HNK-1-Reactive Oligosaccharide, Sulfate-*O*-3GlcA β 1-4Xyl β 1-MU, Synthesized by Cultured Human Colorectal Cancer Cells

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23 — Human colorectal cancer cells were incubated with medium containing
4-methylumbelliferyl- β -D-xyloside (Xyl-MU). The cells synthesized Xyl-MU-
derivatives which were detected in the culture medium by gel-filtration high-
performance liquid chromatography. These included a Xyl-MU-induced
glycosaminoglycan and its biosynthetic intermediates, Gal β 1-4Xyl β 1-MU and
Gal β 1-3Gal β 1-4Xyl β 1-MU, and other Xyl-MU-induced oligosaccharides, not
related to Xyl-MU-induced glycosaminoglycan, were also synthesized. One of
these oligosaccharides, sulfate-*O*-3GlcA β 1-4Xyl β 1-MU, reacted with HNK-1, a
mouse monoclonal antibody raised against human natural killer cells. Human
neural cells and skin fibroblasts have also been reported to synthesize HNK-1-
reactive sugar chains. Since HNK-1-reactive sugar chains are known to be
involved in cell adhesion in the nervous system, the present results suggest that
epithelium-derived colorectal cancer cells might also be able to utilize them in cell
adhesion. — human colorectal cancer cell; 4-methylumbelliferyl- β -D-
xyloside; HNK-1-reactive oligosaccharide; epithelial cell; cell adhesion
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Abbreviations: Xyl-MU, 4-methylumbelliferyl- β -D-xyloside; GAG, glycosaminoglycan; GAG-MU, Xyl-
MU-induced glycosaminoglycan; Xyl, xylose; Gal, galactose; GlcA, glucuronic acid; SA, sialic acid; MEM,
minimum essential medium; PA, 2-aminopyridine; HPLC, high-performance liquid chromatography.

It has been reported that cultured cells can synthesize glycosaminoglycan (GAG) chains utilizing β -xyloside as a primer when they are cultured with β -xyloside (Okayama et al. 1973; Schwartz et al. 1974; Fukunaga et al. 1975; Robinson et al. 1975; Kato et al. 1978; Kolset et al. 1986; Sobue et al. 1987; Lugemwa and Esko 1991; Fransson et al. 1992). We have previously reported that 4-methylumbelliferyl- β -D-xyloside (Xyl-MU) derivatives were synthesized by human skin fibroblasts cultured in medium containing Xyl-MU. These included a Xyl-MU-induced glycosaminoglycan (GAG-MU), and its biosynthetic intermediates, Gal β 1-3Gal β 1-4Xyl β 1-MU and Gal β 1-4Xyl β 1-MU (Takagaki et al. 1991). Xyl-MU-induced oligosaccharides, unrelated to GAG-MU, such as SA α 2-3Gal β 1-4Xyl β 1-MU (Freeze et al. 1993; Takagaki et al. 1996), Xyl β 1-4Xyl β 1-MU (Izumi et al. 1994), GlcA1-4Xyl β 1-MU (Nakamura et al. 1994), sulfate-*O*-3GlcA β 1-4Xyl β 1-MU (Shibata et al. 1995) and sulfate-*O*-3Xyl β 1-MU (Tazawa et al. 1998) were also synthesized by cultured human skin fibroblasts. Especially, sulfate-*O*-3GlcA β 1-4Xyl β 1-MU had the HNK-1 carbohydrate moiety (sulfate-*O*-3GlcA) at the non-reducing terminal, which is characteristically expressed on a series of neural cell adhesion molecule (Mcgarra et al. 1983), and on also some glycolipids in the nervous system (Chou et al. 1986; Ariga et al. 1987). The HNK-1 carbohydrate moiety binds to laminin (Mohan et al. 1990), P-selectin and L-selectin (Needham and Schnaar 1993), and plays important roles in cell-cell and cell-substrate interaction. Recently, cDNAs encoding the glucuronyltransferase (Oka et al. 1992, 2000; Terayama et al. 1998) and the sulfotransferase (Bakker et al. 1997) involved in the biosynthesis of HNK-1 carbohydrates have been cloned. Furthermore, transfection of cDNA of the glucuronyltransferase involved in the biosynthesis of HNK-1-reactive sugar chain into Chinese hamster ovary cells induced not only expression of the HNK-1 epitope on the cell surface but also marked

morphological changes of the cells (Terayama et al. 1997). This epitope was considered to be related to the nervous and immune system, however, we revealed in the previous report that it was also produced by fibroblast, which are mesenchymal cells (Shibata et al. 1995). Therefore, it was indicated that the HNK-1 carbohydrate epitope would be expressed on various cells and participate in the characteristics of the cells.

