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Effect of Monensin on the Synthesis of β-D-Xyloside-Initiated Glycosaminoglycan and Its Linkage Region Oligosaccharides in Human Skin Fibroblasts[1]

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Human skin fibroblasts were cultured with a fluorogenic xyloside, 4-methylumbelliferyl-β-D-xyloside (Xyl-MU) as an initiator, and the effects of monensin, which destroys the normal structure of the Golgi complex, on the synthesis of Xyl-MU-initiated glycosaminoglycan (GAG-MU) and its linkage region oligosaccharides were investigated. When the cells were incubated with Xyl-MU in the presence of monensin, the synthesis of GAG-MU was inhibited. In addition, the synthesis of Galβ1-3Galβ1-4Xylβ1-MU as an intermediate of GAG-MU was inhibited, whereas the synthesis of Galβ1-4Xylβ1-MU, which is formed prior to Galβ1-3Galβ1-4Xylβ1-MU, was not. These results indicate that inhibition of GAG-MU synthesis by monensin occurs at the point where the second galactose is joined to Galβ1-4Xylβ1-MU.

Key words: glycosaminoglycan, 4-methylumbelliferyl-β-D-xyloside, human skin fibroblasts, monensin.

Sulfated 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 tissue is not fully understood, it is probably initiated by the transfer of xylose from UDP-xylose to serine residues in the appropriate proteoglycan core protein, followed by stepwise addition of one glucuronic acid and two galactose residues by a series of glycosyltransferase reactions (*2, 3*). 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 (*1-3*).

Recently, the authors have obtained various kinds of 4-methylumbelliferyl-β-D-xyloside (Xyl-MU)-initiated oligosaccharides, in addition to Xyl-MU-initiated GAG (GAG-MU) (*4-7*), by incubating human skin fibroblasts in the presence of Xyl-MU. Two of these derivatives, Galβ1-3Galβ1-4Xylβ1-MU and Galβ1-4Xylβ1-MU, were found to be related to the proteoglycan linkage region structures (*8*). It has also been reported that novel oligosaccharides, SAα2-3Galβ1-4Xylβ1-MU (*9, 10*), sulfate-*O*-3GlcAβ1-4Xylβ1-MU (*11*), and GlcAβ1-4Xylβ1-MU (*12*), can be elaborated from Xyl-MU. Although SAα2-3Galβ1-4Xylβ1-MU is different from intermediates of GAG-MU synthesis, the precursor would be the same (Galβ1-3Xylβ1-MU) as that for Galβ1-4Galβ1-3Xylβ1-MU. Therefore, the SAα2-3Galβ1-4Xylβ1-MU may be synthesized in competition with Galβ1-4Galβ1-3Xylβ1-MU. Further detailed analyses of the relationships among these oligosaccharides may yield information about the regulation of GAG-MU biosynthesis.

In this study, we investigated the effect of monensin, which inhibits the biosynthesis of sugar chains (*13, 14*), on the biosynthesis of GAG-MU and other oligosaccharides derived from Xyl-MU in cultured human skin fibroblasts.

MATERIALS AND PROCEDURES

Materials—Minimum essential medium and fetal bovine serum were obtained from Gibco (Grand Island, NY). 4-Methylumbelliferyl-β-D-xyloside (Xyl-MU) was purchased from Sigma (St. Louis, MO). Monensin was obtained from Wako Pure Chemical (Osaka). Pronase P from *Streptomyces griseus* was purchased from Kaken Kagaku (Tokyo). The Xyl-MU-initiated oligosaccharides (Galβ1-3Galβ1-4Xylβ1-MU, Galβ1-4Xylβ1-MU, SAα2-3Galβ1-4Xylβ1-MU, GlcAβ1-4Xylβ1-MU, and sulfate-*O*-3GlcAβ1-4Xylβ-MU) were prepared as described previously (*8, 10-12*).

