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Characterization of β-D-xyloside-initiated glycosaminoglycan synthesized by human skin fibroblasts in the presence of tunicamycin

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Human skin fibroblasts were incubated with a fluorogenic xyloside, 4-methylumbelliferyl-β-D-xyloside (Xyl-MU), in the presence or absence of tunicamycin. The xyloside-initiated glycosaminoglycans (GAG-MUs) were isolated from the culture medium, and their structures characterized. When the cells were incubated with Xyl-MU in the presence of 0.2 μg ml^{-1} tunicamycin, the synthesis of GAG-MU was increased about three fold, compared with the control value in the absence of tunicamycin (cells exposed to Xyl-MU alone). The structures of GAG-MUs synthesized in the presence or absence of tunicamycin were compared by HPLC analysis using gel-filtration and ion-exchange columns, enzymatic digestion, and unsaturated disaccharide composition analysis. The data indicated that cells incubated with tunicamycin produced more undersulfated and shorter GAG-MUs than cells without tynicamycin. These results suggest that tunicamycin inhibits the elongation and sulfation of glycosaminoglycan (GAG) chains and that, as a result, GAG-MUs with shorter chains and undersulfated residues, but possessing a large number of GAG chains, are synthesized in the presence of tunicamycin.

***Keywords*: cultured human skin fibroblasts, glycosaminoglycan, 4-methylumbelliferyl-β-D-xyloside, tunicamycin**

Introduction

It has been reported that addition of a β-xyloside such as *p*-nitrophenyl-β-D-xyloside, 4-methylumbelliferyl-β-D-xyloside (Xyl-MU) or benzyl-β-D-xlyoside, to cell culture medium induces elongation of glycosaminoglycan (GAG) chains, which is initiated by the β-xyloside acting as a primer [1–9]. Previously we characterized the Xyl-MU-initiated GAG (GAG-MU) produced by human skin fibroblasts cultured in the presence of Xyl-MU [10]. Furthermore, it was found that some intermediates of GAG-MU, such as Galβ1-3Galβ1-4Xylβ1-MU and Galβ1-4Xylβ1-MU [10], were synthesized in addition to GAG-MU. It has also been reported that novel oligosaccharides, SAα2-3Galβ1-4Xylβ1-MU [11, 12], GlcAβ1-4Xylβ1-MU [13], sulfate-*O*-3GlcAβ1-4Xylβ1-MU [14], and Xylβ1-4Xylβ1-MU [15] were produced. The newly detected oligosaccharides were not directly related to GAG-MU production, but were derived from Xyl-MU. Freeze *et al.* [11] showed that the synthesis of SAα2-3Galβ1-4Xylβ1-MU, one of the newly detected oligosaccharides, was related to glycolipid biosynthesis. We have also demonstrated that the synthesis of SAα2-3Galβ1-4Xylβ1-MU influences GAG-MU biosynthesis [12]. However, the relationship between the biosynthesis of GAG-MU and that of the various other oligosaccharides derived from Xyl-MU is not understood.

Tunicamycin, an antibiotic substance, specifically inhibits lipid-linked *N*-acetylglucosamine formation, and inhibits the synthesis of various glycoconjugates [16–18]. Several recent studies have demonstrated that tunicamycin partially inhibits the synthesis of glycosaminoglycan in cultured chick embryo [16, 19]. However, the detailed structure of the GAG synthesized was not reported. Here we describe the effects of tunicamycin on the biosynthesis of GAG and other oligosaccharides synthesized from Xyl-MU in cultured human skin fibroblasts. It was found that the level of GAG-MU biosynthesis increased when tunicamycin was added to the culture medium. Therefore, the detailed structures of the two GAG-MUs synthesized in the presence or absence of tunicamycin were compared. In addition, the relationships among GAG and the other oligosaccharides synthesized from Xyl-MU were investigated along with their modulation by tunicamycin.