In the present study, we cultured human colorectal cancer cells in media containing Xyl-MU, and analyzed the resultant Xyl-MU-derivatives. The colorectal cancer cells synthesized GAG-MU and Xyl-MU-induced oligosaccharides, containing sulfate-*O*-3GlcA β 1-4Xyl β 1-MU. We have demonstrated that epithelium-derived colorectal cancer cells have the ability to synthesize HNK-1-reactive sugar chains, which is similar to that synthesized by human neural cells and skin fibroblasts.

MATERIAL AND METHODS

Chemicals

Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle medium and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co. (Tokyo). MEM containing non-essential amino acids and penicillin-streptomycin solution (penicillin 100 mU/ml and streptomycin 100 mg/ml) were purchased from Life Technologies Inc. (Grand Island, NY, USA). Fetal bovine serum was purchased from JRH Biosciences (Lenexa, KS, USA). Xyl-MU was purchased from Nacalai Tesque Inc. (Kyoto). 2-Aminopyridine (PA) was the same as that reported previously (Takagaki et al. 1990). β -Xylosidase (from *Aspergillus niger*), sulfatase (from *Helix pomatia*), alkaline phosphatase (from calf intestine) and saccharo-1,4-lactone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex G-15 was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). The mouse monoclonal antibody HNK-1 was purchased from

Cosmo Bio Co. (Tokyo). β -Glucuronidase was purified from rabbit liver using p-nitrophenyl- β -D-glucuronide as a substrate (Nakamura et al. 1990). The Xyl-MU-induced oligosaccharide standards were the same as those reported previously (Takagaki et al. 1991, 1996; Nakamura et al. 1994; Shibata et al. 1995). Standard PA-Xyl was purchased from Takara Shuzo Co. (Kyoto). PA-glucuronic acid was synthesized and purified according to the method of Takemoto et al. (1985).

Culture conditions

The human colorectal cancer cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo). DLD-1, COLO 320DM, CCK-81 and WiDr were established from tumor tissues obtained from patients with adenocarcinoma of the colon. CaR-1 was derived from a human rectal adenocarcinoma. COLO 201 was established from ascites fluid obtained from a patient with adenocarcinoma of the colon. DLD-1, COLO 320DM and COLO 201 cells were grown in RPMI 1640 medium. CCK-81, CaR-1 and WiDr cells were grown in Dulbecco's modified Eagle medium, Eagle's MEM and MEM containing non-essential amino acids, respectively. All media contained 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cells were cultured at 37°C in a humidified air atmosphere containing 5% CO₂. The cells were plated at a density of 7×10^5 /100-mm plastic dish (Corning Glass Works, Corning, NY, USA), and cultured for 72 hours in medium containing 0.2 mM Xyl-MU, and the culture medium was recovered.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was performed using a Hitachi model L-6200 system, connected to a Hitachi F-1050, fluorescence detector (Hitachi Co., Tokyo). Xyl-MU-derivatives were detected and

quantified by their fluorescence at an excitation wavelength of 325 nm and an emission wavelength of 380 nm. Xyl-MU-derivatives were separated by gel-filtration using a Shodex OHpak SB-803 HPLC column (8×300 mm, Shoko Co., Tokyo) eluting with 0.2 M NaCl at a column temperature of 30°C and a flow rate of 0.5 ml/min. Reverse-phase HPLC was performed using a Shodex C18-5B column (4.6×250 mm, Shoko Co.) with a linear gradient of distilled water-acetonitrile. The PA-sugars were identified by their relative retention time on an Ultrasphere ODS column (4.6×250 mm, Beckman Instruments, Inc., Palo Alto, CA, USA) with 1% acetonitrile in 0.25 M sodium citrate buffer (pH 4.0) as the eluting solvent (Takagaki et al. 1990).

