J. Biochem. 119, 697-702 (1996)

Characterization and Biological Significance of Sialylα2-3Galactosylβ1-4Xylosylβ1-(4-Methylumbelliferone) Synthesized in Cultured Human Skin Fibroblasts[1]

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Received for publication, October 9, 1995

Human skin fibroblasts were incubated in the presence of a fluorogenic xyloside, 4-methylumbelliferyl-β-D-xyloside (Xyl-MU), then the cultured medium was recovered, concentrated with a lyophilizer, and dialyzed against distilled water. The structures of the Xyl-MU derivatives purified from the dialyzable fraction were investigated. In addition to established glycosaminoglycans-MU (GAGs-MU), Gal-Gal-Xyl-MU, Gal-Xyl-MU, sulphate-GlcA-Xyl-MU, GlcA-Xyl-MU, and Xyl-Xyl-MU, which were induced by Xyl-MU, an oligosaccharide having fluorescence was purified using a combination of gel filtration, ion-exchange chromatography and high-performance liquid chromatography, then subjected to carbohydrate composition analysis, enzyme digestion, Smith degradation, ^{1}H-NMR, and ion-spray mass spectrometric analysis. From the data obtained, the oligosaccharide was considered to have the structure SAα2-3Galβ1-4Xylβ1-MU. The amount of MU-oligosaccharide in the cell culture increased with time and was dependent on the amount of Xyl-MU added. Its production was also different from that of Gal-Gal-Xyl-MU and Gal-Xyl-MU, which are biosynthetic intermediates of GAG-MU. Addition of CDP, an inhibitor of sialyltransferase, to the cell culture medium increased the secretion of GAG-MU. These results suggest that SA-Gal-Xyl-MU production may be related to the regulation of GAG-MU biosynthesis.

Key words: cultured human skin fibroblast, glycosaminoglycan, 4-methylumbelliferyl-β-D-xyloside, SAα2-3Galβ1-4Xylβ1-(4-methylumbelliferone).

Sulphated glycosaminoglycans (GAGs) are covalently attached to the core protein through a linkage region, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser (*1*). Although the biosynthesis of GAGs in living tissue is not yet fully understood, it is probably initiated by transfer of xylose from UDP-xylose to the serine residue of the core protein, followed by stepwise addition of individual monosaccharides from UDP-sugars by a series of glycosyltransferase reactions. The sulphate groups are incorporated after formation of the GAG backbone, sometimes in concert with other polymer-modification reactions (*2-5*).

β-Xylosides such as 4-methylumbelliferyl-β-D-xyloside (Xyl-MU) have been shown to stimulate the synthesis of GAGs by cultured cells from a variety of tissues (*6*). It is very likely that exogenous β-xylosides act as artificial initiators of GAG chain elongation, in competition with the endogenous xylosylated core protein. As a result, the Xyl-MU-induced GAG (GAG-MU) is secreted into the medium. These GAGs bear 4-methylumbelliferone at their reducing termini (*6-9*).

Recently, the authors have characterized the MU-derivatives produced by cultured human skin fibroblasts grown in the presence of Xyl-MU. We found that synthetic intermediates of GAG-MU, such as Galβ1-3Galβ1-4Xylβ1-MU and Galβ1-4Xylβ1-MU (*10*), in addition to GAG-MU, were synthesized. We have also reported that novel oligosaccharides, GlcAβ1-4Xylβ1-MU (*11*), sulphate-*O*-3GlcAβ1-4Xylβ1-MU (*12*), and Xylβ1-4Xylβ1-MU (*13*), which are unrelated to GAGs, are elaborated from Xyl-MU. Freeze *et al.* found that many Xyl-MU-induced oligosaccharides are produced in cultures of Chinese hamster ovary cells and human melanoma cells (*14*). However, none of these oligosaccharides correspond to those found in cultures of human skin fibroblasts. It is not known whether this is due to differences in cell type or the analytical methods employed. Therefore, detailed structural studies of these Xyl-MU derivatives are important.

