Inc., Berkeley, CA, USA).

RESULTS

Plasma KS concentrations: The age groups in the control and patient (MPS and ML except MPS IV) populations were divided into newborn, 0–5 years, 5–10 years, 10–15 years, 15–20 years, and over 20 years (Table 1A).

The level of plasma KS varied with age in both control and patient populations (Table 1A). In the control samples, the plasma KS values in each age group did not show the normal distribution pattern, the lowest KS level was observed in the newborn period (mean 41 ng/ml). The mean value of plasma KS level in the control

population peaked between 5 and 10 years (mean 239 ng/ml) and thereafter gradually became lower. In the patient samples, the lowest KS level was also observed in the newborn period (mean 104 ng/ml). Thereafter, mean plasma KS levels were over 600 ng/ml in the 5–10 years group and subsequently over 600 ng/ml in all age groups. The mean values of plasma KS in each age group showed significant differences between the control and patient groups, as shown in the fourth column of Table 1A.

There was a clear discrimination for an individual patient sample when it was compared to the age-matched control group. Plasma KS values in four newborn patients were above the mean + 2SD (78.2 ng/ml) of the age-matched controls. The four newborn patients were two diagnosed with MPS I, one with MPS VII and one with ML II (plasma KS 87, 145, 89, and 98 ng/ml, respectively).

The level of plasma KS was also compared between each type of MPS and ML and the age-matched controls (Table 1A and Figure 1). Plasma KS levels in MPS I showed that 16 out of 18 (88.8%) patients had above the mean + 2SD of the age-matched controls (Figure 1A). Thirteen of 15 (86.7%) patients with a severe form and all three patients with an attenuated form showed plasma KS levels above the mean + 2SD. MPS II patients had the highest mean KS in plasma among all types of MPS and ML patients (mean 742 ng/ml). All patients except one with a severe form (27 out of 28: 96.4%) had plasma KS values above the mean + 2SD of the age-matched controls (Figure 1B). Plasma KS values in 16 of 20 (80%) MPS III patients were above the mean + 2SD of the age-matched controls (Figure 1C). Twelve of 15 (86.7%) patients with a severe form and 2 of 3 patients with an attenuated form showed plasma KS levels above the mean + 2SD. All three MPS VI patients revealed plasma KS levels above the mean + 2SD (Figure 1E). None of three MPS VII patients with an attenuated phenotype had plasma KS levels above the mean + 2SD, while two patients less than 2 months old with hydrops fetalis and developmental delay showed plasma KS levels above the mean + 2SD (89.1 and 427 ng/ml). Plasma KS values in 9 of 11 (82%) ML patients, ranging between 71 and 1034 ng/ml, were above the mean + 2SD of the age-matched controls. Finally, 85.9% of individual values in other MPS and ML patients except MPS IV plotted above the mean + 2SD of the age-matched controls. Fifty of 62 (80.6%) MPS IV patients had plasma KS levels above the mean + 2SD of the age-matched controls (Figure 1D). These findings suggest that the magnitude of plasma KS elevation in these patients was comparable to that in MPS IV patients.

In the samples from the other 59 LSD patients, the mean KS value was 130 ng/ml (Figure 1F) and only 6 patients (10.2%) had plasma KS values above the mean + 2SD of the age-matched controls. Therefore, the current method does not seem to be suitable for screening of other LSD patients.