Purification of GAG-MU

GAG-MU was purified as follows: After adding 0.2 M NaCl, containing 5% cetylpyridinium chloride, to the recovered culture medium, it was incubated at 4°C for 12 hours, and then, centrifuged. MgCl₂ (0.2 M) was added to the resulting precipitate, followed by 4 volumes of NaCl-saturated ethanol. The final precipitate was analyzed as purified GAG-MU.

Enzymic digestions

The Xyl-MU-induced oligosaccharide was digested with β -glucuronidase in a 0.1 M sodium acetate buffer (pH 4.5) at 37°C for 12 hours, as described previously (Nakamura et al. 1990); sulfatase digestion was performed in a 0.1 M sodium acetate buffer (pH 4.5) at 37°C for 4 hours; alkaline phosphatase digestion was performed in a 0.1 M Tris-HCl buffer (pH 8.0) at 37°C for 4 hours, and β -xylosidase digestion was performed in a 0.1 M sodium acetate buffer (pH 4.0) at 37°C for 6 hours. The digestion products were analyzed by gel-filtration HPLC using a Shodex OHpak SB-803 HPLC column.

Smith degradation

Smith degradation of the Xyl-MU-induced

oligosaccharide was performed as described previously (Shibata et al. 1995). The degradation product was hydrolyzed, pyridylaminated (Takemoto et al. 1985), and then analyzed by HPLC using an Ultrasphere ODS column.

Analytical methods

In order to determine their sugar composition, sample was hydrolyzed in 2 N HCl at 100°C for 4 hours and then pyridylaminated (Takemoto et al. 1985). The resulting pyridylaminated monosaccharides were identified and quantified by HPLC using an Ultrasphere ODS column (Takagaki et al. 1990). Mass spectra of the sample was obtained using an ion-spray mass spectrometer (Sciex API-III, Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization source (Shibata et al. 1995).

Solid-phase binding assay

Binding of the Xyl-MU-induced oligosaccharides to the HNK-1 monoclonal antibody was determined as described previously (Shibata et al. 1995). A Corning® ELISA plate was coated with 0, 5, 10 or 20 µg of HNK-1 monoclonal antibody (dissolved in 100 µl of 0.1 M carbonate buffer, pH 9.6), and incubated at 37°C for 3 hours. Then, the plate was washed with PBS and blocked with 100 µl of PBS containing 2% bovine serum albumin at 37°C for 2 hours. The plate was washed with PBS 3 times. The Xyl-MU-induced oligosaccharide and GlcAβ1-4Xylβ1-MU (40 pmol each), dissolved in 100 µl PBS, were then added and the plate was incubated at 4°C for 24 hours, followed by washing with PBS containing 0.05% Tween 20 3 times. In order to solubilize the Xyl-MU-induced oligosaccharides binding to HNK-1, 50 µl of 0.1 M glycine-HCl buffer (pH 2.5) was added and the plate was incubated at 4°C for 48 hours. To estimate the HNK-1-binding Xyl-MU-induced oligosaccharides, the recovered Xyl-MU-induced oligosaccharides were subjected to HPLC.

RESULTS

Production of Xyl-MU-derivatives by cultured human colon cancer cells

CCK-81 human colon cancer cells were incubated with medium containing 0.2 mM Xyl-MU for 72 hours. The medium was then recovered and analyzed by gel-filtration HPLC. Several peaks having fluorescence from Xyl-MU were detected. They were identified by comparison with the retention times of Xyl-MU-derivatives, purified from human skin fibroblast culture medium. Peaks 1, 2, 3, 4, 5 and 6 were thought to be GAG-MU, SAα2-3Galβ1-4Xylβ1-MU, sulfate-O-3GlcAβ1-4Xylβ1-MU, GlcAβ1-4Xylβ1-MU, Galβ1-3Galβ1-4Xylβ1-MU and Galβ1-4Xylβ1-MU, respectively (Fig. 1). Since sulfate-O-3GlcAβ1-4Xylβ1-MU is known to react with HNK-1 (Shibata et al. 1995), we have confirmed the ability of CCK-81 to synthesize this oligosaccharide, by purifying it to homogeneity and analyzing its structure.

