Brief Report

Effect of benzyl-*N*-acetyl-α-galactosaminide on KL-6 mucin expression and invasive properties of a human pancreatic carcinoma cell line

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ABSTRACT: KL-6 mucin is a type of MUC1 mucin and its aberrant expression has been shown to be associated with aggressive metastasis and poor clinical outcome in tumors. The present study is to investigate the effects of benzyl-*N*-acetyl-α-galactosaminide (GalNAc-*O*-bn), an *O*-glycosylation inhibitor, on KL-6 mucin expression and invasive properties of a human pancreatic carcinoma cell line, Suit-2 cells. Expression profiles of KL-6 mucin in the cells pretreated with or without 5 mM GalNAc-*O*-bn for 48 h were examined by Western blotting and immunocytochemical staining and invasive properties were examined by transwell chamber assay. Western blotting and immunocytochemical staining showed that the expression profiles of KL-6 mucin changed significantly after GalNAc-*O*-bn treatment. Meanwhile, the invasive ability of Suit-2 cells decreased significantly after GalNAc-*O*-bn treatment ($p < 0.05$). These results suggest that glycosylation of KL-6 mucin may be closely related to aggressive behaviors of pancreatic cancer cells like metastasis and invasion.

Keywords: KL-6 mucin, *O*-glycosylation, Benzyl-*N*-acetyl-α-galactosaminide, Invasion, Pancreatic carcinoma

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1. Introduction

Patients with pancreatic cancer still have a poor prognosis, a 5-year survival rate of ~3% and a median survival of < 6 months (*1*), although recent efforts have improved cancer prevention, screening, and therapy. The major problem in the management of postsurgical cases is failure to control cancer metastases, which results from a lack of early detection and effective treatment.

MUC1 is a polymorphic, highly glycosylated, type I transmembrane glycoprotein expressed by ductal epithelial cells of secretory organs, including the pancreas, breast, lung, and gastrointestinal tract, that is overexpressed and aberrantly glycosylated in most cases of adenocarcinoma (*2*). The deduced amino acid sequence of MUC1 mucin reveals four distinct domains: an NH_2-terminal domain consisting of a hydrophobic signal sequence, a highly *O*-glycosylated tandem-repeat domain, a transmembrane domain, and a cytoplasmic domain (*3*). A number of studies have suggested that overexpression of MUC1 plays an important role in pancreatic cancer metastasis and that MUC1 seems to be an attractive target for treatment of pancreatic cancer (*4,5*). Therefore, having MUC1 target monoclonal antibody (mAb) with high specificity and affinity may represent an effective strategy.

KL-6 mucin, a type of MUC1 categorized as cluster 9, is recognized by KL-6 mAb, and its epitope includes sialo-oligosaccharide moiety in MUC1 molecules (*6,7*). This mucin was first established in the serum of patients with intestinal pneumonia but has recently been detected in various cancer tissues (*8,9*). Previous immunohistochemical studies by the current authors have shown that overexpression of KL-6 mucin was associated with worse tumor behaviors such as invasion and metastasis in ampullary carcinoma, primary

colorectal carcinoma, and metastatic liver cancer tissues (*10-12*). In addition, the relationship between expression of KL-6 mucin and metastatic potential has also been reported in colorectal carcinoma cell lines (*13*). However, the role of glycosylation in KL-6 mucin in tumor behaviors remains to be elucidated. Reports have indicated that extension of *O*-glycosylation in cultured cells can be blocked in the presence of benzyl-*N*-acetyl-α-galactosaminide (GalNAc-*O*-bn) (*14-16*). The present study aimed to evaluate the effect of the *O*-glycosylation inhibitor GalNAc-*O*-bn on the expression profiles of KL-6 mucin and the invasive properties of the human pancreatic carcinoma Suit-2 cell line.

2. Materials and Methods

2.1. *Reagents*

GalNAc-*O*-bn, trypsin, cell lysis regents, trypan blue, and culture media were purchased from Sigma-Aldrich Japan, Tokyo, Japan. KL-6 mAb was provided by Eisai Co., Ltd, Tokyo, Japan. The Histofine SAB-PO kit and BD BioCoat™ Tumor Invasion System were from Nichirei Corporation, Tokyo, Japan and BD Biosciences, San Jose, CA, USA, respectively.