In the present study using cultured human skin fibroblasts, we purified the main Xyl-MU-induced oligosaccharide secreted into the medium and analyzed its structure. The results indicated that its structure was SAα2-3Galβ1-4Xylβ1-MU, the same as that secreted from Chinese hamster ovary and human melanoma cells (*14*). Furthermore, we investigated in detail the characteristics of the

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (Nos. 05274107, 05680518, and 60238285), and from the Karohji Medical Foundation, the Mizutani Foundation for Glycoscience, and the Nakatomi Foundation.

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Abbreviations: MU, 4-methylumbelliferone; Xyl-MU, 4-methylumbelliferyl-β-D-xyloside; Xyl, xylose; Gal, galactose; SA, sialic acid; GlcA, glucuronic acid; GAG, glycosaminoglycan; PA, 2-aminopyridine.

synthesized SAα2-3Galβ1-4Xylβ1-MU.

EXPERIMENTAL PROCEDURES

Materials—Minimum essential medium and fetal bovine serum were obtained from Gibco (Grand Island, NY, USA). Sialidase (*Clostridium perfringens*), β-galactosidase (*Aspergillus niger*), 4-methylumbelliferyl-β-D-xyloside (Xyl-MU), 4-methylumbelliferyl-β-D-lactoside, 6′-*N*-acetylneuramin-lactose, 3′-*N*-acetylneuramin-lactose, and CDP were obtained from Sigma Chemical (St. Louis, MO, USA). Sephadex G-15 and DEAE-Sephacel were purchased from Pharmacia LKB Biotech. (Uppsala, Sweden). Other reagents and chemicals were obtained from commercial sources.

Cell Culture—Human skin fibroblasts were cultured by the method described previously (*10*). The cells were plated at a density of 2×10^5/10-cm dish with minimum essential medium including 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Fibroblasts at passages 4–6 were used for the study. Confluent cultured fibroblasts were incubated for 3 days with 0.5 mM Xyl-MU.

Purification of the Major MU-Induced Oligosaccharide—For isolation of the oligosaccharide, spent culture medium (20 liters) was pooled, lyophilized, and dialyzed against distilled water for 48 h using a Visking tube. The dialyzable fraction was concentrated with a lyophilizer, then applied to a Sephadex G-15 column (4.2×132 cm), which was equilibrated and eluted with 0.1 M acetic acid at a flow rate of 50 ml/h, and 25-ml fractions were collected. The fluorescence intensity of the eluate was monitored at excitation and emission wavelengths of 325 and 380 nm, respectively. The fractions exhibiting fluorescence were collected and applied to a column of DEAE-Sephacel (2.3×40 cm), which was equilibrated with distilled water. Elution was performed with a linear gradient of 0–0.9 M NaCl at a flow rate of 50 ml/h, and 10-ml fractions were collected.

The fractions exhibiting fluorescence were collected and subjected to preparative gel-filtration HPLC with a Shodex OHpak KB-2004 column (2×30 cm, Shoko, Tokyo) using 0.2 M NaCl at a flow rate of 3.0 ml/min, and 3.0-ml fractions were collected. The fractions containing the oligosaccharide, which was detected by its fluorescence, were recovered after HPLC and purified by reverse-phase HPLC with a Shodex C18-10F column (2×25 cm, Shoko). Gradient elution using 0–60% acetonitrile in distilled water was performed at a flow rate of 3.0 ml/min, and 3.0-ml fractions were collected. A liquid chromatograph (Hitachi L-6200) equipped with a fluorescence spectrophotometer (Hitachi F-1050) was used. Fluorescence was detected at 325 nm (excitation wavelength) and 380 nm (emission wavelength).