Purification of the Xyl-MU-induced oligosaccharide

The Xyl-MU-induced oligosaccharide was purified from the recovered medium by gel-filtration chromatography on a Sephadex G-15 column, followed by reverse-phase HPLC, as described previously (Shibata et al. 1995).

Carbohydrate composition of the Xyl-MU-induced oligosaccharide

After acid hydrolysis and pyridylation of the purified Xyl-MU-induced oligosaccharide, the resulting PA-monosaccharides were identified and quantified by HPLC using an Ultrasphere ODS column. The Xyl-MU-induced oligosaccharide was composed of 4-methylumbelliferone, xylose and glucuronic acid in molar ratios of 1.0 : 0.78 : 0.96, respectively. No galactose, glucosamine, or galactosamine was detected (Table 1).

Fig. 1. Gel-filtration HPLC analysis on a Shodex OHpak SB-803 column of medium recovered from CCK-81 human colon cancer cell cultures. Medium recovered after 72-hours of incubation with Xyl-MU was subjected to gel-filtration HPLC on a Shodex OHpak SB-803 column (8×300 mm). The eluate was monitored with a fluorescence detector at excitation and emission wavelengths of 325 and 380 nm, respectively. The arrows denote the purified Xyl-MU-derivatives recovered from the culture medium of human skin fibroblasts:

1, GAG-MU; 2, SA α 2-3Gal β 1-4Xyl β 1-MU; 3, sulfate-*O*-3GlcA β 1-4Xyl β 1-MU; 4, GlcA β 1-4Xyl β 1-MU; 5, Gal β 1-3Gal β 1-4Xyl β 1-MU; 6, Gal β 1-4Xyl β 1-MU.

Vo., void volume; Vt., total bed volume.

TABLE 1. Carbohydrate composition of 4-methylumbelliferyl β -D-xyloside-induced oligosaccharide

Component	Molar ratio
4-Methylumbelliferone	1.0
Xylose	0.78
Galactose	N.D.
Glucuronic acid	0.96
Hexosamine	N.D.

Data are expressed as molar ratios relative to 4-methylumbelliferone. N.D., not detected.

Enzymic digestion of the Xyl-MU-induced oligosaccharide

The purified Xyl-MU-induced oligosaccharide was digested by various enzymes and then subjected to gel-filtration HPLC as described in Materials and Methods. Its retention time did not alter after digestion with β -glucuronidase (Fig. 2b) or alkaline phosphatase (data not shown). However, after sequential digestion with sulfatase, β -glucuronidase, and β -xylosidase, the retention times of the resultant digestion products corresponded to those of

GlcA-Xyl-MU (Fig. 2c), Xyl-MU (Fig. 2d), and 4-methylumbelliferone (data not shown). These results showed that the sequence of the Xyl-MU-induced oligosaccharide was sulfate-GlcA-Xyl-MU.

Smith degradation of the Xyl-MU-induced oligosaccharide

In order to determine the position of the sulfate linkage on glucuronic acid residue, a sample of the purified Xyl-MU-induced oligosaccharide was subjected to Smith degradation. The product was then hydrolyzed, pyridylaminated and analyzed by HPLC, as described in Materials and Methods. PA-glucuronic acid was detected (data not shown). If the sulfate had been linked to glucuronic acid at any position other than the C-3, it would have been cleaved and thus not detected as PA-glucuronic acid. Therefore, this finding indicates that the oligosaccharide's structure was sulfate-*O*-3GlcA β 1-4Xyl β 1-MU. Ion spray mass spectrometric analysis showed that the mass number of the Xyl-MU-induced oligosaccharide was the same as that of sulfate-*O*-

Fig. 2. Gel-filtration HPLC analysis of the Xyl-MU-induced oligosaccharide, after incubation with various enzymes. The column used was a Shodex OHpak SB-803 column (8×300 mm), and the products were eluted with 0.2 M NaCl at a flow rate of 0.5 ml/min. The eluate was monitored with a fluorescence detector. a, before enzymic digestion; b, after incubation with β -glucuronidase; c, after incubation with sulfatase; d, after sequential incubation with sulfatase followed by β -glucuronidase. The arrows denote the positions of Xyl-MU derivatives: 1, the Xyl-MU-induced oligosaccharide; 2, GlcA-Xyl-MU; 3, Xyl-MU.