Materials and methods

Materials

Minimal essential medium and fetal bovine serum were obtained from Gibco (Grand Island, NY). Pronase P from

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Streptomyces griseus was obtained from Kaken Kagaku (Tokyo, Japan). Chondroitin 6-sulfate from shark cartilage, dermatan sulfate from pig skin, hyaluronic acid from human umbilical cord, chondroitinase ABC, *Streptomyces* hyaluronidase, Flavobacterium heparinum heparitinase, and standard unsaturated disaccharides were from Seikagaku Corp. (Tokyo, Japan). Xyl-MU was purchased from Sigma Chem. Co. (St. Louis, MO). The Xyl-MU-initiated oligosaccharides (Galβ1-3Galβ1-4Xylβ1-MU, Galβ1-4Xylβ-MU, SAα2-3Galβ1-4Xylβ1-MU, GlcAβ1-4Xylβ1-MU, and sulfate-*O*-3GlcAβ1-4Xylβ1-MU) were prepared as described previously [10, 12–14]. [^{3}H]Glucosamine (specific activity, 40 Ci mmol^{-1}) was obtained from ICR Radiochemicals (Irvine, CA). Tunicamycin was obtained from Wako Pure Chem. Co. (Osaka, Japan). Sephadex G-25 was purchased from Pharmacia LKB Biotech (Uppsala, Sweden). Other reagents were of the highest grade from commercial sources.

Cell cultures

Human skin fibroblasts were cultured by the method described previously [10]. The cells were plated at a density of 2×10^5 per 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. To investigate the biosynthesis of GAG and oligosaccharides derived from Xyl-MU, confluent cultured fibroblasts were incubated with 0.5 mM Xyl-MU. To investigate the biosynthesis of GAG as a proteoglycan sugar chain, confluent cultured fibroblasts were incubated with 5 μCi ml^{-1} [^{3}H]glucosamine. Other reagents to be added to the medium were prepared as described below. Specifically, a stock solution of tunicamycin (0.5 mg ml^{-1} in 25 mM NaOH) was prepared, and then the solution was added to the medium so that the final concentration was 0.2 μg ml^{-1}.

Preparation of GAG-MU and ^{3}H-labelled GAG

GAG-MU was extracted from the medium by the method described in our previous paper [20]. In brief, confluent fibroblasts were incubated for 72 h with 0.5 mM Xyl-MU. The spent culture medium was pooled, lyophilized, and passed through a Sephadex G-25 column to remove low-molecular-mass materials. Fractions eluted from the column were digested with pronase P in 0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$, at 50 °C for 24 h. The digestion was terminated by heating for 5 min in boiling water. After centrifugation, four volumes of ethanol saturated with NaCl were added to the supernatant. The resulting precipitates were dissolved in 0.04 M NaCl and precipitated with 2% cetylpyridinium chloride solution. Subsequently, the collected precipitates were dissolved in 2 M $MgCl_2$ and passed through a Sephadex G-25 column using water as the eluent. Fractions eluted from the column were pooled and used as GAG-MU. GAG-MU was quantified using a fluorescence spectrophotometer (Hitachi 204-R, Hitachi, Tokyo, Japan) with an excitation wavelength of 325 nm and an emission wavelength of 380 nm.

^{3}H-Labelled GAG (^{3}H-labelled hyaluronic acid and chondroitin sulfate/dermatan sulfate) was prepared according to the method described in our previous paper [21]. In brief, confluent fibroblasts were incubated for 72 h with 5 μCi ml^{-1} [^{3}H]glucosamine in the absence of Xyl-MU. The medium was collected and digested with pronase P at 45 °C for 18 h. The resulting medium was concentrated and mixed with a one-fourth volume of 50% trichloroacetic acid, and then the mixture was cooled and centrifuged. To the supernatant was added four volumes of ethanol saturated with NaCl, and the glycosaminoglycan fraction was collected by centrifugation. The precipitate was dissolved in water and subjected to high-performance liquid chromatography (HPLC) to separate and quantify ^{3}H-labelled hyaluronic acid and ^{3}H-labelled chondroitin sulfate/dermatan sulfate.

HPLC

A high-performance liquid chromatograph (Hitachi L-6200, Hitachi) connected to a fluorescence detector (Hitachi F-1050) or a UV-detector (Hitachi L-4200) was used. For analysis of Xyl-MU derivatives, gel-filtration HPLC was performed using a Shodex OHpak SB-803 column (8×300 mm, Shoko, Tokyo, Japan) with 0.2 M NaCl as the solvent at the flow rate of 0.5 ml min^{-1} and a column temperature of 30 °C. Ion-exchange HPLC was performed using a TSKgel DEAE-5PW column (7.5×75 mm, Tosoh, Tokyo, Japan) with a linear gradient of 0–1 M NaCl in 40 min at a flow rate of 1 ml min^{-1} and a column temperature of 30 °C. For detection of Xyl-MU derivatives, an excitation wavelength of 325 nm and an emission wavelength of 380 nm were used.