Urine KS concentrations: The age groups in the control and patient (MPS and ML except MPS IV) populations were divided into 0–5 years, 5–10 years, 10–15 years, 15–20 years, and over 20 years (Table 1B). The level of urine KS changed with age in control and patient populations (Table 1B). In the control samples, the urine KS values in each age group did not show the normal distribution pattern; the lowest urine KS level was observed in the over 20 years group (mean 0.13 mg/g creatinine),

while the highest value was in the 10–15 years group (mean 0.29 mg/g creatinine; $p < 0.001$). In the patient samples, the lowest urine KS level was also observed in the over 20 years group (mean 0.28 mg/g creatinine), while the highest was in the 10–15 years group (mean 0.6 mg/g creatinine). The mean KS values in both control and patient populations over 20 years of age were decreased at 0.13 and 0.28 mg/g creatinine, respectively. Throughout all the age groups, the patient group had

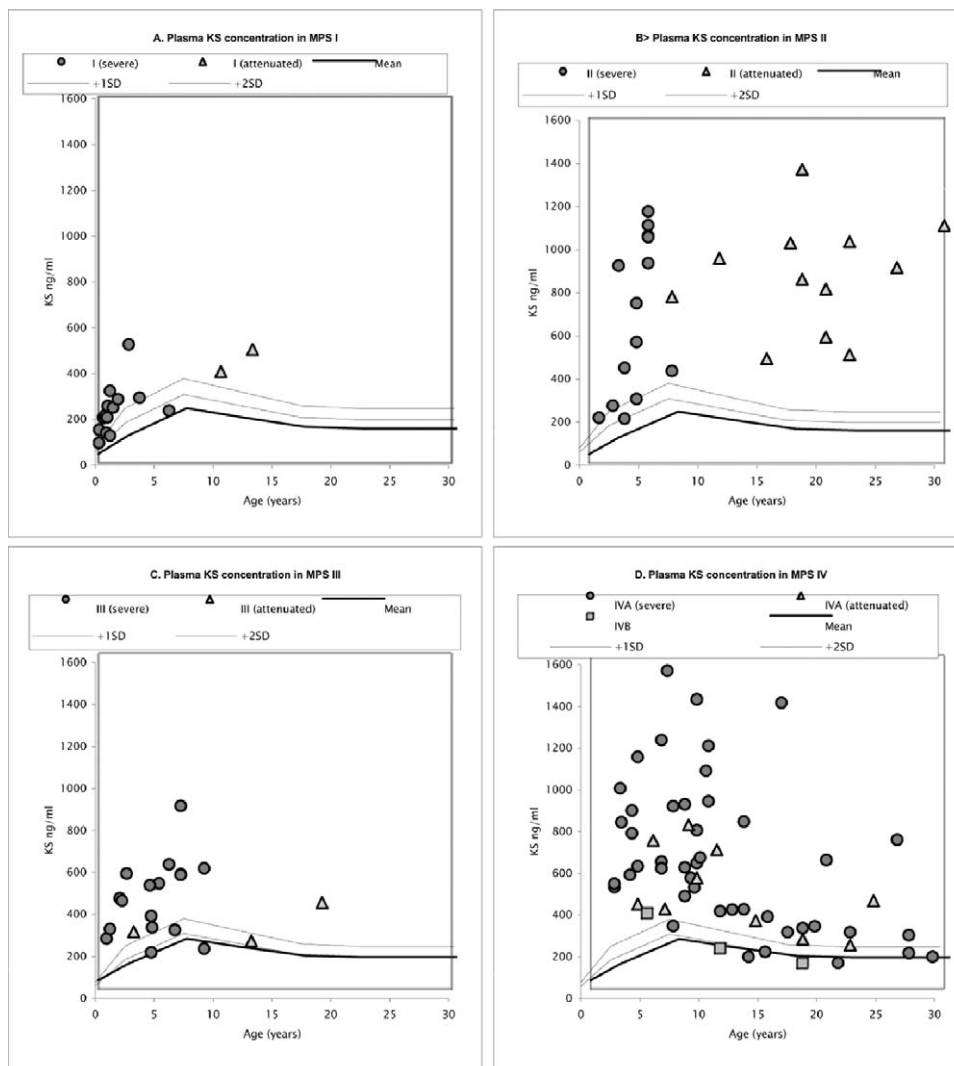


Figure 1 Concentrations of KS in plasma of patients with MPS, ML and other LSDs, and normal individuals. Results of all specimens from each individual were plotted with respect to age. The age was shown up to 30 years in each disease group