3GlcA β 1-4Xyl β 1-MU (563, data not shown).

Solid-phase binding assay of the Xyl-MU-induced oligosaccharide

Binding of the Xyl-MU-induced oligosaccharide to HNK-1 was determined using a solid-phase binding assay. The affinity of sulfate-*O*-3GlcA β 1-4Xyl β 1-MU for HNK-1 was found to be concentration-dependent, but GlcA β 1-

Fig. 3. Time course for the synthesis of the HNK-1-reactive oligosaccharide synthesized by CCK-81 cells. Medium, recovered after various incubation periods, was subjected to gel-filtration HPLC on Shodex OHpak SB-803 column. The fluorescence intensities of the HNK-1-reactive oligosaccharide peaks were plotted for each incubation period.

4Xyl β 1-MU did not react with HNK-1 (data not shown).

Time course for the synthesis of the HNK-1-reactive oligosaccharide by CCK-81

The synthesis of the HNK-1-reactive oligosaccharide by CCK-81 was monitored by gel-filtration HPLC as described in MATERIAL AND METHODS. The synthesis of this HNK-1-reactive oligosaccharide was found to increase with prolonged incubation (Fig. 3).

Synthesis of the HNK-1-reactive oligosaccharide and GAG-MU by human colorectal cancer cells

We examined the ability of 6 human colorectal cancer cell lines to synthesize sulfate-*O*-3GlcA β 1-4Xyl β 1-MU and GAG-MU, after 72 hours of incubation with Xyl-MU. Sulfate-*O*-3GlcA β 1-4Xyl β 1-MU and purified GAG-MUs were subjected to gel-filtration HPLC and quantified by the intensity of their fluorescence. There were differences both in the synthesized amounts and their molecular sizes of GAG-MUs synthesized by colorectal cancer cell lines (Fig.

Fig. 4. Synthesis of GAG-MUs by human colorectal cancer cell lines. GAG-MUs were purified by sequential precipitation with cetylpyridinium chloride and ethanol, as described in MATERIALS AND METHODS. GAG-MUs were purified from equivalent volumes of recovered media incubated with the 6 human colorectal cancer cell lines, and then analyzed by gel-filtration HPLC on a Shodex OHpak SB-803 column.

a, DLD-1; b, COLO 320DM; c, COLO 201; d, CCK-81; e, CaR-1; f, WiDr; V_o , void volume.

TABLE 2. *Xyl-MU-derivatives synthesized by human colorectal cancer cells*

	(pmol/10 000 cells)		Sulfate-GlcA-Xyl-MU/GAG
	Sulfate-GlcA-Xyl-MU	GAG-MU	
CCK-81	1386	45	30.84
WiDr	459	307	1.50
DLD-1	411	374	1.10
COLO320DM	166	159	1.04
CaR-1	181	702	0.26
COLO201	141	1078	0.13
Human skin fibroblast	9507	13 026	0.73

4). Together with the amounts of sulfate-*O*-3GlcA β 1-4Xyl β 1-MU and GAG-MUs synthesized by colorectal cancer cell lines, their ratios are summarized in Table 2. The amounts of both Xyl-MU-derivatives were smaller than those synthesized by human skin fibroblasts. Compared to the other cell lines, CCK-81 synthesized the largest amount of sulfate-*O*-3GlcA β 1-4Xyl β 1-MU. Analysis of the sulfate-

O-3GlcA β 1-4Xyl β 1-MU: GAG-MU ratios indicated that there were three different groups. Group one was a cell line whose ratio was about 31 (CCK-81); group 2 comprised 3 cell lines (WiDr, DLD-1, and COLO 320DM), whose ratios were similar to that of human skin fibroblasts. The third group consisted of 2 cell lines whose ratios were 0.1~0.2 (CaR-1 and COLO 201). The cell line which had the greatest ability to

synthesize sulfate-*O*-3GlcA β 1-4Xyl β 1-MU (CCK-81) had the poorest ability to synthesize GAG-MU. Same results were replicated in the analysis of the medium from other dishes.