2.2. *Cells and culture conditions*

Human pancreatic carcinoma Suit-2 cell line was obtained from JCRB Cell Bank, Tokyo, Japan. The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2 in air at 37°C. For maintenance and subculture, cells in the exponential phase were treated with 0.25% trypsin solution containing 0.02% EDTA. Cells reaching 80% of confluence were collected and subjected to the following experiment.

2.3. *Cell proliferation assay*

Cells were reseeded in 96-well plates (3×10^3 cells per well) incubated with 0, 1, 2, 4, 8, and 16 mM of GalNAc-*O*-bn for 48 h. Cell proliferation was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay as described elsewhere (*17*). Absorbance was detected at 550 nm with 750 nm as a reference wavelength.

2.4. *Western blotting*

Cells treated with or without 5 mM of GalNAc-*O*-bn for 48 h at 37°C were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, containing 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin) with occasional sonication. After centrifuging at 12,000 × g for 30 min at 4°C, cell extracts (10 μg protein each) were subjected to SDS-PAGE (8% gel) and then electrotransferred onto polyvinylidene difluoride membranes. After blocking with 20 mM Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween-20 overnight at 4°C, the membranes were incubated with KL-6 mAb (1:750 dilution) for 1 h at room temperature. After they were washed three times, the membranes were incubated with horse radish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Reactivity was visualized by enhanced chemiluminescence using the ECL Western Blotting Starter Kit (RPN2108; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Data were obtained from three independent experiments.

2.5. *Immunocytochemistry*

Cells (1×10^4) treated with or without GalNAc-*O*-bn were seeded on MAS-coated slides (Matsunami, Inc., Osaka, Japan) and incubated for 4 h. Cells were then fixed with 3.7% paraformaldehyde for 30 min followed by treatment with 0.2% Triton X-100 for 10 min. Next, immunohistochemical staining by KL-6 mAb was done as described before (*10*). Briefly, cells were blocked with normal goat serum for 30 min at room temperature, and then incubated with or without KL-6 antibody (1:200 dilution) for 60 min at room temperature. After the incubation of biotin-labeled secondary antibody, detection of KL-6 mucin was achieved by the biotin-streptavidin-peroxidase complex method using Histofine SAB-PO kit. 3,3'-Diaminobenzidine was used as the chromogen, and haematoxylin was used as a counterstain.

2.6. *Transwell chamber assay*

Motility and the invasive abilities of cells were assessed in 24-well transwell plates (Corning, NY, USA). Cells were pre-incubated with or without 5 mM of GalNAc-*O*-bn for 48 h at 37°C in a CO_2 incubator and then detached and resuspended in serum-free RPMI-1640. A suspension of cells (2×10^5 cells/mL) was placed on matrigel-coated filters and control filters, respectively. The lower chambers were filled with 0.75 mL of RPMI-1640 medium supplemented with 5% FBS. Cells were allowed to migrate for 22 h at 37°C. Cells that invaded the matrigel and reached the opposite surface of the filter were stained with a Diff-Quik kit (Dade Behring, Newark, DE, USA) in accordance with the manufacturer's instructions. The invaded cells were quantified by counting the number of cells in eight random microscopic fields per filter at a magnification of ×100. Each data point was calculated from two separate experiments performed

in triplicate. A p value less than 0.05 was considered statistically significant.

3. Results and Discussion

Control of metastatic pancreatic cancer remains a major objective in pancreatic cancer treatment. The overexpression of MUC1 mucin plays an important role in the process of pancreatic cancer metastasis and invasion. MUC1 targeting mAb with high specificity and affinity might represent a useful targeted therapy. KL-6 mucin is a type of MUC1 recognized by KL-6 mAb, and its epitope includes sialo-oligosaccharide moiety in MUC1 molecules (*6-9*). Many studies have shown that overexpression of KL-6 mucin may be associated with worse tumor behaviors such as invasion and metastasis in many kinds of cancers (*6,10-12*). Since MUC1 is a highly *O*-glycosylated cell surface glycoprotein, GalNAc-*O*-bn, an *O*-glycosylation inhibitor, was used to inhibit *O*-linked oligosaccharide of KL-6 mucin. GalNAc-*O*-bn is a synthetic analogue of *N*-acetylgalactosamine and inhibits elongation of *O*-glycans. The inhibition is competitive and instead of monosaccharide transfer in which GalNAc is bound to serine or threonine, the elongation of the *O*-glycan chain occurs in benzyl-*N*-acetyl-α-galactosaminide molecules (*18,19*).