^{3}H-Labelled GAG was analysed with a TSKgel SAX column (6×150 mm, Tosoh) at a column temperature of 30 °C and a flow rate of 0.8 ml min^{-1}. The column was washed with 50 mM sodium acetate buffer, pH 6.0, for 10 min and eluted with the same buffer containing a linear gradient of 0–2 M NaCl from 10 min to 45 min, and then eluted with the same buffer containing 3 M NaCl from 45 min to 80 min. Fractions of 0.8 ml were collected, and the ^{3}H radioactivity of each fraction was measured using a liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan).

Unsaturated disaccharides were analyzed with a Shodex NH-5B column (4.6×250 mm, Shoko) with a linear gradient of 16–470 mM NaH_2PO_4 in 60 min at a flow rate of 1 ml min^{-1} and a column temperature of 45 °C. The detection of unsaturated disaccharides was performed by absorbance at 232 nm.

Enzymatic digestion

GAG-MU was digested successively with the following enzymes: chondroitinase ABC (0.1 M Tris-HCl buffer, pH 8.0)

[22], *Flavobacterium heparinum* heparitinase (0.1 M potassium phosphate buffer, pH 6.8) [23], and *Streptomyces* hyaluronidase (0.1 M sodium acetate buffer, pH 6.0) [24], at 37 °C for 2 h.

Results

Effects of tunicamycin on GAG-MU production

Human skin fibroblasts were cultured with Xyl-MU in the presence of tunicamycin. Aliquots of the culture medium were taken at various incubation times to monitor quantitative changes in the production of GAG-MU, as described in Materials and Methods (Figure 1). After 72 h in the presence of tunicamycin, the amount of GAG-MU synthesized was about three times greater than that synthesized in its absence. Therefore, various concentrations of tunicamycin were tested to determine its effects on GAG-MU synthesis. The results revealed that the maximum amount of product was obtained in the presence of about 0.2 $\mu g\,ml^{-1}$ tunicamycin (Figure 2). Thus, it was concluded that tunicamycin accelerates the synthesis of GAG-MU in human skin fibroblasts.

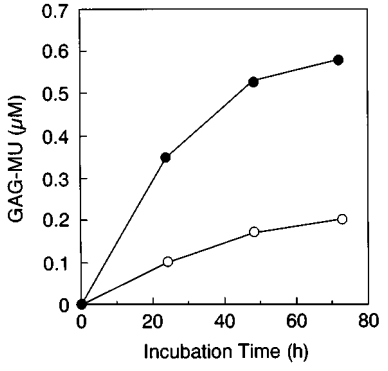

Figure 1. Time course of effects of tunicamycin on the synthesis of GAG-MU. Human skin fibroblasts were incubated with 0.5 mM Xyl-MU for up to 72 h in the absence (○) or presence (●) of 0.2 $\mu g\,ml^{-1}$ tunicamycin. After incubation, Xyl-MU-initiated GAG (GAG-MU) was extracted from the medium and measured as described in the text.

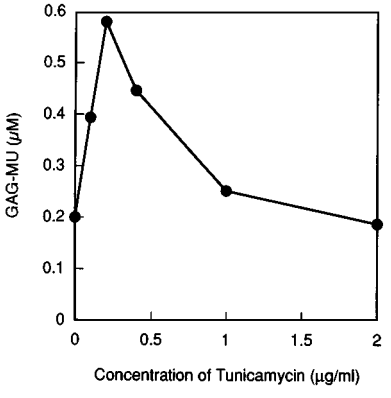

Figure 2. Effects of tunicamycin concentration on the synthesis of GAG-MU. Human skin fibroblasts were incubated with 0.5 mM Xyl-MU for 72 h in the presence of various concentrations of tunicamycin. Secreted GAG-MU was measured as described in the text.