DISCUSSION

Previous reports have shown that neural cells can synthesize a glycolipid (Chou et al. 1986; Ariga et al. 1987) and a glycoprotein (McGarry et al. 1983; Kruse et al. 1984, 1985; Bollensen and Schachner 1987) which are both capable of reacting with a mouse monoclonal antibody raised against human natural killer cells (HNK-1). The glycolipid was found to have sulfate-*O*-3GlcA at its non-reducing terminal. Mesenchymal cells are also capable of synthesizing HNK-1-reactive sugar chains, since we found that human skin fibroblasts can produce sulfate-*O*-3GlcA β 1-4Xyl β 1-MU (Shibata et al. 1995). In order to determine whether epithelial cells have the ability to synthesize HNK-1-reactive sugar chains, we cultured CCK-81 human colorectal cancer cells in Xyl-MU-containing medium for 72 hours. After the incubation, the medium was recovered and subjected to gel-filtration HPLC. Several Xyl-MU-derivatives were detected (Fig. 1) and in consideration for their retention time, GAG-MU,

SA α 2-3Gal β 1-4Xyl β 1-MU, sulfate-*O*-3GlcA β 1-4Xyl β 1-MU, GlcA β 1-4Xyl β 1-MU, Gal β 1-3Gal β 1-4Xyl β 1-MU and Gal β 1-4Xyl β 1-MU were likely to be synthesized by the colon cancer cells. To clarify whether the colon cancer cells have the ability to synthesize the HNK-1-reactive sugar chains, purification and structural analysis of the Xyl-MU-induced oligosaccharide likely to be sulfate-*O*-3GlcA β 1-4Xyl β 1-MU was performed. The results of the carbohydrate composition analysis, enzymic digestion and Smith degradation elucidated that the Xyl-MU-induced oligosaccharide synthesized by CCK-81 cells has the same structure as sulfate-*O*-3GlcA β 1-4Xyl β 1-MU synthesized by human skin fibroblasts, and the Xyl-MU-induced oligosaccharide was also reactive with HNK-1. We have demonstrated that five other colorectal cancer cell lines also had the ability to synthesize HNK-1-reactive sugar chains. Although the quantity of the HNK-1-reactive oligosaccharide synthesized by colorectal cancer cell lines was less than that synthesized by human skin fibroblasts, we have conclusively demonstrated that epithelium-derived colorectal cancer cells also have the ability to synthesize HNK-1-reactive sugar chains.

Xyl-MU-derivatives are thought to be

Fig. 5. Suggested pathway for the biosynthesis of Xyl-MU-derivatives synthesized by cultured cells.

synthesized predominantly through the GAG-MU pathway, as shown in Fig. 5. However, the CCK-81 cells, which synthesized more HNK-1-reactive sugar chain than the other colorectal cancer cell lines, synthesized the lowest amounts of GAG-MU, therefore, this is not the main pathway in this cell line.

It is not understood why Xyl-MU-induced oligosaccharides, which are unrelated to GAG-MU synthesis, are synthesized by cultured cells. However, the HNK-1-reactive glycolipid and its sugar chain have been shown to inhibit the growth and adhesion of neurites and astrocytes (Kumemund et al. 1988) and to be ligands for selectins (Needham and Schnaar 1993; Friederichs et al. 2000). It is possible that HNK-1-reactive sugar chains might also be involved in cell growth and adhesion in human skin fibroblasts and colorectal cancer cells. Chondroitin sulfate proteoglycans have been reported to inhibit the adhesion of cells to fibronectin or collagen (Yamagata et al. 1989). Therefore, it is likely that these cells synthesize both types of cell-adhesion-related molecules.

The ability of the 6 human colorectal cancer cell lines to synthesize HNK-1-reactive sugar chains varied by an order of magnitude, from 1386 pmol/10 000 cells for CCK-81 cells, to 141 pmol/10 000 cells for COLO 201 cells. If this sugar chain is involved in cell adhesion in these tumor cells, then it is interesting to speculate that the difference in HNK-1-reactive sugar chain synthesis might be related to their metastatic and invasive potential in vivo. And, it may be concerned with the difference of the biological behavior containing metastatic and invasive potential that which type of celladhesion-related molecules is dominantly produced.

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