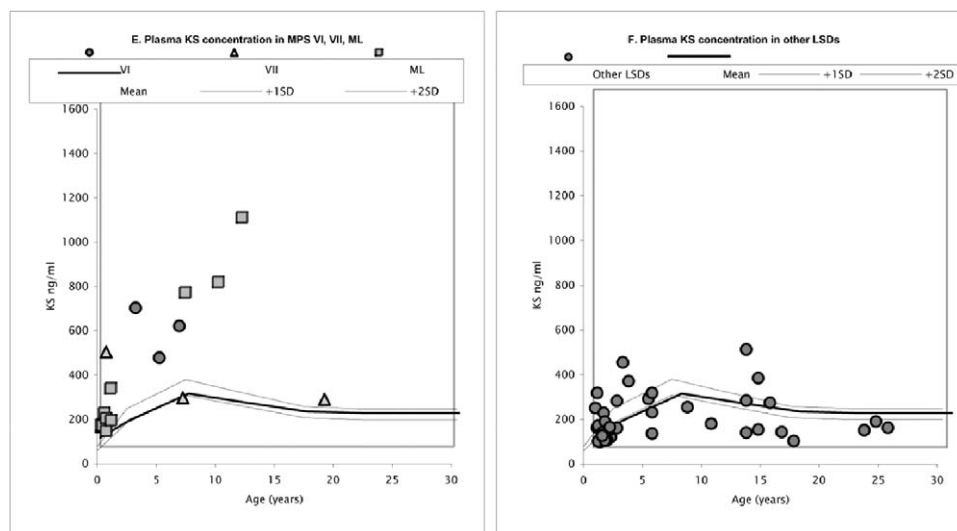


Figure 1 Continued

a significantly higher urine KS level compared with the age-matched control group, as shown in the fourth column of Table 1B.

When each type of MPS and ML and the age-matched controls (Table 1B and Figure 2) were compared, urine KS levels in MPS I showed that 6 out of 34 (17.6%) patients had above the mean + 2SD of the age-matched controls (Figure 2A). Twelve of 34 (35.3%) MPS II patients had urine KS levels above the mean + 2SD of the age-matched controls (Figure 2B). Urine KS values in 6 of 32 (18.8%) MPS III patients were above the mean + 2SD of the age-matched controls (Figure 2C). Only 1 of 7 MPS VI patients revealed urine KS level above the mean + 2SD (Figure 2E). One of 9 MPS VII patients showed urine KS levels above the mean + 2SD. Urine KS values in 5 of 11 (45.5%) ML patients were above the mean + 2SD of the age-matched controls. Overall, 24.4% of individual values in other MPS and ML patients plotted above the mean + 2SD of the age-matched controls, suggesting that urine KS concentration is not a suitable indicator as a screening test for these types.

In contrast, the mean values in MPS IVA and MPS IVB were 9.1 and 2.6 mg/g creatinine with 76 of 78 (97.4%) MPS IVA and 6 of 7 (85.7%) MPS IVB patients above the mean + 2SD of the age-matched controls (Figure 2D). Comparing plasma and urine KS levels in each type demonstrated that there is a difference in the blood KS to urine KS ratio between MPS IV patients and other MPS and ML patients.

Additionally, 6 of 20 (30%) other LSD patients had urine KS level above the mean + 2SD of the age-matched controls (Figure 1D).

To confirm that the elevation observed in MPS and ML patients is derived specifically from KS, the specificity of the immunoassay for KS was assessed by determining the cross-reactivity (%) to structurally related compounds. The KS assays all showed cross-reactivity of <0.1% against HS, DS and CS compared with the same

concentration of KS (Figure 3). We also pretreated the plasma and urine specimens with keratanase II and repeated the ELISA. No KS was detected in any enzyme-treated sample, suggesting that elevation of KS measured by the current ELISA assay is derived from KS but not from other GAGs.