Characterization of GAG-MU synthesized in the presence of tunicamycin

The chemical structure of GAG-MU synthesized and secreted in the presence of tunicamycin was compared with that of GAG-MU synthesized in the absence of tunicamycin (the control). The molecular masses of both GAG-MUs were analysed by gel-filtration HPLC (Shodex OHpak SB-803) (Figure 3), and the GAG-MU synthesized in the presence of tunicamycin showed a slower retention time than that of the control, indicating that its molecular mass was lower than that of GAG-MU synthesized without tunicamycin.

GAG-MU synthesized in the presence of tunicamycin was digested with *Streptomyces* hyaluronidase, heparitinase and chondroitinase ABC, and each digest was examined by HPLC using a Shodex OHpak SB-803 column (data not shown). GAG-MU was completely digested by chondroitinase ABC, but not by the other enzymes, indicating that the GAG-MU was a galactosaminoglycan composed of chondroitin sulfate and dermatan sulfate, and similar types of GAG-MU synthesized without tunicamycin.

The charge of each GAG-MU was analysed by ion-exchange HPLC (TSKgel DEAE-5PW) (Figure 4). The results revealed that the elution of GAG-MU synthesized in the presence of tunicamycin was faster and broader than that of the GAG-MU synthesized in its absence, indicating a reduction in the negative charge of the GAG chain. Then, after digestion of both GAG-MUs with chondroitinase ABC, the resulting unsaturated disaccharides were analysed by HPLC (Table 1). GAG-MU synthesized without tunicamycin contained only 2.1% unsulfated disaccharides (ΔDi-0S), compared with 22.7% for the GAG-MU synthesized in the presence of tunicamycin. These results indicate that GAG-MU synthesized in the presence of tunicamycin has a lower degree of sulfation than that synthesized without it.

Effects of tunicamycin on GAG synthesis of proteoglycan

To investigate whether the effect of tunicamycin is specific for GAG-MU, human skin fibroblasts were incubated with [^{3}H]glucosamine without Xyl-MU, and then the amount of GAG synthesized as a proteoglycan sugar chain in the presence or absence of tunicamycin was analysed (Table 2). The data showed that tunicamycin did not inhibit incorporation of [^{3}H]glucosamine into the hyaluronic acid fraction, but that it markedly inhibited incorporation of the radioactivity into the chondroitin sulfate and dermatam sulfate fractions. These results indicate that acceleration of the synthesis of GAG chains by tunicamycin is specific to Xyl-MU-initiated GAG (GAG-MU).

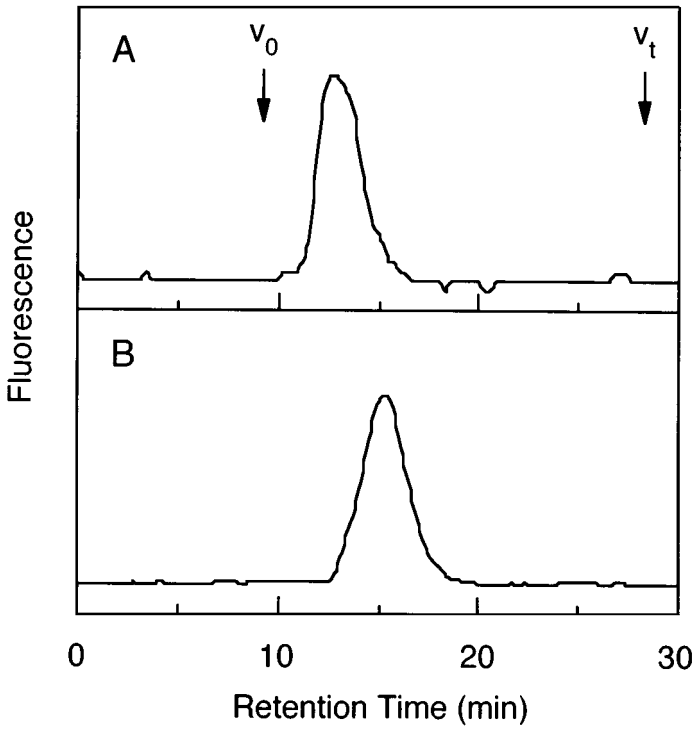

Figure 3. Gel-filtration HPLC analysis of GAG-MUs. Human skin fibroblasts were incubated with 0.5 mM Xyl-MU for 72 h in the absence (A) or presence (B) of 0.2 μg ml^{-1} tunicamycin, and secreted GAG-MUs were prepared as described in the text. GAG-MUs were analysed by gel-filtration HPLC on a Shodex OHpak SB-803 column (8 × 300 mm) with 0.2 M NaCl at a flow rate of 0.5 ml min^{-1}. The eluate was monitored with fluorescence detector.