Chemical Analysis—To determine their sugar composition, samples were hydrolyzed for 2 h at 100°C in 2 N HCl for neutral sugars and uronic acid, or for 8 h at 100°C in 4 N HCl for amino sugars. They were then labeled with a fluorescent reagent, 2-aminopyridine (PA), as described previously (*15*). The resulting PA-monosaccharide was identified by analysis using an Ultrasphere ODS column (0.46×25 cm, Beckman Instruments, Palo Alto, CA, USA) with 0.25 M sodium citrate buffer (pH 4.0) containing 1% acetonitrile as the eluent at a flow rate of 0.5 ml/min at 40°C (*15*). Fluorescence was detected at 320 nm (excitation wavelength) and 400 nm (emission wavelength) after release from oligosaccharide by hydrolysis for 1 h at 80°C in 50 mM HCl. Sialic acid was labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and analyzed with a PALPAK Type R column (0.46×25 cm, Takara Shuzo, Kyoto) using acetonitrile/methanol/distilled water=9 : 7 : 84 (v/v/v) as the eluent at a flow rate of 0.9 ml/min at 30°C (*16*). Fluorescence was detected at 373 nm (excitation wavelength) and 448 nm (emission wavelength). Sulphate and phosphate analysis was performed by the rhodizonate method (*17*) and the malachite green-ammonium molybdate method (*18*), respectively.

Digestion with Glycosidases—The oligosaccharide was digested successively with sialidase (0.1 M sodium acetate buffer, pH 5.0) (*19*) and β-galactosidase (0.1 M sodium acetate buffer, pH 4.0) (*20*). After incubation, the reactions were terminated by boiling for 5 min.

Periodate Oxidation—Periodate oxidation was performed as follows according to the method of Oegema *et al.* (*21*). The sample was dissolved in 0.1 ml of 0.02 M $NaIO_4$/0.05 M sodium acetate buffer (pH 4.8) and kept in the dark at 4°C for 20 h. Then, excess periodate was destroyed with 5 ml of ethylene glycol.

Ion-Spray Mass Spectrometry—Mass spectra were obtained on an API-III triple-quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an atmospheric-pressure ionization source, as described previously (*22*). The samples were dissolved in 0.5 mM ammonium acetate-acetonitrile (50 : 50) and injected at 2 μl/min with a micro-HPLC syringe pump (pump 22, Harvard Apparatus, MA, USA). In positive and negative modes, scanning was done from m/z 300 to 1,200 during the 1-min scan (six cycles). In MS/MS analysis, argon was used as the collision gas and the collision energy was 40 eV.

Nuclear Magnetic Resonance Spectroscopy—The sample was exchanged with deuterium by repeated drying under vacuum from 99.8% D_2O (twice), and 99.95% D_2O (twice). Then, the sample was dissolved in 99.95% D_2O for ^{1}H-NMR analysis. The ^{1}H-NMR spectrum was obtained with a JNM EX-270 (JEOL, Tokyo) at 70°C.

RESULTS

Isolation of the Major MU-Induced Oligosaccharide—Human skin fibroblasts were cultured for 72 h in the presence of 0.5 mM Xyl-MU. The spent culture medium (20 liters) was pooled, lyophilized, and dialyzed against distilled water. The dialyzable fraction was recovered and concentrated with a lyophilizer, then applied to a Sephadex G-15 column. The MU-derivatives were weakly bound through hydrophobic interactions to the resin of a Sephadex G-15 column. The major oligosaccharide fraction, which was eluted around V_t, was then recovered (see Fig. 1 in Ref. *12*). Next, the major oligosaccharide fraction was loaded on a DEAE-Sephacel column (Fig. 1). The oligosaccharide was absorbed in DEAE-Sephacel and eluted with NaCl at about 0.25 M. After recovery of the fraction indicated with a bar in Fig. 1, it was further purified by preparative HPLC. The fractions exhibiting fluorescence were recovered and subjected to preparative gel-filtration HPLC (Shodex OHpak KB-2004), and the fraction indicated with a bar in Fig. 2

was recovered. Furthermore, the major oligosaccharide fraction was subjected to reverse-phase HPLC (Shodex C18-10F) and eluted with acetonitrile (Fig. 3). A symmetrical peak was detected, and this was used as the purified preparation of the major MU-derivative oligosaccharide for further studies after concentration.