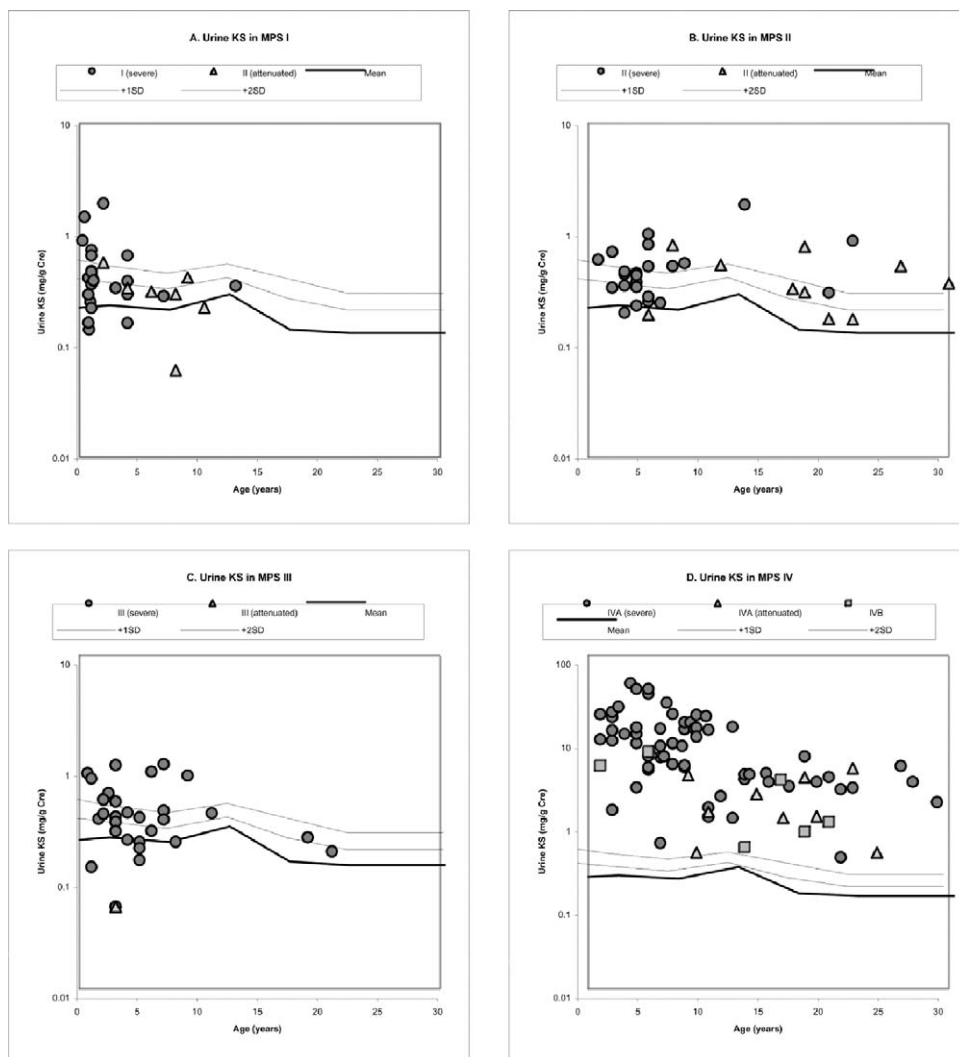


Figure 2 Concentrations of KS in urine of patients with MPS, ML and other LSDs, and normal individuals. Results of all specimens from patients and normal individuals were plotted on a semilogarithmic scale with respect to age. The scale of the KS concentration is different in MPS IVA. The age was shown up to 30 years in each disease group