Cell Cultures—Human skin fibroblasts were cultured by the method described previously (*8*). 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. To investigate the biosynthesis of GAG and oligosaccharides derived from Xyl-MU, confluent cultured fibroblasts were incubated with

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Abbreviations: Xyl-MU, 4-methylumbelliferyl-β-D-xyloside; GAG, glycosaminoglycan; GAG-MU, Xyl-MU-initiated glycosaminoglycan(s); Xyl, xylose; Gal, galactose; GlcA, glucuronic acid; SA, sialic acid; Ch6S, chondroitin 6-sulfate; DS, dermatan sulfate; HA, hyaluronic acid; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide.

0.5 mM Xyl-MU. Monensin for addition to the medium was prepared as described below. Specifically, a stock solution of monensin (0.35 mg/ml in DMSO) was prepared, and this was added to the medium to give a final concentration of 0.35 μg/ml (*15*).

Preparation of GAG-MU—GAG-MU was extracted from the medium by the method described in the preceding paper (*8*). In brief, 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 precipitates obtained with cetylpyridinium chloride were then collected. Subsequently, the collected precipitates were dissolved in 2 M $MgCl_2$ solution 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) with an excitation wavelength of 325 nm and an emission wavelength of 380 nm.

HPLC—A high-performance liquid chromatograph (Hitachi L-6200, Hitachi) connected to a fluorescence detector (Hitachi F-1050) 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) with 0.2 M NaCl as the solvent at a flow rate of 0.5 ml/min 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.

Electrophoresis on Cellulose Acetate Membrane—Cellulose acetate membrane electrophoresis was carried out using Separax (Jookoo, Tokyo) in 0.1 M pyridine-0.47 M formic acid buffer, pH 3.0, at 1 mA/cm for 30 min, and the membrane was stained with 0.05% alcian blue in 70% ethanol (*16*).

RESULTS

Human Skin Fibroblasts Were Cultured for 72 h with Xyl-MU in the Presence or Absence of Monensin—After incubation, GAG-MU in the medium was isolated and analyzed by electrophoresis on a cellulose acetate membrane (Fig. 1). In the absence of monensin, GAG-MU gave a broad band at the same position as authentic dermatan sulfate. This band was determined to be a mixture of dermatan sulfate and chondroitin sulfate, on the basis of its sensitivity to digestion with chondroitinase ABC and chondroitinase AC II (data not shown). However, the band of GAG-MU was not observed in the presence of monensin. These results indicated that monensin completely inhibited the synthesis of GAG-MU in human skin fibroblasts.

To study the influence of monensin on Xyl-MU-initiated oligosaccharide synthesis, the cells were incubated with Xyl-MU in the presence of the inhibitor. After 72 h of incubation, an aliquot of the culture medium was subjected to gel-filtration HPLC using a Shodex OHpak SB-803 column (Fig. 2), and some Xyl-MU-initiated oligosaccharides (Galβ1-4Xylβ1-MU and GlcAβ1-4Xylβ1-MU) were detected. Their structures were confirmed by carbohydrate composition analysis, enzymic digestion, and ion-spray mass spectrometric analysis (data not shown).

This allowed comparison of the quantitative changes in the production of Xyl-MU-initiated GAG and oligosaccharides in the presence and absence of monensin. The results (Fig. 3) showed that monensin prevented the synthesis of GAG-MU, Galβ1-3Galβ1-4Xylβ1-MU, SAα2-3Galβ1-4Xylβ1-MU, and sulfate-*O*-3GlcAβ1-4Xylβ1-MU, and increased the secretion of GlcAβ1-4Xylβ1-MU and Galβ1-4Xylβ1-MU into the medium.

DISCUSSION

The mode of biosynthesis of the GAG chains of proteoglycans has been studied (*1-3*), and it has been concluded that the formation of GAG chains is initiated by *O*-D-xylosylation of selected serine residues in the appropriate