First, the inhibitory effects of GalNAc-*O*-bn on Suit-2 cell proliferation were examined using MTT assay. Inhibition of Suit-2 cell proliferation was not observed in the presence of up to 16 mM GalNAc-*O*-bn (data not shown). Therefore, a concentration of 5 mM was used in the following experiments as reported previously (*18*). Cell viability, as judged by trypan blue exclusion, was also unaffected in the presence of 5 mM GalNAc-*O*-bn (data not shown).

Using the KL-6 antibody, KL-6 mucin expression was next detected in Suit-2 cells with or without GalNAc-*O*-bn treatment by means of Western blotting. As shown in Figure 1A, KL-6 mucin was detected with variations in molecular weight. Notably, the intensity of the major band around 209 kDa was significantly lower after treatment with 5 mM GalNAc-*O*-bn. In contrast, the intensity of other two bands with a molecular weight of around 110 and 178 kDa, respectively, seemed to be higher in GalNAc-*O*-bn treated cells than in the untreated cells. To examine the subcellular expression of KL-6 mucin, Suit-2 cells with or without GalNAc-*O*-bn treatment were subjected to immunocytochemical staining. Before GalNAc-*O*-bn treatment, strong KL-6 mucin stains were observed in Suit-2 cells in the entire cell, including its membrane and cytoplasm (Figure 1B). After GalNAc-*O*-bn treatment, KL-6 mucin stains decreased significantly especially in the cell membrane. Cell morphology was also found to have changed after GalNAc-*O*-bn treatment (Figure 1B).

One of the earliest steps in metastasis is the invasion of the basement membrane. Next evaluated was the effect of GalNAc-*O*-bn on the motility and invasive ability of Suit-2 cells migrating through a matrigel-coated polycarbonate membrane. As shown in Figure 2, the invasive potential of Suit-2 cells decreased significantly after 5 mM GalNAc-*O*-bn treatment. The percentage of cells penetrating the matrigel-coated polycarbonate filters without or with GalNAc-*O*-bn treatment was 93.5% and 10.3%, respectively ($p < 0.05$).

The present study suggests that GalNAc-*O*-bn, an *O*-glycosylation inhibitor, may significantly alter the expression profiles of KL-6 mucin, especially on the cell surface, and it may alter the invasive ability of Suit-2 cells. Since KL-6 mAb recognizes sialo-oligosaccharides in addition to part of the core polypeptide of KL-6 mucin (*8*), the decrease in KL-6 mucin expression as a result of treatment with GalNAc-*O*-bn may be due to the insufficient elongation of oligosaccharide chains. Although the present study did

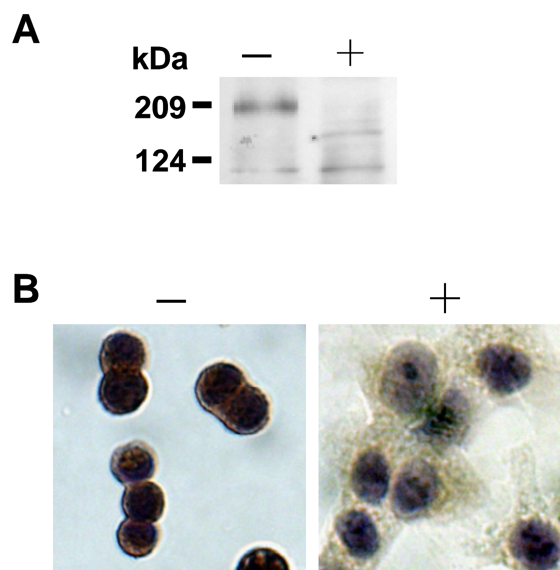

Figure 1. Western blotting and immunocytochemical analyses for KL-6 mucin expression in Suit-2 cells treated with (+) or without (–) GalNAc-*O*-bn. Suit-2 cells were pretreated with or without 5 mM GalNAc-*O*-bn for 48 h at 37°C, and then subjected to Western blotting (A) and immunocytochemical analysis (B) as described in Materials and Methods. Original magnification of B, ×400.