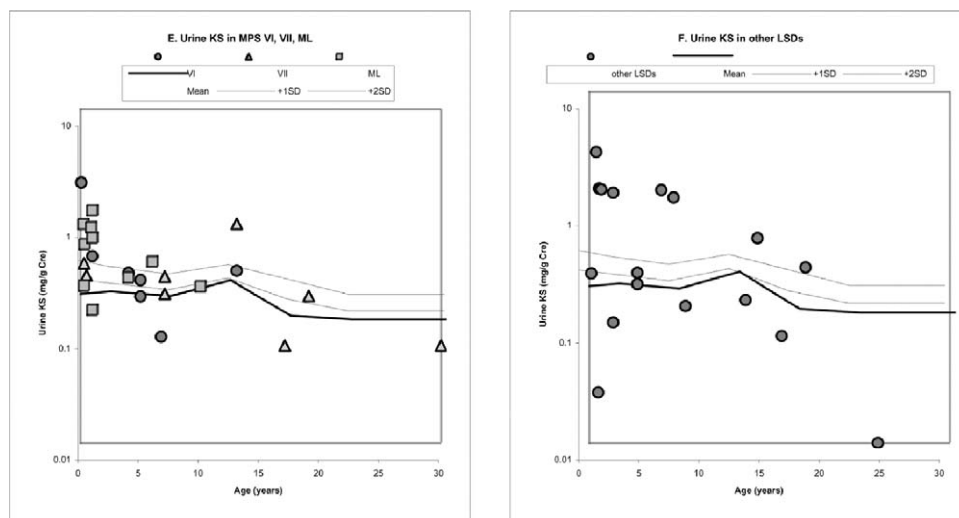


Figure 2 Continued

HPLC analyses: Eleven blood and 16 urine specimens of LSD patients were analysed by HPLC. Each specimen was analysed by the sandwich ELISA assay to confirm an elevated KS concentration. The unsaturated disaccharide isomers formed by sample digestion with keratanase II were detected by HPLC when the

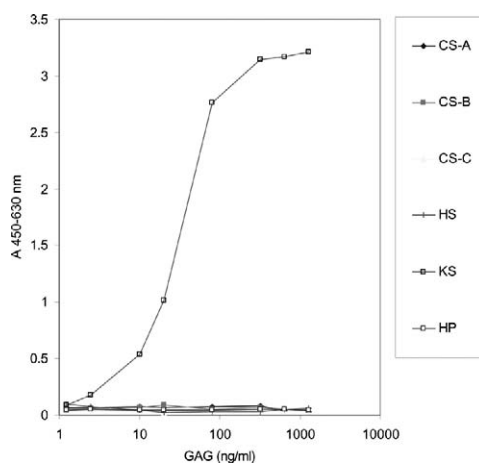


Figure 3 Cross-reactivity of KS-ELISA to KS-related GAGs. The absorbances were plotted against the concentration of each GAG sample. Abbreviations: CS-A, chondroitin sulphate A (Seikagaku # 400658); CS-B, chondroitin sulphate B (Seikagaku # 400660); CS-C, chondroitin sulphate C, (Seikagaku # 400670); HS, heparan sulphate (Seikagaku # 400700); KS, keratan sulphate (Seikagaku # 400610); HP, heparin (Seikagaku # 400610)

mono- and di-sulphated disaccharide isomers were eluted with increasing salt. Total KS was calculated as shown in Table 2 for plasma and urine samples. Tables 2A and 2B show the results of KS concentration obtained by ELISA and by HPLC. The HPLC results of plasma samples showed elevated KS in all types of MPS and ML specimens compared with normal controls. HPLC analysis for urine KS showed 10–40 times higher concentrations in MPS IVA and IVB patients and 2–7 times higher concentrations in other MPS and ML patients as compared to controls. The ratio of ELISA to HPLC in plasma revealed the higher value in normal controls as compared with the patients, while that in urine was lower in the control as compared with the MPS IVA patients. The regression plot between the ELISA and HPLC assays showed a correlation coefficient of 0.855 ($p < 0.0001$) in plasma and 0.925 ($p < 0.0001$) in urine. Thus, the HPLC analyses confirmed the elevation of KS in MPS and ML specimens.