Structure of the Major MU-Induced Oligosaccharide— The major purified MU-induced oligosaccharide was characterized with regard to its carbohydrate and other constituents. The oligosaccharide was composed of 4-methylumbelliferone, xylose, galactose, and sialic acid in a molar ratio of 1.00:0.73:0.82:0.64, but contained no glucuronic acid, *N*-acetylgalactosamine, sulphate or phosphate. It was considered that the oligosaccharide consisted of 4-methylumbelliferone, xylose, galactose, and sialic acid in equimolar amounts.

The molecular weight of this oligosaccharide was determined by MS analysis with an ion-spray mass spectrometer. Consequently, a pseudo-molecular ion was observed at m/z 760.0 $[M-H]^-$ by MS analysis in the negative mode (Fig. 4A). This ion was consistent with a structure containing one 4-methylumbelliferone, one sialic acid, one hexose, and one pentose. Next, the structure of this oligosaccharide was analyzed on the basis of its fragmenta-

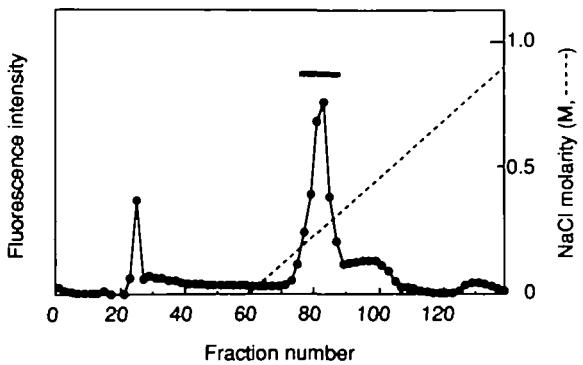

Fig. 1. **Ion-exchange chromatography on DEAE-Sephacel.** The recovered fractions from Sephadex G-15 chromatography of the dialyzable fraction of the culture medium were applied to a DEAE-Sephacel column (2.3×40 cm), which was equilibrated with distilled water. Elution was performed with a linear gradient of 0-0.9 M NaCl at a flow rate of 50 ml/h, and 10-ml fractions were collected. The eluate was monitored with a fluorescence detector. The fractions indicated by the horizontal bar were collected and used for further purification.

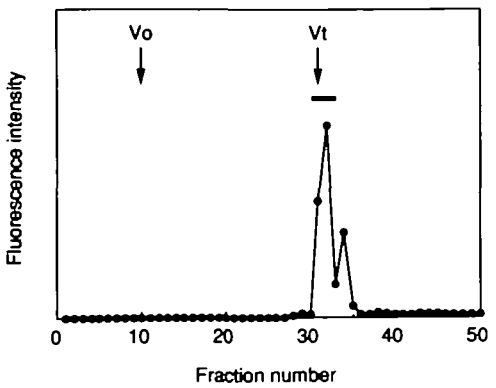

Fig. 2. **Gel-filtration HPLC on a Shodex OHpak KB-2004.** HPLC was performed using a Shodex OHpak KB-2004 column (2×30 cm) with 0.2 M NaCl at a flow rate of 3.0 ml/min, and 3.0-ml fractions were collected. The eluate was monitored with a fluorescence detector. The fractions indicated by the horizontal bar were collected and used for further purification.

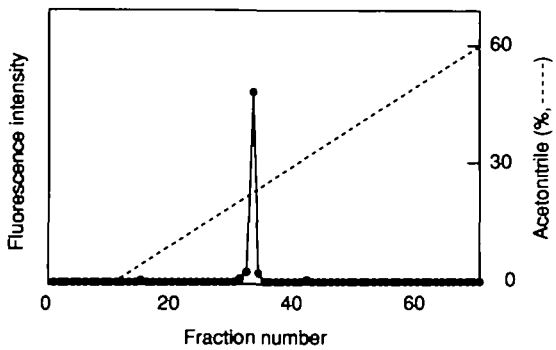

Fig. 3. **Reverse-phase HPLC on Shodex C18-10F.** HPLC was performed using a Shodex C18-10F column (2×25 cm) with a linear gradient of distilled water-acetonitrile at a flow rate of 3.0 ml/min, and 3.0-ml fractions were collected. The eluate was monitored with a fluorescence detector. The fractions were collected and used for analysis as the purified oligosaccharide.