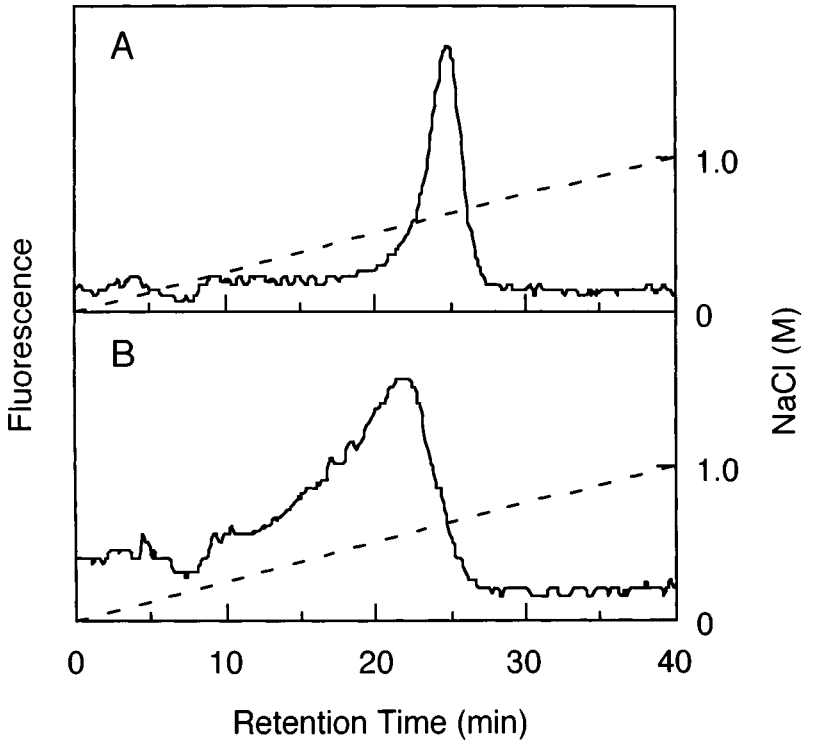

Figure 4. Ion-exchange HPLC analysis of GAG-MU. GAG-MU synthesized in the absence (A) or presence (B) of 0.2 μg ml^{-1} tunicamycin was prepared as described in the text. HPLC was performed using TSKgel DEAE-5PW (7.5 × 75 mm) column with a linear gradient of 0–1 M NaCl in 40 min at a flow rate of 1 ml min^{-1}. The eluate was monitored with a fluorescence detector.

Table 1. Relative composition of GAG-MU from skin fibroblasts incubated with tunicamycin. Human skin fibroblasts were incubated for 72 h with 0.5 mM Xyl-MU in the absence (−) or presence (+) of 0.2 μg ml^{-1} tunicamycin. GAG-MU was prepared as described in the text. After GAG-MU was digested with chondroitinase ABC, the digests containing unsaturated disaccharides were measured for UV absorption at 232 nm on HPLC. HPLC was carried out on a Shodex NH-5B column as described in the text.

GAG-MU	*ΔDi-0S (%)*	*ΔDi-4S (%)*	*ΔDi-6S (%)*	*ΔDi-diS (%)*
Tunicamycin (−)	2.1	20.6	75.2	2.2
Tunicamycin (+)	22.7	33.7	43.6	0

Table 2. Incorporation of [^{3}H]glucosamine into glycosaminoglycan chains of proteoglycan from human skin fibroblasts incubated with tunicamycin. Human skin fibroblasts were incubated for 72 h with 5 μCi ml^{-1} [^{3}H]glucosamine in the absence (−) or presence (+) of 0.2 μg ml^{-1} tunicamycin. Glycosaminoglycan fractions were prepared and separated with a TSKgel SAX column as described in the text. The results were expressed as ^{3}H CPM per 10^5 cells. Percentage inhibition of radioactivity incorporation into each glycosaminoglycan fraction = [1 − (cpm with tunicamycin/cpm without tunicamycin)] × 100.