DISCUSSION

There are well-known relationships between types of MPS and GAGs that accumulate (Neufeld and Muenzer 2001). Elevation of KS level in blood or urine was considered the hallmark of MPS IVA. To our knowledge, this is the first report demonstrating that MPS disorders other than MPS IV can be associated with elevated blood and urine KS levels in addition to the GAGs originating from the respective enzyme defect. The presence of excessive amounts of KS is also observed in ML patients.

The majority of KS is produced in the cartilage tissue and secreted in blood. MPS IVA is caused by deficiency of the enzyme directly involved in KS catabolism, leading to accumulation of undegraded KS in cartilage tissues. The resulting accumulation of undegraded KS damages the cartilage proteoglycans and increases KS levels in the body fluids. The extent of elevated KS in blood and urine in MPS IVA correlates positively with clinical severity (Tomatsu *et al* 2004).

The mechanism by which the KS is elevated in all other types of MPS and ML is unclear since the current theory on the pathway of KS metabolism cannot explain this phenomenon. Several hypotheses can be offered to explain elevation of blood KS in other MPS and ML patients: (a) the synthesis of KS may be stimulated by storage of other GAG(s); (b) the elevation of KS may be a secondary consequence of the bone dysplasia and damage to the cartilage tissue produced by accumulation of the other GAGs; (c) KS may be co-deposited with the other accumulated GAGs, which inhibits the interaction between KS and enzymes catabolizing KS; and/or; (d) alterations in the extent and distribution of fucosylation, sialylation and sulphation on KS secondary to GAG accumulation may make KS resistant to degradation. KS derived from articular cartilage contains sialic acid and fucose. Sialic acid residues, present as chain caps, and fucose residues inhibit degradation of KS molecules (Tai *et al* 1994). All of these factors may contribute.

Comparisons of KS in urine showed a less striking elevation in all MPS disorders compared with blood, except in MPS IV in which the KS substrate is a direct result of the enzyme deficiency. The lower elevation of KS in urine specimens of other

Table 2A HPLC analyses of plasma from MPS and ML patients

No.	Type	Age (y)	KS (ng/ml) (by ELISA)	KS (µg/ml) (by HPLC)	Ratio ELISA/HPLC
1	I	2.0	679	6	0.11
2	II	5.0	1100	5.1	0.21
3	II	5.0	1050	3.59	0.29
4	IIIA	2.0	419	2.88	0.15
5	IIIC	13.0	227	2.11	0.11
6	IVA	16.2	1370	4.46	0.3
7	IVA	6.5	1530	7.75	0.2
8	VI	3.0	625	2.12	0.29
9	VII	0.5	427	3.42	0.12
10	MLII	0.9	263	1.87	0.14
11	MLIII	12.0	1030	3.88	0.27
12	Normal	3.0	166	0.3	0.55
13	Normal	5.0	209	0.4	0.52
14	Normal	41.0	127	0.13	0.98
15	Normal	40.0	113	0.12	0.94

MPS and ML patients compared to the elevation in blood could possibly be explained by amounts of KS aggregated with other GAGs or unknown factors in the bloodstream too large to be cleared in the urine. In this scenario, undegraded KS that is not filtered out by the kidney is retained in the blood, leading to a less