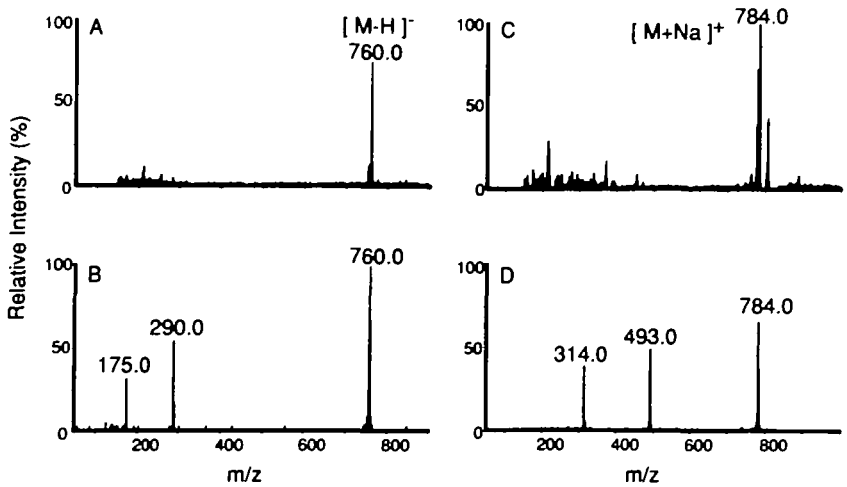

Fig. 4. **Ion-spray mass spectra.** (A) Negative-mode mass spectrum of the main Xyl-MU-induced oligosaccharide, and (B) product ions on fragmentation by MS/MS analysis using m/z 760 as the precursor ion (A). (C) Positive-mode mass spectrum of the main Xyl-MU-induced oligosaccharide, and (D) product ions on fragmentation by MS/MS analysis using m/z 784 as the precursor ion (C).

tion pattern on MS/MS analysis. The pseudo-molar ion at m/z 760.0 was selected as the precursor ion and subjected to fragmentation, MS/MS analysis, using weak collision energy. Two product peaks with mass numbers of 175.0 and 290.0 were obtained and identified as $[MU-H]^-$ and $[(SA-18)-H]^-$, respectively (Fig. 4B). In the positive mode, a pseudo-molecular ion was observed at m/z 784.0 $[M+Na]^+$ (Fig. 4C). In this mode, two peaks with mass numbers of 314.0 and 493.0 were obtained and identified as $[(Gal\text{-}Xyl)+H]^+$ and $[(Gal\text{-}Xyl\text{-}MU)+Na]^+$, respectively (Fig. 4D). From the above results and the constitution of the linkage region between glycosaminoglycan and the core protein, its structure was considered to be Gal-Xyl-MU with sialic acid links to either galactose or xylose.

This was confirmed by the 270-MHz ^{1}H-NMR spectrum. The acetyl group derived from sialic acid was observed at 1.98 ppm, and furthermore doublets of xylose ($J=7.6$ Hz) and galactose ($J=7.4$ Hz) derived from H-1 were observed at 4.52 and 4.62 ppm, respectively (data not shown).

To examine the sequence structure of the oligosaccharide, sequential degradation of glycosidases was carried out. The oligosaccharide was incubated with β-galactosidase, then subjected to gel-filtration HPLC on a Shodex OHpak KB-803 column (Fig. 5B). However, its elution position did not change relative to that of the control (Fig. 7A). The peak of the oligosaccharide was shifted to a position corresponding to Gal-Xyl-MU after sialidase digestion (Fig. 5C). Upon digestion of the oligosaccharide with sialidase initially and then with β-galactosidase (Fig. 5D), the oligosaccharide peak shifted to a position corresponding to Xyl-MU, thus confirming α-linking of sialic acid to galactose. Furthermore, its sugar sequence was concluded to be SAα-Galβ-Xylβ-MU.