	Tunicamycin		*Percentage inhibition (%)*
	−	+	
	(cpm)		
Hyaluronic acid	1 452 000	1 436 000	1
Chondroitin sulfate/ dermatan sulfate	660 000	132 000	80

Effects of tunicamycin on Xyl-MU-initiated oligosaccharide production

Human skin fibroblasts were incubated with Xyl-MU (control). After 72 h of incubation, and aliquot of the culture medium was subjected to gel-filtration HPLC using a Shodex OHpak SB-803 column (Figure 5). In addition to GAG-MU, many Xyl-MU-initiated oligosaccharides (Galβ1-3Galβ1-4Xylβ1-MU, Galβ1-4Xylβ1-MU, SAα2-3Galβ1-4Xylβ1-MU, GlcAβ1-4Xylβ1-MU, and sulfate-*O*-3GlcAβ1-4Xylβ1-MU) were detected. To study the influence of tunicamycin on this Xyl-MU-initiated oligosaccharide synthesis, the cells were incubated with Xyl-MU in the presence of tunicamycin for 72 h. The culture medium was sampled to monitor the quantitative changes in the production of Xyl-MU-initiated oligosaccharides using HPLC (Figure 6). The results showed that the amount of SAα2-3Galβ1-4Xylβ1-MU was decreased in the presence of tunicamycin in comparison with that in its absence.

Discussion

It has been reported that addition of a β-xyloside to cell culture medium induces elongation of GAG chains, becaue of β-xyloside acts as a primer. It has also been observed that Xyl-MU initiates GAG synthesis by human skin fibroblasts in culture [10]. Tunicamycin, an antibiotic substance, specifically inhibits the formation of dolichol-diphospho-*N*-acetylglucosamine, an intermediate in the biosynthesis of *N*-asparaginyl-linked glycoprotein [16–18]. In the present study, human skin fibroblasts synthesized approximately three times more GAG-MU in the presence of tunicamycin. The synthesized GAG-MU was found to be undersulfated, and had a smaller average molecular mass.

Hart and Lennarz [19] showed that tunicamycin partially inhibited the synthesis of β-D-xyloside-linked chondroitin sulfate. Therefore, the molecular structures of GAG-MU synthesized in the presence and absence of tunicamycin were compared. The results indicate that cells incubated with tunicamycin produced more undersulfated and shorter GAG-MU than cells cultured without it. In this report, the amount of GAG-MU is presented in terms of molarity, as estimated by fluorescence derived from the MU of Xyl-MU. These findings suggest that tunicamycin inhibits the elongation and sulfation of GAG chains, and that as a result, a larger number of GAGs having shorter and undersulfated chains are synthesized.

A principal question is the mechanism by which tunicamycin accelerates the synthesis of GAG-MU. It was found that some Xyl-MU-initiated oligosaccharides, including two biosynthetic intermediates of GAG-MU, Galβ1-4Xylβ1-MU and Galβ1-3Galβ1-4Xylβ1-MU, were synthesized by cultured human skin fibroblasts [10, 12–14]. On the basis of the structure of these oligosaccharides, a metabolic pathway for the synthesis of some of the oligosaccharides initiated from Xyl-MU and GAG is shown in Figure 7. We have reported that SAα2-3Galβ1-4Xylβ1-MU is the main Xyl-MU-initiated oligosaccharide synthesized in cultured human skin fibroblasts, in competition with Galβ1-3Galβ1-4Xylβ1-MU [12]. This suggests that SAα2-3Galβ1-4Xylβ1-MU production may be related to the regulation of GAG-MU biosynthesis. Indeed, the data described here also indicate that the amount of SAα2-3Galβ1-4Xylβ1-MU synthesized was decreased in the presence of tunicamycin. Therefore, the increase in the amount of GAG-MU synthesized may be due to inhibition of SAα2-3Galβ1-4Xylβ1-MU synthesis.