Table 2B HPLC analyses of urine from MPS and ML patients

No.	Type	Age (y)	KS (mg/gCre) (by ELISA)	KS (mg/g Cre) (by HPLC)	Ratio ELISA/HPLC
1	I	2	2.6	22.5	0.12
2	I	2	1.9	26.2	0.07
3	II	4	0.63	13.1	0.05
4	II	2	0.7	12.6	0.06
5	II	13	1.86	14	0.13
6	IIIA	4	1.61	13.7	0.12
7	IIIA	3	1.03	16.4	0.06
8	IIIB	8	0.65	8.57	0.08
9	IIIB	10	0.64	7.81	0.08
10	IVA	10	46.2	125	0.36
11	IVA	4	39.7	119	0.33
12	IVA	2	18.2	107	0.17
13	IVB	5	7.02	41.6	0.17
14	MLIII	10	1.25	13.7	0.09
15	GMI	5	3.41	58.1	0.06
16	Fucosidosis	2	1.37	26.6	0.05
17	Normal	4	0.35	5.82	0.06
18	Normal	6	0.25	5.9	0.04
19	Normal	8	0.45	3.36	0.13
20	Normal	13	0.31	3.17	0.1

remarkable increase in urine. This hypothesis (compatible with (c) and (d) above) may explain why previous studies by thin-layer chromatography could not detect any increase of urine KS in other mucopolysaccharidoses.

Most MPS and ML patients have severe bone dysplasia as in MPS IV. Therefore, elevated KS in the blood of other MPS and ML patients could relate to underlying bone disease, especially cartilage tissues, agreeing with hypothesis (b). Since the bone disease is difficult to treat, the successful reduction of the KS could provide more specificity for the bone pathology of MPS disease than the originally directly stored substrates HS or DS. It would be useful to further investigate the KS levels in the blood as a biomarker of other mucopolysaccharidoses.

The elevation of blood KS was observed in all types of MPS and ML patients compared with the age-matched controls. Whether KS concentrations are also elevated in affected newborn babies is an important question from the viewpoint of early detection of the diseases. Although MPS and ML are progressive disorders that often take years to present clinically, there is considerable evidence from both humans (Crow *et al* 1983; Wiesmann *et al* 1980) and animal models (Crawley *et al* 1997) that biochemical storage commences early in gestation and is well advanced by the time of birth. Other evidence from previous studies using lysosomal membrane proteins also indicates that the storage process begins early in gestation and can be well advanced by birth. The preliminary data from cat and dog MPS models also showed findings similar to those in human MPS patients, such as elevation of KS and age-dependency of KS (data not shown). The presence of increased blood KS in all four newborns of the patient group in the present study suggests that, at least in these individuals, the KS is stored during fetal life. Although these preliminary results support the usefulness of KS as a screening marker at an early stage of MPS and ML, further studies on the newborn population will be required to confirm the usefulness of KS. Interestingly, other patients with fucosidosis, GM₁-gangliosidosis, sialidosis, or galactosialidosis also showed an increase in urinary KS. It is well known that those disorders store predominantly oligosaccharide derivatives. This observation suggests that sulphated oligosaccharides derived from KS also accumulate in these disorders. Future studies will examine the correlation between blood and urinary KS levels for these disorders.

The specificity of the assay for KS was demonstrated using keratanase II treatment. The HPLC assay method for KS was compared with ELISA and showed that the ELISA-based method produced lower levels. This discrepancy in KS concentrations between ELISA and HPLC may have several explanations. The anti-KS monoclonal antibody used here recognizes more than six oligosaccharides, while HPLC can detect disaccharides produced by keratanase II digestion. The anti-KS monoclonal antibody recognizes polysulphated KS, binding more tightly to sulphated sugar but binding less to low-sulphated KS. These findings accounted for higher KS values in HPLC as compared to ELISA. The ratio of KS by ELISA to KS by HPLC in plasma of MPS and ML was constant between 0.1 and 0.3, while the KS ratio in plasma of controls showed a higher value. This may reflect the differences in age, sulphation and molecular size of KS between patients and controls. Further studies are needed to clarify the mechanism in a larger number of samples.

On the basis of these results, we propose that blood and/or urine KS levels may be useful markers for early diagnosis and for monitoring clinical responses to treatment in most MPS and ML individuals. We are currently evaluating whether other GAGs such as heparin sulphate, chondroitin sulphate and dermatan sulphate can be used as adjunctive markers that may increase the sensitivity for this purpose.

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S.T. and K.O. contributed equally to this work.

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