From the analytical results described above, the oligosaccharide appeared to be Gal-Xyl-MU with a sialic acid residue. Therefore, its structure was most likely to be SAα2-3Galβ1-4Xylβ-MU or SAα2-6Galβ1-4Xylβ1-MU. To examine the position of the sialic acid linkage to the galactose residue, the oligosaccharide was subjected to periodic acid oxidation (0.02 M $NaIO_4$/0.05 M sodium acetate buffer, pH 4.8, at 4°C for 20 h), hydrolysis with 2 N HCl at 100°C for 2 h, then pyridylamination, and the recovery values of PA-galactose were measured by HPLC analysis on an Ultrasphere ODS column. If the sialic acid

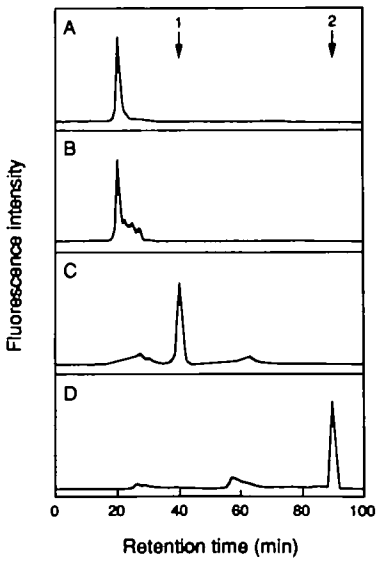

Fig. 5. **Analysis by HPLC of the main Xyl-MU-induced oligosaccharide before and after digestion with various glycosidases.** The column used was a Shodex OHpak KB-803 (8×300 mm), which was eluted with 0.2 M NaCl at flow rate of 1 ml/min. The eluate was monitored with a fluorescence detector. A, before enzymic digestion; B, after digestion with β-galactosidase; C, after digestion with sialidase; D, after digestion with β-galactosidase following sialidase digestion. The arrows denote the positions of Xyl-MU derivatives: 1, Gal-Xyl-MU; 2, Xyl-MU.

TABLE I. **Recovery of galactose after periodate oxidation.**

Oligosaccharide	PA-galactose[a]
3′-*N*-Acetylneuramin-lactose	91
6′-*N*-Acetylneuramin-lactose	5
MU-induced oligosaccharide	100

[a]Highest recovery set at 100.

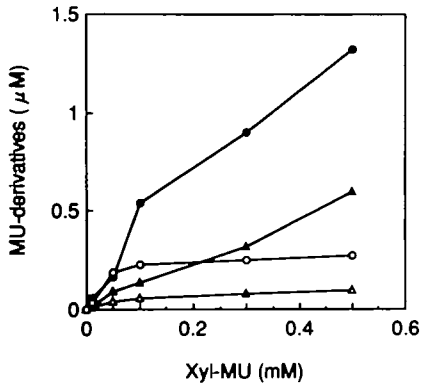

Fig. 6. **Effect of Xyl-MU concentration on Xyl-MU-derivatives secreted into the medium by cultured human skin fibroblasts.** Human skin fibroblasts were incubated for 72 h in the absence (control) or presence of Xyl-MU (0.01 to 0.5 mM). After incubation, Xyl-MU-derivatives in the medium were analyzed by HPLC using a Shodex OHpak KB-803 column. ○, GAG-MU; ●, SA-Gal-Xyl-MU; ▲, Gal-Gal-Xyl-MU; △, Gal-Xyl-MU.