Another likely explanation for the effect of tunicamycin is that it reduces the pool of proteoglycan core protein through inhibition of *N*-linked oligosaccharide synthesis. This is indirectly supported by the fact that while GAG synthesis of proteoglycan core protein was inhibited about

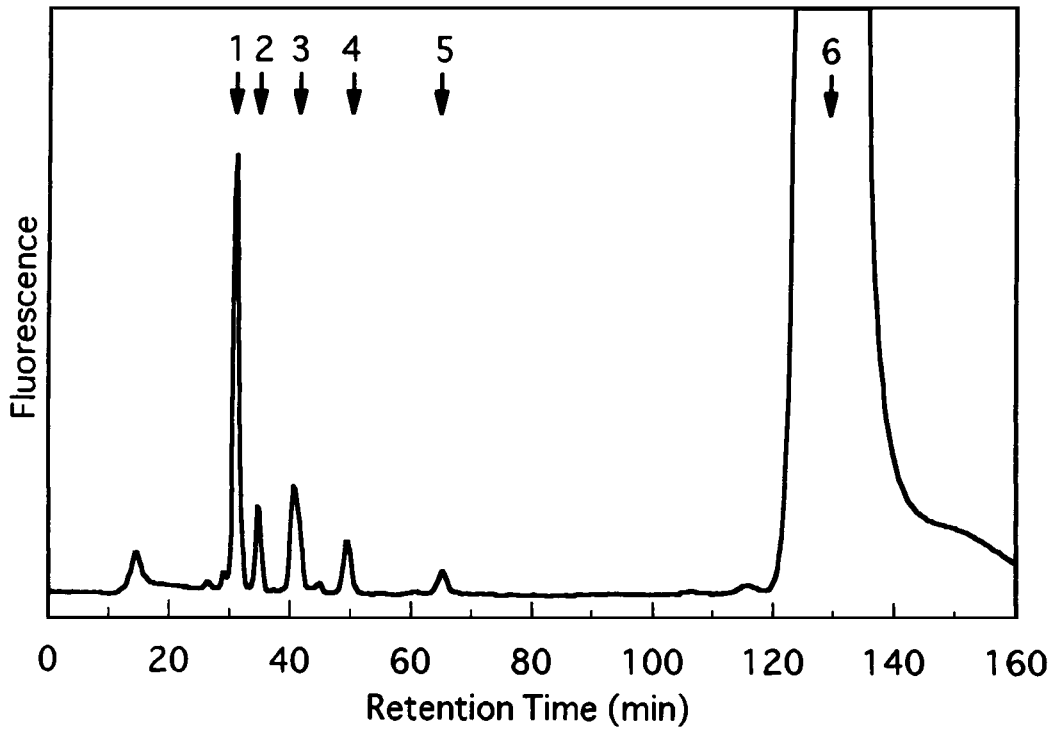

Figure 5. Gel-filtration HPLC analysis of Xyl-MU-initiated oligosaccharides. Human skin fibroblasts were incubated with 0.5 mM Xyl-MU. After a 72 h incubation, aliquots of the culture medium were subjected to gel-filtration HPLC using a Shodex OHpak SB-803. The chromatographic conditions were the same as those described in Figure 3. The arrows denote the Xyl-MU-initiated oligosaccharides: 1, SAα2-3Galβ1-4Xylβ1-MU; 2, sulfate-*O*-3GlcAβ1-4Xylβ1-MU; 3, GlcAβ1-4Xylβ1-MU; 4, Galβ1-3Galβ1-4Xylβ1-MU; 5, Galβ1-4Xylβ1-MU; 6, Xylβ1-MU.