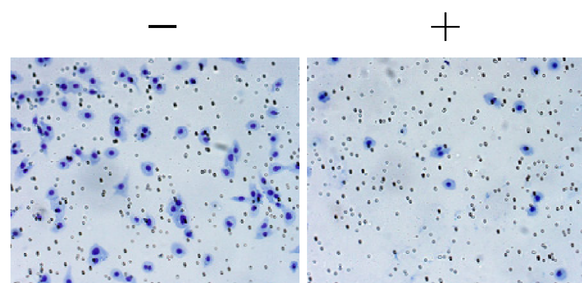

Figure 2. Inhibitory effect of GalNAc-*O*-bn on Suit-2 cell invasion. Suit-2 cells pretreated with (+) or without (–) 5 mM GalNAc-*O*-bn were placed on matrigel-coated chambers and incubated for 22 h at 37°C. Original magnification, ×100.

not examine the changes in levels of expression of the KL-6 mucin core polypeptide for GalNAc-*O*-bn-treated and untreated cells, the current findings suggest that an extracellular domain containing sialo-oligosaccharide chains of KL-6 mucin may play a role in the invasive ability of the cells.

GalNAc-*O*-bn has been reported to inhibit *O*-glycosylation in other cell surface glycoprotein such as brush border glycoprotein sucrase-isomaltase (*20*) and to cause morphological changes with an accumulation of GalNAc terminal glycoproteins that may be mucin precursors at the cell surface (*21*). Further study is needed to clarify the detailed mechanism by which GalNAc-*O*-bn acts on KL-6 mucin expression and the underlying role of KL-6 mucin in the metastatic progression of the pancreatic carcinoma. These experiments may provide *in vitro* evidence for the *O*-glycosylation of KL-6 mucin playing a role in the invasion of cancer cells and portend that therapeutic strategies targeting oligosaccharide moieties of KL-6 mucin should be useful in the treatment of aggressive pancreatic cancer.