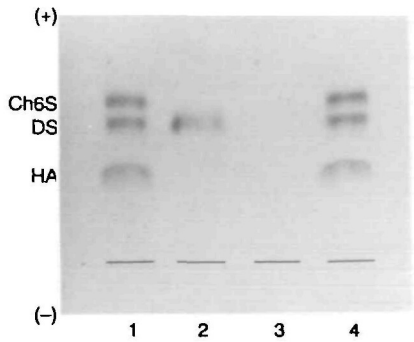

Fig. 1. **Electrophoresis of GAG-MUs on a cellulose acetate membrane.** Human skin fibroblasts were incubated with 0.5 mM Xyl-MU for 72 h in the absence or presence of 0.35 μg/ml monensin, and prepared as described in the text. Electrophoresis was carried out using 0.47 M formic acid-0.1 M pyridine buffer, pH 3.0, at 1 mA/cm for 30 min. The membrane was stained with 0.05% alcian blue in 70% ethanol. 1 and 4, standard mixture of chondroitin 6-sulfate (Ch6S), dermatan sulfate (DS), and hyaluronic acid (HA); 2, GAG-MU in the absence of monensin; 3, GAG-MU in the presence of monensin.

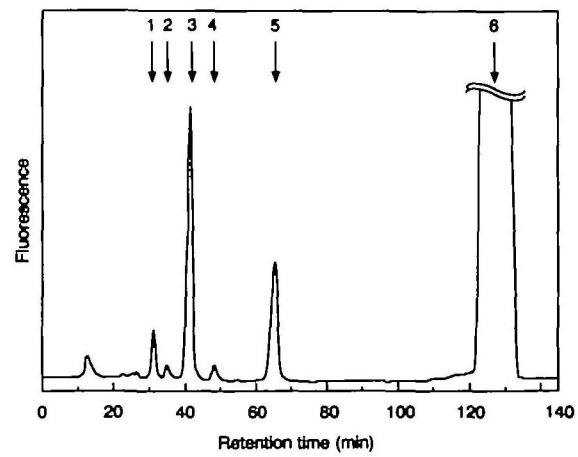

Fig. 2. **Gel-filtration HPLC analysis of Xyl-MU-initiated oligosaccharides.** Human skin fibroblasts were incubated with 0.5 mM Xyl-MU in the presence of 0.35 μg/ml monensin. After a 72-h incubation, aliquots of the culture medium were subjected to gel-filtration HPLC using a Shodex OHpak SB-803 column (8×300 mm) with 0.2 M NaCl at a flow rate of 0.5 ml/min. The eluate was monitored with a fluorescence detector. 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.