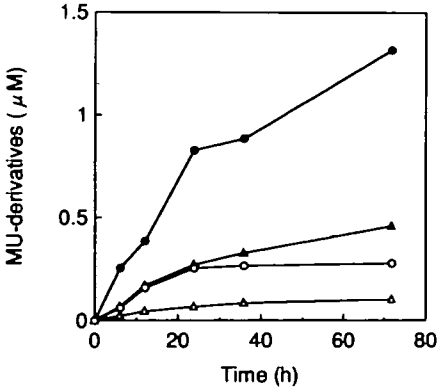

Fig. 7. **Effect of time on amount of Xyl-MU-derivatives secreted into the medium by cultured human skin fibroblasts.** Human skin fibroblasts were incubated with 0.5 mM Xyl-MU for up to 72 h. After incubation, Xyl-MU-derivatives in the medium were analyzed by HPLC using a Shodex OHpak KB-803 column. ○, GAG-MU; ●, SA-Gal-Xyl-MU; ▲, Gal-Gal-Xyl-MU; △, Gal-Xyl-MU.

TABLE II. **Effects of CDP on synthesis of MU-derivatives.** Cultures of human skin fibroblasts were preincubated for 30 min with medium containing 1 mM CDP. The preincubated medium was removed and the cultures were incubated for 72 h in medium containing the same CDP concentration and 0.5 mM Xyl-MU. Each of the MU-derivatives was determined by HPLC (Shodex OHpak KB-803) as described in "EXPERIMENTAL PROCEDURES."

	GAG-MU	Sia-Gal-Xyl-MU	Gal-Gal-Xyl-MU	Gal-Xyl-MU
			(μM)	
Control	0.29	1.39	0.63	0.10
CDP	0.46 (159)[a]	1.30 (93)	0.88 (140)	0.12 (120)

[a]Numbers in parentheses represent percentage of control (0 μM CDP).

had been at the C-6 position of galactose, the galactose would have been cleaved and thus not detected as PA-galactose. The results are shown in Table II. For standard 6′-*N*-acetylneuramin-lactose, which has 1,6-substituted galactose, the recovery of PA-galactose was significantly low. On the other hand, for standard 3′-*N*-acetylneuramin-lactose, which has 1,3-substituted galactose and the oligosaccharide, the recovery of PA-galactose was very similar. In comparison with the recovery for the standard, it was clearly demonstrated that the sialic acid was linked at the C-3 position of the galactose moiety of the oligosaccharide. Therefore, its structure was confirmed to be SAα2-3Galβ1-4Xylβ1-MU.

Characterization of SA-Gal-Xyl-MU Synthesis—The relationship between the oligosaccharide (SA-Gal-Xyl-MU), two biosynthetic intermediates (Gal-Gal-Xyl-MU and Gal-Xyl-MU) of GAG-MU, and GAG-MU was then investigated. First, confluent human skin fibroblasts were incubated with various concentrations of Xyl-MU (0.01 to 0.5 mM). After incubation for 72 h, aliquots of the culture medium were taken and subjected to HPLC (Shodex OHpak KB-803) (Fig. 6). Addition of 0.1 mM Xyl-MU to the culture medium produced a maximal concentration of GAG-MU, which was not increased by exposure of the fibroblasts to higher concentrations of Xyl-MU. On the other hand, the concentration of SA-Gal-Xyl-MU and Gal-Gal-Xyl-MU in the medium increased in proportion to Xyl-MU concentration.

Subsequently, the effect of incubation time (0 to 72 h) was investigated using 0.5 mM Xyl-MU (Fig. 7). The amount of GAG-MU in the medium became constant after 24 h. However, the amount of SA-Gal-Xyl-MU increased with prolonged incubation time. These data showed that the amounts of these MU-derivatives secreted into the medium were correlated with both the Xyl-MU concentration and incubation time. Also under both conditions the amounts of SA-Gal-Xyl-MU increased markedly and almost linearly with time, and the change was different from that of Gal-Gal-Xyl-MU and Gal-Xyl-MU.