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References

1. Schneider G, Siveke JT, Eckel F, Schmid RM. Pancreatic cancer: basic and clinical aspects. Gastroenterology 2005; 128:1606-1625.
2. Kim YS, Gum J, Brockhausen I. Mucin glycoproteins in neoplasia. Glycoconjugate J 1996; 13:693-707.
3. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nature Rev 2004; 4:45-60.
4. Kohlgraf KG, Gawron AJ, Higashi M, Meza JL, Burdick MD, Kitajima S, Kelly DL, Caffrey TC, Hollingsworth MA. Contribution of the MUC1 tandem repeat and cytoplasmic tail to invasive and metastatic properties of a pancreatic cancer cell line. Cancer Res 2003; 63:5011-5020.
5. Luttges J, Feyerabend B, Buchelt T, Pacena M, Kloppel G. The mucin profile of noninvasive and invasive mucinous cystic neoplasms of the pancreas. Am J Surg Pathol 2002; 26:466-471.
6. Ogawa Y, Ishikawa T, Ikeda K, Nakata B, Sawada T, Ogisawa K, Kato Y, Hirakawa K. Evaluation of serum KL-6, a mucin-like glycoprotein, as a tumor marker for breast cancer. Clin Cancer Res 2006; 6:4069-4072.
7. Kohno N, Inoue Y, Hamada H, Fujioka S, Fujino S, Yokoyama A, Hiwada K, Ueda N, Akiyama M. Difference in sero-diagnostic values among KL-6-associated mucins classified as cluster 9. Int J Cancer 1994; 8:81-83.
8. Kohno N, Akiyama M, Kyoizumi S, Hakoda M, Kobuke K, Yamakido M. Detection of soluble tumor-associated antigens in sera and effusions using novel monoclonal antibodies, KL-3 and KL-6, against lung adenocarcinoma. Jpn J Clin Oncol 1988; 18:203-216.
9. Kohno N. Serum marker KL-6/MUC1 for the diagnosis and management of interstitial pneumonitis. J Med Invest 1999; 46:151-158.
10. Tang W, Inagaki Y, Kokudo N, Guo Q, Seyama Y, Nakata M, Imamura H, Sano K, Sugawara Y, Makuuchi M. KL-6 mucin expression in carcinoma of the ampulla of Vater: Association with cancer progression. World J Gastroenterol 2005; 11:5450-5454.
11. Guo Q, Tang W, Inagaki Y, Midorikawa Y, Kokudo N, Sugawara Y, Nakata M, Konishi T, Nagawa H, Makuuchi M. Clinical significance of subcellular localization of KL-6 mucin in primary colorectal adenocarcinoma and metastatic tissues. World J Gastroenterol 2006; 12:54-59.
12. Xu HL, Inagaki Y, Tang W, Guo Q, Wang FS, Seyama Y, Midorikawa Y, Gai RY, Kokudo N, Sugawara Y, Nakata M, Makuuchi M. Elevation of serum KL-6 mucin levels in patients with cholangiocarcinoma. Hepatogastroenterology 2008; 88: *in press*.
13. Guo Q, Tang W, Inagaki Y, Kokudo N, Sugawara Y, Karako H, Nakata M, Makuuchi M. Subcellular localization of KL-6 mucin in colorectal carcinoma cell lines: association with metastatic potential and cell morphology. Oncol Rep 2007; 17:1057-1060.
14. Delacour D, Gouyer V, Leteurtre E, Ait-Slimane T, Drobecq H, Lenoir C, Moreau-Hannedouche O, Trugnan G, Huet G. 1-Benzyl-2-acetamido-2-deoxy-alpha-*D*-galactopyranoside blocks the apical biosynthetic pathway in polarized HT-29 cells. J Biol Chem 2003; 278:37799-37809.
15. Gouyer V, Leteurtre E, Delmotte P, Steelant WFA, Krzewinski-Recchi MA, Zanetta JP, Lesuffleur T, Trugnan G, Delannoy P, Huet G. Differential effect of GalNAcα-*O*-bn on intracellular trafficking in enterocytic HT-29 and Caco-2 cells: relation with the glycosyltransferase expression pattern. J Cell Sci 2001; 114:1455-1471.
16. Huang J, Byrd JC, Yoor WH, Kim YS. Effect of benzyl-α-GalNAc, an inhibitor of mucin glycosylation, on cancer-associated antigens in human colon cancer cells. Oncol Res 1992; 4:507-515.
17. Zhang L, Hu JJ, Du GH. Establishment of a cell-based assay to screen insulin-like hypoglycemic drugs. Drug Discov Ther 2008; 2:229-233.
18. Satoh S, Hinoda Y, Hayashi T, Burdick MD, Imai K, Hollingsworthman MA. Enhancement of metastatic properties of pancreatic cancer cells by MUC1 gene encoding an anti-adhesion molecule. Int J Cancer 2000; 88:507-518.
19. Paszkiewicz-Gadek A, Porowska H, Lemancewicz D, Wolczynski S, Gindzienski A. The influence of *N*- and *O*-glycosylation inhibitors on the glycosylation profile of cellular membrane proteins and adhesive properties of carcinoma cell lines. Int J Mol Med 2006; 17:669-674.
20. Alfalah M, Jacob R, Preuss U, Zimmer KP, Naim H, Naim HY. *O*-linked glycans mediate apical sorting of human intestinal sucrase-isomaltase through association with lipid rafts. Curr Biol 1999; 9:593-596.
21. Kuan SF, Byrd JC, Basbaum C, Kim YS. Inhibition of mucin glycosylation by aryl-*N*-acetyl-a-galactosaminides in human colon cancer cells. J Biol Chem 1989; 264:19271-19277.

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