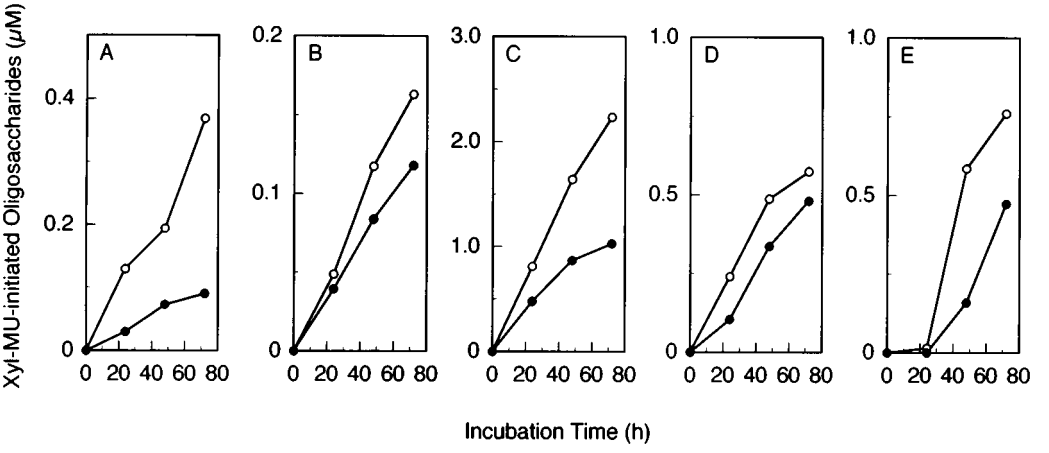

Figure 6. Time course of the effects of tunicamycin on Xyl-MU-initiated GAG and oligosaccharide synthesis. Human skin fibroblasts were incubated with 0.5 mM Xyl-MU for up to 72 h in the absence (○) or presence (●) of 0.2 μg ml^{-1} tunicamycin. After incubation, Xyl-MU-initiated oligosaccharides were identified and quantified as shown in Figure 5. A, Galβ1-3Galβ1-4Xylβ1-MU; B, Galβ1-4Xylβ1-MU; C, SAα2-3Galβ1-4Xylβ1-MU; D, sulfate-*O*-3GlcAβ1-4Xylβ1-MU; E, GlcAβ1-4Xylβ1-MU.

80% by tunicamycin, Xyl-MU-initiated GAG synthesis was actually increased. Together, these two results suggest that the proteoglycan core protein, and not the enzymes required for GAG synthesis, became limiting in the presence of tunicamycin. Since the observed amount of Xyl-MU-initiated GAG synthesis is the result of the Xyl-MU directly competing with endogenous proteoglycan core protein for the GAG biosynthesis machinery, it is expected that any decrease in proteoglycan core protein would lead to increased Xyl-MU-initiated GAG synthesis, which produces shorter and undersulfated chains. Further detailed studied of the relationships among these Xyl-MU-derivatives may yield

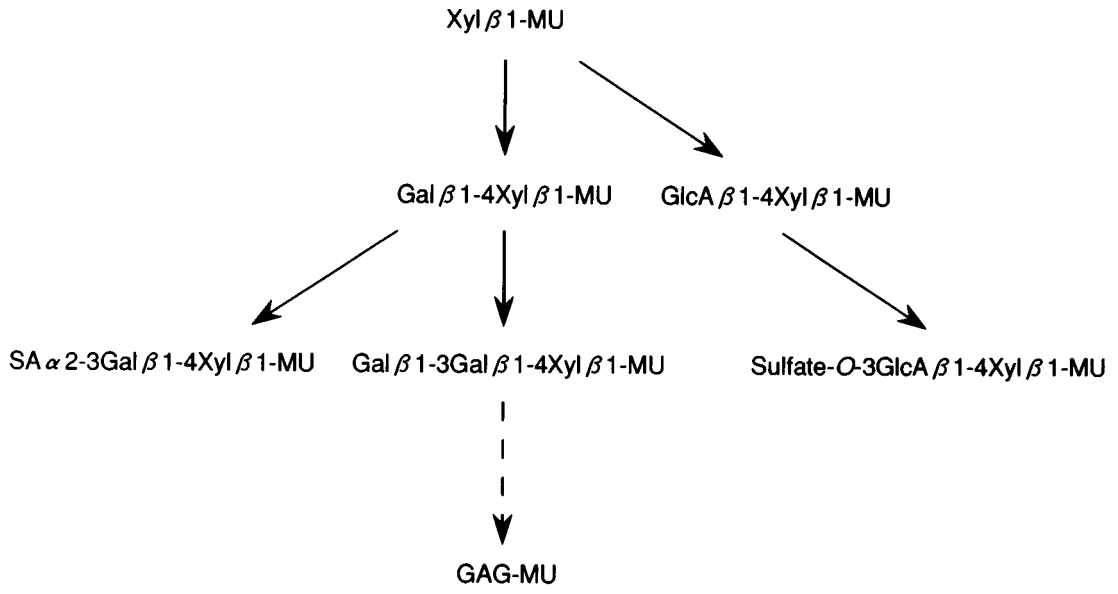

Figure 7. Proposed pathways for synthesis of Xyl-MU-initiated GAG and oligosaccharides in human skin fibroblast cells.

information about the regulation of GAG-MU biosynthesis.

Acknowledgments

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