Next, the effects of CDP, a known inhibitor of sialyltransferase (*23*), on the MU-derivatives secreted into the medium were investigated. CDP decreased the secretion of SA-Gal-Xyl-MU, but increased that of GAG-MU, Gal-Gal-Xyl-MU, and Gal-Xyl-MU (Table II). These results suggest that addition of the α-2,3-sialic acid unit to Gal-Xyl-MU inhibits the addition of the β-1,3-galactose unit to Gal-Xyl-MU, and that this may be related to regulation of GAG-MU biosynthesis.

DISCUSSION

These experiments demonstrated that incubation of human skin fibroblasts in the presence of Xyl-MU resulted in the synthesis of many fluorogenic oligosaccharides. The xyloside acted as an artificial chain initiator, and the xyloside-induced oligosaccharides were secreted into the medium. Using a large amount of medium (20 liters), the major Xyl-MU-induced oligosaccharide was purified for structural analysis. First, analysis of its sugar constitution showed that the oligosaccharide consisted of xylose, galactose, and sialic acid in equimolar amounts. Second, from MS analysis, MS/MS analysis, ^{1}H-NMR analysis, and enzyme digestion, its structure was considered to be Galβ1-4Xylβ1-MU linked with sialic acid, representing the linkage region between GAGs and the core protein. Finally, periodate oxidation confirmed that its structure was SAα2-3Galβ1-4Xylβ1-MU.

Very few studies have examined the oligosaccharides produced by cells incubated in the presence of artificial acceptors. Recently, Freeze *et al.* detected a novel Xyl-MU-induced oligosaccharide, SAα2-3Galβ1-4Xylβ1-MU, that was produced by human melanoma cells and Chinese hamster ovarian cells (*14*). They studied the relationship between the oligosaccharide and glycolipid biosynthesis and considered that the former played an important regulatory role. In this study, the same oligosaccharide was found in the culture medium of human skin fibroblasts. Therefore, it is likely that SAα2-3Galβ1-4Xylβ1-MU biosynthesis generally occurs in various cells. Furthermore, the influence of a sialyltransferase inhibitor, CDP, was studied, and the results indicated that GAG-MU synthesis was actually increased in the presence of CDP. Therefore, it is likely that SA-Gal-Xyl-MU production may inhibit GAG-MU biosynthesis.

The mode of biosynthesis of the GAG chains of proteoglycans has been studied (*2–5*), and it has been concluded that the formation of GAG chains is initiated by *O*-D-xylosylation of selected serine residues in the appropriate proteoglycan core protein, followed by the addition of two galactose units. The resulting Galβ1-3Galβ1-4Xyl trisaccharide sequence serves as an acceptor for the first glucuronic acid monosaccharide unit. The GAG chain proper is then generated by stepwise alternating transfer of *N*-acetylhexosamine and glucuronic acid residues from the corresponding UDP-monosaccharide derivatives to the nonreducing terminal. Recently, we have obtained various kinds of Xyl-MU-induced oligosaccharides by incubating human skin fibroblasts in the presence of Xyl-MU (*10–13*). Two of these derivatives, Gal-Gal-Xyl-MU and Gal-Xyl-MU, were related to the proteoglycan linkage region structures. Although SA-Gal-Xyl-MU was different from intermediates of GAG-MU, the precursor would be the same Gal-Xyl-MU as for Gal-Gal-Xyl-MU. Recently, Etchison *et al.* (*24*) reported that GAG core-specific galactosyltransferase I and α-2,3-sialyltransferase are co-located within the same Golgi compartment. Therefore, the SA-Gal-Xyl-MU may be synthesized in competition with Gal-Gal-Xyl-MU. Further detailed analyses of the relationships among these intermediates may yield information about the regulation of GAG-MU biosynthesis.

We thank Dr. H. Matsue and Dr. K. Takaya (Aomori Prefectural Research Center for Industrial Technology) for ^{1}H-NMR analysis and Dr. Y. Umeda (Takara Shuzo Co., Ltd., Biotechnology Research Laboratory) for ion-spray mass analysis.

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