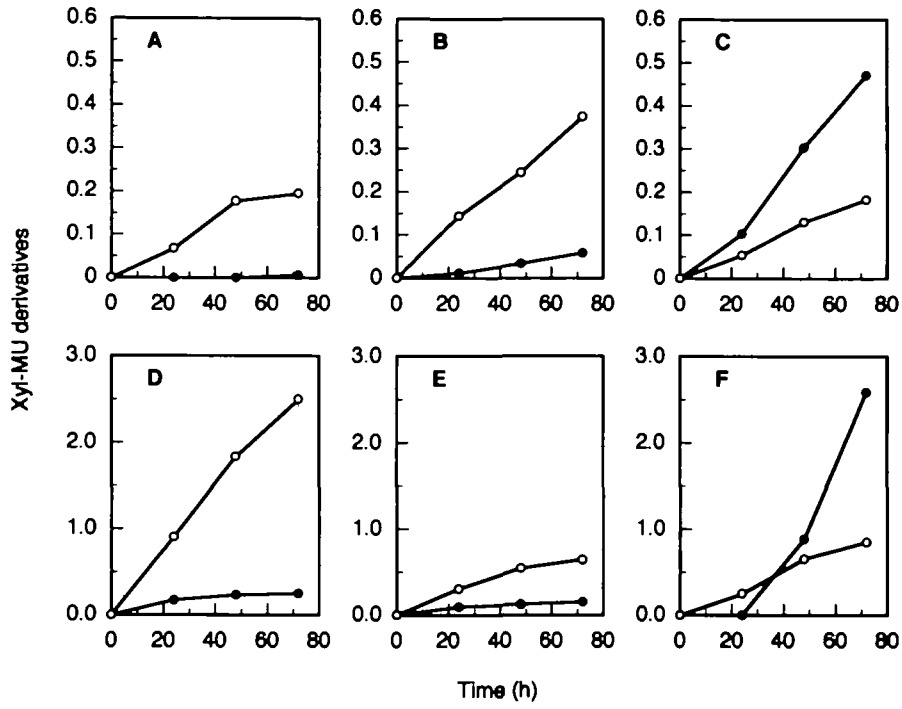

Fig. 3. **Time course of the effects of monensin on Xyl-MU-initiated GAG and oligosaccharide synthesis.** Human skin fibroblasts were incubated with 0.5 mM Xyl-MU for up to 72 h in the absence (○) or presence of 0.35 μg/ml monensin (●). After incubation, Xyl-MU-initiated oligosaccharides were identified and quantified as shown in Fig. 2, and GAG-MU was measured as described in the text. A, GAG-MU; B, Galβ1-3Galβ1-4Xylβ1-MU; C, Galβ1-4Xylβ1-MU; D, SAα2-3Galβ1-4Xylβ1-MU; E, sulfate-*O*-3GlcA-β1-4Xylβ1-MU; F, GlcAβ1-4Xylβ1-MU.

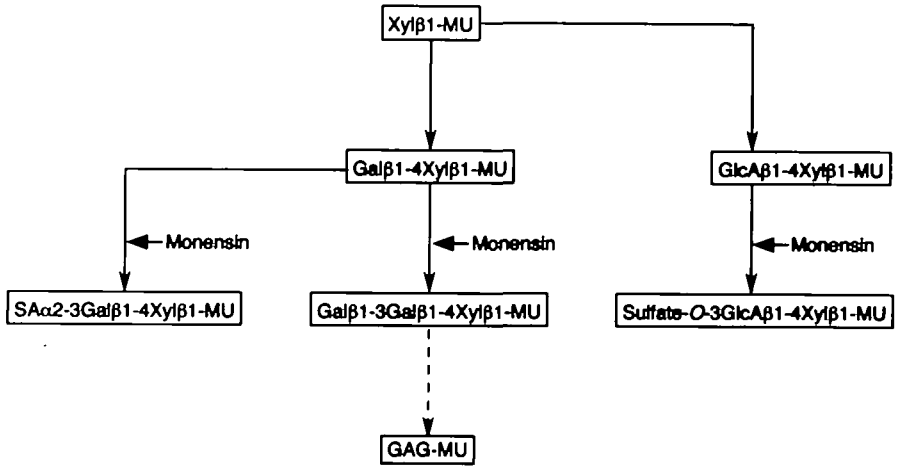

Fig. 4. **Proposed pathways for synthesis of Xyl-MU-initiated GAG and oligosaccharides in human skin fibroblast cells.**

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-initiated oligosaccharides by incubating human skin fibroblasts in the presence of Xyl-MU (*8-12*). 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 was deduced, as shown in Fig. 4. Two of these derivatives, Galβ1-4Xylβ1-MU and Galβ1-3Galβ1-4Xylβ1-MU, are biosynthetic intermediates of GAG-MU (*4*). We added monensin to the culture medium, and studied its effects on the synthesis of Xyl-MU derivatives. Monensin, which is known to affect the assembly of sugar chains near the Golgi apparatus (*17, 18*), inhibited almost completely the synthesis of GAG chains initiated from Xyl-MU. In addition, the synthesis of Galβ1-3Galβ1-4Xylβ1-MU as an intermediate of GAG-MU was also inhibited by monensin, whereas the synthesis of Galβ1-4Xylβ1-MU, which is formed prior to Galβ1-3Galβ1-4Xylβ1-MU, was not. The results presented here indicate that inhibition of the synthesis of GAG-MU by this inhibitor occurs at the point where the second galactose is joined to Galβ1-4Xylβ1-MU (Fig. 4).

In the biosynthesis of SAα2-3Galβ1-3Galβ1-4Xyl-MU and sulfate-*O*-3GlcAβ1-4Xylβ1-MU, the addition of SA or sulfate was also inhibited. However, it is not clear that sialyltransferase and sulfotransferase exist at the same locations as galactosyltransferase II in the Golgi apparatus. Further detailed analyses of the relationships among these

MU-derivatives may yield information about the regulation of GAG-MU biosynthesis.

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